Special Focus on Age-related Macular Degeneration, Original Article

Characterization of age-related macular degeneration in Indian donor eyes

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Purpose: The purpose of this study was to test the reliability of fundus stereomicroscopy in postmortem eyes to assign severity of age-related macular degeneration (AMD) using the Minnesota grading and confirmation by histology using Alabama and Sarks grading scales and to assess the incidence of AMD pathology in donor eyes from a South Indian population. **Methods:** Eyes (199) from 153 donors (55–95 years) after obtaining fundus images were processed for histology. Fundus images were graded according to the Minnesota grading system based on drusen size, area of depigmentation, and atrophy. At least one eye from each donor displaying the AMD phenotypes were subjected to histological examination. The fundus grading was correlated with histology and the stages of AMD assigned for early AMD by the Alabama AMD grading system and for both early and advanced AMD by the Sarks classification. **Results:** Stereoscopic examination of the fundus found that 10 of the 153 donors had features of early AMD and 3 advanced AMD. Following histological examination, one of the early AMD eyes was reclassified as advanced AMD. Early AMD features that were observed on histology included soft drusen (>63 μm), basal laminar deposits, photoreceptor outer segment degeneration, disorganization of retinal pigment epithelium (RPE), Bruch's membrane thickening. Advanced AMD features observed in histology are extensive atrophy of RPE, choroidal neovascularization and disciform scar formation**. Conclusion:** Identification of either early or advanced AMD using stereomicroscopic assessment (SMA) showed high sensitivity and specificity. However, misclassification between AMD stages can occur when only SMA is used.

Key words: Age-related macular degeneration, donor eyes, fundus images, histopathology, retinal pigment epithelium

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Age-related macular degeneration (AMD) involves complex pathological changes in the retina with multifactorial etiology,[1] which results in irreversible loss of central vision. The degenerative changes involving retina, Bruch's membrane (BM) and choroid occur predominantly in elderly people over 50 years of age.[2-4] AMD has been classified in different epidemiological studies using grading systems as exudative, nonexudative, early, and advanced AMD. Advanced AMD is further classified as atrophic or neovascular.[5] Although both result in visual impairment, the neovascular form is responsible for 90% of eyes with vision loss as the scarring that accompanies this process causes marked disorganization of the macular retina.[6] The widely followed grading systems for AMD clinically are the Wisconsin's age-related maculopathy grading system and age-related eye disease study (AREDS).[7] However, a practical stereomicroscopic assessment (SMA) for AMD classification of donor eyes is required to make use of the donor retinal tissue for a variety of analysis such as histo/immunopathology, gene expression, and/or protein profile, etc., when the correct donor history is not available.

An early approach to characterize AMD in donor eyes was made by Sarks,[8] who used histopathological methods combined with the known AMD history of the donors. Sarks classification described normal (Group I), aging (Group II), early AMD (Group III, IV), and advanced AMD (Group V, VI). In comparison, the Alabama grading system (AGS)[9] was carried out on the basis of history and postmortem fundus examination, followed by confirmation with histopathological analysis (Grade 0 – normal, grade 1 – aging, grade 2 – to 4 – early AMD). However, the AGS is restricted to early AMD, whereas in the Minnesota grading system (MGS),[10] the postmortem fundus was graded into level 1 (normal including "aging"), level 2 (early AMD), level 3 (intermediate AMD), and level 4 (advanced AMD) along with the known history of the donors.

The exact pathogenesis of AMD and the key mediators involved in this disease are not well understood,[11] and it is also unclear how normal age-related changes (AGS grade 1) are related to the progression towards early AMD. Several studies have investigated the role of certain genes in the pathogenesis

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of AMD, such as complement factor H, ApoE, inflammatory cytokines, chemokines, and toll-like receptors.[12] While studies of genomic DNA are valuable, expression analysis of particular disease phenotypes would provide further molecular insights of how such genotypes are manifested in the various phenotypes observed in AMD populations worldwide. With these considerations in mind and as animal models are not fully reflective of AMD in humans,[13,14] for the study of molecular mechanisms of AMD, postmortem human eyes represent a valuable source of material for advanced study.

In India, more than 40,000 donor eyes have been collected by various eye banks during the year 2010. These eyes are a good source of human retinal tissue, and it is therefore necessary to develop a reliable method to identify their AMD phenotype by examination of the postmortem fundus, especially when the history of the donors was not available to the investigators. In this report, we describe a method of grading AMD in donor eyes using a SMA mapped to the MGS, followed by confirmation using histopathological grading according to the AGS and Sarks classification. Our method proved useful for identification and classification of donor eyes as normal, aging, or with early and advanced AMD and indicates the incidence of these AMD stages in a cross-sectional study of AMD pathology in donor eyes from a South Indian population.

Methods

Eyes from 153 donors (age ranging from 55 to 95 years) were procured during the year 2010 from the Eye Bank of the host institute. However, 100 eyes from 50 donors could not be graded due to a poor view of the macula due to concomitant disease and/or postmortem artifacts. Among the remaining 103 donors, paired eyes were available from 96 donors of age group 55–95 years with one eye obtained from the remaining seven donors of age group 75–95 years. A total of 199 eyes were examined and no previous history regarding AMD except age, sex, and diabetic condition of the donors was available. The donor eyes were enucleated within 4 h of death, maintained at 4°C, and made available for the study within 24 h after removal of the cornea for transplantation. All tissues were handled in accordance with the Declaration of Helsinki. The cause of death of the donors included in the study were cardiac arrest (61), respiratory distress (14), myocardial infarction (53), road traffic accident (6), cerebrovascular accident (5), asthma (2), epilepsy (1), stroke (1), liver failure (1), kidney failure (7), and old age trauma (2). The written consent for the use of the eyes in research was obtained from the deceased or next of kin.

Stereomicroscopic postmortem fundus assessment

Each globe was cut circumferentially at the pars plana to remove the anterior segment-lens complex and the fundus examined and imaged using a stereo-zoom dissection microscope (Leica) with fiber optic epi-illumination. Images were captured at 10X magnification (eye piece 10X, objective 1.0X zoom) using a digital camera (Nikon CoolPix 4100).

The digital images of the postmortem fundus were transferred to a personal computer and graded as per the MGS classification. For standard size reference, a stage micrometer (having 2 mm subdivided into 20 divisions) was used for calibration instead of the 1000 µm ruby spheres used in MGS. The images were imported to Adobe illustrator (ver. 11.0), and a digital grid template was applied as in the MGS, which in fact followed the grid originally used in AREDS manual of operation (2001) with three concentric, center, inner, and outer circles measuring 1000, 3000, and 6000 µm in diameter, respectively, corresponding to coverage of the foveal, parafoveal, and perifoveal regions, respectively. Five additional circles of 63, 125, 180, 360, and 660 µm were created in the inner circle of the grid to have sufficient size reference for the measurement of drusen [Fig. 1a]. The center circle of the grid was superimposed and expanded to 1 mm of the stage micrometer, and the diameters of the inner and outer circles were proportionately changed by the illustrator software. The optic nerve head (~1.5 mm) was used as a reference to confirm the final grid dimension and the grid overlaid on the postmortem fundus image, centrally placed on the fovea. Each fundus was graded according to the type, size, approximate number and location of drusen, pigmentary changes in retinal pigment epithelium (RPE), and presence and location of geographic atrophy.

Soon after SMA, the posterior segments from 15 eyes were fixed in 10% freshly prepared buffered formalin for histological studies.

Histopathological analysis

The formalin fixed posterior segment of the eye cup was cut with a sharp blade to separate the macular region from the peripheral retina. One cut was made at 1 mm nasal to the optic disc and another cut 9.5 mm temporal to the disc and macula to include the whole macular region and the nerve head. The macular region along with optic nerve head was processed using the standard histological procedure. Eight µm sections were stained with haematoxylin and eosin. The stained sections were graded based on the nature of the drusen, basal laminar deposit (BLamD), and changes in RPE and photoreceptor outer segment as per Sarks classification[8] and AGS[9] by two authors of this study, one of them was unaware of the SMA of the donors [Table 1].

The accuracy of the MGS grade obtained by SMA for each eye was then compared to its histopathological grade (AGS, SC) to determine the sensitivity and specificity of our grading based on SMA. Sensitivity, specificity, positive and negative predictive values were calculated using the formula included in the Table 2. In order to determine the level of disagreement between SMA and histopathological grading, McNemar Chi-square test was used for early and advanced AMD. Further, in order to understand the level of correlation between SMA and histopathological grading, Kappa statistics was used as in Table 2.

Results

Stereomicroscopic postmortem fundus assessment

One hundred and ninety-nine eyes from 153 donors were evaluated by our method for identification of AMD following the MGS grading. No signs of AMD or age-related changes were observed in the macula of 62 eyes from 32 donors below 64 years of age. Age-related changes were commonly encountered in 70 eyes from 35 donors above 65 years of age and consisted of hard drusen of <63 µm and hypopigmentation of RPE as shown in Fig. 1a and Table 1 (S. no. 1–7).

Of the 67 eyes from 36 donors aged 75 and above, 10 eyes had early AMD and 3 eyes had advanced AMD features. Early AMD features included drusen ≤ 125 µm in the parafoveal and perifoveal region of macula along with hypopigmentation of the RPE [Table 1, S.no. 8–11, Fig. 2].

Three donors had advanced AMD in at least one eye [Table 1, S. no.13–15]. In one donor, the more advanced eye had geographic atrophy and the contralateral eye had drusen [Table 1, S.no. 13]. In the other two donors, one eye of each had choroidal neovascularization (CNV) [Table 1, S.no.14, 15, Fig. 2c] and the contralateral eyes had drusen >63 µm.

Symmetry of early and advanced AMD features

Five donors [Table 1, S.no. 8–11] classified as early AMD exhibited symmetrical bilateral drusen (>63 µm), and the

Table 1: Grading of the donor eyes by postmortem fundus examination and histopathology

S. No	Age (yrs)/ sex	Stereomicroscopic assessment as per MGS	Histopathology on the basis of		Grade
			AGS	SC	
1	68 M	hard drusen (<63 μm), hypopigmented RPE	hard drusen (~25 μm), hypopigmented RPE	hard drusen (~25 μm), hypopigmented RPE	Aging
2	70 F	hypopigmented RPE	drusen (~40 μm)	drusen (~40 μm)	Aging
3	78 F	hypopigmented RPE	hypopigmented RPE	hypopigmented RPE	Aging
4	80 F	hypopigmented RPE	drusen (~48 μm), thickened BM, hypopigmented RPE	drusen (~48 μm), thickened BM, hypopigmented RPE	Aging
5	82 F	hypopigmented RPE	hypopigmented RPE	hypopigmented RPE	Aging
6	85 M	hypopigmented RPE	drusen (~22 μm), hypopigmented RPE	drusen (~22 μm), hypopigmented RPE	Aging
7	89 F	hypopigmented RPE	hard drusen (~10 μm)	hard drusen (~10 μm)	Aging
8	75 F	drusen (>63 μm), hypopigmented RPE	soft drusen (>63 μm, ≤125 μm), absence of POS, thickened BM, disorganized RPE	soft drusen (>63 μm), absence of POS, thickened BM, disorganized RPE	Early AMD
9	80 F (a)	drusen (>63 μm, ≤125 μm) hypopigmented RPE	soft drusen (>63 μm), absence of POS, thickened BM	soft drusen (>63 μm, ≤125 μm), absence of POS, thickened BM	Early AMD
10	82 M	drusen (>63 μm, ≤125 μm), hypopigmented RPE	continuous BLamD, soft drusen, shortened POS, thickened BM, mild disorganized RPE	continuous BLamD, soft drusen, shortened POS, thickened BM, mild disorganized RPE	Early AMD
11	84 F	drusen (>63 μm, ≤125 μm), hypopigmented RPE	BLamD, drusen (>63 μm, 125 μm), shortened POS, disorganized RPE	BLamD, drusen (>63 μm, ≤125 μm), shortened POS, disorganized RPE	Early AMD
12	81 M*	drusen (>63 μm), hypopigmented RPE	NA	drusen (>63 μm), CNV	Advanced AMD
13	72 M	geographic atrophy	NA	CNV, RPE and PO atrophy	Advanced AMD
14	81 F	disciform scar	NA	CNV	Advanced AMD
15	95 M	disciform scar	NA	CNV with disciform scar, RPE and PO atrophy	Advanced AMD

MGS - grading system (Olsen *et al*., 2004) includes Level 1 - Normal including aging, Level 2 - Early AMD, Level 3 - Intermediate AMD, Level 4 - Advanced AMD. AGS - Alabama grading system (Curcio *et al*., 1998), includes Grade 0 - Normal, Grade 1 - Aging, Grade 2-4 - Early AMD. SC - Sarks Classification includes Group I - Normal, Group II - Aging, Group III, IV - Early AMD, Group V, VI - Advanced AMD. M - Male, F - Female, NA - Not applicable (advanced AMD grade was not available in AGS classification); ND - Not done (the eye was not processed for histopathology). "a" refers to one eye and "b" refers to the fellow-eye of the same donor

Table 2: Specificity and sensitivity of SMA Vs histopathological grading

Stereo-microscopic postmortem fundus assessment (MGS)	Histopathology grading AGS and SC		Percentage agreement	McNemar Chi-square test (P)	Kappa statistics (P)
	AMD phenotypes	Normal			
AMD phenotypes	8 (a)	0 (b)	100.0%	Not applicable	1.00(0.0001)
Normal	0 (c)	7 (d)			
	Early AMD	Advanced AMD			
Early AMD	4 (a)	1 (b)	87.5%	0.317	0.75(0.014)
Advanced AMD	0 (c)	3 (d)			

Sensitivity = [a/(a+c)] x 100, Specificity = [d/(b+d)] x 100, Positive predictive value (PPV) = [a/(a+b)] x 100, Negative predictive value (NPV) = [d/(c+d)] x 100

pattern and density of drusen were similar in both eyes of each pair. Two donors had asymmetrical features; one eye with advanced AMD (geographic atrophy/CNV) and the other with early AMD [Table 1, S.no 13, 14].

Histopathological grading

Fifteen eyes (seven aging, five early AMD, and three advanced AMD as classified by SMA) were processed for histological analysis. Eyes classified as MGS Level 1 showed aging changes such as the presence of hard drusen <63 μm, RPE hypopigmentation, and Bruch's membrane thickening [Table 1, S.no: 1–7 & Fig. 1b].

Of the 10 eyes identified as early AMD in SMA, 5 eyes were examined histologically. In these, three were soft drusen > 63 μm, continuous BLamD, thickening of Bruch's membrane, mild disorganization of RPE and attenuation of the photoreceptor outer segments [Table 1, S.no: 8–11 & Fig. 2b].

One eye [Table 1, S.no. 12], which was classified as early AMD by SMA (MGS Level 2), on histology showed advanced AMD (Sarks group IV) with CNV that breached Bruch's membrane.

All three eyes identified as advanced AMD by postmortem SMA evaluation (MGS Level 4) were confirmed by histological analysis, on the basis of extensive atrophy of RPE and photoreceptor cells, CNV and disciform scar [Table 1, S.no: 12–15 & Fig. 2d]. One of the eyes categorized as geographic atrophy in SMA, on histology, was found to have CNV in addition to RPE and photoreceptor atrophy [Table 1, S.no: 13].

Sensitivity and specificity

The sensitivity and specificity of AMD identification between SMA of the postmortem fundus and histopathological examination was carried out. Both positive and negative predictive values were 100% between the two methods in distinguishing normal from AMD. The sensitivity and specificity between postmortem fundus and histopathology grading is 100 and 75% for early and advanced AMD, respectively, with a positive predictive value of 80% and a negative predictive value of 100%. Further, in the comparison between both methods in the identification of early and late AMD features, there is no significant difference between SMA and histopathological grading on the basis of McNemar Chi-Square test [Table 2]. Kappa statistics was performed. This yielded a perfect agreement between SMA and histopathology in identifying AMD and normal aging features (*P*-value = 1) and a substantial agreement between SMA and histopathology in identifying the early and late AMD features (*P*-value = 0.75) [Table 2].

The detailed characterization of AMD in donor eyes based on grading by both stereomicroscopic postmortem fundus assessment and histopathological analysis is provided in Table 3.

Discussion

In this study, we have employed three established grading systems to classify the donor eyes into normal versus aging and early versus advanced AMD in Indian donor eyes, even though the clinical history of the deceased was not available. The characterization was done in the postmortem fundus as per the MGS, which followed the AREDS classification[7,10] and was compared to histopathological grading of the same eyes using the AGS[9] for early AMD and the Sarks classification for both early and advanced AMD.[8] The postmortem fundus was evaluated by SMA for the presence of drusen (number, size, and location), geographic atrophy and/or disciform scar, followed by confirmation by histopathological analysis noting features such as size of drusen, hyalinization (thickening) of Bruch's membrane, atrophy of the RPE, missing photoreceptor outer/inner segment, and CNV.

In the MGS, the AMD-related pathology of the postmortem fundus was graded on the basis of donor history prior to confirmation of the AMD changes through histopathology.[10] The AGS evaluated the postmortem fundus of the donors with the history of AMD and confirmed histopathology only for early AMD.[9] In Sarks classification, AMD was graded by histopathology for both early and advanced AMD, correlated with the clinical history of donors.[8] The most ideal method would be to procure the donor eyes along with their clinical history. However, such information is not made available to the eye banks in India, either because the pre-existing AMD is mostly undiagnosed in the Indian population or the family may not be aware of the pre-existing clinical condition in the deceased. Therefore, our strategy was designed to make use of the above three methods to distinguish normal fundus from those with AMD as well as to identify early and advanced AMD by postmortem fundus examination followed by confirmation through histopathology.

Of 199 eyes studied, four eyes from four donors were graded as early AMD and three eyes from three donors as advanced AMD by both MGS and AGS/Sarks classification. One exception was that one donor eye identified as early AMD in postmortem fundus was confirmed as advanced AMD through histopathology. The AMD phenotypes were observed in donors above the age of 75. The remaining eyes (90.4%) from donors between 65 and 75 years were found to have normal aging as per AGS and Sarks. All other donor eyes (<65 years) showed macula free of any detectable AMD features through postmortem examination. Though this is not a population-

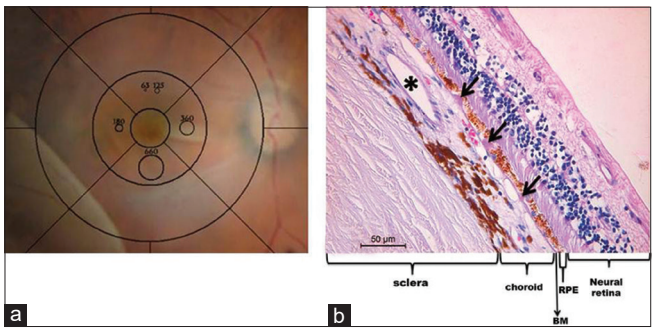

Figure 1: (a) Postmortem fundus image of the 80-year donor Retina [Table 1, S.no-4] with a grid, showing features of age-related changes, hypopigmentation of retinal pigment epithelium (RPE). (b) Same donor eye section [Table 1, S.no-4] showing the presence of choroidal vessel (*) and hard drusen (arrows) confirming donor eye having the normal aging features

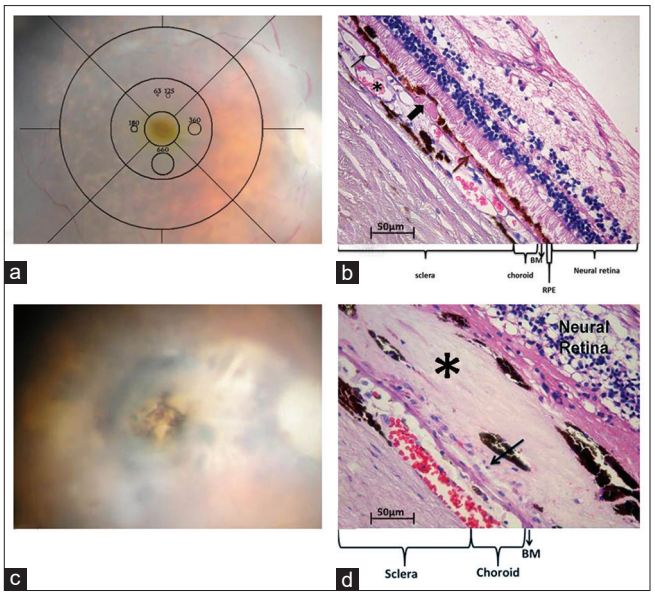

Figure 2: (a) Postmortem fundus image of the 82-year donor [Table 1, S.no-10] with 15 drusen (>63 µm). (b) Same donor eye section showing "early AMD" features - soft drusen (bold arrow) with shortening of overlying POS, continuous BLamD (brown arrow), BM thickening (black arrow), and disorganized RPE. *choroidal vessel. (c) Postmortem fundus image of a 95-year donor [Table 1, S.no 15] showing CNV with disciform scar in the macula. (d) Same donor eye section showing disciform scar (asterisk) with extensive atrophy of photoreceptor and RPE, confirming that the donor is "advanced AMD"

based study, we have observed the proportion of eyes among donors above 55 years in the postmortem fundus examination: 31% no change, 62% age-related changes, 5% early AMD, and 1.5% late AMD. In general, this trend is comparable to the observation in population-based prevalence studies in both northern and southern India.[15,16]

In postmortem fundus grading, we were unable to characterize certain features such as subretinal hemorrhage related to advanced AMD, since hemorrhage could be due to postmortem changes, as described earlier by Olsen and Feng (2004).[10] Certain eye diseases mimicking AMD may have also contributed to misclassification and assignment of donor eyes to specific AMD categories. For example, Doyne honeycomb macular dystrophy exhibit AMD features like drusen, degenerative changes in RPE, geographic atrophy, and neovascularization. Such conditions are more easily distinguished by *in vivo* clinical assessment. Earlier age of onset, specific features that are visible with clinical fundus examination and the use of investigations such as electrophysiology and genetic testing can help distinguish inherited retinal macular diseases from AMD.[17] There are also a number of distinct late AMD phenotypes (type 1, type 2, RAP, and PCV), but owing to the few neovascular AMD samples in our population and the fact that the lesions had progressed to dense disciform scars that involved the entire outer retina, we were unable to make such phenotypic distinctions.

Conclusion

The need for obtaining well-preserved human ocular tissue for understanding the molecular mechanism of retinal diseases cannot be overemphasized. In spite of the color change and opacification of the neural retina caused by formalin fixation, we have achieved a good correlation between the SMA features of the postmortem fundus and subsequent histopathology. Therefore, based on the high sensitivity and specificity, and the symmetrical AMD features in paired donor's eyes, we propose that our method is valid for selecting human retinal tissue for further investigation at the molecular level, even when the clinical history of the donor is not available. In future, we hope to make use of the procedure described in selection of donor retinal tissue for studies to compare the transcriptome and proteome profiles of normal aging and early AMD and thereby gain further insight of the changes that occur when healthy aging progresses to AMD.

Table 3: Comparison of stereomicroscopic postmortem fundus assessment with histopatholgical grading

Stages	Normal	Aging	Early AMD	Advanced AMD
SMA (postmortem fundus)				
RPE hypo/depigmentation	N	Y	Y	N
Drusen <63 μm	N	Y	Y	N
Drusen >63 μm	N	N	Y	Y
GA	N	N	N	Y
CNV	N	N	N	Y
Disciform scar	N	N	N	Y
Patchy BLamD	N	Y	N	N
Continuous BLamD	N	N	Y	N
Drusen <63 μm	N	Y	Y	N
Histological features				
Drusen >63 μm	N	N	Y	Y
Disorganization of RPE	N	Y	Y	Y
POS missing	N	N	Y	Y
BM thickening	N	Y	Y	Y
Intercapillary tuft	N	N	Y	Y
Attenuation of photoreceptors	N	N	N	Y
RPE atrophy	N	N	N	Y
CNV	N	N	N	Y
Disciform scar	N	N	N	Y

Y=presence of the features; N=absence of the features; RPE - Retinal pigment epithelium, BM - Bruch's membrane

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Conflicts of interest

There are no conflicts of interest.

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