Relationship Between the Second Reflective Band on Optical Coherence Tomography and Multifocal Electretinography in Age-Related Macular Degeneration

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Submitted: January 6, 2013
Accepted: March 18, 2013
Citation: Wu Z, Ayton LN, Guymer RH, Luu CD. Relationship between the second reflective band on optical coherence tomography and multifocal electretinography in age-related macular degeneration. Invest Ophthalmol Vis Sci. 2013;54:2800–2806. DOI:10.1167/iovs.13-11613

PURPOSE. The second hyper-reflective band on spectral domain optical coherence tomography (SD-OCT) has been suggested to correlate with the photoreceptor inner segment ellipsoids (ISe). The purpose of our study was to determine the relationship between the intensity of the ISe band and retinal function measured by multifocal electretinography (mFERG) in patients with early age-related macular degeneration (AMD).

METHODS. A high-resolution horizontal line scan through the fovea on SD-OCT and an mFERG recording were performed in one eye of 29 early AMD and 31 control participants. The relative intensity of the ISe band within 1000 μm of the fovea was quantified using ImageJ. The relationships between the relative intensity of the ISe band and the mFERG response parameters (P1 amplitude and implicit time) within the three central hexagons along the horizontal axis were determined.

RESULTS. In normal participants, the relative intensity of the ISe band was significantly correlated with age (r = −0.654, P < 0.001) and also exhibited a topographic variation. On average, the relative intensity of the ISe band was significantly lower in patients with early AMD (1.77 ± 0.26) compared to control subjects (1.95 ± 0.27, P < 0.001) of a similar age range. The relative intensity of the ISe band was correlated significantly with the mFERG P1 implicit time (r = −0.745, P < 0.001), but not P1 amplitude (r = 0.144, P = 0.281).

CONCLUSIONS. The relative intensity of the ISe band reduced with age and further in early AMD. The relative intensity was significantly correlated with mFERG P1 implicit time.

Keywords: age-related macular degeneration, optical coherence tomography, multifocal electrotetinography, IS/OS, inner segment ellipsoids

Research and development of novel interventions for early age-related macular degeneration (AMD) currently is hampered by the lack of measureable markers of early disease. Such markers are required to provide an assessment of disease severity and serve as outcome measures of disease progression. The identification of early structural changes on high-resolution optical coherence tomography (OCT) and its correlation with functional changes can provide novel biomarkers of early disease.

Emerging evidence suggests that the second hyper-reflective band seen on high-resolution OCT, previously referred to as the junction between the inner and outer segments (IS/OS) of the photoreceptors, actually corresponds with the anatomic location of the ellipsoid portion of the photoreceptor inner segments (ISe).1–3 The ellipsoids of inner segments are densely packed with mitochondria, and have an important metabolic function in generating a response to light stimuli. In addition, the mitochondria also have an important function in guiding light towards the photopigments located in the outer segments.4,5 There currently is great interest in assessing the integrity of the ISe band due to a close association between the integrity of the band and visual function (including reduced visual acuity and retinal sensitivity measured by microperimetry) in different diseases6–15 and following treatment.16–19

The integrity of the ISe band has been limited mostly to qualitative assessment by noting either the presence of disruption, or the presence of the band itself.20 However, Hood et al. showed that the intensity of the ISe band was reduced in patients with achromatopsia or cone dystrophy.21 This study suggested that functional changes occur despite a near normal–appearing integrity of the ISe band on visual inspection. Although to our knowledge this was the first study to examine the intensity of the ISe band, it did not examine quantitatively the relationship between the intensity of the ISe band and degree of retinal function change.

To date, to our knowledge no studies have examined quantitatively the intensity of the ISe band with age or in AMD, and determined its relationship with retinal function. The purpose of our study was to determine the relationship between the intensity of the ISe band on spectral domain optical coherence tomography (SD-OCT) and retinal function as measured by multifocal electrotetinography (mFERG) in subjects with early AMD.

METHODS

Our study was approved by the Human Ethics Committee of the Royal Victorian Eye and Ear Hospital (RVEEH) and was conducted in accordance with the tenets of the Declaration of
Helsinki. Written informed consent was obtained from all participants after a thorough explanation of the study and tests involved.

Participants

Participants with AMD were recruited from the medical retina clinic at RVEEH and private ophthalmology practices. Control participants were recruited from the spouses and friends of the AMD participants. Inclusion criteria for AMD patients were being over 50 years of age, having early AMD in at least one eye. This was defined by drusen larger than 125 μm, with or without pigmentary changes, and a visual acuity of 20/30 or better in the study eye. The fellow eye had at least early AMD, but also could have late stage AMD. Exclusion criteria for all participants were bilateral late-stage AMD, including central geographic atrophy (GA) and choroidal neovascularization (CNV), significant corneal or media opacity, diabetes, uncontrolled hypertension (systolic > 150 mm Hg and diastolic > 90 mm Hg), amblyopia, neurologic or systemic disease that could compromise vision, medications that are known to affect retinal function (e.g., hydroxychloroquine), physical and/or mental impairment, or inability to sign a consent form. Control participants were excluded if any evidence of macular changes in the fundus suggestive of AMD were noted, including the presence of any hard drusen. When both eyes met the inclusion criteria in both groups, the eye with the better visual acuity was chosen as the study eye.

OCT Imaging

All participants underwent simultaneous spectral domain OCT and confocal scanning laser ophthalmoscopy using the Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany). A single high-resolution horizontal line scan was obtained after averaging 100 frames using the automatic real time (ART) mode.

Multifocal Electroretinography

Recordings of the mfERG were performed monocularly using the Visual Evoked Response Imaging System (VERIS Science 6: ElectroDiagnostics Imaging, Inc., Redwood City, CA) and Dawson-Trick-Litzkow (DTL) thread electrodes. Pupils were dilated using 1 drop of 1% tropicamide and 1 drop of 2.5% phenylephrine, and recordings commenced only when pupil dilation was at least 7 mm. A fixation monitoring system (FMS) delivered the test stimulus consisting of 61 retinal-scaled hexagons, which alternated between white and black frames on a pseudorandom m-sequence (m = 14) at a rate of 75 Hz. Luminance of the white hexagons was set at 5.53 cd.s/m² and the background luminance was set at 200 cd.m⁻². The stimuli were adjusted for optimal focus and corrected refractive error by the participant through manual adjustment of the refractor unit. The recorded signal was filtered using a band pass filter between 10 and 100 Hz, and was amplified 100,000 times (model 12; Grass NeuroData, Quincy, MA). Segments that were contaminated with blinks or eye movements were discarded and rerecorded. The fellow eye that was not tested was occluded with an eye patch.

Analysis of the IsE Band

Light reflectance profiles (LRP) were obtained using ImageJ (Version 1.46r; National Institutes of Health, Bethesda, MD), an image processing and analysis software, using a selection of 5 pixels laterally and 20 pixels vertically on the region of interest (corresponding to approximately 29 μm laterally and 77 μm vertically within the retina). A small horizontal dimension of the OCT scan was chosen to account for the curvature of the retina, since peak intensities shift with horizontal displacement of sample selection, where larger horizontal samples may lower the value of the peak intensity through averaging values of lower intensity at a different location (Fig. 1). To avoid a possible effect of drusen on the reflective properties of IsE, only regions where the IsE band exhibited no disruption from drusen within 50 μm of the sampled region were chosen for analysis. The values of peak intensities of the first and second reflective bands were recorded, and the relative intensity of the IsE band was determined as a ratio of the second to the first reflective band (corresponding to external limiting membrane [ELM]). This was chosen, since the ELM is nonneural layer and also had a consistent intensity with eccentricity.

The relative intensity of the IsE band was sampled manually at the fovea, and approximately 500 and 1000 μm from the fovea nasally and temporally (Fig. 2). These regions were chosen due to their correspondence with the central three hexagons along the horizontal axis of the mfERG stimulus grid. All measurements were performed by a single examiner.

Statistical Analyses

The mfERG responses of the central three hexagons along the horizontal axis were used to determine the relationship between the relative intensity of the IsE band and retinal function. No spatial averaging was applied to the mfERG analysis. The N1-P1 response amplitude density and P1 implicit time of the first order kernel of the three central hexagons along the horizontal axis was obtained for each subject. The average of the relative intensity of the IsE band at the five sampled locations was calculated. Pearson correlation coefficient and multiple linear regression analysis were used to determine the relationship between mfERG parameters, average relative intensity of the IsE band and age. To compare the IsE band relative intensity between AMD and control groups, a subset of normal subjects (n = 13) whose age was within the age range of the AMD group (ranged from 56–90 years) were selected. The means of the IsE band intensity between the AMD and this group of normal subjects at 5 retinal locations were compared using a General Linear Model and post hoc Bonferroni tests.

RESULTS

A total of 29 participants with early AMD (70.2 ± 10.2 years; range, 56–90 years) and 31 control subjects (52.3 ± 17.9 years; range, 23–83 years) was recruited for this study; and all patients exhibited good central fixation. Among the control subjects, 13 subjects of similar age (mean 69.6 ± 6.4 years; range, 60–83) as the AMD group were selected for comparison. Of the early

FIGURE 1. Vertical light reflectance profile plots showing peak intensities corresponding to the IsE and ELM in two different retinal locations (represented by black and gray rectangular sampled regions on the OCT image, corresponding with the plots).
AMD participants, 27 had bilateral early AMD and 2 had late AMD in the fellow eye.

The intensity of the ISe, ELM and other retinal layers at different retinal eccentricities in a normal subject is shown in Figure 3. The intensity of the ELM and other retinal layers appeared to be relatively constant with varying eccentricity, whereas the intensity of the ISe band displayed a regional variation, reaching a minimum at the fovea.

In normal subjects, the relative intensity of the ISe band decreased significantly with age ($r = -0.634$, $P < 0.001$, Fig. 4). A significant increase in P1 implicit time also was observed with age ($r = 0.547$, $P = 0.001$), but not with response amplitudes ($r = 0.169$, $P = 0.363$).

Linear regression analysis was performed and the results showed that the relative intensity of the ISe band was significantly and inversely correlated with the mfERG P1 implicit time ($r = -0.745$, $P < 0.001$, Fig. 5). However, there was no significant association between the relative intensity of the ISe band and N1-P1 response amplitude ($r = 0.144$, $P = 0.281$, Fig. 5).

![Figure 2](image1.png)

**Figure 2.** Diagram illustrating the locations of the ISe and ELM band sampled, and the corresponding mfERG. Five representative vertical light reflectance profiles and their corresponding ISe/ELM ratios are shown. The central hexagon subtends approximately $1.39^\circ$, capturing the cone-rich fovea. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer.

![Figure 3](image2.png)

**Figure 3.** LRP obtained from an OCT image (left) and the intensity of different retinal layers at various retinal eccentricities from a representative normal subject is shown (right). The intensity ISe band diminishes towards the fovea, while the intensity of the ELM and other retinal layers remains relatively constant at all eccentricities.
The results of the multiple linear regression analysis showed that the relative intensity of the ISe band was associated significantly with age, and mfERG P1 implicit time to a greater extent (see Table).

On average, the relative intensity of the ISe band was significantly lower in subjects with early AMD (1.77 ± 0.26) compared to control participants (1.95 ± 0.27, \( P < 0.001 \)) of a similar age range. Comparing the intensity at each retinal location, the ISe band was reduced significantly at all eccentricities (\( P < 0.005 \)), except the central point (\( P = 0.625 \), Fig. 6).

A similar analysis also was performed on the intensity of the ISe band as a ratio of the outer nuclear layer (ISe/ONL) and inner nuclear layer (ISe/INL) to examine whether changes were occurring uniquely to the ISe band. The ratio of ISe/ONL and ISe/INL were similarly reduced in subjects with early AMD compared to control participants (Fig. 6). Additionally, the ratio between any combination of the intensity of the ELM, ONL, and INL (i.e., ONL/ELM, INL/ELM, and ONL/INL) were not significantly different between the two groups at all retinal locations (\( P \geq 0.318 \)). Furthermore, the ISe/ONL and ISe/INL ratio also were correlated significantly with mfERG P1 implicit time (\( r = -0.608, P < 0.001 \) and \( r = -0.629, P < 0.001 \), respectively), but not N1-P1 amplitude (\( r = 0.152, P = 0.245 \) and \( r = 0.161, P = 0.219 \), respectively).

A representative OCT scan is shown in Figure 7, illustrating qualitatively and quantitatively the difference in the intensity of the ISe band in a control and early AMD subject.

**DISCUSSION**

In our study, we found that the relative intensity of the ISe band was associated significantly with the mfERG implicit time after controlling for age and mfERG P1 amplitude. Subjects with early AMD exhibited reduced intensity of the ISe band and increased mfERG implicit time compared to control participants. There was no significant association between the ISe band relative intensity and mfERG amplitude. These novel findings suggested that severity of early AMD could be inferred by careful analysis of the intensity of the ISe band.

The second hyper-reflective band traditionally has been assigned to the IS/OS junction. However, recent histologic data suggested that the second hyper-reflective band aligns consistently with the inner segment ellipsoids of the photoreceptors. It also has been shown that the age-related maculopathy susceptibility 2 (ARMS2) gene, which encodes a mitochondrial protein localized to the ellipsoids of the photoreceptor inner segments, is associated strongly with AMD. Thus, the reduction in the relative intensity of the ISe band and the increase in the mfERG P1 implicit time in subjects with AMD observed in our study are consistent with mitochondrial dysfunction, supporting previous suggestions of the role of mitochondrial-related pathways in AMD.

The relative intensity of the ISe band in this study was correlated significantly with retinal function as measured by mfERG implicit time measurements, but not response amplitudes. The poor correlation with response amplitudes is not surprising, since response amplitudes are much less sensitive than implicit time to early functional deficits. In our study, we used the ELM as a reference for the determination of the ISe band relative intensity (ISe/ELM ratio). A reference is required, since the SD-OCT images are adjusted automatically for optimal image brightness. To our knowledge, the only previous study
by Hood et al. that examined the relative intensity of the ISe band used a large sampling area as a reference.\textsuperscript{22} The sampling area encompassed the entire retinal region from the retinal nerve fiber layer (RNFL) to Bruch’s Membrane (BM), extending \pm 2.75 mm in width. However, the presence of anatomic changes present in our participants including drusen and RPE changes would alter the brightness of the reference if a large sampling region were used. Therefore, the ELM was chosen as a reference instead, due to the knowledge that it remains relatively unaffected in normal eyes and eyes exhibiting early disease.\textsuperscript{28} Since the reflection of the ELM originates from the zonulae adherentes between the photoreceptors and Müller cells,\textsuperscript{29} an increased reflectivity of this band is not likely to originate from anatomic changes of the ELM, such as an increase in the number of zonulae adherents. Instead, it may be a result of an increase in overall image brightness.

To examine whether the changes in the ISe/ELM ratio occurs uniquely to the changes in intensity of the ISe band, we also examined the ISe/ONL and ISe/INL ratio. Comparison between early AMD and control subjects revealed a similar reduction in ISe/ONL and ISe/INL ratio in early AMD subjects. Additionally, the ratio between any combination of the ELM, ONL, and INL intensity was not significantly different between early AMD and control subjects. Collectively, the relative intensity of the ISe appears to be reduced in early AMD, while the relative intensity of the ELM, ONL, and INL appears relatively unaffected. This is consistent with previous findings by Hood et al.,\textsuperscript{21} where additional analyses confirmed that changes in relative intensity were unique to the ISe band. This finding suggests that other layers also could be used potentially as a reference for the relative intensity of the ISe band. However, we have chosen the ELM in this study, since it is a nonneural layer, unlike other neural layers, such as ONL and INL, whose intensity may be more likely to be altered with pathologic changes.

Unlike the intensity of the ELM, the intensity of the ISe band varied with eccentricity and reached a minimum at the fovea. This may be attributed to the difference in packing density of the mitochondria in the ISe in different regions of the retina. The ISe of cones at the fovea are long and thin, densely packed in a way that is suggested to confer an optical benefit,\textsuperscript{1,4} and may explain the lowered reflectivity. The increased intensity of the ISe band with increasing eccentricity most likely corresponds with the increasing packing density of the mitochondria in the ISe as the cones become shorter and wider.\textsuperscript{4} Rods, which are absent at the fovea, also contribute to the intensity of the ISe band as noted by others.\textsuperscript{21,30} Although their relative contribution is unknown, the intensity of the ISe band may be dominated by cones, since their ellipsoids are substantially larger than those in rods.\textsuperscript{4}

The relative intensity of the ISe band was not significantly different at the fovea between subjects with early AMD and controls. A possible explanation for this is due to the difference in optical properties of the ISe band at the fovea, and renders it as a poor candidate region for measuring the intensity of the ISe band.

The novel findings of our study have wide-reaching application in the early diagnosis of retinal disease and the monitoring of disease progression. Future developments that will allow automated measurements of intensity of the ISe band encompassing the entire scan potentially will be more accurate given its regional variation. However, the regional variation of the ISe band intensity itself may provide further insight into the earliest pathology of AMD once the pathways are elucidated further. Furthermore, the use of rod-mediated retinal function tests may give further insight into the relative contribution of rod and cone photoreceptors to the ISe band. Longitudinal studies will be important to determine changes in relative intensity over time.

Strengths of our study included the consistency of image analysis by a single examiner, and using quantitative methods to correlate retinal structure and function. Limitations of our study included a small sample size, variability caused by sampling a small region of the ISe band, and only comparing a small number of retinal regions without high spatial correspondence. In addition, the relationship between retinal
function and the width of the ISe band was not explored. A weakness of this study was the inability to mask the diseases status during the analysis of the OCT image.

In conclusion, we have demonstrated that the relative intensity of the ISe band reduces with age and is reduced further in patients with early AMD. There is a significant correlation between the ISe relative intensity and retinal function, as measured by mfERG. This has implications not only for the role of mitochondria in early AMD, but also presents the possibility of using the ISe band intensity as a biomarker of disease severity and progression.

Acknowledgments
Supported by the National Health and Medical Research Council (NH&MRC) Project Grant (1027624), Macular Degeneration Foundation Research Grant, NH&MRC practitioner fellowship (#529905; RHG). Centre for Eye Research Australia (CERA) receives Operational Infrastructure Support from the Victorian Government and is supported by a NHMRC Centre for Clinical Research Excellence Award (#529923).

Disclosure: Z. Wu, P; L.N. Ayton, None; R.H. Guymer, P; C.D. Luu, P

References

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