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Primary retinal pathology in multiple sclerosis as detected by optical coherence tomography

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Optical coherence tomography studies in multiple sclerosis have primarily focused on evaluation of the retinal nerve fibre layer. The aetiology of retinal changes in multiple sclerosis is thought to be secondary to optic nerve demyelination. The objective of this study was to use optical coherence tomography to determine if a subset of patients with multiple sclerosis exhibit primary retinal neuronopathy, in the absence of retrograde degeneration of the retinal nerve fibre layer and to ascertain if such patients may have any distinguishing clinical characteristics. We identified 50 patients with multiple sclerosis with predominantly macular thinning (normal retinal nerve fibre-layer thickness with average macular thickness $<$5th percentile), a previously undescribed optical coherence tomography defined phenotype in multiple sclerosis, and compared them with 48 patients with multiple sclerosis with normal optical coherence tomography findings, 48 patients with multiple sclerosis with abnormal optical coherence tomography findings (typical for multiple sclerosis) and 86 healthy controls. Utilizing a novel retinal segmentation protocol, we found that those with predominant macular thinning had significant thinning of both the inner and outer nuclear layers, when compared with other patients with multiple sclerosis ($P < 0.001$ for both), with relative sparing of the ganglion cell layer. Inner and outer nuclear layer thicknesses in patients with non-macular thinning predominant multiple sclerosis were not different from healthy controls. Segmentation analyses thereby demonstrated extensive deeper disruption of retinal architecture in this subtype than may be expected due to retrograde degeneration from either typical clinical or sub-clinical optic neuropathy. Functional corroboration of retinal dysfunction was provided through multi-focal electroretinography in a subset of such patients. These findings support the possibility of primary retinal pathology in a subset of patients with multiple sclerosis. Multiple sclerosis-severity scores were also significantly increased in patients with the macular thinning predominant phenotype, compared with those without this phenotype ($n = 96$, $P = 0.006$). We have identified a unique subset of patients with multiple sclerosis in whom there appears to be disproportionate thinning of the inner and outer nuclear layers, which may be occurring as a primary process independent of optic nerve pathology. *In vivo* analyses of retinal layers in multiple sclerosis have not been previously performed, and structural demonstration of pathology in the deeper retinal layers, such as the outer nuclear layer, has not been previously described in multiple sclerosis. Patients with inner and outer nuclear layer pathology

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have more rapid disability progression and thus retinal neuronal pathology may be a harbinger of a more aggressive form of multiple sclerosis.

Keywords: optical coherence tomography; retinal segmentation; multiple sclerosis; outer nuclear layer; multiple sclerosis-severity score

Abbreviations: MTP = macular thinning predominant; OCT = optical coherence tomography; RNFL = retinal nerve fibre layer

Introduction

Multiple sclerosis is thought to be an immune-mediated disorder of the central nervous system and is the most common non-traumatic cause of neurological disability in early to middle adulthood (Anderson *et al.*, 1992). While the precise aetiology of multiple sclerosis remains elusive, pathological hallmarks of multiple sclerosis lesions include breakdown of the blood–brain barrier, demyelination, gliosis, axonal degeneration and neuronal loss (Prineas, 2001; Frohman *et al.*, 2006). While originally designated an inflammatory demyelinating disorder of the CNS, early descriptions of multiple sclerosis report prominent axonal and neuronal pathology (Marburg, 1906; Putnam, 1936). In recent times, axonal and neuronal pathology in multiple sclerosis have regained considerable focus improving our understanding of the biological underpinnings of the multiple sclerosis disease process.

Although cortical demyelinating lesions account for 26–59% of all brain lesions in multiple sclerosis (Brownell and Hughes, 1962; Lumsden, 1970), the pathophysiological basis of neuronal injury and neuronal loss in multiple sclerosis remains unclear. Neuronal atrophy or loss in multiple sclerosis may be caused by either retrograde degeneration (Rawes *et al.*, 1997; Shindler *et al.*, 2008) or anterograde trans-synaptic degeneration (Madigan *et al.*, 1996). Prior observations also provide evidence to suggest that, in some instances, the mechanism of grey matter tissue injury in multiple sclerosis may differ from that of white matter tissue injury, and that grey matter injury may be the derivative of a primary neuronal pathobiology (Bo *et al.*, 2003; Moll *et al.*, 2008).

Optical coherence tomography (OCT) is a non-invasive ocular imaging technology that uses near-infrared light to produce cross-sectional or 3D images of the retina (Huang *et al.*, 1991; Hrynchak and Simpson, 2000; Frohman *et al.*, 2008). This allows evaluation of retinal structures at very high resolution ($<10\,\mu m$) (Hsu *et al.*, 2003). Thus far, OCT imaging in multiple sclerosis has primarily focused on evaluation of the retinal nerve fibre layer (RNFL), the innermost retinal layer. The RNFL is principally composed of unmyelinated axons, which originate from the ganglion cells located in the ganglion cell layer beneath the RNFL (Fig. 1). By measuring RNFL thickness, OCT provides an objective estimation of axonal integrity. Furthermore, OCT allows measurement of total macular thickness. Since the macula is neuronally enriched, OCT-derived metrics of macular thickness and volume are sometimes inferred as providing estimates of retinal neuronal integrity (Burkholder *et al.*, 2009). To date, OCT research in multiple sclerosis has predominantly focused on characterization of the impact of retrobulbar optic nerve demyelination upon proximal axonal and neuronal retinal architecture. The prevailing hypothesis is that optic nerve demyelination results in retrograde axonal degeneration, culminating in ganglion cell death (Shindler *et al.*, 2008). Until now, little consideration has been given to the possibility that a primary process targeting retinal neurons independent of optic neuropathy may also be operative in multiple sclerosis, in a way that is potentially analogous to the early targeting of the grey matter compartment in multiple sclerosis (Geurts *et al.*, 2005; Calabrese *et al.*, 2007*a*, *b*).

The retina is anatomically isolated and represents a unique unmyelinated model within which to study multiple sclerosis associated neurodegeneration and inflammation, as well as the distal effects of demyelination. Furthermore, retinal architecture can be assessed rapidly and non-invasively by OCT. In this cross-sectional case–control study, we sought to use spectral domain OCT imaging to determine if a subset of patients with clinically definite multiple sclerosis may exhibit evidence implicating primary retinal neuronal layer pathology, and to ascertain if such patients have any corresponding and distinguishing clinical characteristics. Utilizing a novel retinal segmentation protocol, we show that a subset of patients with multiple sclerosis have significant thinning of both the inner nuclear layer and outer nuclear layer. These data are consistent with a recent post-mortem analysis showing the loss of not only retinal ganglion cells, but also of inner nuclear layer neurons (bipolar, horizontal and amacrine cells) in the retinas of patients with multiple sclerosis (Green *et al.*, 2010). Our investigation corroborates our hypothesis that in some patients with multiple sclerosis, *in vivo* primary retinal neuronal pathology extends to the outer retinal layers, and may occur independently of optic nerve, tract or ganglion cell-related pathology.

Materials and methods

Patients

Subjects were recruited from the Johns Hopkins Multiple Sclerosis Centre, the University of Pennsylvania Multiple Sclerosis Centre and the University of Texas-Southwestern Multiple Sclerosis Centre. All patients with multiple sclerosis had their diagnosis confirmed by their treating neurologist, based on McDonald criteria (Polman *et al.*, 2005). Study subjects were divided into four groups. Group 1 consisted of patients with multiple sclerosis whose OCT scans demonstrated predominantly macular thinning. Macular thinning predominant (MTP) patients were defined as having the OCT combination of average macular thickness <5th percentile, with ipsilateral normal average RNFL thicknesses (between the 5th and 95th percentiles), in one or both eyes, in the absence of a history of acute optic neuritis in affected eyes (Fig. 2 depicts OCT findings in a patient with MTP). As none of

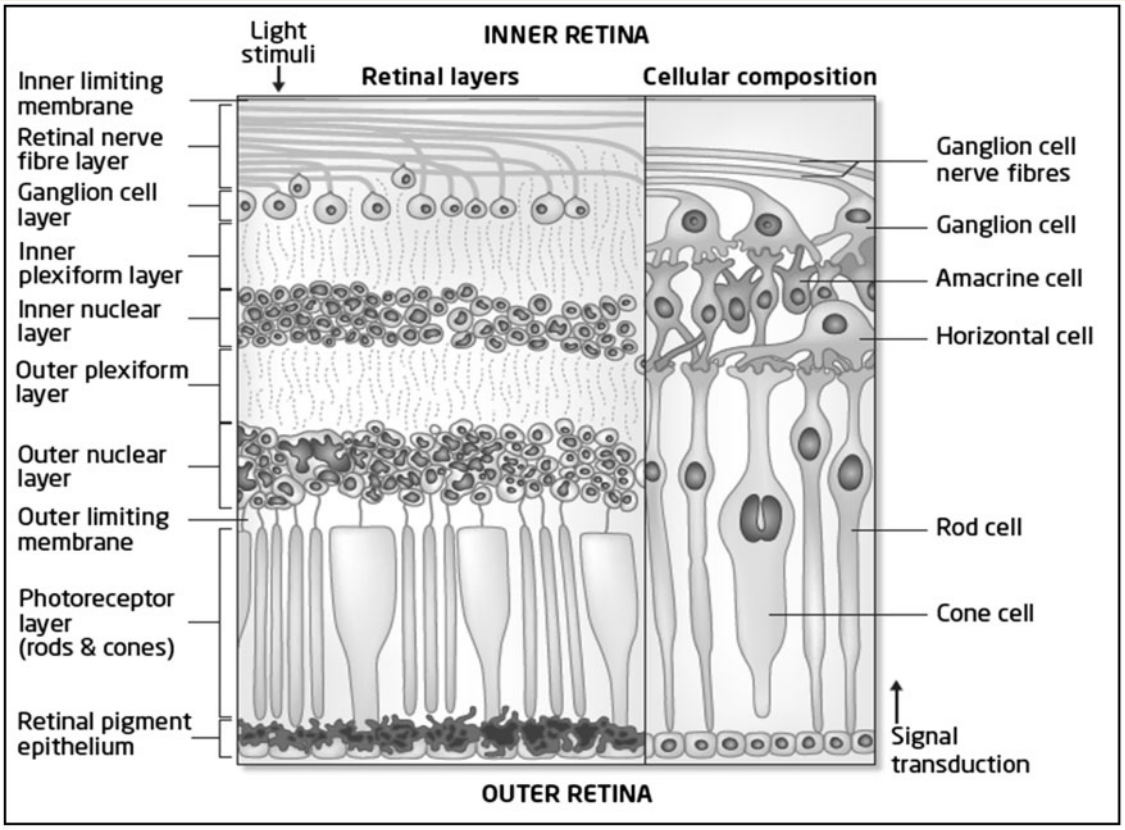

Figure 1 An illustration of the layers of the retina. Note that the direction of signal transduction is directed from the photoreceptors (rods and cones) at the outer aspect of the retina towards the RNFL at the inner aspect of the retina. The fibres of the RNFL exit the retina at the optic disc, where they form the optic nerve. Also note the origin of the nerve fibres of the RNFL from the ganglion cells in the ganglion cell layer. Optic nerve demyelination results in RNFL degeneration, which in turn leads to ganglion cell body death. Reproduced with permission from Saidha *et al*. (2010).

the identified patients with MTP had primary progressive multiple sclerosis, patients with primary progressive multiple sclerosis were not included in the comparator groups. The majority of patients with MTP were recruited prospectively at the Johns Hopkins Multiple Sclerosis Centre. In order to estimate the prevalence of patients with multiple sclerosis with this OCT phenotype as accurately as possible, a minority of patients were also identified retrospectively. The prevalence of the MTP phenotype in patients with multiple sclerosis was determined by calculating the total number of patients with multiple sclerosis with the MTP phenotype on Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, California) and the total number of all patients with multiple sclerosis (excluding primary progressive multiple sclerosis), who had ever had a Cirrus HD-OCT examination performed at the Multiple Sclerosis Centre within the Neuroimmunology Division at Johns Hopkins. Group 2 was composed of patients with multiple sclerosis whose OCT scans demonstrated abnormalities consistent with those typically observed in multiple sclerosis, as has been previously described (Parisi *et al.*, 1999; Trip *et al.*, 2005; Fisher *et al.*, 2006; Pulicken *et al.*, 2007; Burkholder *et al.*, 2009). Patients with abnormal OCTs were defined by the presence of RNFL thinning, with or without concomitant ipsilateral macular thinning (Fig. 3 illustrates OCT findings in patients with abnormal OCTs). Group 3 consisted of patients with multiple sclerosis whose OCT scans were normal, with both RNFL and average macular thicknesses between the 5th and 95th percentiles. Patients with known ophthalmologic disorders, other neurological disorders in addition to multiple sclerosis, diabetes or hypertension, that may otherwise affect OCT measurements, were excluded from the study. Scans performed within a 3 month period of an acute optic neuritis event were also excluded, in order to minimize the effect of optic disc swelling on OCT measurements. Group 4 consisted of healthy controls without history of ocular or neurological disease that were recruited from among Johns Hopkins and the University of Texas-Southwestern Medical Centre staff and unaffected family members of patients with multiple sclerosis. The Johns Hopkins University, University of Pennsylvania and University of Texas-Southwestern Medical Centre Institutional Review Board approval was obtained for all study protocols. All recruited participants provided written informed consent. The study was performed in accordance with Health Insurance Portability and Accountability Act guidelines.

Clinical data

Patient demographics were recorded on study subjects. Multiple sclerosis classification was recorded based on recognized multiple sclerosis subtype definitions—relapsing remitting multiple sclerosis, secondary progressive multiple sclerosis, primary progressive multiple sclerosis and relapsing progressive multiple sclerosis (Lublin and Reingold, 1996). The Expanded Disability Status Scale (Kurtzke, 1983) score was determined by a Neurostatus certified examiner the same day as OCT examination. Patient history and personal medical records

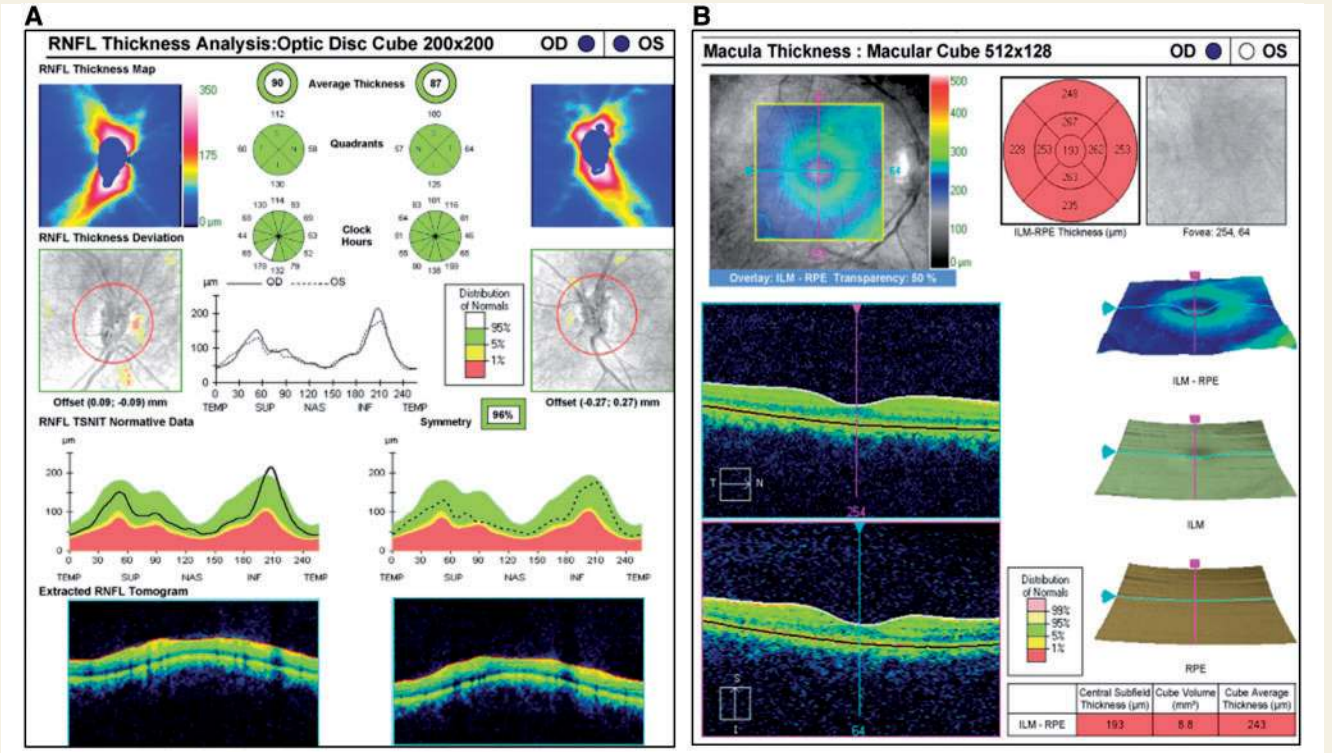

Figure 2 An OCT RNFL (**A**) and macular (**B**) report generated by Cirrus HD-OCT with software 4.0 (Carl Zeiss Meditec, Dublin, CA) in a patient with multiple sclerosis without history of optic neuritis. The upper middle section of (**A**) shows the average RNFL thickness for the right eye (OD) and the left eye (OS), as well as the quadrant and clock-hour measures of RNFL thickness for each eye. Note that the average RNFL thickness, the quadrant and clock-hour measures are represented in colours, which correspond to the normal distribution of RNFL thickness values. The average RNFL thickness (as well as quadrants and sectors) in each eye is represented in green (indicating values within normal range). (**B**, top right) Quadrant measurements of retinal thickness (between the inner limiting membrane and the retinal pigment epithelium: ILM-RPE). These are represented in colours that correspond to the normal distribution of macular thickness values. The central macula represents the foveola, with the four quadrants immediately surrounding this (inner macula) representing the parafoveola. Note that the average macular thickness (cube average thickness) indicated in the bottom right chart (as well as all of the macular quadrant thicknesses) are represented in red, indicating values $<1\%$ of what would be expected compared with an age-matched reference population. The macular scan of the left eye in the same patient (not shown) is similar to that of the right eye in (**B**). The combination of OCT findings described fulfil our criteria for a patient with MTP. Please note that this represents a patient with an average macular thickness <1st percentile from this group. Those with a normal average RNFL thickness and average macular thickness between the 1st and 5th percentile also fulfil our criteria for a patient with MTP but are not illustrated. ILM = inner limiting membrane; RPE = retinal pigment epithelium.

were reviewed in order to determine disease duration, co-morbidities, current treatment, prior treatment, history of corticosteroid use and history of acute optic neuritis events, including their date and side affected. Study subjects were screened for ophthalmic symptoms in a blinded fashion with a standard visual questionnaire. Based on disease duration and Expanded Disability Status Scale scores, multiple sclerosis-severity scores (Roxburgh *et al.*, 2005) were determined for participants with multiple sclerosis. Only Expanded Disability Status Scale data, multiple sclerosis-severity scores data and data on ophthalmic symptoms for those patients attending the Johns Hopkins Multiple Sclerosis Centre were used in analyses.

Optical coherence tomography

Retinal imaging was performed using the Cirrus HD-OCT (model 4000) with software version 5.0. OCT images were obtained with the Optic Disc Cube 200×200 protocol, which consists of 200 horizontal scan lines (each composed of 200 A-scans) that form a $6 \times 6 \times 2$ mm volume cube. Segmentation software determines the location of the inner limiting membrane and the outer boundary of the RNFL at each A-scan to create a 2D map of the thickness of the RNFL in this peripapillary region. Software automatically determines the centre of the optic disc and samples the RNFL thickness in a circumpapillary circle of 1.73 mm radius around the optic disc. The average RNFL thickness around that circle samples all axons leaving the optic disc. Furthermore, software automatically determines the optic disc area. Macular data were obtained using the Macular Cube 512×128 protocol (128 horizontal scan lines each composed of 512 A-scans and one central vertical and horizontal scan composed of 1024 A-scans) which forms a $6 \times 6 \times 2$ mm volume cube. Different segmentation software identifies the inner limiting membrane and the inner boundary of the retinal pigment epithelium in this cube to create a 2D map of retinal thickness in the macular region. The analysis software uses this segmentation to provide total macular volume over the cube, average macular thickness over the cube, and average thickness over nine macular subfields (foveola, four inner macular

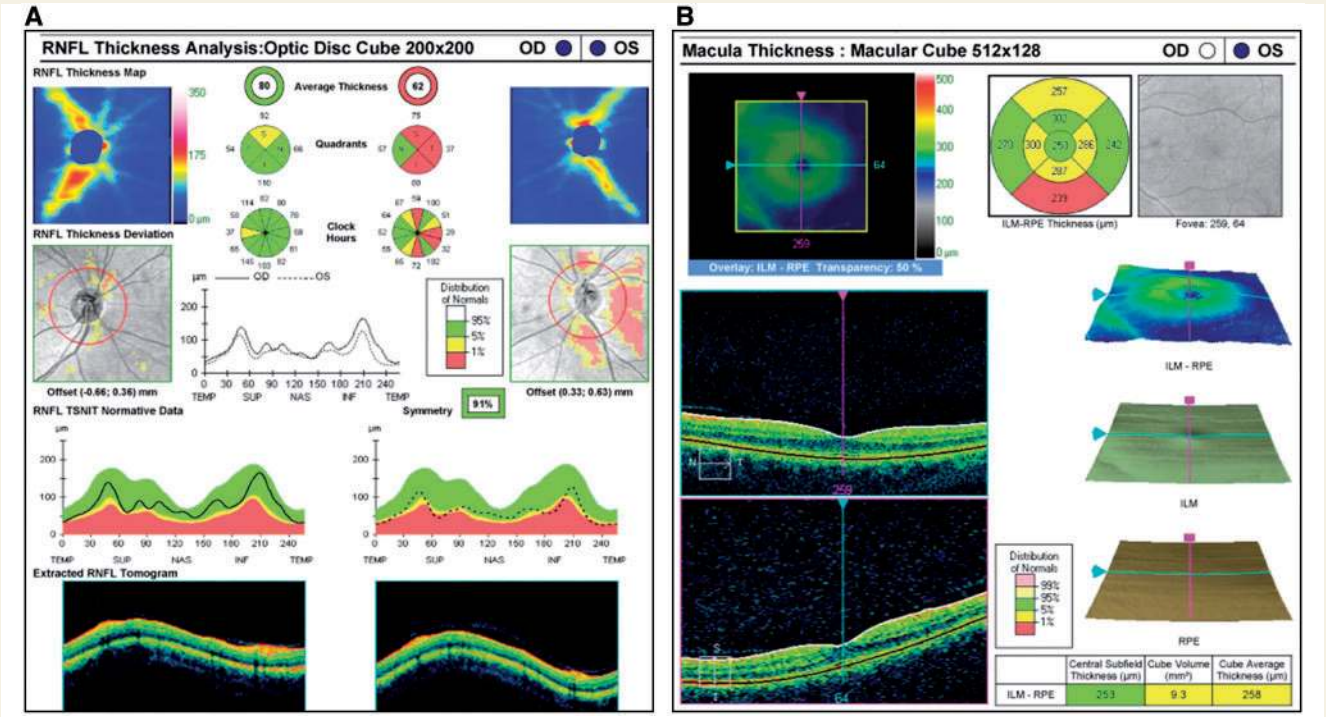

Figure 3 An OCT RNFL (**A**) and macular (**B**) report generated by Cirrus HD-OCT with software 4.0 (Carl Zeiss Meditec, Dublin, CA, USA) in a patient with multiple sclerosis with a prior history of left-sided optic neuritis. Note that the average RNFL thickness in the right eye (OD) is represented in green (indicating a value within normal range), while the average RNFL thickness (as well as multiple quadrants and sectors) in the left eye (OS) is represented in red, indicating a reduced RNFL thickness (red denotes values <1% of what would be expected when compared with a reference population). (**B**, top right) Quadrant measurements of retinal thickness in the left eye (between the inner limiting membrane and the retinal pigment epithelium: ILM-RPE). Note the thickness reductions in the inner and outer macula, thought to be indicative of neuronal loss (mostly thought to reflect ganglion cell body loss), while the corresponding reduction in RNFL thickness in the same eye of this patient (**A**) is thought to represent loss of ganglion cell axons. (**B**, bottom right) The average macular thickness (cube average thickness), represented in yellow (yellow denotes values between 1 and 5% of what would be expected compared with a reference population) indicating a reduction in average macular thickness in that eye. This OCT exemplifies those abnormalities on OCT typically expected to be observed in the setting of multiple sclerosis and is an example of an OCT from the multiple sclerosis with abnormal OCT cohort. Reproduced with permission from Saidha *et al*. (2010). ILM = inner limiting membrane; RPE = retinal pigment epithelium.

quadrants and four outer macular quadrants) as defined by the Early Treatment Diabetic Retinopathy Study (ETDRS Research Group, 1985).

OCT scanning was performed by trained technicians at each centre, who monitored scans to ensure fixation was reliable. Mydriatic drops were not administered for scan acquisition, as mydriasis has been shown to have little impact on measurements or reproducibility in patients with pupillary diameters of 5 mm or more (Fisher *et al.*, 2006; Zaveri *et al.*, 2008). Scans with a signal strength less than 7 (maximum of 10) were excluded from analysis. At the time of scanning the examining technician ran Cirrus HD-OCT analysis and confirmed that automatic optic disc centring was correct.

For each eye, the OCT software used an automated, computerized algorithm to compare measurements of average RNFL thickness and average macular thickness with a normative database of age-matched control subjects (for patients aged 18 years or older). This algorithm assigned these measurements a rank against a normal distribution percentile scheme derived from the database of age-matched controls, such that these measurements were designated into the following categories: normal (5–95th percentile), below normal (<5th percentile), markedly below normal (<1% percentile) or supra-normal (>95th percentile). The internal Cirrus HD-OCT normative database consists of 284 subjects with a similar gender distribution, with an age range of 18–84 years (mean age: 46.5 years).

The macular scans were further analysed in a blinded fashion using prototype segmentation software that identified the outer boundary of the RNFL (using a different method than in the peripapillary scans), the outer boundary of the inner plexiform layer, and the outer boundary of the outer plexiform layer (Fig. 4). The segmentation software does not distort the retinal layers, retaining and detecting the true curvature of the retina during the segmentation process. The difference between the RNFL and the inner plexiform-layer segmentations yields the combined thickness of the ganglion cell bodies and the inner plexiform layer. These two layers are difficult to distinguish from each other, but the combined thickness is considered to be representative of the health of the ganglion cell layer. The difference between the inner and outer plexiform-layer segmentations yields the combined thickness of the inner nuclear layer and the outer plexiform layer. These two layers were not distinguished by our segmentation software, but it seemed reasonable to consider the combined thickness as representative of the health of the inner nuclear layer, since the outer plexiform layer is considered to be a relatively thin retinal layer (Hogan *et al.*, 1971). The difference between the outer

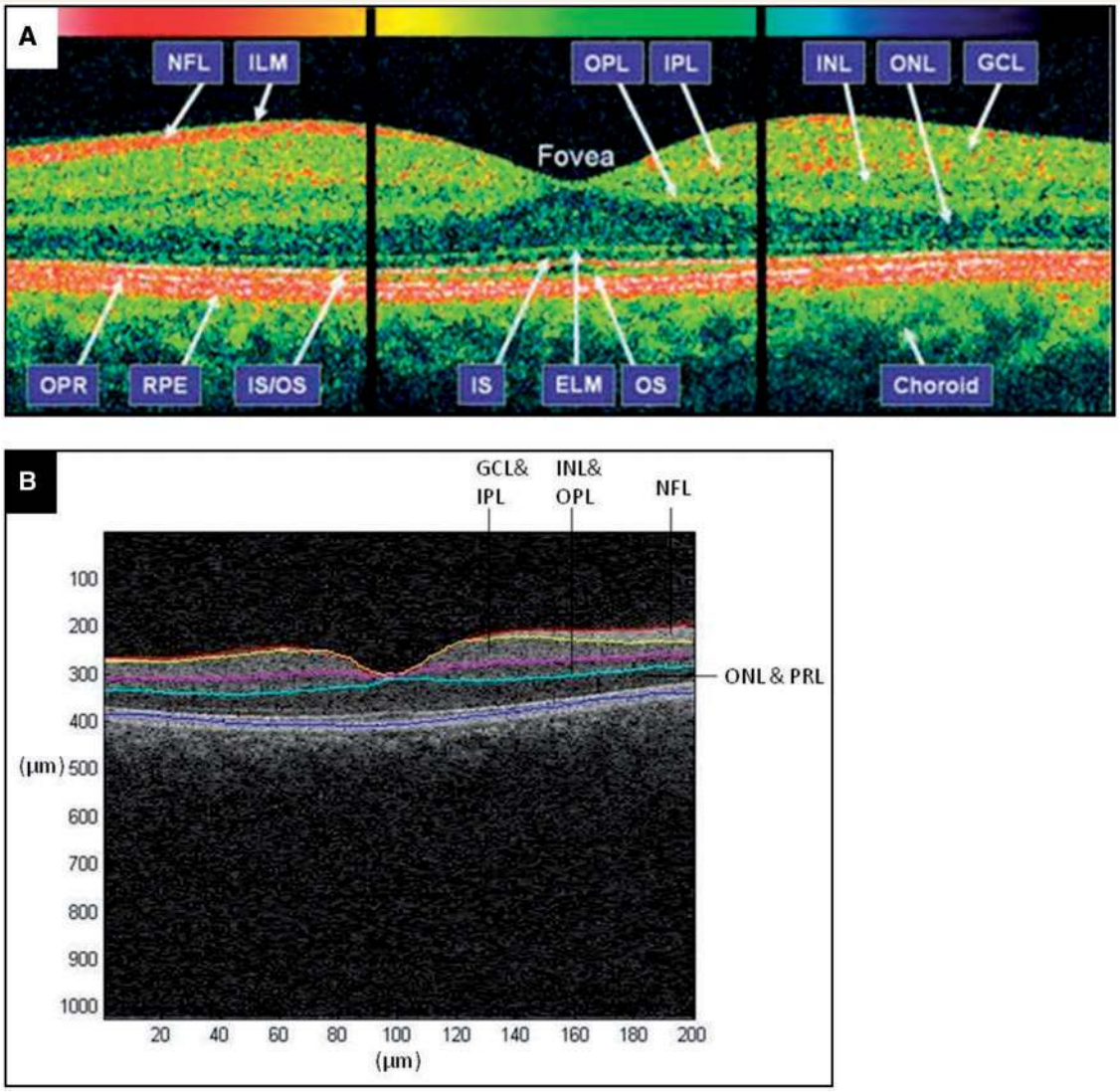

Figure 4 (**A**) A Cirrus HD-OCT B-scan with false colour scheme, the generation of which is based upon differences in tissue reflectivity within different retinal layers. Note that the ganglion cell layer (GCL) and inner plexiform layer (IPL) are virtually indistinguishable from one another. (**B**) A grey-scale Cirrus HD-OCT B-scan. The different colour lines correspond to the retinal layer boundaries as identified during our segmentation process as follows: red line = inner limiting membrane; yellow line = outer boundary of the RNFL; purple line = outer boundary of IPL; green line = outer boundary of the outer plexiform layer (OPL), blue line = retinal pigment epithelium (RPE). IS = inner photoreceptor segments; IS/OS = IS/OS junction; OPR = outer photoreceptors; OS = outer photoreceptor segments; PRL = photoreceptor layer; RPE = retinal pigment epithelium; ELM = external limiting membrane.

plexiform-layer segmentation and the retinal pigment-epithelium segmentation identified by the regular Cirrus algorithm yields the combined thickness of the outer nuclear layer and the inner and outer photoreceptor segments. The outer nuclear layer (which contains the cell bodies and nuclei of the rods and cones), is considered to be thicker than the photoreceptor segments layer (Hogan *et al.*, 1971), and the combined thickness of these layers is thus considered mostly representative of the health of the outer nuclear layer. In all cases, these average thicknesses were measured in an annulus of inner radius 0.54 mm and outer radius 2.4 mm, a region where retinal layers appear thickest on average in normal maculae.

In order to allow determination of Cirrus HD-OCT segmentation reproducibility, the two operators from the Johns Hopkins Multiple Sclerosis Centre who performed the bulk of the OCTs in this study each performed a macular scan of one eye in an independent cohort of 20 healthy subjects and 23 patients with multiple sclerosis consecutively on the same day. Segmentation of all acquired macular scans was performed as described above.

Visual function testing

Refracted visual function was tested with retro-illuminated eye charts in a darkened room, prior to OCT examination. Full contrast Early Treatment Diabetic Retinopathy Study charts and low contrast Sloan letter charts (2.5 and 1.25% contrast; at 2 m) were used. Standard testing protocols were employed. Testing was performed monocularly, with subjects using their habitual distance spectacles or contact lenses.

Charts were scored letter-by-letter and the number of letters correctly read was recorded.

Other ophthalmological assessments

Complete ophthalmic examination was conducted in a subset of patients in the MTP group. The patients were selected based on their availability and willingness to undergo further evaluations. The ophthalmic examination included slit lamp assessment, contact lens biomicroscopy, intraocular pressure measurement, dilated fundus examination with scleral indentation, fundus photography, macular microperimetry, Goldmann visual field testing (Goldmann visual field perimeter; Haag Streit, Berne, Switzerland) and multifocal electroretinography (Veris v.4.9; EDI Inc, San Mateo, CA, USA).

The cone multifocal electroretinography was recorded under light-adapted conditions and performed with 103 scaled hexagons conforming to International Society for Clinical Electrophysiology of Vision guidelines (Hood *et al.*, 2008), as previously described (Hood, 2000). Briefly, a patterned stimulus of concentric hexagons was presented on a screen in front of the patient and the patient was instructed to fixate at the centre of the pattern. Responses from right and left eyes were recorded simultaneously using Burian–Allen corneal electrodes. The 103 hexagons were arranged to match the cortical magnification factor. The normal response range has been previously established for the six distinct rings of hexagons. The resultant multifocal electroretinography responses were examined for local changes in amplitude and latency, and were categorized as abnormal if there were evident reductions in amplitude or increases in latency.

Statistical analyses

Statistical analysis was completed on STATA Version 11 (StataCorp, College Station, TX, USA), using one eye of participants to avoid bias due to inter-eye correlation. *T*-test was used to compare clinical and OCT segmentation data between the study groups examined in this study, as the examined variables followed a normal distribution. Intra-class correlation coefficients on inter-rater reproducibility were used to determine the reproducibility of the OCT-segmentation methods used. Intra-class correlation coefficients were computed in Statistical Package for the Social Sciences (SPSS) Version 12, using a 2-way random model for absolute agreement.

Results

Fifty patients with the MTP OCT phenotype (OCT combination of normal RNFL thickness, with average macular thickness <5th percentile) were identified and compared with 96 age-matched non-MTP patients with multiple sclerosis (patients with abnormal OCTs, $n = 48$; patients with normal OCTs, $n = 48$) and 86 healthy controls. Of those patients with multiple sclerosis with the MTP phenotype, 31 (62%) had an OCT with an average macular thickness <1st percentile, while 19 (38%) had an OCT with average macular thickness between the 1st and 5th percentiles, in conjunction with normal RNFL thicknesses. The OCT phenotype characterizing patients with MTP was unilateral in 66% of patients with MTP and bilateral in 34% of patients with MTP. Of 891 patients who had Cirrus HD-OCT scans performed at the Division of Neuroimmunology at Johns Hopkins Hospital, 449 patients had clinically definite multiple sclerosis. Of these, 45 had the MTP phenotype, equating to a prevalence of 10%. Thirteen of these were identified retrospectively, for which data on multiple sclerosis-severity scores, visual acuity and ophthalmic symptoms were unavailable in a minority. Demographics of our study cohorts and a summary of statistics are given in Table 1. Average macular thickness was significantly lower in patients with MTP (253 μm) compared with healthy controls ($n = 86$ 284 μm, $P < 0.001$), patients with abnormal OCTs ($n = 48$, 260 μm, $P = 0.007$) and patients with normal OCTs ($n = 48$, 278 μm, $P < 0.001$). Although average RNFL thickness was significantly lower in patients with MTP (86 μm) compared with healthy controls (94 μm, $P < 0.001$) and patients with normal OCTs (93 μm, $P < 0.001$), it was significantly higher in patients with MTP compared with patients with abnormal OCTs (71 μm, $P < 0.001$). A summary of average RNFL thickness, average macular thickness and OCT segmentation statistics is provided in Table 2.

The intra-class correlation coefficients for inter-rater reproducibility were high for Cirrus HD-OCT segmentation measurements in healthy controls and patients with multiple sclerosis. Intra-class correlation coefficient 95% confidence intervals (CIs) were narrow. Findings of Cirrus HD-OCT segmentation reproducibility data are summarized in Table 3.

All OCT retinal-layer segmentation measurements (Table 2) were significantly lower in patients with MTP compared with healthy controls and patients with normal OCTs. While average ganglion cell-layer thickness was significantly higher in patients with MTP compared with patients with abnormal OCTs ($P < 0.001$), thicknesses of deeper retinal layers, including the inner nuclear layer and outer nuclear layer, were significantly lower in patients with MTP compared with patients with abnormal OCTs ($P < 0.001$ for both). Conversely, there was no significant difference for these latter measurements between non-MTP patients with multiple sclerosis (either patients with normal OCTs or patients with abnormal OCTs) and healthy controls. The patients with MTP were further divided into two sub-groups (those with an average macular thickness <1st percentile, and those with an average macular thickness between the 1st and 5th percentiles). Even those patients with MTP with an average macular thickness between the 1st and 5th percentiles demonstrated significantly higher ganglion cell-layer thicknesses ($P = 0.006$) and significantly lower inner ($P = 0.003$) and outer nuclear layer ($P = 0.02$) thicknesses compared with patients with abnormal OCTs (Supplementary Table 1).

In Pearson correlation analyses between peripapillary RNFL thickness and temporal quadrant RNFL thickness, with thickness of the retinal layers on segmentation, we found strong correlations between peripapillary RNFL ($r = 0.76$) and temporal quadrant RNFL thickness ($r = 0.72$) and ganglion cell-layer thickness in non-MTP patients with multiple sclerosis. In patients with MTP, peripapillary RNFL thickness ($r = 0.31$) and temporal quadrant RNFL thickness ($r = 0.51$) correlated less well with ganglion cell-layer thickness. Temporal quadrant RNFL thickness ($r = -0.29$) correlated weakly, but inversely, with outer nuclear layer thickness in patients with MTP with multiple sclerosis, while peripapillary RNFL thickness did not correlate with outer nuclear layer thickness ($r = -0.16$). Neither peripapillary RNFL thickness nor temporal quadrant RNFL thickness correlated with inner nuclear layer

Table 1 Summary of demographics and clinical characteristics

	All MTP	MTP (<1%)	MTP (1–5%)	Non-MTP MS	MSN	MSA	HC
Subjects, *n* (%)	50	31	19	96	48	48	86
Relapsing remitting	43 (86)	26 (83.9)	17 (89.5)	75 (78.1)	40 (83.3)	35 (72.9)	
Relapsing progressive	2 (4)	2 (6.5)	0 (0)	0 (0)	0 (0)	0 (0)	
Secondary progressive	5 (10)	3 (9.7)	2 (10.5)	21 (21.9)	8 (16.7)	13 (27.1)	
Age at OCT scan, yr (SD)	41.8 (10.9)	42.1 (11.0)	41.2 (10.9)	43.1 (10.7)	44.2 (10.5)	42.1 (11.0)	35.0 (9.3)
Range	20–59	20–59	21–58	20–65	28–65	20–64	22–55
Sex, *n* (%)							
Male	35 (70.0)	24 (77.4)	11 (57.9)	69 (80.2)	34 (70.8)	35 (72.9)	57 (66.3)
Female	15 (17.4)	7 (22.6)	8 (42.1)	17 (19.8)	14 (29.2)	13 (27.1)	29 (33.7)
Average disease duration (SD)[a]	9.0 (8.8)	9.0 (8.0)	8.8 (10.6)	11.7 (8.6)	10.3 (6.9)	13.2 (9.9)	
Average EDSS (SD)[a]	3.3 (1.5)	3.5 (1.3)	3.1 (1.9)	3.1 (2.0)	3.1 (2.1)	3.1 (2.0)	
Average MSSS (SD)[a]	5.1 (2.2)	5.4 (1.8)	4.5 (2.7)	3.8 (2.4)	4.0 (2.5)	3.7 (2.4)	
All MTP MDSS versus all MS, MSN, MSA, *P*-value				0.006	0.03	0.006	
MTP (<1%) MDSS versus all MS, MSN, MSA, *P*-value				0.002	0.01	0.002	
MTP (1–5%) MDSS versus all MS, MSN, MSA, *P*-value				0.33	0.53	0.30	
Eyes with history of AON, *n* (%)	0 (0)	0 (0)	0 (0)	30 (31%)	5 (10.4)	25 (52.1)	
Letters read correctly at 100% (SD)[+]	57.1 (7.8)	56.5 (9.1)	58.1 (4.7)	56.3 (13.5)	59.5 (11.1)	53.0 (15.1)	63.1 (6.8)
Letters read correctly at 2.5% (SD)[+]	24.7 (10.5)	22.9 (10.9)	27.8 (9.4)	25.0 (13.7)	29.4 (12.5)	20.2 (13.3)	32.4 (8.3)
Letters read correctly at 1.25% (SD)[+]	12.5 (10.9)	10.9 (11.1)	15.4 (10.4)	11.1 (10.9)	14.5 (11.6)	7.1 (8.2)	22.0 (10.5)

Visual-acuity data was not available for nine of the patients with MTP who were identified retrospectively, five of the patients with normal OCTs (MSN) and five of the patients with abnormal OCTs (MSA). MSA = patients with multiple sclerosis with abnormal OCT scans demonstrating abnormalities typical of those seen in multiple sclerosis; MSN = patients with multiple sclerosis with normal OCT scans; MTP (<1%) = MTP scan with average macular thickness <1st percentile; MTP (1–5%) = MTP scan with average macular thickness between the 1st and 5th percentiles. EDSS and MSSS data were not available for patients recruited from centres other than Johns Hopkins.

[a]All MT: *n* = 43, MTP (<1%): *n* = 28; MTP (1–5%): *n* = 15; All MS: *n* = 96; MSN: *n* = 48; MSA: *n* = 48.

[+]All MT: *n* = 41; MTP (<1%): *n* = 26; MTP (1–5%): *n* = 15, All MS: *n* = 86, MSN: *n* = 43, MSA: *n* = 43, Healthy controls: *n* = 57.

AON = acute optic neuritis; EDSS = Expanded Disability Status Scale; MS = multiple sclerosis; MSA = patients with multiple sclerosis with abnormal OCT scan; MSN = patients with multiple sclerosis with normal OCT scan; MSSS = multiple sclerosis severity score.

thickness in patients with MTP or with inner or outer nuclear layer thicknesses in non-MTP patients with multiple sclerosis.

To investigate whether OCT metrics (average RNFL thickness, average macular thickness and OCT-segmentation measurements) in our study were affected by optic disc area, we ran a regression model adjusting for disc area (Supplementary Table 2). None of the *P*-values between the comparator groups for these measurements changed in significance, other than the average RNFL thickness comparison between MTP multiple sclerosis and non-MTP patients with multiple sclerosis, which went from 0.04 to 0.10 (reflecting no significant difference in RNFL thickness between MTP and all non-MTP patients with multiple sclerosis when adjusting for disc area).

With regards to clinical characteristics, we found that the average multiple sclerosis severity score was significantly higher in patients with MTP ($n = 43$, multiple sclerosis-severity score = 5.1) compared with patients with normal OCTs ($n = 48$, multiple sclerosis-severity score = 4.0, $P = 0.03$) and patients with abnormal OCTs ($n = 48$, multiple sclerosis-severity score = 3.7, $P = 0.006$). However, in sub-group analyses of the patients with MTP, average multiple sclerosis-severity score scores were only significantly higher in those patients with MTP with average macular thickness <1st percentile, while there was no difference between multiple sclerosis-severity score in those patients with MTP with average macular thicknesses between the 1st and 5th percentiles and patients with normal OCTs and patients with abnormal OCTs.

Tweny-five of 39 MTP patients with multiple sclerosis (64%) with available ophthalmic symptom data complained of ophthalmic symptoms not typical of those generally described as sequelae of acute optic neuritis, including photophobia, excessive glare, nyctalopia and photopsia. Ophthalmic symptoms for symptomatic patients with MTP are summarized in Table 4.

Exposure to disease modifying therapies (β-interferon, glatiramer acetate and natalizumab) at the time of OCT scanning did not differ significantly between the MTP multiple sclerosis group and the comparator multiple sclerosis groups (χ^2-analysis between MTP and non-MTP patients with multiple sclerosis; $P = 0.3$), nor did history of prior corticosteroid exposure differ between these groups (χ^2-analysis between MTP and non-MTP patients with multiple sclerosis; $P = 0.9$). Additionally, the number of disease-modifying therapies exposed to in the past did not differ significantly between the study groups (*t*-test analysis between MTP and non-MTP patients with multiple sclerosis; $P = 0.62$).

High contrast (100%) letter acuity scores and 1.25% letter acuity scores were all significantly lower in MTP, patients with abnormal OCTs and patients with normal OCTs compared with healthy controls. In MTP and patients with abnormal OCTs 2.5% letter acuity scores were significantly lower compared with healthy

Table 2 Comparison of OCT between patients with multiple sclerosis with MTP, patients with multiple sclerosis without MTP and healthy controls

Comparison of Cirrus-HD OCT between MTP MS											
Patients, non-MTP MS patients and healthy controls	MTP group mean (SD)	MSN group mean (SD)	MSA group mean (SD)	Non-MTP MS mean (SD)	HC group Mean (SD)	MTP versus HC, *P*-value	MTP versus MSN, *P*-value	MTP versus MSA, *P*-value	MSN versus HC, *P*-value	MSA versus HC, *P*-value	MTP versus non-MTP MS, *P*-value	Non-MTP MS versus HC, *P*-value
Un-segmented peripapillary OCT												
ARNFLT (μm)	86.1 (8.6)	92.6 (10.0)	70.8 (8.3)	81.7 (14.3)	93.8 (10.3)	0.001	0.001	0.001	0.53	0.001	0.04	0.001
AMT (μm)	253.0 (8.8)	277.6 (11.4)	259.7 (14.7)	268.8 (15.9)	283.6 (13.9)	0.001	0.001	0.007	0.01	0.001	0.001	0.001
Segmented macular OCT												
AT GCL + IPL (μm)	69.0 (6.8)	76.7 (6.7)	62.9 (8.2)	69.8 (10.2)	83.1 (7.0)	0.001	0.001	0.001	0.001	0.001	0.58	0.001
AT RNFL + GCL + IPL (μm)	95.4 (9.8)	107.4 (8.9)	87.6 (12.5)	97.5 (14.7)	115.8 (8.6)	0.001	0.001	0.001	0.001	0.001	0.35	0.001
AT INL + OPL (μm)	60.8 (4.0)	65.6 (5.1)	66.1 (5.2)	65.9 (5.1)	64.8 (4.7)	0.001	0.001	0.001	0.36	0.08	0.001	0.11
AT ONL + PRL (μm)	111.4 (7.1)	119.7 (6.6)	119.1 (6.7)	119.4 (6.6)	119.8 (9.1)	0.001	0.001	0.001	0.96	0.67	0.001	0.76

AMT = average macular thickness; ARNFLT = average RNFL thickness; AT = average thickness; GCL = ganglion cell layer; HC = healthy control; INL = inner nuclear layer; IPL = inner plexiform layer; MSA = patients with multiple sclerosis with abnormal OCT scans demonstrating abnormalities typical of those seen in multiple sclerosis; MSN = patients with multiple sclerosis with normal OCT scans; non-MTP = MSN & MSA; ONL = outer nuclear layer; OPL = outer plexiform layer; PRL = photoreceptor segments layer.

Table 3 Inter-rater reproducibility for Cirrus-HD OCT-segmentation parameters in an independent cohort of healthy controls and patients with multiple sclerosis

	Healthy controls *n* = 20		MS patients *n* = 23	
	ICC	95% CI	ICC	95% CI
AT GCL + IPL	0.99	0.99–0.99	0.99	0.99–0.99
AT RNFL + GCL + IPL	0.99	0.99–0.99	0.99	0.99–0.99
AT INL + OPL	0.91	0.79–0.96	0.94	0.86–0.97
AT ONL + PRL	0.93	0.84–0.97	0.92	0.83–0.97

AT = average thickness; CI = confidence interval; GCL = ganglion cell layer; ICC = intra-class correlation; INL = inner nuclear layer; IPL = inner plexiform layer; ONL = outer nuclear layer; OPL = outer plexiform layer; PRL = photoreceptor segments layer.

Table 4 Summary of ophthalmic symptoms in 25/39 symptomatic patients with multiple sclerosis with MTP

Ophthalmic symptoms	Number of MTP patients with symptoms
Photophobia and excessive glare	7
Photophobia and excessive glare and nyctalopia	6
Photophobia and excessive glare and nyctalopia and photopsia	3
Nyctalopia alone	3
Photopsia alone	2
Photophobia and nyctalopia and photopsia	1
Photophobia and excessive glare and photopsia	1
Excessive glare and nyctalopia and photopsia	1
Excessive glare alone	1

controls, but there was no significant difference between 2.5% letter acuity scores between patients with normal OCTs and healthy controls. In sub-group analyses of patients with MTP, 100% and 1.25% letter acuity scores were significantly lower in both MTP sub-groups compared with healthy controls (Supplementary Table 1).

In those who underwent complete ophthalmic assessment ($n = 7$), there was no evidence of optic disc swelling or macular oedema seen on contact lens biomicroscopy. Funduscopic examination did not reveal any clinical signs of vasculitis, vitritis (no vitreous haze or cells) or intermediate uveitis (snow-balls, snow-banking or exudates in the peripheral retina and pars plana). There was no clinical evidence suggestive of maculopathy or optic neuropathy in this subset of patients (except for slight pallor of the right optic nerve head in two patients who had the MTP OCT phenotype contralaterally). Multifocal electroretinography was diffusely abnormal with attenuated amplitudes (<5th percentile) of the P1 waveform (measured from the N1 trough to the P1 peak) of the first-kernel order across the majority of rings bilaterally in five of seven patients, suggesting retinal dysfunction in these patients. P1 amplitudes may be reduced as a result of damage to the ON bipolar cells, the outer plexiform layer and the cone photoreceptors (Hood, 2000). All waves of the first-kernel order showed normal latencies in all patients. Multifocal

Table 5 Summary of the amplitudes of the P1 waveform in the multifocal electroretinography of a subset of patients with MTP ($n = 7$), showing abnormally reduced amplitudes in both eyes of five patients

Ring Zone in degrees Normal amplitude in (nV/deg^2)[a]		Ring #1 <2.4° 108	Ring #2 From 2.4° to 5.2° 60	Ring #3 From 5.2° to 11.6° 41	Ring #4 From 11.6° to 19.6° 30	Ring #5 From 19.6° to 29.8° 25	Ring #6 From 29.8° to 44.4° 23	MTP pattern
Patient 1	OD	136	**54**	**39**	**27**	25	25	No
	OS	**106**	**37**	**28**	**23**	**20**	**22**	Yes
Patient 2	OD	111	76	**36**	**19**	**21**	23	No
	OS	**88**	**43**	**32**	**25**	**21**	**21**	Yes
Patient 3	OD	**107**	**58**	**38**	**28**	**24**	25	Yes
	OS	**47**	**41**	**31**	**22**	**19**	**20**	Yes
Patient 4	OD	118	81	47	34	36	23	Yes
	OS	138	85	49	35	25	23	No
Patient 5	OD	142	63	49	40	33	34	Yes
	OS	116	**59**	44	35	29	29	Yes
Patient 6	OD	**104**	**54**	**34**	**26**	**23**	24	Yes
	OS	**107**	**51**	**40**	30	27	30	Yes
Patient 7	OD	**92**	**53**	**37**	**26**	25	27	Yes
	OS	**87**	**52**	**31**	**25**	**21**	23	Yes

Two patients (Patients 4 and 5) demonstrated predominantly normal amplitudes. nV/deg^2 = nanovolts per squared degree; OD = right eye; OS = left eye. Please note that in Patients 1 and 2 there was evidence of pallor in the right eye of each of these patients (possibly reflective of optic neuropathy), however, multifocal electroretinography amplitudes were more greatly reduced in those eyes with the MTP designation.

[a]Cut-off for normal (all values below these are <5th percentile), abnormal amplitudes (<5th percentile) are represented in boldface.

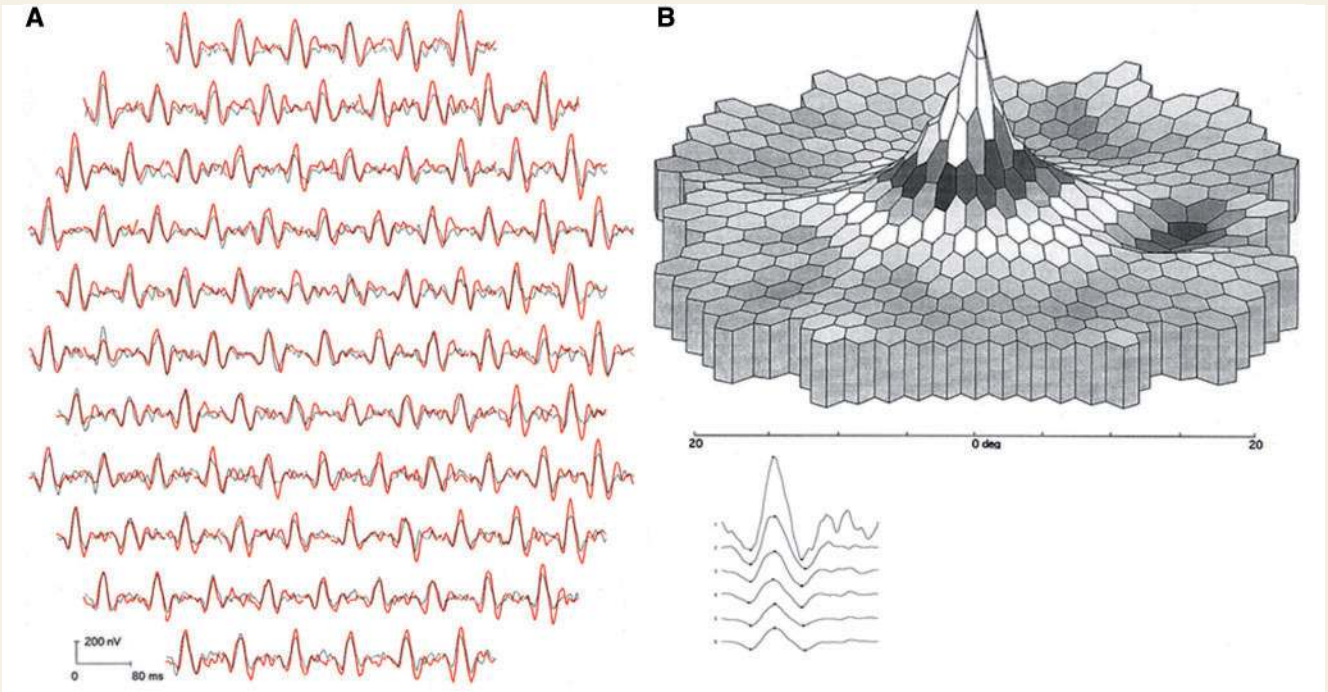

Figure 5 A representative multifocal electroretinography trace array from a patient with multiple sclerosis with MTP (right eye), demonstrating mild-moderate diffuse reduction in amplitudes is depicted on the left. Abnormally reduced amplitudes (black waveforms) are superimposed with normal amplitudes (red waveforms). A 3D plot (*top right*) from the same patient is shown, demonstrating the uniform distribution of the reduced amplitudes within the central 20° of the visual field. A graphical representation of the first order kernel waveforms in the same patient from the tested six annular subfields (0–44.40) centred on the fovea is also shown (*bottom right*).

electroretinography results are summarized in Table 5 and an example of an abnormal multifocal electroretinography from an MTP patient is depicted in Fig. 5.

Goldmann visual field testing demonstrated normal responses for all isopters along all meridians in each eye of Patients 5, 6 and 7. Patient 1 had mild ($<10^{\circ}$) constriction for all isopters along the superior meridians bilaterally. Patients 2 and 3 had mild to moderate ($>10^{\circ}$ but $<25^{\circ}$) constriction for all isopters along all meridians. Patient 4 showed normal responses for all isopters along all meridians in the right eye and mild constriction for all isopters along five meridians in the supero-temporal field. No patients had severe ($>25^{\circ}$) visual field loss.

Discussion

Results of this study demonstrate that in a subset of patients with multiple sclerosis, the retinal macula, rather than the optic nerve and RNFL, is preferentially affected as corroborated by OCT derived detection of thinning specific to the inner and outer nuclear layer, with relative sparing of the ganglion cell layer. These findings are conspicuous in that while the composition of the retina is principally neural tissue, it is devoid of myelin, suggesting that the histopathological substrate in this novel patient group is a likely derivative of mechanisms that are distinctive from those that characterize the more typical targeting of the multiple sclerosis disease process in the anterior visual system. This is a concept that has not been previously explored through quantitative *in vivo* retinal assessment in multiple sclerosis. To the best of our knowledge, the MTP OCT phenotype described in this study has not been previously elucidated in multiple sclerosis. The finding that average RNFL thickness in patients with MTP was lower compared with healthy controls and patients with multiple sclerosis with normal OCTs, but higher in patients with MTP than in patients with multiple sclerosis with OCT abnormalities typical of multiple sclerosis, suggests that a more proximal (macular neuronal) mechanism of injury may be operative in this newly identified patient sub-group. Observed RNFL (axonal) thinning in these patients may be the result of anterograde degeneration, initiated by a primary pathological process occurring within the deeper layers of the retina. An alternative possibility that warrants careful consideration is that patients with MTP may constitute a group of patients with multiple sclerosis with sub-clinical optic neuropathy with a greater propensity to nuclear rather than ganglion cell loss, as a culmination of retrograde degeneration. Our results validate a possible association between more marked macular thinning in the MTP group and a corresponding risk of accelerated clinical disease progression. If the documented changes in the retinal nuclear layers of these patients are the consequence of retrograde degeneration, then it may be plausible to postulate that patients with multiple sclerosis, who suffer greater retrograde degeneration, are prone to more accelerated disease progression.

A salient finding in our study that argues in favour of a primary retinal process in patients with MTP, is the determination that average inner and outer nuclear layer thicknesses were significantly lower in patients with MTP than non-MTP patients with multiple sclerosis (both patients with abnormal and normal OCTs). Further, there was little or no correlation between thicknesses of these layers and peripapillary RNFL thickness or temporal quadrant RNFL thickness. While patients with abnormal OCTs (52% of whom had suffered from optic neuritis) had significantly lower ganglion cell-layer thicknesses than patients with MTP, there was no difference in inner and outer nuclear layer thicknesses between this group and healthy controls (nor was there any difference in thickness of these layers between patients with normal OCTs and healthy controls). Thus, in the group of patients with abnormal OCTs there appeared to be preservation of the deeper retinal layers, despite clear evidence of ganglion cell-layer thinning and a history of prior optic neuropathy. While thinning of the ganglion cell layer may be secondary to retrograde changes, thickness changes in the inner and outer nuclear layer do not appear to occur on a similar basis, since it was not observed in non-MTP patients with multiple sclerosis (refer to Fig. 6 for a summary of the key OCT-segmentation findings). To our knowledge, retrograde degeneration of retinal layers secondary to optic atrophy has not been demonstrated to occur in animal models. In animals, optic nerve transection does result in ganglion cell loss (Hollander *et al.*, 1984; Levkovitch-Verbin *et al.*, 2001) and reorganization of the inner nuclear layer (Williams *et al.*, 2001), however, atrophy or dysfunction distal to the ganglion cells has not been observed.

In order to better understand the physiological consequences of the pathological changes we observed in retinal architecture, we performed multifocal electroretinography in a subset of patients with MTP. Multifocal electroretinography (along with full field electroretinography) waveforms are thought to receive little or no contribution from retinal ganglion cells, and predominantly reflect the health of the outer retina. Abnormalities were detected in five of seven patients providing functional corroboration for the relevance of our detected OCT-segmentation structural abnormalities. It is known from prior studies comparing multifocal electroretinography to qualitative spectral domain OCT assessment that there is disconnect between these two tests, and that both tests are not abnormal in ~50% of cases in the setting of a retinal disorder (Dale *et al.*, 2010). Furthermore, multifocal electroretinography is expensive, time-consuming, invasive and potentially painful. For these reasons multifocal electroretinography was only performed in a subset of patients with MTP. In the future there may be benefit in studying this more extensively. Interestingly, optic atrophy resulting from section or optic nerve compression (Dawson *et al.*, 1982; Seiple *et al.*, 1983; Kaufman and Celesia, 1985) does not produce the same changes in electroretinography waveforms as has been previously demonstrated to occur in patients with multiple sclerosis, raising the suggestion from previous researchers that detected electroretinography abnormalities in multiple sclerosis may be independent of optic nerve pathology (Gills, 1966; Papakostopoulos *et al.*, 1989) or perhaps represent a primary multiple sclerosis-related retinal process (Gills, 1966). More recently, bright flash *a*-wave, cone *b*-wave and rod-cone *b*-wave implicit times have been demonstrated to be significantly delayed, and the amplitude of the sums of photopic oscillatory potentials also significantly reduced in patients with multiple sclerosis, without correlation with visual acuity, contrast sensitivity, colour vision or visual fields (Forooghian *et al.*, 2006). These electroretinography abnormalities

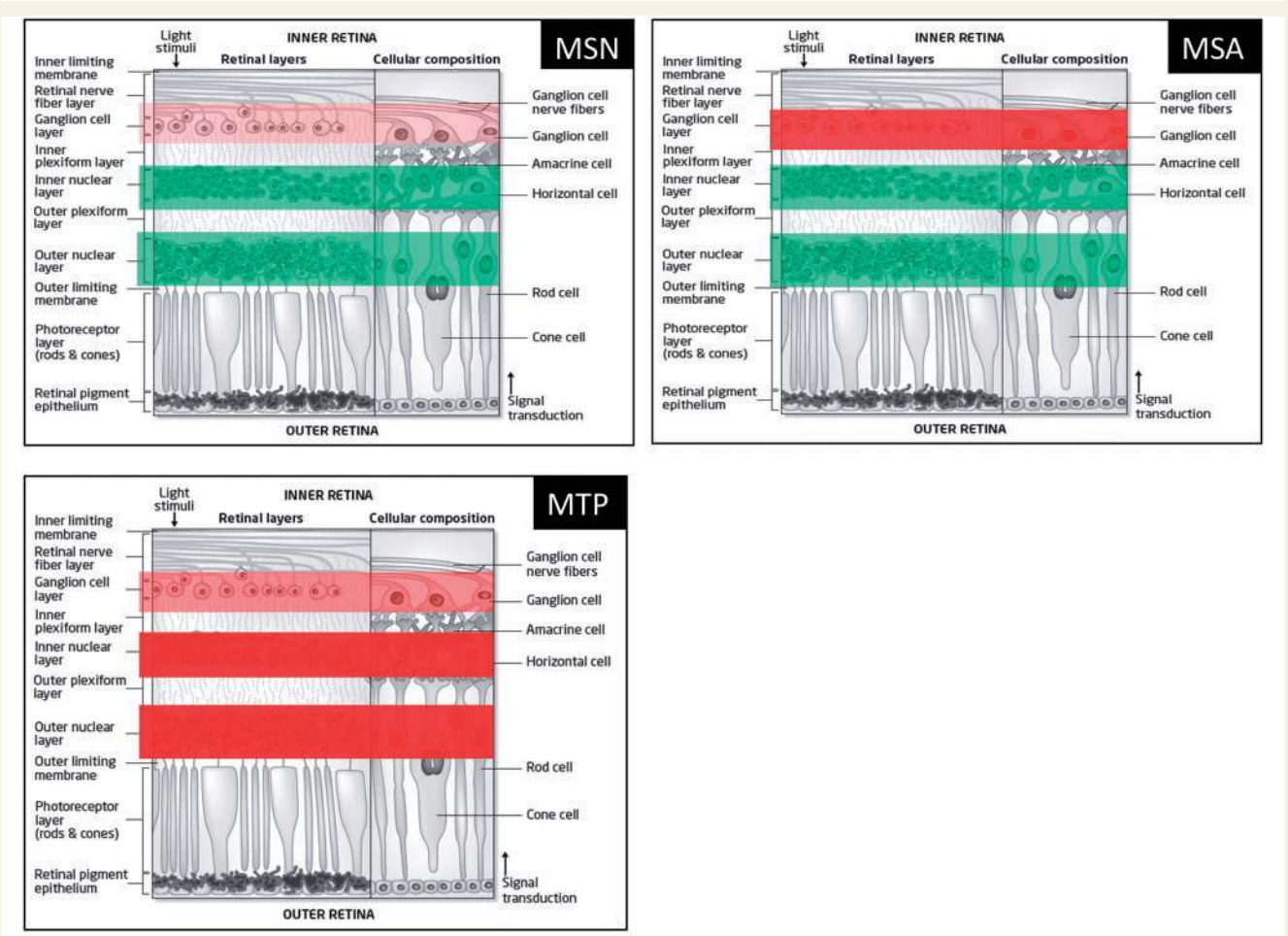

Figure 6 Schematic illustrations to summarize our key OCT-segmentation findings. Areas represented in different shades of red correspond to different degrees of thinning, whilst areas represented in green correspond to areas of normal thickness. The ganglion cell layer is mildly thinned in the patients with normal OCTs (MSN), moderately thinned in the MTP group and severely thinned in the patients with abnormal OCTs (MSA). Inner and outer nuclear-layer thicknesses are normal in the patients with normal and abnormal OCTs, but severely reduced in the MTP group. Despite severe thinning of the ganglion cell layer in the patients with abnormal OCTs (MSA), within which the majority of eyes had a prior history of clinical optic neuritis, there was no thinning of the inner or outer nuclear layers. The absence of thinning in the inner and outer nuclear layers of the patients with normal and abnormal OCTs implies that thinning in these layers may not be a typical sequela of optic neuropathy (either clinical or sub-clinical). Therefore, thinning in these layers in the MTP group suggests that such changes may be the result of a primary retinal process.

in some patients with multiple sclerosis imply dysfunction in several retinal layers, but particularly so in the photoreceptor layer.

Previous studies assessing retinal pathology in multiple sclerosis have demonstrated extensive qualitative atrophy of the RNFL and ganglion cell layer in >70% of multiple sclerosis eyes (Kerrison *et al.*, 1994; Green *et al.*, 2010). Furthermore, prominent qualitative pathological atrophy of the inner nuclear layer has recently been demonstrated in 40% of eyes of patients with multiple sclerosis (Green *et al.*, 2010), suggesting that inner neuronal pathology is not restricted to the ganglion cell layer in the eyes of patients with multiple sclerosis. Qualitative analyses of the plexiform layers and deeper retinal structures in multiple sclerosis eyes are lacking. Quantitative pathological ultra-structural retinal analysis has not been previously performed in multiple sclerosis eyes, predominantly on account of post-mortem retinal detachment and degradation. Likewise, clinicopathological correlation of ultra-structural changes in the retinas of patients with multiple sclerosis are lacking for the same reasons. The majority of retinal pathological descriptions in multiple sclerosis are limited by being restricted to end-of-life analysis, and may not inform us of the mechanism or order in which such changes arose. To our knowledge this is the first study to assess *in vivo* changes in retinal layers of multiple sclerosis eyes (other than the RNFL), and to demonstrate that multiple sclerosis may structurally affect deeper retinal layers, such as the outer nuclear layer. Such is the predilection for the multiple sclerosis disease process to affect the optic nerves that, on post-mortem analysis, 94–99% of patients with multiple sclerosis are noted to have multiple sclerosis lesions in the optic nerves, irrespective of a history of acute optic neuritis (Ikuta and Zimmerman, 1976; Toussaint *et al.*, 1983). If the findings of this study are validated that a primary retinal pathology defines a discrete pathological subset of patients with multiple sclerosis, then it

is possible that there are patients with multiple sclerosis who may have retinal changes resulting from bi-directional pathological processes (mixed pathology). Since our definition of MTP required the RNFL thickness to be concomitantly between the 5th and 95th percentiles, such potential patients were not captured for analysis in this study. Perhaps OCT segmentation in the future may allow us to parse out such changes in multiple sclerosis eyes, and ultimately examine the corresponding clinical characteristics of this subset in larger cohorts.

In conjunction with the unique visual symptoms experienced by many of the patients with MTP, they also appear to exhibit more rapid disease progression than non-MTP patients with multiple sclerosis, as evidenced by their significantly higher multiple sclerosis-severity scores. It must be noted however that this association was only present in those with more marked macular thinning in the MTP group (those with an average macular thickness $<$1st percentile). This is an important finding, as it represents yet another distinguishing characteristic of patients with MTP, implying that MTP patients with multiple sclerosis, in particular those with more marked macular thinning, may accumulate disability more rapidly than non-MTP patients with multiple sclerosis.

This study has a number of limitations that merit detailed discussion. Since it was our primary goal to identify MTP patients with multiple sclerosis using OCT (a previously undescribed OCT phenotype in multiple sclerosis), we elected to define MTP *a priori* based on an OCT metric definition. As mentioned above, it is possible that patients with multiple sclerosis may suffer from bi-directional pathology resulting in retinal changes. This study was not designed to assess or capture this group of patients with multiple sclerosis. Since there was no precedence for a study of this nature, we felt that our definition of MTP *a priori* was an appropriate preliminary step in studying this entity. However, in so doing we may have potentially introduced selection bias into the study. To address this potential flaw, we determined the total number of patients with multiple sclerosis who had ever had Cirrus HD-OCT scans performed at our centre (for both clinical and research purposes) and retrospectively identified all additional potential MTP patients with multiple sclerosis from our total pool of patients with multiple sclerosis. With a prevalence of 10%, it seems unlikely that patients with MTP simply represent general population outliers for average macular thickness. However, our determination of prevalence may also be an underestimate, since 40% of patients with multiple sclerosis have been shown to suffer from inner nuclear layer cell loss at post-mortem analysis (Green *et al.*, 2010). Contributory to this may be our imprecision in identifying patients with multiple sclerosis who have a profile of mixed retinal pathology. Our study represents only a first step towards the identification of a potentially important cohort of patients with multiple sclerosis with a distinctive retinal macular pathology. Moving forward, we anticipate greater refinement in the method of defining the MTP group designation, and it is likely that this study will be strengthened and extended via replication in the future.

This study was also not designed to determine the potential prevalence of the MTP OCT phenotype in healthy controls. This is something that needs to be addressed in the future and will probably require a large normative database of healthy individuals in order to do so.

Although the OCT retinal-layer-segmentation method used in this study is new, we feel the reproducibility analyses support its validity as an investigational tool for future studies. Currently our method of retinal segmentation is incapable of separating the ganglion cell layer from the inner plexiform layer, the inner nuclear layer from the outer plexiform layer or the outer nuclear layer from the photoreceptor segment layers. Since post-mortem studies (Kerrison *et al.*, 1994; Green *et al.*, 2010) have shown thinning of the ganglion cell-layer and inner nuclear layers in multiple sclerosis, we suspect that the majority of reduction in the OCT-segmentation parameters in our study relate to neuronal pathology. In any case, it would perhaps not be unexpected that inner nuclear layer or outer nuclear layer pathology/thinning may also be associated with plexiform layer sequelae and vice versa, or that outer nuclear layer pathology may be associated with photoreceptor segment layer changes and vice versa. Notwithstanding the limitations associated with the OCT-segmentation algorithm, they do not militate against the findings of our investigation. It is however an issue that needs to be clarified once advances in segmentation algorithms allow, in order to more accurately define precisely what retinal disturbances may be occurring. Improvements in layer recognition during the segmentation process will assist with this endeavour in the future.

To date, OCT measurement of total macular volume has been inferred in some studies to provide estimates of retinal neuronal integrity, since the macula is relatively enriched by neurons (Burkholder *et al.*, 2009). One must be cautious in interpreting total macular volume in such a manner, as total macular volume is derived from a total retinal thickness measurement and the area that has been sampled by OCT (Saidha *et al.*, 2010). Therefore any isolated loss of the RNFL may also potentially reduce total macular volume. The segmentation algorithms used in this study probably provide more accurate estimates of retinal neuronal integrity than traditional total macular volume measurements. Spectral domain OCT segmentation has been previously examined in ophthalmological disorders highlighting the utility of OCT in the assessment of deeper retinal layers. For example, photoreceptor loss (identified through OCT segmentation) has been demonstrated to occur in patients with retinitis pigmentosa, with preservation of inner nuclear layer thickness in comparison to healthy controls (Hood *et al.*, 2009).

Our results suggest that multiple sclerosis targets the anterior-visual system at multiple levels including the optic nerve (with subsequent axonal and neuronal degeneration in the retina) and the retina itself (involving discrete pools of neurons). These findings are potentially analogous to the emerging pathological principle that cerebral grey matter pathology may occur in multiple sclerosis, both secondary to and independent of, white matter pathology (Bo *et al.*, 2003; Moll *et al.*, 2008). While the ultimate mechanism by which multiple sclerosis may produce primary retinal pathology is unclear, evidence implicates immune-mediated injury cascades. For instance, post-mortem demonstration of

retinal inflammation by immunohistochemistry in a subset of multiple sclerosis eyes has recently been shown through the identification of human leucocyte antigen-DR cells with the phenotype of microglia, as well as astroglial cell activation (Green *et al.*, 2010). The emerging role of B cells in multiple sclerosis pathogenesis (Corcione *et al.*, 2004) has led to the reappraisal of multiple sclerosis as a primarily T cell mediated disorder. Antibody targets in multiple sclerosis are not restricted to myelin antigens (Derfuss *et al.*, 2009). Retinal periphlebitis occurs in up to 20% of multiple sclerosis cases (Rucker, 1972; Kerrison *et al.*, 1994; Sepulcre *et al.*, 2007) suggesting myelin may not be necessary for establishing or maintaining retinal inflammation in multiple sclerosis, emphasizing breakdown of the blood-retinal barrier in multiple sclerosis. Indeed, anti-retinal antibodies directed against arrestin and α-enolase have been described in multiple sclerosis (Gorczyca *et al.*, 2004; Forooghian *et al.*, 2007) and other autoimmune disorders (Hooks *et al.*, 2001; Gorczyca *et al.*, 2004; Forooghian *et al.*, 2007).

Atrophy of the retina has been shown to be associated with total brain weight (Green *et al.*, 2010). Supporting evidence that intra-ocular pathologies may reflect more global CNS pathology in multiple sclerosis is derived from the demonstration that OCT-detected-RNFL thinning in multiple sclerosis correlates with white matter brain atrophy (Gordon-Lipkin *et al.*, 2007; Grazioli *et al.*, 2008; Siger *et al.*, 2008; Villoslada *et al.*, 2008). In light of these factors, it may be worthwhile investigating if MTP patients with multiple sclerosis have any abnormalities in magnetic resonance imaging derived estimates of brain structure volumes.

In summary, we have identified a unique subset of patients with multiple sclerosis with primary retinal pathology in whom there appears to be disproportionate thinning of the inner and outer nuclear retinal layers. That multiple sclerosis may primarily target the retina, independent of processes occurring in the optic nerves, constitutes a novel conceptualization further broadening of the heterogeneity of this inflammatory, demyelinating, white and grey matter disorder of the central nervous system. Our elucidation of an abnormal pattern of retinal architecture in multiple sclerosis, with a predominant macular topographical distribution, may further our understanding of the pathobiological underpinnings of multiple sclerosis. Further, patients with greater inner and outer nuclear-layer pathology appear to have a predilection toward a more accelerated rate of disability progression, signifying a potential relationship between this pathological profile and the clinical course of multiple sclerosis.

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Supplementary material

Supplementary material is available at *Brain* online.

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