Longitudinal evidence for anterograde trans-synaptic degeneration after optic neuritis

Running title: Trans-synaptic degeneration after optic neuritis

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Abstract

In multiple sclerosis (MS), microstructural damage of normal-appearing brain tissue is an important feature of its pathology. Understanding these mechanisms is vital to help develop neuroprotective strategies. The visual pathway is a key model to study mechanisms of damage and recovery in demyelination. Anterograde trans-synaptic degeneration across the lateral geniculate nuclei has been suggested as a mechanism of tissue damage to explain optic radiation (OR) abnormalities seen in association with demyelinating disease and optic neuritis (ON), although evidence for this has relied solely on cross-sectional studies.

We therefore aimed to assess 1) longitudinal changes in the diffusion properties of ORs after ON suggesting trans-synaptic degeneration; 2) the predictive value of early optic nerve MRI measures for late ORs changes; 3) the impact on visual outcome of both optic nerve and brain post-ON changes.

Twenty-eight consecutive patients with acute ON and eight healthy controls were assessed visually (logMAR, colour vision, and Sloan 1.25%, 5%, 25%) and by MRI, at baseline, three, six, and twelve months. MRI sequences performed (and metrics obtained) were: i) Optic nerve fluid-attenuated inversion-recovery (optic nerve cross-sectional area); ii) Optic nerve proton density (PD) fast spin-echo (optic nerve PD-lesion length); iii) Optic nerve post-gadolinium T1-weighted (gadolinium-enhanced lesion length); and iv) Brain diffusion-weighted imaging (to derive OR fractional anisotropy [FA], radial diffusivity [RD], and axial diffusivity [AD]). Mixed-effects and multivariate regression models were performed, adjusting for age, gender, and OR lesion load. These identified changes over time and associations between early optic nerve measures and one-year global OR/clinical measures.

The FA in patients' ORs decreased (p=0.018) and RD increased (p=0.002) over one year following ON, whereas OR measures were unchanged in controls. Also, smaller cross-sectional areas of affected optic nerves at three months post-ON predicted lower FA and higher RD at one year (p=0.007) in the ORs, whereas none of the inflammatory measures of the optic nerve predicted changes in ORs. Finally, greater gadolinium-enhanced lesion length at baseline and greater optic nerve PD-lesion length at one year were associated with worse visual function at one year (p=0.034 for both). Neither the cross-sectional area of the affected optic nerve after ON nor the damage in ORs was associated with one-year visual outcome.

Our longitudinal study shows that, after ON, there is progressive damage to the ORs, greater in patients with early residual optic nerve atrophy, even after adjusting for OR lesions. These findings provide evidence for trans-synaptic degeneration.

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Introduction

Disability accumulation in multiple sclerosis (MS) is thought to arise from both microstructural normal-appearing central nervous tissue damage and visible inflammatory changes (Fernando *et al.*, 2005). A detailed understanding of the damage mechanisms in MS is crucial to help the development of biomarkers of tissue degeneration, which could be used in clinical trials to monitor neuroprotective treatment effects. The visual pathway, from the optic nerve to the visual cortex, and including the optic tracts, lateral geniculate nuclei and optic radiations, provides a well-characterised neural system and unique setting to study these mechanisms *in vivo* (Toosy *et al.*, 2014).

Inflammatory, demyelinating optic neuritis (ON) typically presents with the triad of dyschromatopsia, visual loss and painful eye movements. ON can be used as a model for relapse in multiple sclerosis (MS) to explore the mechanisms of damage, recovery and persistent deficits (Toosy *et al.*, 2014). The acute ON lesion begins with focal inflammatory demyelination, giving rise to conduction block and loss of vision. Recovery of vision is associated with resolution of inflammation, oedema, remyelination (incomplete) and redistribution of sodium channels (Smith and McDonald, 1999). In the post-acute phase there may be persistent neuroaxonal loss and optic nerve atrophy (Hickman *et al.*, 2002; Hickman *et al.*, 2004; Jenkins *et al.*, 2011).

There is accumulating evidence from MRI studies for changes in post-geniculate visual pathways in association with ON. For example structural (Audoin *et al.*, 2006; Jenkins *et al.*, 2011) and functional (Toosy *et al.*, 2005; Jenkins *et al.*, 2010, 2010b) cortical changes have been described after ON. The optic radiations (ORs) have also been investigated using diffusion tensor imaging (DTI), which provides information about voxel-level microstructural

properties (Basser *et al.*, 1994; Pierpaoli and Basser, 1996; Beaulieu, 2002). Cross-sectional studies of ORs following ON have demonstrated (i) reduced connectivity metrics in the ORs after ON compared with healthy controls (Ciccarelli *et al.*, 2005), (ii) increased tissue damage in the ORs, reflected by decreased fractional anisotropy (FA) and axial diffusivity (AD), and increased radial diffusivity (RD), after ON, compared with healthy controls (Reich *et al.*, 2009; Kolbe *et al.*, 2012; Balk *et al.*, 2015) (iii) that MS patients with smaller retinal nerve fibre layer (RNFL) thickness had greater damage in the ORs as shown by lower FA and greater RD, suggestive of trans-synaptic degeneration (Reich *et al.*, 2009); (iv) that MS patients with ON have more OR abnormalities than MS patients without a history of ON (Rocca *et al.*, 2013; Gabilondo *et al.*, 2014). These findings could be interpreted as reflecting a trans-synaptic degenerative process, where impaired synaptic input to lateral geniculate nucleus (LGN) neurons leads to secondary pathology in the ORs, although the data collected (analyses performed) have been cross-sectional hence the inferences made have been indirect.

In order to better assess trans-synaptic degeneration, a longitudinal analysis is required after an incident ON to track the OR changes over time. We therefore conducted a prospective study of a cohort of acute ON patients followed up over one year with MRI and addressed three aims: 1) To characterize the longitudinal diffusion properties in the ORs after acute ON over one year; 2) To determine whether earlier ON structure characteristics predicts later OR diffusion changes, suggestive of a trans-synaptic lag effect; 3) To investigate how ON and OR MRI metrics correlate with visual function one year after the ON.

We hypothesized that changes in the diffusion properties of the ORs can be predicted by changes in earlier optic nerve structure and can relate to clinical measures of visual function.

Methods

Subjects

Subjects with their first episode of acute unilateral ON and no previous neurological history were consecutively recruited from Moorfields Eye Hospital. Healthy volunteers were also recruited. Participants whose scans showed incidental inflammatory lesions after recruitment were included. Assessments were performed for each subject at baseline (within one month of onset of ON), 3, 6 and 12 months later. All subjects underwent MRI; ON patients additionally had visual function assessments. Also, in ON patients, the presence of a second relapse suggestive of MS (but not the topography of the second relapse) during the 12-month follow-up was recorded. The local Ethics Committee approved the study and all subjects provided informed written consent.

Visual function

A retroilluminated Early Treatment Diabetic Retinopathy Study (ETDRS) Chart was used to measure best corrected visual acuity, recorded as the 4m logarithm of the minimum angle of resolution (logMAR). Poorer visual acuity is reflected by higher logMAR scores. When no letters could be correctly identified, a score of 1.7 was assigned. The Humphrey automated field analyser was used to assess visual fields using the 30-2 programme (Allergan-Humphrey Inc., San Leandro, Ca., USA). The global visual field mean deviation (HMD) was calculated. More negative error scores indicate worse vision. Colour vision (CV) was measured using the Farnsworth-Munsell 100-hue test (Farnsworth, 1943), and scored as the square root of the error score. If the visual acuity was too poor to attempt the test, an error score of 36.6 was assigned (Optic Neuritis Study Group, 1991). Higher error scores indicate worse vision. Low contrast acuity was assessed using Sloan charts (Ferris *et al.*, 1982), at

25%, 5% and 1.25% contrast. The scoring system was identical to standard logMAR scoring, with higher scores indicating worse vision (Optic Neuritis Study Group, 1991).

MRI studies

MRI data were acquired using a 1.5T GE Signa Echospeed MRI (Milwaukee, WI) scanner with maximum gradient strength of 33mTm^{-1}. All individuals were scanned at baseline, three months, six months and at 12 months.

The MRI protocol was the follow-up study of Jenkins et al (Jenkins *et al.*, 2011), where baseline data were reported. For the present study, we processed the images corresponding to three-month, six-month, and 12-month follow-up time points. The protocol included:

Optic nerve structural scans:

1) Coronal-oblique proton density (PD) fast spin-echo sequence (repetition time [TR] = 2300ms, echo time [TE] = 68ms, 2 excitations, echo train length = 8, matrix size = 512 × 384, field of view [FOV] = 24 × 18cm, 16 contiguous 3mm slices).

2) Coronal-oblique fluid-attenuated inversion-recovery (FLAIR) imaging (TR = 2500ms, TE = 12.7ms, inversion time [TI] = 995ms, 6 excitations, echo train length = 6, matrix size = 512 × 384, FOV = 24 × 18cm, 16 contiguous 3mm slices).

3) Post-triple-dose gadolinium-enhanced coronal-oblique fat-saturated T1-weighted spin-echo was acquired (TR = 600ms, TE = 20ms, 1 excitation, matrix size = 256 × 192, FOV = 24 × 18cm, 16 contiguous 3mm slices).

Optic nerve images were processed to obtain:

1) Lesion length (only in patients, at all time points), by an experienced (and blinded) neuroradiologist (K.M.).

2) Optic nerve cross-sectional area (all individuals, at all time points), from five contiguous slices anterior from the orbital apex (Hickman *et al.*, 2001), using a semiautomated contouring technique (Plummer, 1992), by a blinded observer (T.J.), as a marker of optic nerve axonal loss (Trip *et al.*, 2006).

3) Gadolinium-enhanced lesion length (only in patients and only at baseline)

Brain structural and functional scans:

1) Visual fMRI task-dependent scans acquired in four scanning sessions, as previously described (Jenkins *et al.*, 2010).

2) Diffusion-weighted imaging scans of the optic radiations and occipital lobes. We used an optimized single-shot, cardiac-gated, diffusion-weighted echo-planar imaging sequence, as previously described (Jenkins *et al.*, 2010, 2010b, 2011). Diffusion-weighting gradients were applied along 61 distributed directions (Cook *et al.*, 2007), with b = 1200sec/mm^2, which was optimized for white matter. Seven interleaved non-diffusion-weighted b0 scans were also acquired. One additional b0 volume was acquired, covering the whole brain to assist co-registration of partial brain diffusion data to whole-brain fMRI data (see below), which was necessary for tractography. Head motion– and eddy-current–induced distortions were corrected and the diffusion tensor was then calculated on a pixel-by-pixel basis, using FSL tools (http://www.fmrib.ox.ac.uk/fsl).

3) Axial oblique, proton-density (PD), dual echo, fast spin echo of the whole brain, as previously described (Jenkins *et al.*, 2010).

Processing of OR images was as follows:

1) Diffusion-weighted images: the ORs were reconstructed using the FSL probabilistic tractography algorithm (http://www.fmrib.ox.ac.uk/fsl/fdt/fdt_probtrackx.html). First level fMRI contrast images for all subjects were combined and two spherical regions of interest (ROIs) of 3.5mm radius were created centred on the global maximal coordinates for each LGN (MNI coordinates: right 22 -24 -4 left -22-26 -4). ROIs underwent normalisation to each subject's native space, co-registration to each subject's partial brain diffusion data, via the whole brain b0 intermediate step, and binarisation. These ROIs were then moved in each subject's native diffusion space to sit 8-10 voxels laterally within the apex of Meyer's loops. Visual confirmation that they were placed correctly was performed in each case and probabilistic tractography was performed from these seed points (Jenkins *et al.*, 2010). The reason why fMRI seeds were averaged at the group level was that signal at the individual level was considered too variable, and tractography results would have been unreliable. The mean fractional anisotropy (FA), axial and radial diffusivities (AD and RD) within the tractography-derived tract were obtained for each side, in each subject, at each time-point. Since optic nerve fibres approximately hemi-decussate at the optic chiasm, DTI metrics for left and right OR sides were averaged, after checking there were no significant differences between right and left hemispheres (data not shown).

2) PD images: we used these images to obtain the whole brain and OR lesion load.

3) Visual fMRI task-dependent scans were used to define the seed points needed for tractography of the ORs.

Statistical analysis

There were three stages of analysis:

(1) Evaluation of differences between patients and controls in optic radiations' DTI and optic nerve measures, at baseline and over time:

Differences at baseline: these were examined using multiple linear regression of the DTI or optic nerve variables as response variables, with a group indicator, age, and gender as covariates.

Longitudinal differences over time: changes over time were examined with the measures of interest as response variables in linear mixed models with group, time and group x time interaction as explanatory variables, and with age and gender covariates. The possibility of non-linear trajectories was examined by including a quadratic term in time with corresponding group interaction. For changes in length of optic nerve lesion, OR lesion load at baseline was added as covariate.

(2) Analysis of the relationship between early optic nerve variables and later OR variables. Multivariate multiple regression was used to simultaneously regress those OR DTI variables that had shown change over time on baseline optic nerve area of affected eye, PD-lesion length or gadolinium-enhanced lesion length, entered singly, with age, gender, baseline DTI and OR lesion load as covariates. Models with baseline optic nerve area as explanatory variable were also adjusted for optic nerve area of the unaffected eye. Unless otherwise stated, all results refer to models adjusted for these covariates. Two advantages of the multivariate regression were i) the possibility of a single joint test for an optic nerve predictor to reduce multiple testing: only a significant single joint test, with associations all in the same direction, was taken as evidence that the optic nerve predictor was associated with overall damage to the OR measures. ii) Under missing-at-random (MAR) and multivariate normality assumptions, all available data points contributed to the model to reduce the potential bias from exclusion of patients without complete data on all variables.

Similar multivariate models were fitted for individual optic nerve variables measured at three and six months after the optic neuritis (also adjusting for age, gender, OR lesion load at three or six months, respectively, and clinical variables also at three or six months) to determine temporal associations with OR DTI variables at one year.

(3) To investigate how optic nerve and OR MRI metrics correlate with visual function, we performed two types of analyses, to assess:

i) The relationship between early optic nerve variables and visual function one year after the optic neuritis. Multivariate regression models similar to those described above (2) were fitted. All one-year visual measures (i.e. LogMAR, Humphrey, Colour vision, Sloan charts at 1.25%, 5%, and 25%) were response variables in simultaneously fitted regression models with baseline optic nerve explanatory variables entered singly; and with the following covariates: age, gender, baseline OR lesion load, and baseline values of the visual response variable. Unless otherwise stated, all results refer to models adjusted for these covariates. Joint tests similar to those in (2) were performed to reduce multiple testing. Similar models were fitted for optic nerve explanatory variables measured at three and six months after the optic neuritis. For these models, age, gender, OR lesion load at three or six months respectively, depending on the model, and visual response variables at three or six months were used as covariates.

ii) Cross-sectional associations between optic nerve, optic radiation and visual function variables at one year. Multivariate regression models (again similar to those described above) were fitted. All one-year visual measures (i.e. LogMAR, Humphrey, Colour vision, Sloan charts at 1.25%, 5%, and 25%) were response variables in simultaneous regressions

with one-year optic nerve and OR variables entered singly as explanatory variables. Models were adjusted for age and gender. OR lesion load at one year and visual function at baseline were explored as covariates.

Statistical evidence was considered when p-value was < 0.05. Statistical analysis was conducted using Stata 13.1 (Stata Corporation, College Station, Texas, USA). For the multivariate regressions the SEM (structural equation model) command was used with unstructured covariance and maximum likelihood with missing values.

Results

Twenty-eight ON patients (23 females) with mean (standard deviation (SD)) age of 32.04 (6.53) years, and ten healthy controls (8 females) with mean (SD) age of 30.90 (3.11) years were recruited. Median duration from symptom onset was 22 days (range 7-34). Only 25 patients (20 females) and 8 controls (6 females) had longitudinal data.

1) Differences between patients and controls at baseline and over time

After optic neuritis, the OR FA decreased and RD increased over one year. Patients' affected optic nerve areas also decreased over time.

Tables 1 and **2** show the main characteristics of optic radiations and optic nerve variables at baseline and at each time point, in patients and controls.

i) Differences at baseline

There were no significant adjusted differences between patients' and controls' mean FA, RD, and AD in ORs: adjusted differences, i.e. patients' *minus* controls' measures, (95% CI) were: for FA: 0.007 (-0.021, 0.036), p=0.595; RD: $0.141 \cdot 10^{-4}$ mm^2/s ($-0.270 \cdot 10^{-4}$, $0.552 \cdot 10^{-4}$), p=0.488 for RD; and AD: $0.402 \cdot 10^{-4}$ mm^2/s ($-9.492 \cdot 10^{-4}$, $10.296 \cdot 10^{-4}$), p=0.934.

Patients' affected optic nerve areas were larger than controls' areas: adjusted difference (95% CI): 1.801mm^2 (0.097, 3.505), p=0.039; patients' unaffected optic nerve areas were slightly and non-significantly smaller than controls' optic nerve areas by -0.488 mm^2 (-2.192, 1.216), p=0.569; patients' affected optic nerve areas were larger than patients' unaffected nerve areas by 2.289 mm^2 (1.060, 3.517), p<0.001.

ii) Differences over time

For the ORs, average FA significantly decreased in patients over time (monthly rate of change [95% CI]: $-1.483 \cdot 10^{-3}$ FA units/month [$-2.714 \cdot 10^{-3}$, $-0.251 \cdot 10^{-3}$], p=0.018), whereas no significant longitudinal change occurred in controls ($0.278 \cdot 10^{-3}$ [$-1.457 \cdot 10^{-3}$, $2.013 \cdot 10^{-3}$], p=0.754), although patient vs. control rate difference did not reach statistical significance (p=0.105) (**Figure 1A**). Average RD significantly increased in patients ($0.031 \cdot 10^{-4}$ mm^2/s/month [$0.012 \cdot 10^{-4}$, $0.049 \cdot 10^{-4}$], p=0.001), whereas no significant longitudinal change occurred in controls ($-0.008 \cdot 10^{-4}$ mm^2/s/month [$-0.035 \cdot 10^{-4}$, $0.018 \cdot 10^{-4}$], p=0.549). Additionally, for RD, the rate of change was significantly more positive in patients than in controls by $0.039 \cdot 10^{-4}$ mm^2/s/month ($0.006 \cdot 10^{-4}$, $0.071 \cdot 10^{-4}$), p=0.019 (**Figure 1B**). There was no evidence of non-linear change over time for FA or RD. Finally, average AD in the OR did not significantly change in patients ($0.014 \cdot 10^{-4}$ mm^2/s/month [$-0.005 \cdot 10^{-4}$, $0.034 \cdot 10^{-6}$], p=0.155) or in controls ($-0.003 \cdot 10^{-4}$ mm^2/s/month [$-0.033 \cdot 10^{-4}$, $0.026 \cdot 10^{-4}$], p=0.819) (**Figure 1C**).

In patients, lesion load in the ORs (sum of left and right ORs) significantly increased over time (4.060 mm^3/month [1.566, 6.554], p=0.001). For that reason, similar longitudinal (mixed-effects) models were created but adjusting for lesion load at each time point, not just at baseline. The results were similar to those reported when adjusting for baseline OR lesion load (**Supplementary table 1**).

There was evidence of a non-linear decrease over time in the affected optic nerve area, with the rate of change levelling off from 6 to 12 months (regression coefficients (RCs) [95% CI]: -0.563 mm^2/month [-0.779, -0.348], p<0.001; and 0.026 $mm^2/month^2$ [0.009, 0.043], p=0.002) (**Figure 2A**). The unaffected optic nerve area in patients or optic nerve area of controls did not significantly change over time, either in a linear (RCs [95% CI]: 0.046 mm^2/month [-0.170, 0.261], p=0.678; and 0.084 mm^2/month [-0.279, 0.446], p=0.651, respectively) or in a non-linear way (p=0.956 and p=0.905, respectively). Optic nerve PD-lesion length significantly increased over time in patients (RC [95% CI]: 1.598 mm/month [0.795, 2.402], p<0.001) and there was evidence of a negative non-linear component (RC [95% CI]: -0.066 $mm/month^2$ [-0.129, -0.002], p=0.042), i.e. the increase in change of lesion size slowed down towards the end of the follow-up (**Figure 2B**).

Finally, as expected, in patients, all visual outcomes of the affected eye improved over time, most of them showed a non-linear profile: a rapid initial improvement was followed by a plateau (**Supplementary table 2**).

During follow-up, 11 patients had a second relapse (9 women; mean age [SD]: 32.273 years [7.964]; median OR lesion load at baseline [range]: 102.8 mm^3 [0 to 494.8]), whereas 13

patients did not (11 women; mean age [SD]: 30.846 years [5.145]; median OR lesion load at baseline [range]: 0 mm^3 [0 to 107.2]). In 4 patients, clinical follow-up information was not available. There were no differences in OR FA, RD, or AD, between those who presented a second MS relapse during the follow-up and those who remained as clinically isolated syndromes, either at baseline (p-values = 0.514 (FA), 0.093 (RD), and 0.883 (AD)) or at one-year follow-up (p-values = 0.121 (FA), 0.148 (RD), 0.510 (AD)).

2) Predicting late OR DTI metrics from earlier optic nerve measures

Greater optic nerve atrophy three months after optic neuritis predicted lower OR FA and higher OR RD at one-year

OR FA and RD were entered into the multivariate analysis but not OR AD as this did not show significant change over time. Smaller optic nerve area of the affected eye at three months predicted greater damage in the ORs at one year, i.e. lower FA (RC [95% CI]: 0.535 FA units/mm^2 of optic nerve area [0.207, 0.863], p=0.001) and greater RD (RC [95% CI]: -0.401 $mm^2/s/mm^2$ of optic nerve area [-0.762, -0.039], p=0.030), after one year of having developed the ON (joint test p=0.0069).

Although at six months of follow-up smaller optic nerve areas of the affected eye implied lower FA and greater RD at one-year follow-up, these associations were not significant (joint test p=0.5996). None of the inflammatory parameters of the optic nerve predicted damage in the optic radiations after one year of the ON. **Table 3** shows the standardised regression coefficients obtained from multivariate analyses.

3) Prediction of twelve-month visual outcome

Longer gadolinium-enhanced optic nerve lesion length at baseline predicted worse clinical outcome at one-year after optic neuritis

Table 4 shows the clinical measures at all time points, in patients.

i) Prediction of one-year visual function by earlier optic nerve variables

A longer gadolinium-enhanced optic nerve lesion at baseline predicted worse overall clinical outcomes (for LogMAR, Humphrey, colour vision, Sloan charts at 1.25%, 5%, and 25%) at one-year follow-up (joint test p=0.0386). The standardised regression coefficients are shown in **Table 5**. Greater optic nerve PD-lesion length of the affected side at three and six months predicted worse clinical outcome (in LogMAR, Humphrey, colour vision, and Sloan charts at 1.25%, 5%, and 25%) at one-year follow-up, after adjusting only for age and gender (joint test p=0.0005 and p=0.0292, respectively). However, these associations became non-significant (joint test p=0.1591 and p=0.4042, respectively) after adjusting for clinical measures and OR lesion load at the time point of the predictor (see **Table 5**). Finally, for all these models, being female was associated with better visual function at one year (joint test p<0.001), and worse visual function at baseline did not predict worse overall visual function at one-year follow-up (joint test p=0.1921). Age was not associated with visual function at one-year follow-up (**Supplementary table 3** shows the standardised regression coefficients for visual function at baseline, age and gender in models with only these predictors).

ii) Associations between optic nerve, OR variables and visual outcomes one year after ON

At twelve months after ON, greater optic nerve PD-lesion lengths were associated with worse overall clinical outcome (p=0.0338). No OR DTI metric was significantly associated with visual outcome at one year (**Table 6**).

Discussion

In this longitudinal study, trans-synaptic degeneration occurring after an acute inflammatory insult was investigated by assessing microstructural changes in the ORs over one year following first episode acute ON. Our findings provide strong evidence of anterograde trans-synaptic degeneration in the visual pathway after ON because a) OR FA and RD demonstrate longitudinal degenerative changes over 12 months, even after adjusting for OR lesion load, and b) after the acute optic nerve insult, smaller affected optic nerves at three months predict twelve month OR FA and RD in a manner consistent with a trans-synaptic lag effect, again after adjusting for OR lesion volume. We also found that better visual recovery after ON was associated with milder early inflammation of the optic nerve, whereas early optic nerve atrophy or greater OR damage were not associated with the extent of visual recovery. We will now discuss each of these findings and their implications in more detail.

After ON, there is progressive damage to the ORs that is independent of visible inflammation

This study shows that after ON there is progressive decrease in FA and increase in RD in the ORs that is independent of the presence of visible lesions in the same brain region.

FA reflects the degree of water diffusion anisotropy of a given tissue, that is, the degree of diffusion along the main direction of diffusion (Pierpaoli and Basser, 1996). In our study, a progressive decrease in OR FA implied that after the ON there was an increase in the ability of water molecules to diffuse in all spatial directions, instead of being restricted by the natural tissue boundaries such as axonal structures. This denotes progressive tissue damage in the ORs, as suggested by different ex-vivo studies, where reduced FA can be associated with both demyelination (Mottershead *et al.*, 2003; van Hecke *et al.*, 2009; Zollinger *et al.*, 2011), and axonal loss (Renoux *et al.*, 2006). These results were supported by the presence of a progressive increase in RD in ON patients, implying a gradual increase in the ability of water molecules to diffuse across axonal fibres, reflecting either demyelination (Klawiter *et al.*, 2011; Zollinger *et al.*, 2011) or axonal damage (DeBoy *et al.*, 2007; Klawiter *et al.*, 2011). Previous studies have also reported increases in OR RD in demyelination/ON (Reich *et al.*, 2009; Li *et al.*, 2011; Kolbe *et al.*, 2012; Rocca *et al.*, 2013). Of note, the estimated rates of change in diffusion properties of the ORs and the difference between patients and controls in these rates of change remained unaltered after adjusting for OR lesion load, making it very unlikely that OR lesions have a significant biological influence. Hence, our reported changes in OR FA and RD probably reflect true microstructural changes most likely due to anterograde trans-synaptic degeneration, which could be one of the pathological substrates of the damage in normal-appearing brain tissue, at very early stages of MS (Fernando *et al.*, 2005). In fact, although there may be an association between greater visible inflammation and more important damage in the normal-appearing brain tissue, this damage cannot only be explained by visible lesions, implying that other degenerative mechanisms are probably involved (Fernando *et al.*, 2005).

Trans-synaptic degeneration has been proposed as a mechanism of axonal and neuronal damage in many diseases of the CNS, including MS (Rocca *et al.*, 2013). It results from deafferentation across a synapse (Ghetti *et al.*, 1972; Ghetti and Wisniewski, 1972), and histopathological studies have shown that after the damage (or destruction) of pre-synaptic neuron, both pre-synaptic axon terminal and post-synaptic dendrite are engulfed by astrocytes (Ghetti and Wisniewski, 1972). Following this, myelinated post-synaptic axons start to show Wallerian degeneration (Ghetti *et al.*, 1972). The observed longitudinal changes in OR FA and RD in our ON patients are consistent with this mechanism.

After ON we did not observe significant changes in OR AD over time. This is consistent with some previous studies (Li *et al.*, 2011; Raz *et al.*, 2015) but not others, which have reported increases in OR AD (Reich *et al.*, 2009). Both increases (Reich *et al.*, 2009; Zollinger *et al.*, 2011) and decreases (DeBoy *et al.*, 2007; Kolbe *et al.*, 2012) for AD in demyelination have been reported, hence an overall change in AD may reflect a balance between competing influences making pathological inferences difficult to interpret. Also, previous studies have tended to be cross-sectional, performed in patients with established MS and/or ON. As a result, OR AD measurements may have been influenced by other, more chronic factors related to the underlying disease that would not have had time to affect the measurements in our study of incident ON. Additionally, it should be noted that the diffusion tensor (DT) is an approximation of the complex microstructure determining MR signal in the presence of diffusion weighting. Therefore it is possible that the DT does not capture damage along and across the main fibres of the ORs because the same damage blurs the true directions of the fibres (Wheeler-Kingshott and Cercignani, 2009; Wheeler-Kingshott *et al.*, 2012). Another explanation for the non-significant AD change in our study could be simply lack of power due to inadequate sample size or measurement noise.

Evidence of early optic nerve atrophy after ON predicts more marked diffusion changes in the ORs independent of visible inflammation

The proposed trans-synaptic mechanism of brain damage after ON is reinforced by the finding, in patients, of an association between smaller optic nerve areas of affected eyes three months after ON, and greater damage in the ORs one year after the ON, reflected by a greater decrease in FA, and greater increase in RD. At baseline assessment, the affected optic nerve areas were significantly larger than the unaffected and controls' ones, explained by acute optic nerve swelling secondary to inflammation. As inflammation subsides, the affected optic nerve areas experienced an initial reduction in size –that slowed down in later months– and by twelve months the affected optic nerve areas were smaller than the unaffected and control eyes, implying neuroaxonal loss or optic nerve atrophy (Trip *et al.*, 2006). ON patients with greater optic nerve axonal loss at three months (relatively early after the acute insult) developed greater damage in the ORs at 12 months, supporting the presence of trans-synaptic degeneration. Importantly, these associations were present after adjusting for the OR lesion load, again suggesting a degree of independence between visible white matter inflammation and microstructural changes in the ORs after the ON.

Damage in optic radiations after ON suggesting anterograde trans-synaptic degeneration has been described by some authors (Ciccarelli *et al.*, 2005; Korsholm *et al.*, 2007; Kolbe *et al.*, 2012), but not others. A recent cross-sectional study did not show any correlation between RNFL thickness and OR damage in a cohort of 17 ON patients (Raz *et al.*, 2015). Notably, our longitudinal study conveys a temporal association between optic nerve and OR structure further supporting trans-synaptic degeneration.

Better visual recovery after ON correlates with milder early inflammation of the optic nerve

Longer baseline gadolinium-enhanced lesion length was associated with poorer visual function at twelve months, adjusting for OR lesion volume, age, gender, and baseline visual scores. Also, longer optic nerve lesions (measured on proton density MRI) at three and six months also predicted worse overall clinical outcome at one year, although this became non-significant when adjusting for visual scores and OR lesion load, at both three and six months. Finally, at one-year follow-up greater optic nerve PD-lesion length, probably reflecting greater local secondary degeneration, of either Wallerian or retrograde type, was associated with worse overall clinical outcome, after adjusting for age, gender, and OR lesion load. These results are consistent with previous studies and reflect that the immediate mechanism of visual impairment after an ON and its recovery are mainly related to local changes within the optic nerve, such as swelling, which may compress optic nerve axons (Hickman *et al.*, 2004). Thus, those patients with smaller PD-lesion lengths at one year also had better visual outcomes. Remarkably, those patients with smaller optic nerve areas at any time of the follow-up, though, did not have a worse visual outcome, in line with previous studies that suggested the presence of redundancy of tissue or remodelling of function within the optic nerve (Hickman *et al.*, 2004). Of note, in a previous analysis of our data, when a more conventional regression-type statistical approach was conducted, we did not find a significant association between acute structural measures of the optic nerve and visual function at twelve months (Jenkins *et al.*, 2010b). In the present study, we adopted a multivariate approach with a multiple imputation method to account for missing data. Although multivariate approaches are generally more conservative and only the most robust associations are detected, the fact

that they can also account for missing data can increase the power to detect significant associations (Abdel-Aziz *et al.*, 2015). This probably explains the discrepancy between the two analyses.

In our study, none of the OR metrics were significantly associated with visual outcomes at one year. Other authors have reported some correlations between OR changes and visual function after ON (Reich *et al.*, 2009). However, these correlations had been observed in patients with established MS, probably reflecting a longstanding impact of OR damage on visual function (Reich *et al.*, 2009). Instead, our study was carried out in a population selected for having recently suffered an acute ON as CIS, and therefore with practically no disease duration, meaning that a hypothetical impact of ORs on visual function might not have had enough time to develop. Besides, the results are not consistent across studies (Kolbe *et al.*, 2012), and the role on visual function of post-geniculate nucleus damage following ON needs still to be confirmed.

Neither poorer vision at baseline nor age predicted worse overall visual outcome at one year (as shown in **supplementary table 3**). Interestingly, males showed a clear poorer overall visual outcome at one-year follow-up, as previously described (Costello *et al.*, 2012; Malik *et al.*, 2014; Costello *et al.*, 2015). However, further research is required to determine the causes of this gender difference.

In our study, 11 patients converted to clinically definite MS during the follow-up, whereas 13 patients did not. Remarkably, in spite of those who presented a second relapse having higher OR lesion loads at baseline than those who did not, the baseline values of the diffusion metrics in the ORs were very similar for the two groups. This not only suggests a relative

independence of visible inflammation and diffusivity metrics, but also reinforces the main message of our paper, where we show that over time there is a progressive damage in the ORs that seems to be independent of visible inflammation, and which seems to be reflecting a trans-synaptic degeneration.

Although our study has a longitudinal design that allowed us to model the evolution of changes over time in the ORs and determine their associations with anterior visual pathway structure, there are additional points to consider. We did not apply lesion masks to the ORs when obtaining the OR diffusion metrics. Of note, when we designed this study, we thought that the assessment of the parts of the WM tracts where lesions were nested could also be of interest, since they could also provide important information on tissue microstructure. Additionally, even if we had masked the lesions, we would still not have been able to exclude the local effect of visible lesions on the optic radiations through Wallerian degeneration.

However, in order to ensure that our results were not just driven by OR lesions, we adjusted for the OR lesion loads, as performed by other authors (Balk *et al*., 2015), at baseline and over the follow-up period. Both the change in OR diffusion metrics in ON patients over time and the association between smaller optic nerve at three months and greater OR damage at one-year follow-up remained significant. We also carried out post-hoc analyses in which we re-ran the models described in the first stage of our study but excluded patients with highest OR lesion loads. Namely, we re-ran these models excluding patients with OR lesion loads equal to or greater than 392 mL (90^{th} percentile of OR lesion load at 12 months), and in a separate model excluding patients with OR lesion loads equal to or greater than 172 mL (75^{th} percentile of OR lesion load at 12 months). In these two analyses, patients –unlike controls– again showed a significant decrease in FA and a significant increase in RD, as with the

original analysis of the whole cohort. In addition, neither the direction nor the magnitude of the regression coefficients for FA and RD changed substantially (data not shown). Similarly, when we re-ran the models of the second stage of our analysis using only these sub-samples with lower OR lesion loads, the results still remained significant, i.e. smaller optic nerve areas at three months significantly predicted lower OR FA and higher OR RD at one-year follow-up (data not shown).

Besides, as other authors have also shown, in our study the proportion of the tract volume occupied by lesions was very small, i.e. less than 3%, meaning that the potential impact of these lesions on the diffusion measures was probably negligible. In fact, at baseline, where some of the ON patients already showed white matter lesions in the ORs, the values of OR FA, RD, and AD were very similar in patients and controls (none of the comparisons were statistically significant), again implying that the impact of lesions on the averaged OR diffusivity metrics was minimal.

Finally, even if the results of the first stage of our analysis, i.e. the presence of changes in OR diffusion metrics over time after ON, could potentially have been influenced by OR lesional diffusivity or could have reflected a global progressive neurodegenerative phenomenon, the results of the second part of the analysis, i.e. smaller optic nerve areas predict worse OR FA and RD independently of OR lesion load, still provide robust longitudinal evidence for anterograde trans-synaptic degeneration. Of course, it may well be that newer diffusion related techniques such as Neurite Orientation Dispersion and Density Imaging (NODDI) (Zhang *et al.*, 2012; Grussu *et al.*, 2015) will provide better understanding of microstructural changes underlying brain damage. Future studies using this and other techniques highly sensitive to tissue microstructure, and focusing on not only the ORs but also on other white

matter tracts not related to visual system, could further improve our knowledge of trans-synaptic degeneration.

Furthermore, our multivariate analysis assumes that the directional characteristics of the dependent variables behave in a biologically congruent manner. This is likely to be appropriate for both the DTI metrics, where FA and RD are known to reflect two aspects of the same process, i.e. the diffusion of water molecules in a given tissue, and the clinical variables, which improved over time in a similar non-linear way (**Supplementary table 2**). This suggests that our (conservative) approach of considering as significant only those associations that showed a similar biological direction was appropriate. With this approach, the risk of type I error was smaller than adopting other statistical approaches, hence ensuring that only the most robust associations were reported.

In conclusion, our study provides longitudinal evidence of damage occurring in the ORs following ON, which is independent of white matter inflammation and more pronounced in patients with signs of post-acute optic nerve atrophy. This is strongly supportive of anterograde trans-synaptic degeneration. The lack of association between OR structural metrics and visual outcomes implies that local optic nerve factors played a greater role in this cohort, and perhaps for this relationship our study was underpowered. Future studies could investigate the clinical consequences of the post-synaptic changes that occur after ON, and whether they are due to the accumulation of structural brain damage or whether they are better explained by a disruption of brain structural connectivity secondary to axonal damage. Also, future trials using neuroprotective agents could evaluate the downstream effects on OR structure and whether OR damage could be ameliorated by early intervention to prevent optic nerve atrophy.

Table 1. Optic radiation variables at all time points

	Patients				**Controls**			
	Baseline	**3 months**	**6 months**	**12 months**	**Baseline**	**3 months**	**6 months**	**12 months**
N scanned	28	24	22	20	10 (**c**)	8	8	8
Average FA **(a)** *Mean (SD)*	0.361 (0.031)	0.363 (0.034)	0.350 (0.039)	0.350 (0.038)	0.356 (0.035)	0.376 (0.047)	0.365 (0.024)	0.364 (0.028)
Average RD **(a)** [$x10^{-4}$ $mm^2 \cdot s^{-1}$] *Mean (SD)*	6.104 (0.495)	6.167 (0.573)	6.192 (0.522)	6.476 (0.569)	6.003 (0.340)	5.695 (0.504)	5.879 (0.279)	5.814 (0.355)
Average AD **(a)** [$x10^{-4}$ $mm^2 \cdot s^{-1}$] *Mean (SD)*	10.783 (0.672)	10.945 (0.708)	10.675 (0.534)	11.070 (0.776)	10.465 (0.425)	10.023 (0.297)	10.305 (0.387)	10.288 (0.334)
OR lesion load [mm^3] *Mean (SD)* *Median (range)* **(b)**	63.021 (117.159); 3.5 (from 0 to 494.8), N=28	67.925 (120.559); 9.7 (from 0 to 376.1), N=24	120.2 (195.737); 34.75 (from 0 to 812.9), N=22	131.52 (200.417); 43.05 (from 0 to 796.1), N=20	-	-	-	-

(a) Average values from left and right optic radiations; **(b)** Due to OR lesion load being very positively skewed, median (range) is reported instead of mean (SD); (**c**) Although all 10 controls were scanned at baseline, OR data were only available in 8 of them (6 women, 2 men). *Abbreviations*: AD: axial diffusivity; FA: fractional anisotropy; mL: millilitres; mm: millimetres; N: number of individuals; OR: optic radiation; RD: radial diffusivity; s: seconds; SD: standard deviation;

Table 2. Optic nerve variables at all time points in patients

	Patients			
	Baseline	**3 months**	**6 months**	**12 months**
N scanned	28	24	22	20
Area of affected eye [mm^2] *Mean (SD)*	14.629 (3.205)	12.681 (1.704)	12.376 (1.587)	11.296 (1.772)
Area of unaffected eye [mm^2] *Mean (SD)*	12.340 (1.275)	12.705 (1.911)	12.594 (1.841)	12.882 (1.492)
Length of ON lesion [mm] *Mean (SD)*	22.071 (10.367)	25.250 (11.498)	29.045 (8.899)	31.200 (9.356)
Length of Gd enhancement [mm] *Mean (SD)* **(a)**	23.538 (13.327)	-	-	-

(a) The length of the gadolinium enhancement was only assessed at baseline. *Abbreviations*: N: number of individuals; ON: optic nerve; SD: standard deviation.

Table 3. Prediction of optic radiation damage at one-year follow-up with optic nerve measures at different time points

	Dependent variables		**Joint test p-value**
	FA	**RD (mm^2/s)**	
Optic nerve measure (a)	**Standardised regression coefficients (95% CI) from multivariate analyses**		
Affected optic nerve area at baseline	-0.081 (-0.336, 0.174)	-0.147 (-0.513, 0.219)	0.2569
Affected optic nerve area at 3 months	0.535 (0.207, 0.863)	-0.401 (-0.762, -0.039)	0.0069 **(b)**
Affected optic nerve area at 6 months	0.256 (-0.268, 0.779)	-0.347 (-1.243, 0.549)	0.5996
Lesion length of affected optic nerve at baseline	0.218 (-0.077, 0.513)	-0.469 (-0.874, -0.063)	0.1058
Lesion length of affected optic nerve at 3 months	-0.074 (-0.327, 0.179)	0.048 (-0.288, 0.384)	0.7985
Lesion length of affected optic nerve at 6 months	0.117 (-0.128, 0.361)	-0.096 (-0.502, 0.310)	0.5610
Gd-enhanced lesion length of affected optic nerve at baseline	0.161 (-0.126, 0.449)	-0.276 (-0.663, 0.112)	0.3860

(a) The predictors (optic nerve measures) shown in the table were tested individually (one at a time), together with age, gender, and OR lesion load at corresponding time point, either baseline, three-month follow-up, or six-month follow-up; **(b)** This was the only significant result and the effect of the predictor (affected eye's area at 3 months) had effects on the dependent variables that went in the same biological direction. *Abbreviations:* FA: fractional anisotropy; Gd: gadolinium; OR: optic radiation; RD: radial diffusivity; SE: standard error.

Table 4. Clinical variables at all time points in patients

Clinical variables	Time point							
	Baseline		3 months		6 months		12 months	
	N=28		N=24		N=23		N=24	
	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected
LogMAR scores *Mean (SD)*	0.719 (0.694)	-0.049 (0.103)	0.164 (0.351)	-0.032 (0.136)	0.109 (0.252)	-0.040 (0.109)	0.091 (0.220)	-0.051 (0.078)
Humphrey scores *Mean (SD)*	-19.510 (9.672)	-6.096 (1.561)	-8.221 (3.307)	-5.007 (1.750)	-7.849 (2.846)	-5.001 (1.380)	-7.219 (2.515)	-5.110 (1.285)
Colour vision scores *Mean (SD)*	24.354 (9.914)	10.361 (2.852)	15.071 (6.206)	10.750 (2.763)	14.743 (5.893)	10.865 (3.137)	14.221 (4.549)	11.617 (2.459)
Sloan chart at 1.25% scores *Mean (SD)*	1.624 (0.247)	0.697 (0.288)	1.428 (0.381)	0.762 (0.332)	1.268 (0.417)	0.713 (0.280)	1.080 (0.372)	0.684 (0.275)
Sloan chart at 5% scores *Mean (SD)*	1.297 (0.520)	0.364 (0.324)	0.767 (0.441)	0.374 (0.305)	0.632 (0.392)	0.317 (0.192)	0.605 (0.332)	0.333 (0.212)
Sloan chart at 25% scores *Mean (SD)*	0.829 (0.673)	0.051 (0.134)	0.293 (0.353)	0.062 (0.168)	0.244 (0.278)	0.041 (0.129)	0.211 (0.256)	0.061 (0.108)

Abbreviations: SD: standard deviation. See main text for further explanations about visual function scores.

Table 5. Prediction of clinical outcomes at one-year follow-up with optic nerve measures at different time points

	Dependent variables						**Joint test p-value**
	LogMAR	**Humphrey**	**Colour vision**	**Sloan charts at 1.25%**	**Sloan charts at 5%**	**Sloan charts at 25%**	
Optic nerve measure (a)	**Standardised regression coefficients (95% CI) from multivariate analyses**						
Affected optic nerve area at baseline	-0.257 (-0.655, 0.140)	0.039 (-0.418, 0.496)	0.283 (-0.052, 0.617)	0.136 (-0.222, 0.495)	-0.122 (-0.505, 0.260)	-0.186 (-0.582, 0.210)	0.0095 **(b)**
Affected optic nerve area at 3 months	-0.230 (-0.633, 0.173)	0.216 (-0.107, 0.539)	-0.126 (-0.334, 0.083)	0.174 (-0.213, 0.562)	0.098 (-0.308, 0.504)	-0.138 (-0.545, 0.269)	0.0006 **(b)**
Affected optic nerve area at 6 months	-0.020 (-0.193, 0.152)	0.343 (0.013, 0.674)	-0.079 (-0.362, 0.205)	0.136 (-0.200, 0.471)	0.134 (-0.184, 0.452)	0.042 (-0.219, 0.304)	0.2280
Lesion length of affected optic nerve at baseline	-0.116 (-0.506, 0.274)	-0.122 (-0.557, 0.312)	0.268 (-0.183, 0.718)	0.153 (-0.160, 0.466)	0.185 (-0.142, 0.512)	0.022 (-0.341, 0.386)	0.0908
Lesion length of affected optic nerve at 3 months	-0.007 (-0.342, 0.329)	-0.169 (-0.489, 0.152)	0.032 (-0.180, 0.244)	0.133 (-0.188, 0.454)	0.242 (-0.059, 0.544)	0.084 (-0.253, 0.421)	0.1591
Lesion length of affected optic nerve at 6 months	-0.013 (-0.135, 0.109)	-0.071 (-0.311, 0.169)	0.128 (-0.069, 0.324)	0.070 (-0.187, 0.327)	0.089 (-0.157, 0.336)	0.029 (-0.169, 0.227)	0.4042
Gd-enhanced lesion length of affected optic nerve at baseline	0.205 (-0.152, 0.562)	-0.479 (-0.857, -0.102)	0.584 (0.260, 0.907)	0.104 (-0.217, 0.425)	0.164 (-0.167, 0.496)	0.206 (-0.138, 0.551)	0.0386 **(c)**

(a) The predictors (optic nerve measures) shown in the table were tested individually (one at a time), together with age, gender, and visual function and OR lesion load at the same time point of the main predictor (i.e. optic nerve measure), either baseline, three-month follow-up, or six-month follow-up; **(b)** Although the joint test shows a significant result (at 5% significance level, $p<0.05$), the regression coefficients go in different 'biological' directions, so we have not considered this as a significant result; **(c)** Here the joint test shows a significant result and the regression coefficients go in the same biological direction. *Abbreviations:* CI: confidence interval.

Table 6. Associations between optic nerve and optic radiations variables and visual function at one-year follow-up

	Dependent variables						**Joint test p-value**
	LogMAR	**Humphrey**	**Colour vision**	**Sloan charts at 1.25%**	**Sloan charts at 5%**	**Sloan charts at 25%**	
One-year measures (a)	**Standardised regression coefficients (95% CI) from multivariate analyses**						
Affected optic nerve area	-0.080 (-0.718, 0.559)	-0.015 (-0.662, 0.632)	0.154 (-0.471, 0.779)	0.236 (-0.342, 0.815)	0.194 (-0.430, 0.818)	-0.014 (-0.654, 0.626)	0.1410
Lesion length of affected optic nerve	0.0442 (-0.343, 0.432)	-0.372 (-0.739, -0.004)	0.434 (0.098, 0.770)	0.114 (-0.237, 0.464)	0.140 (-0.233, 0.513)	0.069 (-0.310, 0.448)	0.0338 **(b)**
Average FA	-0.281 (-0.980, 0.419)	-0.111 (-0.890, 0.668)	0.034 (-0.992, 1.060)	-0.016 (-0.729, 0.698)	-0.205 (-0.857, 0.448)	-0.174 (-0.856, 0.508)	0.1193
Average RD	0.101 (-0.350, 0.552)	-0.291 (-0.742, 0.161)	0.143 (-0.407, 0.694)	-0.015 (-0.449, 0.418)	0.023 (-0.391, 0.438)	-0.023 (-0.448, 0.402)	0.0004 **(c)**
Average AD	0.181 (-0.546, 0.907)	-0.373 (-1.086, 0.340)	0.313 (-0.494, 1.119)	-0.105 (-0.711, 0.502)	-0.039 (-0.638, 0.560)	0.012 (-0.623, 0.647)	0.2043

(a) The predictors (optic nerve measures) shown in the table were tested individually (one at a time), together with age, gender, and visual function and OR lesion load at the same time point of the main predictor (i.e. optic nerve measure), either baseline, three-month follow-up, or six-month follow-up; **(b)** Here, all the coefficients pointed at the same biological direction and the joint test showed significant result at 5% significance level; **(c)** Although the joint test showed a significant result, since the coefficients pointed at different biological directions, we ignored this result. *Abbreviations:* AD: axial diffusivity; CI: confidence interval; FA: fractional anisotropy; RD: radial diffusivity.

Figure Legends

Figure 1. Changes over time in DTI measures in the optic radiations

Evolution over time of the DTI measures in the optic radiations over one year, after adjusting for OR lesion load at baseline, in patients (grey) and controls (black). In **A**, FA (dimensionless units) significantly decreased over time in patients, whereas it did not change significantly in controls. In **B**, radial diffusivity (mm^2s^{-1} $x10^{-4}$) significantly increased in patients, whereas it did not significantly change in controls. In **C**, axial diffusivity (mm^2s^{-1} $x10^{-4}$) did not significantly change in patients or controls.

Figure 2. Evolution of optic nerve measures over time

Figure 2A shows the evolution over time of optic nerve areas in patients and in controls. As can be seen, optic nerve areas of affected eyes in patients decreased over time in a non-linear manner. Optic nerve areas of unaffected eyes in patients were consistently smaller than those in controls, although the difference did not reach statistical significance. The monthly rates of change in optic nerve areas in controls and in patients' unaffected eyes were not significantly different from zero. Figure 2B shows the evolution over time of optic nerve lesion length in patients. As can be seen, optic nerve lesion length increased over time in a non-linear manner so that there is a levelling off towards the end of follow-up. Abbreviations: SE: standard error.

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