Aus dem Experimental and Clinical Research Center
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

A Multicenter Study on Outer Retinal Layer Changes in Neuromyelitis Optica Spectrum Disorders

Multicenter-Studie zu Veränderungen der äußeren Netzhautschichten in Neuromyelitis Optica Spektrumerkrankungen

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List of Abbreviations

1mm Study	Macular OCT scan with a diameter of 1mm around the fovea
3mm Study	Macular OCT scan with a diameter of 3mm, omitting a 1mm diameter around the fovea
5mm Study	Macular OCT scan with a diameter of 5mm, omitting a 1m diameter around the fovea
AQP4	Aquaporin-4
AQP4-IgG	Aquaporin-4 Immunoglobin G
AQP4-NON	Aquaporin-4 IgG positive eyes without a history of ON
AQP4-ON	Aquaporin-4 IgG positive eyes with a history of ON
B	Estimate
BRB	Blood-Retina Barrier
Cirrus	Cirrus HD-OCT, Carl Zeiss Meditec Inc., Dublin, CA USA
CNS	Central Nervous System
CROCTINO	Collaborative Retrospective Study on retinal optical coherence tomography in Neuromyelitis Optica
EDSS	Expanded Disability Standard Scale
ERG	Electroretinography
ETDRS	Early Treatment in Diabetes Retinopathy Study
GCC	Ganglion Cell Complex
GCIP	Ganglion Cell and Inner Plexiform
HC	Healthy Control
ICC	Intra-class Correlation Coefficient
INL	Inner Nuclear Layer
NMOSD	Neuromyelitis Optica Spectrum Disorders
ON	Optic Neuritis
ONL	Outer Nuclear Layer
OCT	Optical Coherence Tomography
OPL	Outer Plexiform Layer
MOG	Myelin-oligodendrocyte-glycoprotein
MOGAD	MOG Associated Diseases

MOG-IgG	Myelin-oligodendrocyte-glycoprotein Immunoglobulin G
MOG-ON	MOG-IgG positive eyes with a history of ON
mRFNL	Macular Retinal Nerve Fiber Layer
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
PR	Photoreceptor Layer
pRNFL	Peripapillary Retinal Nerve Fiber Layer
RNFL	Retinal Nerve Fiber Layer
RT	Total Retinal Thickness
SE	Standard Error
Spectralis	Spectralis SD-OCT, Heidelberg Engineering, Heidelberg, Germany
Topcon	Topcon 3D-OCT, Topcon Corp., Tokyo Japan

Abstract (English)

Background: Neuromyelitis optica spectrum disorders (NMOSD) is a debilitating autoimmune disease affecting the central nervous system. It often manifests in optic neuritis (ON) which can lead to neuroaxonal damage to the retina. A primary ON-independent astrocytopathy component of the neurodegeneration has been suggested and may contribute to observable changes in the outer layers of the retina. This dissertation will explore this hypothesis in NMOSD patients in a multicentric cross-sectional cohort of patients recruited internationally.

Method: 539 participants from 20 international centers were retrospectively included. Of these, 197 aquaporin-4 (AQP4)-IgG seropositive patients and 32 patients with myelin-oligodendrocyte-glycoprotein associated disease (MOG-IgG+) were enrolled together with 75 healthy controls (HC). All participants underwent optical coherence tomography (OCT) scans of the retina. OCT scans were then subject to central postprocessing, image segmentation and analyses of the various layers, including the retinal nerve fiber layer, ganglion cell and inner plexiform layer, inner nuclear layer, outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane and the Bruch's membrane.

Results: AQP4-IgG+ patients showed no significant change of OPL (25.02±2.03µm) or ONL (61.63±7.04µm) when compared to the MOG-IgG+ diseased group (OPL: 25.10±2.00µm, ONL: 64.71±7.87µm) or HC (OPL: 24.58±1.64µm, ONL: 63.59±5.78µm). AQP4-IgG+ (19.84±5.09µm, p=0.027) and MOG-IgG+ (19.82±4.78µm, p=0.004) eyes with a positive history of ON displayed parafoveal OPL thinning compared with HC (OPL: 20.99±5.14µm). ONL, photoreceptor layer and retinal pigment epithelium did not differ between any of the groups.

Conclusion: Outer retinal layer loss is not a significant and measurable hallmark of APQ4-IgG+ NMOSD. However, we cannot exclude that there are astrocytic changes in the retina of NMOSD patients which do not manifest with changes in retinal layer thickness. Future advanced imaging techniques or post-mortem studies might be able to shed light on this. Furthermore, longitudinal studies are required to investigate whether there are outer retinal layer changes over time.

Abstract (German)

Hintergrund: Neuromyelitis optica Spektrumerkrankungen (NMOSD) sind eine schwerwiegende Autoimmunerkrankung des zentralen Nervensystems. Sie manifestiert sich häufig mit einer Sehnerventzündung (Optikusneuritis, ON), die neuroaxonale Degeneration der Netzhaut zur Folge haben kann. Eine primäre, von der ON unabhängige Astrozytopathie-Komponente der Neurodegeneration wurde vermutet, welche sich durch Veränderungen in den äußeren Netzhautschichten äußern könnte. In dieser Dissertation wird diese Hypothese seropositiven NMOSD Patient*innen in einer internationalen multizentrischen retrospektiven Querschnittskohorte untersucht.

Methode: 539 Teilnehmer*innen aus 20 internationalen Zentren wurden retrospektiv rekrutiert; davon 197 Aquaporin-Antikörper-seropositive AQP4-IgG+-Patient*innen, 32 Patient*innen mit Myelin-Oligodendrozyten-Glykoprotein–Antikörper-assoziierter Erkrankung (MOG-IgG+) und 75 gesunden Kontrollpersonen (HC). Bei allen Teilnehmenden wurden die Netzhaut mit optischer Kohärenztomographie (OCT) untersucht. Die OCT-Scans unterliefen anschließend zentral einer Schichtsegmentierung und -analyse, die die retinale Nervenfaserschicht, die Ganglienzellen und die innere plexiforme Schicht, die innere Kernschicht, die äußere plexiforme Schicht (OPL), die äußere Körnerschicht (ONL), die äußere Grenzmembran und die Bruch'sche Membran umfasste.

Ergebnisse: AQP4-IgG+ Patient*innen zeigten keine signifikante Veränderung der OPL (25,02±2,03µm) oder ONL (61,63±7,04µm) im Vergleich zu der MOG-IgG+ Gruppe (OPL: 25,10±2,00µm, ONL: 64,71±7,87µm) oder zu der HC Gruppe (OPL: 24,58±1,64µm, ONL: 63,59±5,78µm). AQP4-IgG+ (19,84±5,09µm, p=0,027) und MOG-IgG+ (19,82±4,78µm, p=0,004) Augen mit positiver ON-Anamnese zeigten eine parafoveale OPL-Ausdünnung im Vergleich zu der HC Gruppe (OPL: 20,99±5,14µm). Die ONL, die Fotorezeptorenschicht und das retinale Pigmentepithel unterschieden sich in keiner der Gruppen.

Diskussion: Wir konnten keine Verdünnung der äußeren Netzhautschichten bei APQ4-IgG+ NMOSD nachweisen. Allerdings können wir nicht ausschließen, dass es bei NMOSD-Patienten zu astrozytären Veränderungen der Netzhaut kommt, die sich nicht

auf die retinalen Schichtdicken auswirken. Zukünftige fortschrittliche bildgebende Verfahren oder Post-Mortem-Studien könnten hier Licht ins Dunkel bringen. Darüber hinaus sind longitudinale Studien erforderlich, um zu untersuchen, ob Veränderungen der äußeren Netzhautschichten im Verlauf der Erkrankung auftreten.

1. Introduction

1.1 NMOSD and associated diseases

Neuromyelitis Optica Spectrum Disorders (NMOSD), formerly known as Devic's Disease, are a spectrum of relapsing autoimmune disease of the central nervous system (CNS) with clinical syndromes including, but not limited to, optic neuritis (ON), acute transverse myelitis and area postrema syndrome. (1, 2) Historically, the disease was seen as a topographically restricted form of multiple sclerosis (MS) (3) but in the past 18 years the disease has demarcated from this on account of changes in the diagnostic criterion set by the International Panel of NMO Diagnosis. These changes were rooted in the greater accuracy of various serum antibodies tests and MRI imaging pertaining to the optic nerve and spinal cord. (4) Over the past decade the NMO diagnostic criteria was revised to include the presence of serum antibodies to Aquaporin-4 (AQP4; AQP4-IgG), a serological characteristic found in up to 60-80% of NMOSD patients. (5, 6)

AQP4 is a water channel found in many organ systems. (7) In the CNS they are primarily located in the inner neuroaxonal layers of the retina, specifically at the end feet of the astrocytes in the brain, in the Retinal Nerve Fiber Layer (RNFL) and in Müller cells, a type of glial cell. (8, 9) Müller cells are concentrated around the fovea and span the entire depth of the retina; they also undertake various functions such as water and electrolyte homeostasis and neurotransmitter recycling, amongst others. (10) Of particular interest in the outer retinal layer is also the Henle fiber layer, located in the Outer Plexiform Layer (OPL), which is the layer forming the boundary between the bipolar, horizontal and photoreceptor cells. (11) The outer two thirds make up the Henle Fiber layer and consist of the photoreceptor axons encased by the Müller cell processes; it is here where AQP4 water channels are particularly highly concentrated. (9, 11)

Primary and attack independent astrocytopathy (i.e., in the absence of ON) have been hypothesized to underpin neurodegeneration in NMOSDs and contribute to Müller cell associated parafoveal changes. (12-15) Recent experiments using immortalized Müller Cells have shown that in-vivo AQP4 loss reduces Müller Cell proliferation and damages volume homeostasis leading to a non-inflammatory mechanism of retinal injury. (16)

Additionally, inner neuroaxonal damage, particularly the Peripapillary Retinal Nerve Fiber Layer (pRNFL) and the Ganglion Cell and Inner Plexiform (GCIP) Layer thickness, have been observed and studied extensively with the help of spectral domain Optical Coherence Tomography (OCT) (5, 17).

1.2 OCT and the structure of the human retina

OCT employs “low-coherence interferometry to produce two dimensional images of optical scattering from tissue microstructures up to a resolution of 3.9μm”. (18) This technology has matured over the past 30 years finding uses outside of ophthalmology including in the fields of cardiology and dermatology; a detailed description of the workings of OCT is described by Huang et al. (18) Current OCT devices, known as spectral domain OCT, are set up with a fixed reference mirror in which interferometric reflections are simultaneously analysed by Fourier transformation. (19, 20) This allows fast image acquisition, with up to 100,000 A scans per second, and combined with eye tracking to reduce motion artifacts yields far better image quality than earlier OCT devices. (20) Additionally studies have shown a strong correlation between OCT metrics and visual acuity in NMOSD. (21) Therefore OCT imaging remains crucial in both the diagnosis of the disease as well as tracking its disease progression, not only in NMOSD but in other associated neurological diseases such as MS as well. (5, 22)

The non-invasive nature of OCT diagnostics, along with the speed in which images can be taken has allowed it to become a staple in ophthalmological studies, particularly in the study of NMOSD. Given the rarity of the disease (with incidence varying based on ethnic composition: approximately 1 in 100,000 in white Caucasian populations and a slightly higher rate of 3.5 in 100,000 in east Asian populations and 10 in 100,000 in African American populations), (23) multi-centric study designs are employed in order to obtain samples of a significant magnitude. However, not all OCT devices are comparable on account of differences in hardware configuration and software capabilities; scanning resolutions vary amongst devices and each device uses proprietary software to implement intra-retinal segmentation. As the time of writing, there is no standardization in the software and therefore layer thicknesses determined from different devices may not be considered comparable. (24, 25) Additionally, the continued use of various devices across different centers lead to further rigidities in study designs and implementation.

Device longevity presents an issue; if devices need to be upgraded during a study, backward compatibility of data is paramount. Should patients change health care providers, relocate or data sharing takes place amongst centers, continuity also presents challenges when different devices are employed. It is therefore imperative that OCT studies can be reliably compared across multiple centers to overcome these shortcomings. Procedural harmonization is also important in minimizing variability in OCT scans. To that end, the APOSTEL 2.0 recommendations (26) and the OSCAR-IB Criteria (27) impart a certain level of standardization in how scans are to be reported and manually evaluated.

A simplified diagrammatical approximation of the landmarks discernible in OCT scans is set out in Figure 1 below. The RNFL is the inner most of these layers and is made up of unmyelinated axons of the retinal ganglion cells. (10) The GCIP is a combination of the Ganglion Cell layer and the Inner Plexiform layer, as the two are often indistinguishable in OCT scans and show up as hypo-reflective below the relative hyper-reflective RNFL. The GCIP layer contains the cell bodies of the ganglion cells as well as their synapses from the bipolar and amacrine cells of the retina. (28) These three layers are of great concern as they merge and form the optic nerve head exiting the bulbus as the optic nerve itself; structural changes in theses layers have strong clinical correlations associated with ON and visual impairment aberrations. (17, 29)

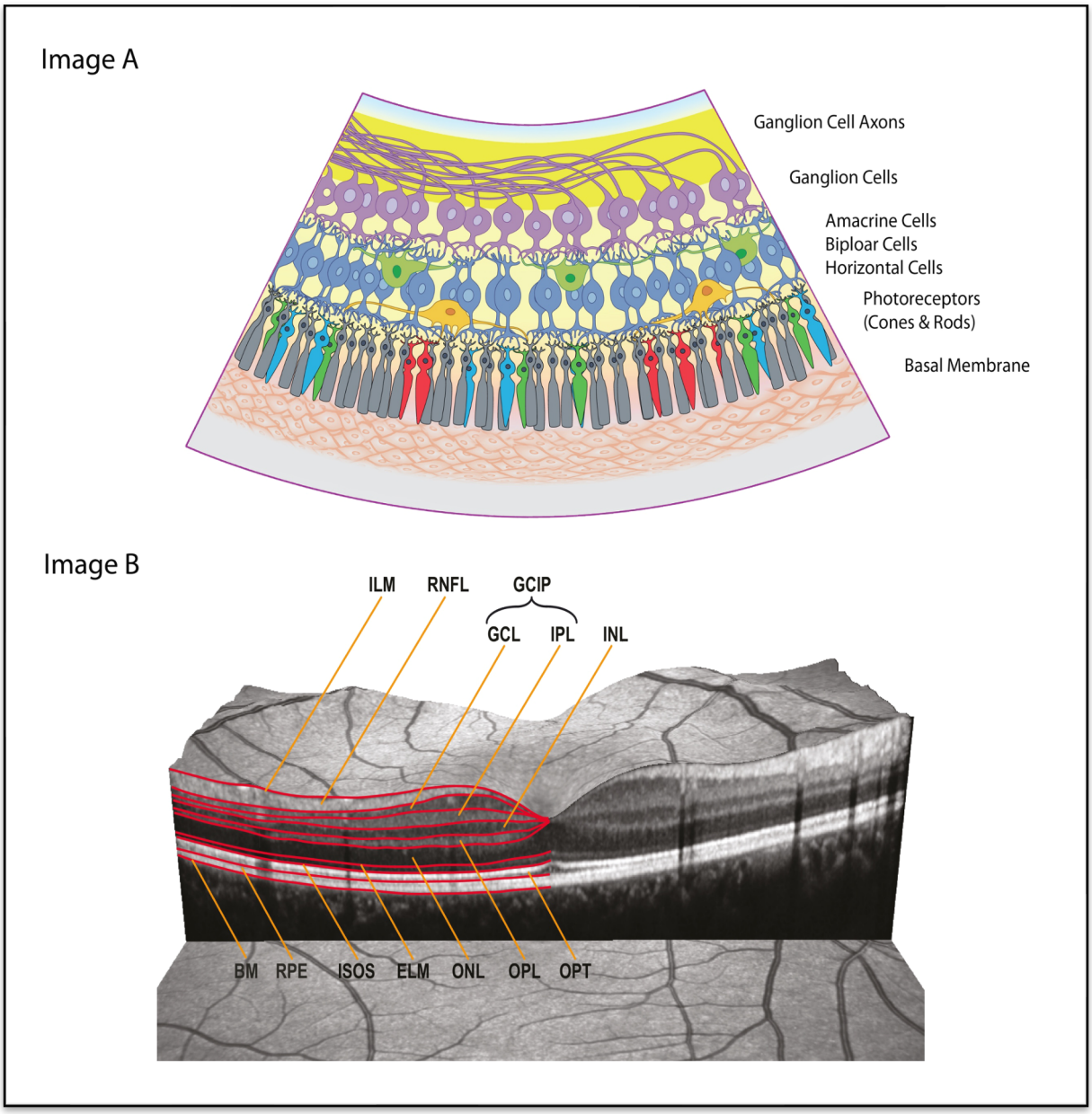

Figure 1: Diagrammatical representation of the human retina

Image A: a simplified representation of the cellular architecture of the retina. Image B: retinal layers as depicted by OCT imaging. Abbreviations: ILM = internal limiting membrane. RNFL = retinal nerve fiber layer, GCIP = ganglion cell and inner plexiform layer (a combination of GCL = ganglion cell layer and IPL = inner plexiform layer). INL = inner nuclear layer. OPL = outer plexiform layer. ONL = outer nuclear layer. ELM = external limiting membrane. OPT = outer photoreceptor tips. ISOS = photoreceptor outer layer / inner layer junction. RPE = retinal pigment epithelium. BM = Bruch‘s membrane. Both images were reproduced under a creative commons attribution 4.0 international license from neurodial.de.

1.3 OCT findings in NMOSD

Recent studies have shown that pRNFL following primary AQP4-IgG induced ON (AQP4-ON) tend to exhibit reductions but this may be masked in the earlier months after such attacks by axonal swelling, thus making diagnosis and quantification of pRNFL loss

difficult; concurrently longitudinal GCIP layer loss has also been observed. (17) The Inner Nuclear Layer (INL) thickness tends to increase following ON, which has been hypothesized to be a result of edema and buildup of macular microcysts, (30) however various studies have proposed contradictory changes in INL thickness in AQP4-ON. (17, 31, 32)

Whilst attack independent astrocytopathy has been observed, studies on these have been contradictory as to where in the retina these take place. Whilst some studies noted that no such changes take place in the pRNFL, mRFNL and GCIP layers (33, 34) others did reveal reductions in mRNFL, GCIP and total retinal thickness (12, 35)

In the outer retinal layers, however, it remains to be seen if similar changes are identifiable and corroborate with clinical changes. To that end, recent studies published by You et al in 2019 (9) and Filippatou et al 2020 (14) have indicated this to be the case, whereby astrocytopathy related outer retinal degeneration in AQP4-IgG seropositive cohorts were observed. (9, 14) However, these studies were limited in their cohort size and homogenous composition and were contradictory in part as to where these changes occur; in You et al, foveal thinning in the Henle Fiber Outer Nuclear Layer as well as the Inner Segment of the photoreceptor layer was observed; You et al also reported a reduction in “b-wave amplitudes in patients with AQP4+ NMOSD in all...scotopic electroretinography” (ERGs), (9) whereas the study published by Filippatou et al described ONL thinning in a 5mm macular scan (excluding fovea). (14)

1.4 MOGAD

Up to 40% of NMOSD patients fulfilling the clinical definition of the disease do not, however, have detectable levels of AQP4-IgG antibodies; in many of these cases, serum levels of Myelin-oligodendrocyte-glycoprotein Immunoglobulin G (MOG; MOG-IgG) are otherwise detectable. (36) MOG is expressed on the outer surface of the oligodendrocytic myelin sheath (32) and MOG-IgG activates cell-mediated and humoral immune demyelinating processes. (13) MOG-IgG associated disease (MOGAD) patients usually present with similar clinical features such as ON and recurrent transverse myelitis, drawing some similarities to the AQP4-IgG positive cohorts. (13, 32, 37, 38) OCT analyses of this cohort also tend to report lower pRNFL and GCIP layer thicknesses. (39)

However, unlike AQP4-IgG seropositive NMOSD, MOGAD is characterized by less frequent and more damaging ON attacks, but with surprisingly better visual outcomes and limited visual acuity loss. (40, 41) Additionally, MOGAD lacks an identifiably astrocytopathic component in its pathology (13, 39, 42); it is now suggested to be a different disease entity altogether and make a particularly good diseased control group. (43, 44)

1.5 Aims

The purpose of this dissertation is to rely on an international multicentric study to identify if attack dependent or independent astrocytopathy in the outer retinal layers of AQP4-IgG seropositive patients, particularly in the foveal and macular ONL, occurs and whether such changes can be correlated to corresponding thinning visible in OCT scans. OCT values will be compared with healthy controls (HC) and MOGAD patients as diseased controls. These results were submitted in October 2021 to the Journal of Neurology, Neurosurgery and Psychiatry and published in February 2022. (45)

2. Methods

2.1 Cohort Design

Patients were recruited internationally as part of the *Collaborative Retrospective Study on retinal optical coherence tomography in Neuromyelitis Optica* (CROCTINO) study. Representing the largest known study of its kind, the CROCTINO study overcomes one of the many common weaknesses of NMOSD studies – being their limited size and homogenous sample population. (46) Consisting of a network of over 20 international centers world-wide and including AQP4-IgG seropositive patients as well as MOGAD patients, the CROCTINO cohort thereby allows world-wide, reliable quantitative and qualitative retinal assessments to be made possible. Further details on the CROCTINO study cohort are reported elsewhere by Specovius et al. (46)

Eligible patients were recruited between 2000 and 2018 as part of CROCTINO. (46) Our published manuscript (45) summarizes the enrollment process in detail; in short, of the original 539 patients recruited by the CROCTINO consortium, 108 patients were excluded on account of missing macular data; of the remaining 431 patients, a further 40 were excluded on account of anomalies in their OCT scans (for example, non-compliance with the OSCAR-IB criteria for OCT quality (27) or due to presence of pathologies such as microcysts in the INL, which tend to occur at a higher rate following ON in NMOSD when compared to other neurological diseases such as MS (47)). The remaining 301 patients were then split based on serology (AQP4-IgG and MOG-IgG) whereby the above noted exclusion criterion further reduced the cohort to its final composition including 197 seropositive AQP4-IgG subjects (317 eyes) and 32 seropositive MOG-IgG subjects (55 eyes). AQP4-IgG and MOG-IgG antibody detection was conducted in each relevant center using, at their discretion, relevant commercially available tests for analysis of serum samples. Clinical data obtained from all patients include antibody serology, disease duration, frequency of ON, location of ON, date of ON, Expanded Disability Standard Scale (EDSS) and treatment received). Additionally, 75 non-age nor sex matched HCs were recruited.

2.2 Optical Coherence Tomography

OCT examinations were conducted using the following devices: Spectralis SD-OCT, Heidelberg Engineering, Heidelberg, Germany (Spectralis), Cirrus HD-OCT, Carl Zeiss Meditec Inc., Dublin, CA USA (Cirrus) and Topcon 3D-OCT, Topcon Corp., Tokyo Japan (Topcon). In each CROCTINO center, two scans from each eye of each patient were collected: (1) a 3.4mm diameter peripapillary ring scan around the optic nerve head for Spectralis SD-OCT (for Cirrus and Topcon devices, this scan was extracted from the optic disc volume scans) and (2) a macular volume scan centered around the fovea. (46) All OCT images for both studies were obtained in line with the OSCAR-IB criteria (27, 48) and the results were presented in line with the APOSTEL 2.0 recommendations. (26) The pRNFL was derived using a device-specific protocol centered around the optic nerve head. The various retinal layers were then segmented semi-automatically and processed using an in-house proprietary software (SAMIRIX). (37)

The following layers were segmented: macular retinal nerve fiber layer (mRNFL), GCIP, INL, OPL, ONL, photoreceptor layer (PR), which spans from the inner photoreceptive segments to the Bruch's Membrane, and the total retinal thickness (RT, calculated as the thickness with the inner limiting membrane as the upper boundary (defined as layer no. 3 per Staurenghi et al) and the Bruch's Membrane as the lower boundary (defined as layer no. 14). (49) Whilst several proprietary intra-retinal algorithms for segmentation of the retinal layers have been employed to reduce what would otherwise be a very manually intensive process, (50, 51) these algorithms are not robust enough to consider variations on account of various pathologies. As a result, manual correction was required in many cases; as reported by Motamedi et al, the current generation of intra-retinal segmentation algorithms are unable to reliably track annual GCIP layer losses in MS (approximately 1.1µm over 2 years), however, they are robust enough to track attack-dependent damage, which are magnitudes greater. (37) Manual correction of the automated segmentation was completed, where required, at the Charité - Universitätsmedizin Berlin. To assure comparability with previously published data on outer retinal layer changes in NMOSD, the macular volume data were further segregated into one of three export protocols: (1) a 5mm diameter cylinder omitting a 1mm diameter around the fovea (5mm study), (2) a 3mm diameter cylinder omitting a 1mm diameter around the fovea (3mm study) and (3) a 1mm mean thickness around the fovea (1mm study). Results are

reported for the 5mm study on Spectralis devices; confirmatory results based on the 3mm and 1mm study as well as for Cirrus and Topcon devices are set out in the supplement.

2.3 Statistical Analysis

OCT data were grouped by: (1) antibody status and (2) ON history. Bifurcation of data by OCT Device sought to mitigate device specific aberrations. In each of the AQP4-IgG, MOG-IgG and HC cohorts, the Student's t test was employed for continuous data. Cross-sectional group comparisons of OCT values were then conducted using linear mixed-effect models with age and sex as fixed effects and center and Patient-ID as random effects. Marginal and conditional coefficients of determination for the models were estimated by pseudo-R^2 for mixed-effect models. Significance was established at $p < 0.05$. Statistical analyses were conducted using R (Version 4.0.0) (RStudio Inc., Boston, MA, USA). (52)

2.4 Excurse: Device comparability

As CROCTINO centers employed three various OCT devices (Spectralis, Topcon and Cirrus devices), we sought to test the comparability of their scanning capabilities in a preliminary study consisting of 16 healthy volunteers from the Charité – Universitätsmedizin Berlin. This study gave us insights into the degree of device agreement and helped shape the design of the CROCTINO outer retinal layer analysis. It was observed that the devices generally deviate significantly enough to warrant further analysis in this area. As such, a deeper analysis is expected to be conducted and published in due course and for the purposes of this dissertation only the preliminary findings will be presented and discussed. This is nonetheless enlightening in respect of the CROCTINO outer retinal layer analysis on account of the heavy emphasis we place on the multi-centric nature of the CROCTINO cohort and the value in data aggregation.

In designing this test, those with chronic neurological disorders or disorders affecting the eyes (such as Glaucoma or diabetic retinopathy), pathological neurovisual conditions, worse best corrected decimal visual acuity of less than 1.0 or eyes with a refractive error greater than +/- 6 diopters were excluded. Test volunteers were subject to one session in both eyes from three OCT devices in June 2018 and the examinations on the three different devices were concluded within one week. Besides OCT scans, data of refractive

error, high contract visual acuity and intraocular pressure measurements were measured from all volunteers to ensure exclusion criteria were met. High contrast visual acuity was evaluated based on Early Treatment in Diabetes Retinopathy Study (ETDRS) charts. The refractive error and intraocular pressure were assessed using a Nidek Tonoref II Autorefractometer Keratometer Tonometer (Nidek Co. Ltd., Aichi, Japan). OCT Scans were processed using the SAMIRIX pipeline and manually corrected in accordance with a standardized set of standard operating procedures, a copy of which can be provided upon request. Thickness reports were generated using the 5mm study export protocol with the following layers: mRFNL, GCIP, GCC, INL, OPL, ONL OPNL, PR and RT.

To assess agreeability and reproducibility in devices and protocols, the Bland-Altman Plots and the intra-class correlation coefficient (ICC) were calculated using the R packages BlandAltmanLeh (53, 54) and ICC (Wolak et al, 2012) respectively. ICC values of greater than 0.9 are regarded as highly reproducible, between 0.8 and 0.9 as moderately reproducible and below 0.8 as insufficiently reproducible (55). Confidence level was set at 0.05.

The mean thickness values of each eye across the three devices are summarized in Table 1. A review of the average thickness of the exported layers indicates that for Spectralis device, larger mRNFL, GCIP, Ganglion Cell Complex (GCC), PR and TM values were seen compared to the other two devices. The INL, ONL and OPNL layers from Cirrus devices were greater while in the OPL, the Topcon devices returned the highest average thickness values (see Figure 2; Table 2 sets out the mean differences in each device pair comparison).

Table 1: Mean thickness of retinal layers across the three measured OCT devices

Retinal layer	Cirrus	Spectralis	Topcon
mRNFL Mean + SD (μm)	31.91 ± 2.57	36.25 ± 2.52	30.24 ± 2.35
GCIP Mean + SD (μm)	77.59 ± 3.52	79.74 ± 3.97	78.79 ± 3.32
GCC Mean + SD (μm)	109.50 ± 5.37	115.99 ± 6.10	109.03 ± 5.09
INL Mean + SD (μm)	41.46 ± 2.52	39.52 ± 2.45	38.10 ± 2.28
OPL Mean + SD (μm)	23.86 ±0.81	24.32 ± 1.37	26.36 ± 0.82

Retinal layer	Cirrus	Spectralis	Topcon
ONL Mean + SD (μm)	67.71± 5.51	65.25 ± 5.88	60.09 ± 5.68
OPNL Mean + SD (μm)	91.57 ± 5.64	89.57 ± 5.50	86.45 ± 5.68
PR Mean + SD (μm)	58.01 ± 1.63	63.78 ± 2.20	54.17 ± 2.05
TM Mean + SD (μm)	322.17 ± 11.94	327.38 ± 12.62	315.13 ± 10.75

Abbreviations: mRNFL = macular retinal nerve fiber layer. GCIP = ganglion cell and inner plexiform layer. GCC = ganglion cell complex. INL = inner nuclear layer. OPL = outer plexiform layer. OPNL = photoreceptor nuclear axonal complex. ONL = outer nuclear layer. PR = photoreceptor layer. TM = total macular thickness. (Own representation)

Table 2: Mean thickness difference between each measured device pair

Retinal layer	Spectralis vs Cirrus	Spectralis vs Topcon	Cirrus vs Topcon
mRNFL Mean Difference ± SD (μm)	4.34 ± 1.34	6.01 ± 1.51	-1.67 ± 1.29
GCIP Mean Difference ± SD (μm)	2.15 ± 1.47	0.95 ± 1.71	1.20 ± 1.31
GCC Mean Difference ± SD (μm)	6.49 ± 1.58	6.95 ± 2.02	-0.46 ± 1.41
INL Mean Difference ± SD (μm)	-1.93 ± 0.91	1.43 ± 0.89	-3.36 ± 0.90
OPL Mean Difference ± SD (μm)	0.46 ± 1.16	-2.04 ± 1.07	2.51 ± 0.56
ONL Mean Difference ± SD (μm)	-2.47 ± 0.89	5.16 ± 1.31	-7.62 ± 1.31
OPNL Mean Difference ± SD (μm)	-2.00 ± 0.92	3.12 ± 1.37	-5.12 ± 1.21
PR Mean Difference ± SD (μm)	5.77 ± 1.24	9.61 ± 1.82	-3.84 ± 1.70
TM Mean Difference ± SD (μm)	5.22 ± 2.09	12.25 ± 3.37	-7.01 ± 2.84

Abbreviations: mRNFL = macular retinal nerve fiber layer. GCIP = ganglion cell and inner plexiform layer. GCC = ganglion cell complex. INL = inner nuclear layer. OPL = outer plexiform layer. OPNL = photoreceptor nuclear axonal complex. ONL = outer nuclear layer. PR = photoreceptor layer. TM = total macular thickness. (Own representation)

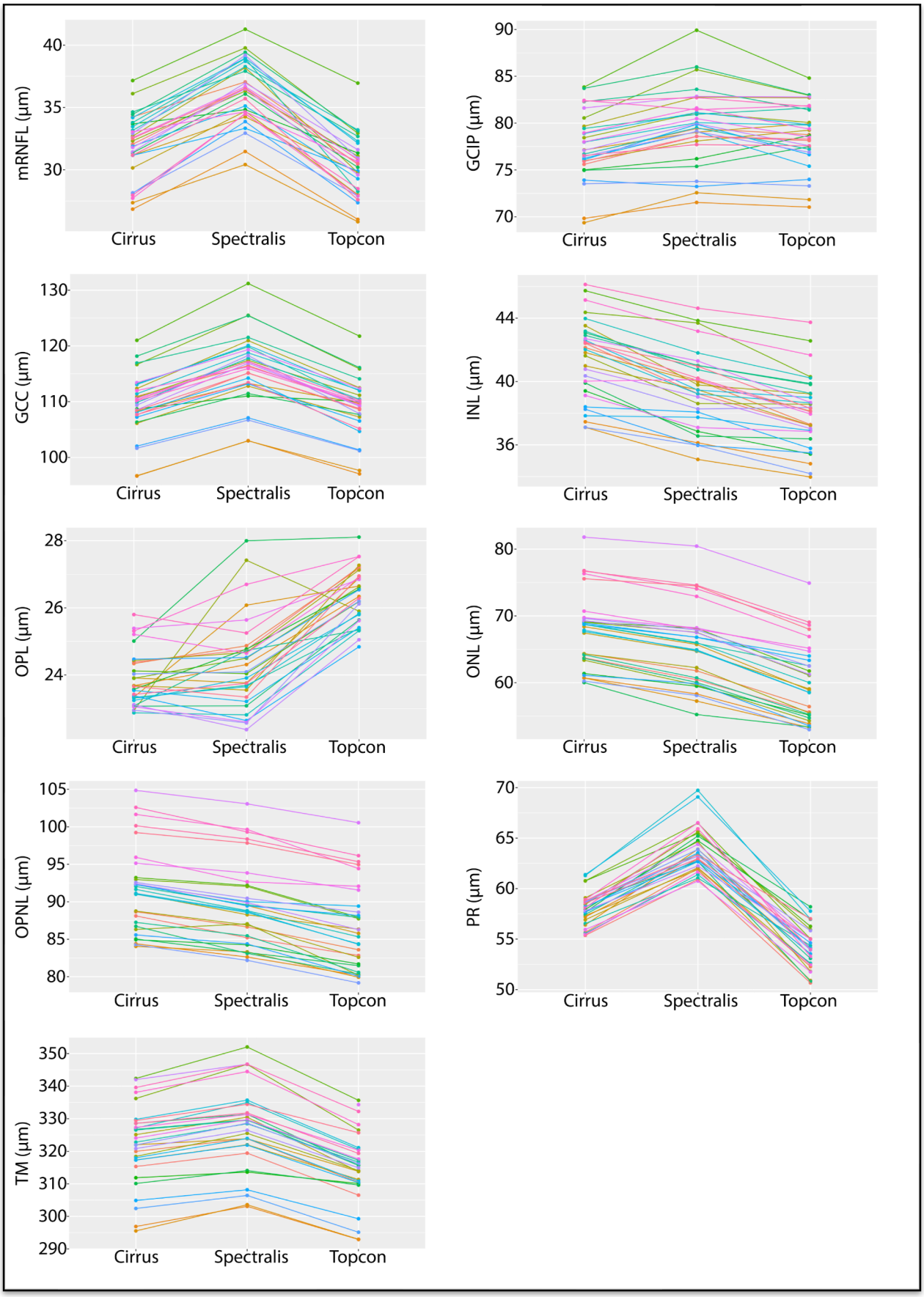
mRNFL (µm)
40
35
30
Cirrus
Spectralis
Topcon
GCIP (µm)
90
85
80
75
70
Cirrus
Spectralis
Topcon
GCC (µm)
130
120
110
100
Cirrus
Spectralis
Topcon
INL (µm)
44
40
36
Cirrus
Spectralis
Topcon
OPL (µm)
28
26
24
Cirrus
Spectralis
Topcon
ONL (µm)
80
70
60
Cirrus
Spectralis
Topcon
OPNL (µm)
105
100
95
90
85
80
Cirrus
Spectralis
Topcon
PR (µm)
70
65
60
55
50
Cirrus
Spectralis
Topcon
TM (µm)
350
340
330
320
310
300
290
Cirrus
Spectralis
Topcon

Figure 2: Spaghetti plots of the average thickness of each retinal layer across the three OCT devices

Each retinal layer is depicted as its own data plot split across the three devices tested (x-axis). Randomised color representation of the average measurements for each specimen eye depicts spread. Abbreviations: mRNFL = macular retinal nerve fiber layer. GCIP = ganglion cell and inner plexiform layer. GCC = ganglion cell complex. INL = inner nuclear layer. OPL = outer plexiform layer. ONL = outer nuclear layer. OPNL = photoreceptor nuclear axonal complex. PR = photoreceptor layer. TM = total macular thickness. (Own representation)

The clinical significance of the inner layers (mRNFL and GCIP) along with possible changes in the ORL (assessed as the combined OPNL) deserves scrutiny; reliable quantification of changes is paramount in disease staging as well as assessing disease progression. These depict a randomization of skew with no systematic shifts. Paired Wilcoxon Signed Rank Tests comparing thickness measurements of device pairs found significant differences for almost all cases (Table 3), except for the GCC in the Topcon vs Cirrus pair and the OPL in the Spectralis vs Cirrus pair. ICC analyses showed poor reliability to mediocre reliability, with ICCs ranging from -0.265 to 0.836 (Table 4).

Table 3: Wilcoxon Signed Rank test for each measured device pair

	Spectralis vs Cirrus	Spectralis vs Topcon	Topcon vs Cirrus
mRNFL	9.313×10^{-10}	9.313×10^{-10}	8.196×10^{-08}
GCIP	2.956×10^{-06}	0.00607	5.613×10^{-05}
GCC	1.233×10^{-06}	1.233×10^{-06}	0.1443
INL	1.501×10^{-06}	1.501×10^{-06}	1.233×10^{-06}
OPL	0.1059	2.216×10^{-06}	1.231×10^{-06}
ONL	9.313×10^{-10}	1.233×10^{-06}	9.313×10^{-10}
OPNL	1.654×10^{-06}	1.233×10^{-06}	9.313×10^{-10}
PR	9.313×10^{-10}	1.233×10^{-06}	1.361×10^{-06}
TM	9.313×10^{-10}	1.863×10^{-09}	1.863×10^{-09}

For the Wilcoxon Signed Rank test a significance level was set at 0.05. Abbreviations: mRNFL = macular retinal nerve fiber layer. GCIP = ganglion cell and inner plexiform layer. GCC = ganglion cell complex. INL = inner nuclear layer. OPL = outer plexiform layer. OPNL = photoreceptor nuclear axonal complex. ONL = outer nuclear layer. PR = photoreceptor layer. TM = total macular thickness. (Own representation)

Table 4: Intraclass correlation coefficient (ICC) and confidence interval (CI) of each retinal layer

	ICC	Lower CI	Upper CI
mRNFL	0.159	-0.045	0.404
GCIP	0.836	0.728	0.910
GCC	0.594	0.398	0.759
INL	0.581	0.382	0.750
OPL	0.007	-0.169	0.245
ONL	0.6272	0.439	0.781
OPNL	0.795	0.667	0.887
PR	-0.265	-0.360	-0.103
TM	0.749	0.601	0.858

Intraclass coefficient (ICC) values measure how reproducible the results are: values of greater than 0.9 are regarded as highly reproducible, between 0.8 and 0.9 as moderately reproducible and below 0.8 as insufficiently reproducible. Abbreviations: mRNFL = macular retinal nerve fiber layer. GCIP = ganglion cell and inner plexiform layer. GCC = ganglion cell complex. INL = inner nuclear layer. OPL = outer plexiform layer. OPNL = photoreceptor nuclear axonal complex. ONL = outer nuclear layer. PR = photoreceptor layer. TM = total macular thickness. (Own representation)

With the help of regression analysis, we saw that the distribution of data against the three device pairs in the OPL vary more widely than in the OPNL segment; variations are significantly reduced as the boundary between the OPL and ONL plays no role in deriving total thickness estimates. These aberrations may be accounted for on account of the various Henle's Fiber Layer morphologies. Accordingly, more recent macular acquisition protocols have been implemented in the vertical axis to due to a postulated reduction in confound of segmentation (56).

To negate any potential systematic errors in comparisons against Spectralis devices, a correction factor was applied to the Cirrus and Topcon mean estimates; this was accomplished using Spectralis as the ground truth. For each Cirrus and Topcon layer, the mean difference to the Spectralis values is incorporated within the calculated values. The results with the correction factors applied appear to be generally more consistent. The ICCs, now more robust and indicate that the results are reproducible (other than OPL and PR), are still somewhat on the lower side, especially as we would generally see agreements in the range of around 0.99 (Table 5).

Table 5: Intra-class correlation coefficient (ICC) and confidence intervals (CI) for each retinal layer with correction factor applied

	ICC	Lower CI	Upper CI
mRNFL	0.848	0.747	0.917
GCIP	0.975	0.854	0.955
GCC	0.954	0.920	0.976
INL	0.933	0.883	0.964
OPL	0.573	0.373	0.744
ONL	0.978	0.962	0.989
OPNL	0.978	0.961	0.988
PR	0.678	0.504	0.814
TM	0.972	0.951	0.985

Potential systematic errors were mitigated using comparisons with results obtained from Spectralis devices as the ground truth. Intraclass coefficient (ICC) values measure how reproducible the results are: values of greater than 0.9 are regarded as highly reproducible, between 0.8 and 0.9 as moderately reproducible and below 0.8 as insufficiently reproducible. Abbreviations: mRNFL = macular retinal nerve fiber layer. GCIP = ganglion cell and inner plexiform layer. GCC = ganglion cell complex. INL = inner nuclear layer. OPL = outer plexiform layer. OPNL = photoreceptor nuclear axonal complex. ONL = outer nuclear layer. PR = photoreceptor layer. TM = total macular thickness. (Own representation)

We assessed rater bias by analyzing only the automatic segmentation profiles and saw that Spectralis computed thicker values across the board. The Paired Wilcoxon Test results also returned non-significant p-values other than in the OPL (p-value = 0.00919)

indicating that the means are not significantly similar. ICC levels were higher for INL, OPL, ONL, OPNL and the total macular volume, however, only the OPNL value was highly reproducible (0.91). Overall, the automatic segmented scans did not appear to show any meaningful improvement in the reliability against the two devices.

Given the general lack of comparability between the devices, and on account of the lack of substantial HC eyes examined using Topcon and Cirrus devices, it was decided that that for the purposes of the CROCTINO outer retinal layer analysis we focus mainly on the Spectralis OCT data (which nevertheless presented a substantial number of test subjects) and analyze the other devices separately.

2.5 Ethics

Written informed consent was obtained from all patients participating in the studies and local ethics committee approvals were obtained by each center in accordance with the Declaration of Helsinki (1964) in its current applicable form. The study also conformed to all relevant best practice guidelines and ethical standards of each center, including the Charité Statute Ensuring Good Scientific Practice of 20 June 2021 (as updated on 29.03.2018).

3. Results

3.1 Cohort Description

197 AQP4-IgG positive patients (317 eyes; age [mean±SD]: 41.83±12.05 years, sex [male N (%)]: 24 (12.2%)) fulfilled the inclusion criteria. We also included 75 HCs (age [mean±SD]: 32.26±9.55 years, sex [male N (%)]: 25 (33.8%)) and 32 MOG-IgG seropositive patients (age [mean±SD]: 36.5±13.73 years, sex [male N (%)]: 10 (31.2%)) as control groups. Detailed summary of the cohort description including descriptive analyses of cohort (HC, seropositive AQP4-IgG and seropositive MOG-IgG) is set out as table 1 in our published manuscript. (45)

Neuroaxonal damage (measured by pRNFL, mRNFL and GCIP OCT values) between AQP4-IgG seropositive and MOGAD cohorts were comparable making MOGAD an ideal diseased control group for the investigation of the outer retinal layers (AQP4-IgG: pRNFL: 78.46±24.13µm, mRNFL: 28.09±6.60µm, GCIP: 65.81±13.03µm; MOGAD: pRNFL: 74.33±23.44µm, mRNFL: 27.62±5.43µm, GCIP: 66.16±11.85µm) (see table 2 and figure 2 respectively in our published manuscript (45) for detailed breakdown of the group comparison between HC, AQP4-IgG and MOG-IgG seropositive patients at baseline using Spectralis devices).

3.2 Results

The results are widely discussed in our published manuscript. (45) In short, it was clear that no significant thinning of OPL and ONL were observed in the AQP4-IgG seropositive cohort, irrespective of a history of ON when compared with HC (B±SE) (OPL: +0.28±0.24µm, p=0.241; ONL: -0.01±0.83µm, p=0.993). Likewise, similar results were seen in MOGAD patients (B±SE) (OPL: -0.01±0.29µm, p=0.986; ONL: 0.69±0.93µm, p=0.457) An examination of ORL changes in the presence or absence of ON revealed that AQP4-ON were not subject to greater levels of in the OPL and ONL when compared with ON negative patients (AQP4-NON) or with HC, despite similar levels of neuroaxonal layer losses seen in the pRNFL and GCIP layers. (45) A comparison between AQP4-IgG cohort and MOGAD cohort revealed that both groups also exhibited comparable neuroaxonal loss in the pRNFL and GCIP layers, irrespective of ON history. Interestingly,

in the 1mm study, we observed OPL thinning around the fovea in AQP4-ON vs HC (B ± SE) (-1.54±0.69μm, p=0.027) as well as MOG-ON vs HC (B ± SE) (-2.51±0.87μm, p=0.004), but no significant differences were seen in AQP4-ON vs MOG-ON (p=0.100). (45)

It should also be noted that significant correlation between ethnicity and immunotherapy on OPL and ONL values were not observed. Additionally, whilst disease duration did not reveal any correlates with OPL (p=0.805) or ONL (p=0.835) values, we cannot exclude time dependent effects which would need to be closely assessed in a longitudinal analysis.

4. Discussion

4.1 Absence of astrocytopathic changes.

The results point to a general macular wide absence of changes in the OPL and ONL in AQP4-IgG seropositive patients versus HC and the MOGAD cohort irrespective of ON history and status. These contrast with previously published data from You et al (9) and Filippatou et al (14), whereby in both studies, measurable changes in the ONL and the inner segment of the photoreceptive layer was observed. We can, however, identify reasons for the divergence which are set out in detail in the published manuscript. (45) We believe that ethnic and age discrepancies in the cohort recruitment of these studies may account for the divergences as well as compounding potential Type I errors.

First, the higher proportion of African American in the study reported by Filippatou et al (14) may be potentially contributory. Studies have reported that African American MS patients are reported to exhibit a more aggressive disease progression, which could also be carried over in similar neuroinflammatory diseases such as NMOSD. (57, 58) And whilst these groups tend to have higher ONL and INL at baseline, they also exhibited faster rates of degeneration during disease progression. (57) Moreover, patients of African American descent were reportedly more likely to suffer from more aggressive ON episodes in both MS and NMOSD which contributes to accelerated rates of thinning in the RNFL and GCIP layers. (59) The lower percentage of African American patients in our cohort (5.6%) potentially resulted in a less profound change in foveal ONL. (14, 60)

Secondly, age may degrade the neuroplastic nature of the CNS. Specifically, the neuroplastic nature of the INL is hypothesized to act as a dam to retrograde trans-synaptic axonal degeneration (i.e., in the direction of the outer retinal layers) following ON. (61) The INL is comprised of the amacrine, horizontal and bipolar cells synapsing together forming a highly plastic synaptic tree. (61) Balk et al reported that axonal degeneration in MS following ON was halted at the INL and no atrophy could be shown beyond this layer even in disease durations of 20 years despite extensive inner retinal layer damage. (61) It therefore remains to be seen if such properties are also present in NMOSD and whether it remains so as the retina ages. It is known that neuroplasticity in the CNS declines with age, and as a corollary, the neuroplastic nature of the INL may also diminish with age.

(48) The highly vascularized nature of the retina, partly in the deep vascular plexus at the boundary between the INL and OPL may lend to its defensive capabilities; (62) it is here where one finds the inner Blood-Retina Barrier (BRB). (63) The inner BRB is composed of the retinal capillary endothelial cells and is structurally supported by astrocytes and Müller Cells. (63) In instances where the blood-retina barrier is compromised, it is conceivable that circulating AQP4-IgG may undermine the protection afforded by the INL and initiate glial dysfunction of the Müller Cells. This may be what was observed in the 1mm Study in the OPL of AQP4-ON and MOG-ON eyes ($p=0.027$ and $p=0.002$, respectively). (45)

The inclusion of the MOGAD cohort offers further insights into a clinically similar, but pathologically distinct disease entity, comparisons of which may shed light into potential changes in the outer retina. Considered a different disease entity to NMOSD, MOGAD presents MS like demyelinating pathologies without a definable astrocytopathy component; the phenotypic overlap between MS and MOGAD itself is at most partial with biological, clinical, and pathological differences. (13, 64) The differentiation between NMOSD and MOGAD at a clinical level is, however, more difficult, with both disease entities presenting with similar phenotypes, most commonly ON leading to substantial neuroaxonal damage after multiple relapses. (32, 42) The severity of ON is, however, a differentiating factor, in which MOGAD ON is characterized with less frequent but more damaging ON episodes that accumulate in neuroaxonal losses in the pRNFL and macular GCIP layers. (13, 32) Interestingly, despite the high relapse rates and severe neuroaxonal damage seen in MOGAD, visual acuity (when tested using high-contrast visual acuity testing) is surprisingly intact when compared to NMOSD. (32, 40) The MOGAD results in this study also seem to indicate no significant changes in attack dependent and independent OPL and ONL values other than, as previously mentioned, in the 1mm study MOG-ON cohort. It remains to be seen if these results speak to other processes occurring in the outer retina independent of attack dependent or independent astrocytopathy or Müller cell dysfunction.

Whilst these results raise additional questions, it does form a basis of further studies to identify if (1) age is a factor in the neuroplasticity of the retina, (2) if ON damages the barrier function of the BRB and (3) if the INL does indeed play a role in halting retrograde

axonal degeneration. Adding a longitudinal aspect would also clarify and quantify the disease progression over time.

Notwithstanding the lack of significant changes seen in the outer retinal layers, attack dependent and independent neuroaxonal damage in the pRNFL and GCIP layers were clearly present in the AQP4-IgG seropositive and MOGAD cohorts in line with recently reported findings. (13, 17, 32, 40, 65). Had outer retinal layer changes been observed, it may have been possible to integrate ONL and OPL measurements as a way of supplementing how disease progression was tracked. Whilst the practice has been to measure pRNFL and GCIP layers as markers of neuroaxonal loss, they are not without problems as studies have seen inconsistent changes, including increases in pRNFL due to tissue swelling on account of edema following ON attacks. (5)

The size and heterogeneity of the study design is one of its primary strengths. As the largest known outer retinal study to date on NMOSD patients, we were able to overcome one of the many limitations of earlier NMOSD studies, that is of a small and homogenous cohort recruitment. We also relied on three different OCT devices in our study. Whilst this compound the complexities of OCT comparisons and a high degree of caution should be warranted as results from differing OCT devices cannot be easily used interchangeably, this study included the three most widely commercially available OCT devices on the market and obtained confirmatory results with each of them. (66) Two of these devices were also used in the studies conducted by You et al (9) and Filippatou et al. (14)

Limitations need also to be discussed. First, the HCs and MOGAD patients were not age and sex matched. (45) For example, retinal thickness tends to decrease with age and males tend to typically exhibit thicker GCIP and TM layers. (37, 45) Additionally, the study did not encompass ERG or functional visual pathway assessments. ERG measures the electrical responses of the retina when stimulated by light; the study by You et al used this method as a way of identifying Müller glial cell dysfunction in the Henle Fiber layer of the retina in AQP4-IgG NMOSD. (9) An inclusion of this diagnostic method would have identified more subtle functional impairment of outer retina layers without associated tissue loss, and not otherwise identifiable with simple EDSS or visual acuity testing. (45) Magnetic Resonance Imaging (MRI) studies were also not conducted on the study participants in tandem with OCT imaging. Whilst brain lesions in NMOSDs are generally

not as frequent as in other neurological diseases such as MS, the presence of such could be a useful distinguishing factor. (67) Additionally MRI imaging of the optic nerve could have served to better correlate OCT results with the clinical manifestation of the disease (for example through visual acuity assessments or EDSS). Lesions and atrophy of the optic nerve using Gadolinium enhanced T1 and T2 weighted sequences are present in attack dependent ON cases. (68-70) Additionally Akaishi et al proposed that optical nerve lesion length tends to strongly correlate with the visual outcomes of AQP4-IgG NMOSD patients. (71) Outer retinal studies are further complicated by various Henle Fiber morphologies which are variable and dependent on OCT beam placement ranging from hyperreflective to indistinct from ONL. (11, 45) When the beam placement results in a 90° angle of incidence to the OPL, the Henle Fiber tends to appear as hyperreflective in the scans and thus results in thicker segmentation profiles. (11) These irregular and variable morphologies introduce a certain element of rater subjectivity in post image correction and analyses, further complicating the ability for OCT scans to be compared consistently. Finally, the Cirrus and Topcon measurements could not be utilized as confirmatory cohorts as there lacked sufficient HC subjects examined by these devices. (45)

4.2 Lack of comparability between OCT devices

The lack of comparability between the devices may be explained on account of how SAMIRIX handles the raw image data. The pipeline is built around two training sets: one for Cirrus and Topcon and another for Spectralis. The raw image file type determines which training set is applied in how the segmentation boundaries of the retinal layers are set. However, given the lack of comparability between Topcon and Cirrus data (Table 2), this must not be the only reason for variations. Furthermore, the mean differences between Cirrus and Topcon in the outer layers (the OPL, ONL and OPNL) showed the greatest mean differences of the three device pairs, even though the same training sets were used. OPL thickness appeared to be distributed randomly across the three devices and could also be explained, again, by the photoreflective properties of the Henle's Fiber layer. The beam place relative to the Henle's Fiber layer results in various manifestations of hypo- and hyper-reflectivities which may explain the why the OPL results appear more randomized than in other layers, an observation made in various other studies including by Oberwahrenbrock et al. (72)

Another area of difference lies in the direction in which the scan protocols were implemented between the devices, as differences can be seen between the Spectralis scans (taken in the vertical position) and those taken with Topcon and Cirrus (in the horizontal positions). Whether that is a perceived difference due to image reconstruction or an actual difference on account of the segmentation algorithm is to be seen. Caldito et al in 2018 (56) published a study indicating that, at least with Spectralis devices, both horizontal and vertical protocols agree excellently on a cohort level, thus the direction of the protocol would not impact greatly on why deviations are seen between Spectralis against the two other devices.

Inter-device comparability studies have been previously published to an extent to highlight the need for standardization in OCT image acquisitions. A similar study conducted by the Johns Hopkins University between Spectralis and Cirrus devices indicated a stronger agreeability between them (66). In that study, both healthy controls (22 subjects) and those with MS (68 subjects) were recruited and the subjects undertook scans with both devices consecutively. The results showed low mean differences for the GCIP, INL, OPNL and PR layers indicating excellent cross platform agreeability. The limit of agreeability was deemed, however, to still be unacceptably wide at an individual level to allow device outputs to be used interchangeably (66). However, other published data tend to present diverging results in this field. Leite et al (73) saw no significant differences between Spectralis, Cirrus and RTVue (Optovue Inc., Fremont California, USA) in detecting macroscopic changes in patients with glaucoma. In a report on the economic value of photographic screening in ophthalmology, Olson et al (74) noted that that global thickness measurements vary greatly between Spectralis, Topcon and Cirrus. Additionally, given that the devices employ various protocols and run differing software in identifying boundaries, disagreements on where to place the lower boundary of the thickness measurement have been reported and present significant problems which results in average errors between the devices of approximately 3μm. Oberwahrenbrock et al (72) yielded generally good ICCs between the same three devices, however the high reproducibility correlated strongly with the layer being analysed: mRNFL and GCIP returned generally strong ICCs (ICC range 0.81-0.99 for mRNFL and 0.86-1.00 for GCIP) but INL and OPL were significantly weaker (ICC range 0.72-0.98 for INL and 0.55-0.87 for OPL). Oberwahrenbrock noted that strong ICCs would ensure comparability in MS studies where significant changes could be observed in the inner layers, particularly the

mRFNL and GCIP layer. However, its strength in assessing ORL remains to be seen given the weaker OPL results.

5. Conclusion

This study concludes that astrocytopathic changes in the ORL is not a component of NMSOD and MOGAD. This potentially relieves diagnostic burdens in practice particularly in the initial diagnosis and the monitoring of disease progression; a shift towards inner layer diagnostic, such as RNFL and GCIP layer measurements could be sufficient in capturing the state of the retina. This is not to say, however, that the state of research on the ORL should stop here. To the contrary, further studies should be undertaken, particularly in respect of longitudinal data to observe if OPL and ONL are damaged later in the disease course and whether this is due to retrograde trans-synapatic axonal degeneration across damaged INL barrier. Whilst only the baseline Spectralis scans were analysed in this study, Topcon and Cirrus scans were also included in the wider CROCTINO study. This afforded the study a far richer constellation of patient data. Had they been integrated into the study questions of intra-device comparability would undoubtedly need to be solved to capitalize on their diagnostic value. We have demonstrated, at least at a small scale, that inter-device comparability remains poor, and the inclusion of multiple devices adds further complexities in multi-centered studies such as CROCTINO. Given the rarity of NMOSD, it would be unfathomable to not lean on the power of multi-centric studies, not only to the extent of greater data aggregation but also as way of opening collegiate collaboration of world-wide experts. It remains to be seen if, in future, calibration and segmentation software may bridge the gap between the devices to further enhance their usability in NMOSD diagnostics.

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Statutory Declaration

I, Angelo Lu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “A Multicenter Study on Outer Retinal Layer Changes in Neuromyelitis Optica Spectrum Disorders / Multicenter-Studie zu Veränderungen der äußeren Netzhautschichten in Neuromyelitis Optica Spektrumerkrankungen” independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (regarding practical work, laboratory regulations, statistical processing) and results (regarding figures, charts, and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

Date Signature

Contributions

I, Angelo Lu contributed the following to the below listed publication:

Publication: **Lu A**, Zimmermann HG, Specovius S, Motamedi S, Chien C, Bereuter C, Lana-Peixoto MA, Fontenelle MA, Ashtari F, Kafieh R, Dehghani A, Pourazizi M, Pandit L, D'Cunha A, Kim HJ, Hyun JW, Jung SK, Leocani L, Pisa M, Radaelli M, Siritho S, May EF, Tongco C, De Sèze J, Senger T, Palace J, Roca-Fernández A, Leite MI, Sharma SM, Stiebel-Kalish H, Asgari N, Soelberg KK, Martinez-Lapiscina EH, Havla J, Mao-Draayer Y, Rimler Z, Reid A, Marignier R, Cobo-Calvo A, Altintas A, Tanriverdi U, Yildirim R, Aktas O, Ringelstein M, Albrecht P, Tavares IM, Bichuetti DB, Jacob A, Huda S, Soto de Castillo I, Petzold A, Green AJ, Yeaman MR, Smith TJ, Cook L, Paul F, Brandt AU, Oertel FC; GJCF International Clinical Consortium for NMOSD. Astrocytic outer retinal layer thinning is not a feature in AQP4-IgG seropositive neuromyelitis optica spectrum disorders. J Neurol Neurosurg Psychiatry. 2022 Feb;93(2):188-195.

Contribution: This publication was a collaborative effort from a consortium of 20 international research centers. Each center was responsible for the recruitment of patients and healthy controls as well as collecting clinical and raw OCT data (total: 1267 scans). Data collation and analysis was centralized in Berlin. The project was conceived and led by a core team at the Charité - Universitätsmedizin Berlin which consisted of FP, HZ, AUB, FCO and Angelo Lu. Angelo Lu performed, using proprietary software, OCT segmentation of the raw OCT data collated by the consortium (and manually corrected these where necessary). Angelo Lu, under the supervision of FCO and HZ completed the statistical analysis, interpreted the results, conducted literary research and prepared drafts and revisions prior to publication. All figures and tables in the publication were created by Angelo Lu on the basis of the statistical evaluations undertaken. The publication was edited with the help of FP, HZ, AUB and FCO. Each co-author provided suggestions, edits and final sign-off on the analysis and publication; this was coordinated by Angelo Lu.

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Signature of doctoral candidate

Publication

Lu A, Zimmermann HG, Specovius S, Motamedi S, Chien C, Bereuter C, Lana-Peixoto MA, Fontenelle MA, Ashtari F, Kafieh R, Dehghani A, Pourazizi M, Pandit L, D'Cunha A, Kim HJ, Hyun JW, Jung SK, Leocani L, Pisa M, Radaelli M, Siritho S, May EF, Tongco C, De Sèze J, Senger T, Palace J, Roca-Fernández A, Leite MI, Sharma SM, Stiebel-Kalish H, Asgari N, Soelberg KK, Martinez-Lapiscina EH, Havla J, Mao-Draayer Y, Rimler Z, Reid A, Marignier R, Cobo-Calvo A, Altintas A, Tanriverdi U, Yildirim R, Aktas O, Ringelstein M, Albrecht P, Tavares IM, Bichuetti DB, Jacob A, Huda S, Soto de Castillo I, Petzold A, Green AJ, Yeaman MR, Smith TJ, Cook L, Paul F, Brandt AU, Oertel FC; GJCF International Clinical Consortium for NMOSD. Astrocytic outer retinal layer thinning is not a feature in AQP4-IgG seropositive neuromyelitis optica spectrum disorders. J Neurol Neurosurg Psychiatry. 2022 Feb;93(2):188-195. doi: 10.1136/jnnp-2021-327412. Epub 2021 Oct 28. PMID: 34711650; PMCID: PMC8785057.

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9	ANNALS OF NEUROLOGY	37,304	9.037	0.044120
10	NEUROLOGY	90,213	8.770	0.103530
11	MOVEMENT DISORDERS	27,638	8.679	0.031140
12	JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY	30,621	8.234	0.028510
13	Neurology-Neuroimmunology & Neuroinflammation	2,232	7.724	0.008400
14	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,992	7.500	0.005960
15	Journal of Stroke	1,247	7.470	0.004240
16	STROKE	66,466	7.190	0.078010
17	Brain Stimulation	6,537	6.565	0.015580
18	NEUROSCIENTIST	5,188	6.500	0.007220
19	Alzheimers Research & Therapy	3,876	6.116	0.011650
20	EPILEPSIA	26,560	6.040	0.029790

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Original research

Astrocytic outer retinal layer thinning is not a feature in AQP4-IgG seropositive neuromyelitis optica spectrum disorders

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ABSTRACT

Background Patients with anti-aquaporin-4 antibody seropositive (AQP4-IgG+) neuromyelitis optica spectrum disorders (NMOSDs) frequently suffer from optic neuritis (ON) leading to severe retinal neuroaxonal damage. Further, the relationship of this retinal damage to a primary astrocytopathy in NMOSD is uncertain. Primary astrocytopathy has been suggested to cause ON-independent retinal damage and contribute to changes particularly in the outer plexiform layer (OPL) and outer nuclear layer (ONL), as reported in some earlier studies. However, these were limited in their sample size and contradictory as to the localisation. This study assesses outer retinal layer changes using optical coherence tomography (OCT) in a multicentre cross-sectional cohort.

Method 197 patients who were AQP4-IgG+ and 32 myelin-oligodendrocyte-glycoprotein antibody seropositive (MOG-IgG+) patients were enrolled in this study along with 75 healthy controls. Participants underwent neurological examination and OCT with central postprocessing conducted at a single site.

Results No significant thinning of OPL (25.02±2.03 µm) or ONL (61.63±7.04 µm) were observed in patients who were AQP4-IgG+ compared with patients who were MOG-IgG + with comparable neuroaxonal damage (OPL: 25.10±2.00 µm; ONL: 64.71±7.87 µm) or healthy controls (OPL: 24.58±1.64 µm; ONL: 63.59±5.78 µm). Eyes of patients who were AQP4-IgG+ (19.84±5.09 µm, p=0.027) and MOG-IgG + (19.82±4.78 µm, p=0.004) with a history of ON showed parafoveal OPL thinning compared with healthy controls (20.99±5.14 µm); this was not observed elsewhere.

Conclusion The results suggest that outer retinal layer loss is not a consistent component of retinal astrocytic damage in AQP4-IgG+ NMOSD. Longitudinal studies are necessary to determine if OPL and ONL are damaged in late disease due to retrograde trans-synaptic axonal degeneration and whether outer retinal dysfunction occurs despite any measurable structural correlates.

INTRODUCTION

Neuromyelitis optica spectrum disorders (NMOSDs) are relapsing autoimmune disorders affecting the central nervous system (CNS).[1] Common clinical attacks in NMOSD include optic neuritis (ON), acute myelitis and area postrema syndrome.[2] Serum autoantibodies to aquaporin-4 (AQP4-IgG) are detectable in 60%–80% of patients with NMOSD .[3 4]

AQP4 is an astrocytic water channel in the CNS.[5] In the retina, astrocytes are mainly located in the inner neuroaxonal layers of the retina, but AQP4 is additionally highly expressed in retinal Müller cells.[6] These glial cells have diverse functions, such as regulation of water homeostasis and neurotransmitter recycling, and are located around the fovea

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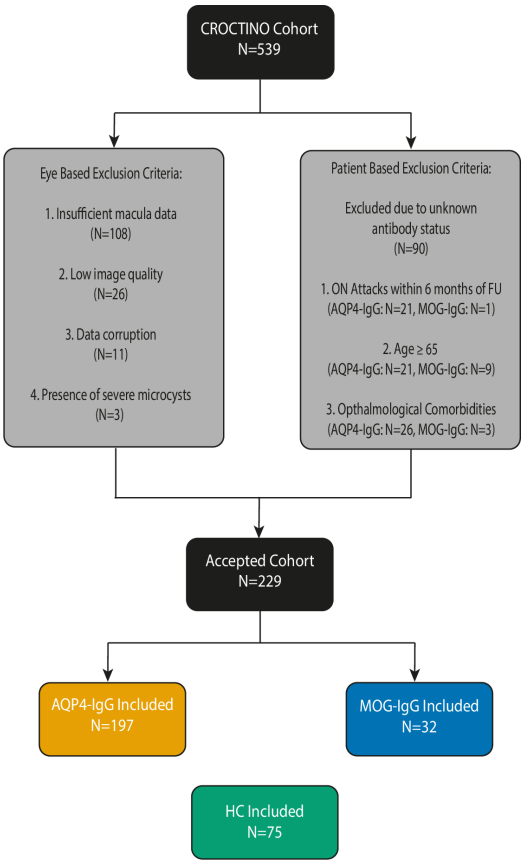

Figure 1 Cohort design and exclusion criteria: from the original 539 patients recruited in the CROCTINO cohort, 108 patients were excluded due to missing macular data. Of the remaining 431 patients in the segmentation cohort, a further 40 patients were excluded due to anomalies in their OCT scans (OSCAR-IB criteria; primarily due to low image quality (26 patients) or the presence of microcysts (3 patients) or other pathologies) or due to data corruption (11 patients). We also excluded patients with unknown antibody status (90 patients). Of the remaining 301 patients, the cohort was split based on AQP4-IgG or MOG-IgG seropositivity and a further set of exclusion criteria were applied based on age (being ≥65 years), ophthalmological comorbidities (eg, glaucoma) and in instances where follow-ups occurred within 6 months of an ON attack. AQP4-IgG, anti-aquaporin-4 antibody; HC, healthy control; MOG-IgG, anti-myelin-oligodendrocyte-glycoprotein antibody; OCT, optical coherence tomography; ON, optic neuritis.

spanning the entire thickness of the retina.[7] Of particular interest is also the Henle Fibre outer nuclear layer (ONL) boundary of the parafovea where AQP4 channels are highly expressed.[8]

A primary and attack-independent astrocytopathy in NMOSD has been suggested to contribute to retinal neurodegeneration and to Müller cell-associated parafoveal changes.[9–13] Recent studies suggested potential astrocytopathy-related outer retinal layer (ORL) thinning in AQP4-IgG seropositive NMOSD but were limited in their sample size and in parts contradictory on the exact layers in which these changes occur.[8 11] It thereby remains unclear if ORLs, especially the ONL are also potentially affected by primary retinal astrocytopathy in AQP4-IgG seropositive NMOSD.

Representing the largest international NMOSD dataset collected so far, the CROCTINO study (*Collaborative Retrospective Study on retinal optical coherence tomography (OCT) in Neuromyelitis Optica*) overcomes one of the common weaknesses of NMOSD studies—being limited to small and homogenous sample populations.[14 15] Using OCT data from over 20 centres worldwide, reliable quantitative and qualitative retinal assessment becomes possible, and controversial questions such as ORL changes in AQP4-IgG seropositive NMOSD can be clarified. Apart from patients who were AQP4-IgG seropositive, the CROCTINO cohort also includes patients with antibodies to myelin-oligodendrocyte-glycoprotein (MOG-IgG); a group that is now believed to be a distinct disease entity.[14 16–18] While clinically similar and undergoing comparable retinal neurodegeneration after ON, MOG-IgG-associated disease (MOGAD) lacks an identifiable astrocytopathy component and is thereby an appropriate diseased control group for patients who were AQP4-IgG seropositive when investigating astrocytic changes.[10 19]

In this study, we investigated if ORL thinning, specifically in the foveal and macular ONL, occurs in patients who were AQP4-IgG seropositive compared with healthy controls (HCs) and with patients with MOGAD as a diseased control group.

METHODS

Cohort design

A total of 539 patients with NMOSD were recruited between 2000 and 2018 as part of CROCTINO (stratified data of centres by device type and number of patients are summarised in the online supplemental file 1).[14] Patients with (1) diseases potentially confounding OCT analyses (including glaucoma, diabetic retinopathy, retinal surgery and ametropia greater than ±6 diopters), (2) a history of ON within the last 6 months before baseline, (3) no evidence of seropositivity for AQP4-IgG or MOG-IgG[20 21] and (4) no macular OCT data were excluded. Cell-based assays were used for the detection of AQP4-IgG and MOG-IgG antibodies in serum samples from all patients. Clinical data (antibody serology, disease duration, frequency of ON, location of ON, date of ON, Expanded Disability Standard Scale and treatment received) were collected from all patients. We also included 75 HCs (recruited from Barcelona, Isfahan, Mangalore and Berlin), who were neither age nor sex matched to either cohort.

Optical coherence Tomography

Retinal examinations were conducted at each centre using the following OCT devices: Spectralis SD-OCT, Heidelberg Engineering, Heidelberg, Germany (Spectralis), Cirrus HD-OCT, Carl Zeiss Meditec Inc, Dublin, California, USA (Cirrus) and Topcon 3D-OCT, Topcon Corp, Tokyo, Japan (Topcon). With respect to each device and each centre, two scans were collected: (1) a 3.4 mm diameter peripapillary ring scan around the optic nerve head for Spectralis SD-OCT (for Cirrus and Topcon devices: extracted from optic disc volume scans), and (2) a macular volume scans, centred on the fovea.[14] Scans were categorised and uploaded onto a central server to be accessed for further processing.

All OCT images fulfilled the OSCAR-IB criteria[22 23] (see figure 1—images from 29 patients not fulfilling these criteria were excluded) and results were presented in line with the

Table 1 Demographic overview

	HC	AQP4-IgG	MOG-IgG
Subjects (N)	75	197	32
Number of eyes (N)	148	317	55
Age (years, mean±SD)	32.3±9.6	41.8±12.1	36.5±13.7
Sex (male, N (%))	25 (33.8)	24 (12.2)	10 (31.2)
EDSS (median (IQR))	–	3.5 (2.0–5.0)	2.0 (1.5–2.5)
Average age at onset (years, median (IQR))	–	32.9 (24.9–42.4)	30.0 (17.6–42.5)
Patients with a history of ON (N (%))	–	142 (72.1)	24 (75.0)
Median number of ON episodes (median, IQR)	–	1.00 (0.00–3.00)	2.00 (1.00–4.00)
Disease duration (years, mean±SD)	–	7.1±6.7	4.8±7.8
Ethnicity (N (%))	White (57 (761)) Asian (16 (21.3)) Hispanic (1 (1.3)) Other (1 (1.3))	White (105 (53.3) Asian (56 (28.4)) African American (11 (5.6)) Other (25 (12.7))	White (19 (59.4)) Asian (13 (40.6))
Current treatment (N (%))		Rituximab (51 (25.9)) Azathioprine (42 (21.3)) Mycophenolate Mofetil (31 (15.7)) Methotrexate (4 (2.0)) Other or missing (69 (35.0))	Rituximab (6 (18.8)) Azathioprine (6 (18.8)) Prednisone (6 (18.8)) Mycophenolate mofetil (5 (15.6)) Other or missing (9 (28.1))
OCT device (N (%))	Spectralis (75 (100))	Spectralis (139 (70.6)) Cirrus (38 (19.3)) Topcon (20 (10.2))	Spectralis (25 (78.1)) Cirrus (3 (9.4)) Topcon (4 (12.5))

Cirrus: Cirrus HD-OCT, Carl Zeiss Meditec Inc, Dublin, California, USA; Spectralis: SD-OCT, Heidelberg Engineering, Heidelberg, Germany;Topcon: Topcon 3D-OCT, Topcon Corp, Tokyo Japan.
AQP4-IgG, anti-aquaporin-4 antibody; EDSS, Expanded Disability Standard Scale; HCs, healthy controls; MOG-IgG, anti-myelin-oligodendrocyte-glycoprotein antibody; N, number of subjects; ON, optic neuritis.

APOSTEL V.2.0 recommendations.[24] Peripapillary retinal nerve fibre layer (pRNFL) thickness was derived using a device-specific protocol and centred around the optic nerve head. Segmentation of all layers in macular volume scans were performed semiautomatically and processed with an in-house proprietary software (SAMIRIX).[25] For the purposes of this study, the macular retinal layers were segmented in the following layers: macular retinal nerve fibre layer (mRNFL), ganglion cell and inner plexiform layer (GCIP), inner nuclear layer (INL), outer plexiform layer (OPL), ONL, the outer plexiform and nuclear layer (OPNL), photoreceptor layer (PR, inner photoreceptor segments to Bruch's membrane) and the total retinal thickness (RT, calculated as the thickness consisting of the RNFL (defined as layer no. 3 per Staurenghi *et al*[26]) to the Bruch's membrane (layer no. 14). All scans were checked and, where necessary, manual correction of the automatic segmentation was conducted using SAMIRIX by experienced raters (FCO, CB and SS for ring scans, HZ, FCO and AL for macular scans) at a single site at the Charité—Universitätsmedizin Berlin. To assure comparability with previously published data on ORL changes in NMOSD, the macular volume data were further segregated into one of three export protocols: (1) a 5 mm diameter cylinder omitting a 1 mm diameter around the fovea (5 mm study), (2) a 3 mm diameter cylinder omitting a 1 mm diameter around the fovea (3 mm study) and (3) a 1 mm mean thickness around the fovea (1 mm study). Results are reported for the 5 mm study on Spectralis devices; confirmatory results based on the 3 mm and 1 mm study as well as for Cirrus and Topcon devices are set out in the online supplemental file 1.

Statistical methods

Data were stratified in cohorts by (1) antibody status and (2) ON history (contralateral eyes of patients with a history of unilateral ON are classified not fulfilling the ON history criteria). The data were further bifurcated by OCT device (Spectralis, Cirrus or Topcon) to mitigate any device-specific aberrations. For continuous cohort data (age, average age at onset and disease duration) on each of the AQP4-IgG, MOG-IgG and HC cohorts, the Student's t-test was employed. Cross-sectional group comparisons of the OCT values were conducted using linear mixed-effect models with age and sex as fixed and centre and patient-ID as random effects; where necessary, models were corrected for age and sex. Marginal and conditional coefficients of determination for the models were estimated by pseudo-R^2 for mixed-effect models. Significance was established at $p<0.05$. Statistical

Table 2 Group comparison between HC and patients who were AQP4-IgG and MOG-IgG seropositive at baseline (Spectralis devices only)

				AQP4-IgG vs HC			AQP4-IgG vs MOG-IgG			MOG-IgG vs HC		
	HC	AQP4-IgG	MOG-IgG	B	SE	P	B	SE	P	B	SE	P
Number of eyes	148	317	55									
pRNFL in µm (mean±SD)	99.17±9.76	78.46±24.13	74.33±23.44	−20.22	2.86	<0.001	0.34	4.33	0.937	−29.40	2.75	<0.001
mRNFL in µm (mean±SD)	35.25±3.13	28.09±6.60	27.62±5.43	−6.12	0.69	<0.001	−0.15	1.38	0.913	−6.98	0.66	<0.001
GCIP in µm (mean±SD)	80.62±6.14	65.81±13.03	66.16±11.85	−14.74	1.45	<0.001	−2.18	2.95	0.461	−15.16	1.33	<0.001
INL in µm (mean±SD)	39.64±2.51	39.85±3.57	41.55±4.14	0.34	0.39	0.384	−1.93	0.87	0.028	1.79	0.53	0.001
OPL in µm (mean±SD)	24.58±1.64	25.02±2.03	25.10±2.00	0.28	0.24	0.241	−0.21	0.44	0.634	−0.01	0.29	0.986
ONL in µm (mean±SD)	63.59±5.78	61.63±7.04	64.71±7.87	−0.01	0.83	0.993	−1.77	1.80	0.327	0.69	0.93	0.457
OPNL in µm (mean±SD)	89.23±6.95	86.65±7.21	89.81±8.61	−0.41	0.85	0.634	−1.54	1.85	0.406	−0.14	0.93	0.878
PR in µm (mean±SD)	80.80±2.38	80.35±2.94	81.49±3.59	−0.30	0.33	0.363	−0.07	0.68	0.923	0.20	0.39	0.610
RT in µm (mean±SD)	324.47±13.24	300.76±20.11	306.6±17.99	−20.16	2.37	<0.001	−6.61	4.77	0.169	−18.91	2.49	<0.001

AQP4-IgG, anti-aquaporin-4 antibody; B, estimate; GCIP, ganglion cell and inner plexiform layer; HC, healthy control; INL, inner nuclear layer; MOG-IgG, anti-myelin-oligodendrocyte-glycoprotein antibody; mRNFL, macular retinal nerve fibre layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OPNL, outer plexiform and nuclear layer; PR, photoreceptor layer; pRNFL, peripapillary retinal nerve fibre layer; RT, total retinal thickness.

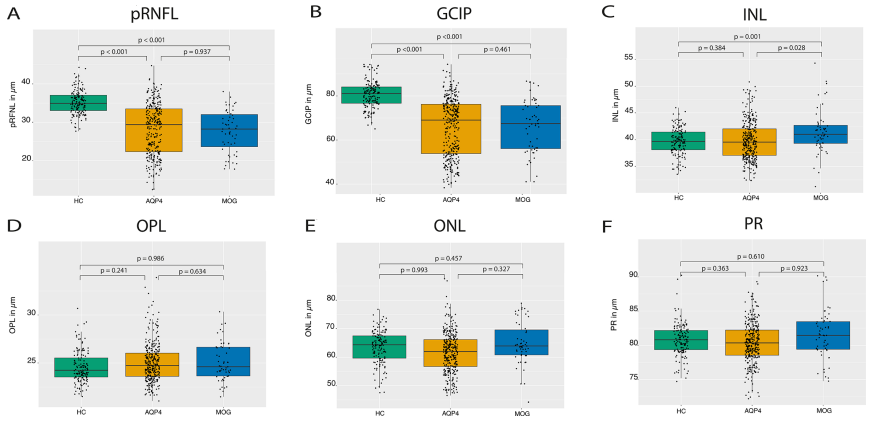

Figure 2 Group comparison of HC and patients who were AQP4-IgG and MOG-IgG seropositive at baseline: boxplots of mean OCT values with individual eyes (jitter) in HC (left, green), patients with AQP4-IgG (middle, yellow) and patients with MOG-IgG (right, blue). (A) pRNFL; (B) GCIP; (C) INL; (D) OPL; (E) ONL; and (F) PR. AQP4, aquaporin-4; HC, healthy control; GCIP, ganglion cell and inner plexiform layer; INL, inner nuclear layer; MOG, myelin-oligodendrocyte-glycoprotein; OCT, optical coherence tomography; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptive layer; pRNFL, peripapillary retinal nerve fibre layer

analyses were conducted using R (V.4.0.0) (RStudio Inc, Boston, Massachusetts, USA).[27]

RESULTS

Cohort description

In total, 197 patients who were AQP4-IgG seropositive fulfilled the inclusion criteria (figure 1, table 1). We also included 75 unmatched HCs and 32 patients who were MOG-IgG seropositiveas control groups.

Neuroaxonal damage measured by pRNFL, mRFNL and GCIP was comparable in patients who were AQP4-IgG seropositive (pRNFL: 78.46±24.13 µm, mRNFL: 28.09±6.60 µm, GCIP: 65.81±13.03 µm) and MOG-IgG seropositive (pRNFL: 74.33±23.44 µm, mRNFL: 27.62±5.43 µm, GCIP: 66.16±11.85 µm) making MOGAD a highly relevant comparative disease control group for our investigation of ORLs (table 2).

Limited outer retinal changes in AQP4-IgG seropositive NMOSD

No significant thinning of macular OPL and ONL in patients who were AQP4-IgG seropositive (irrespective of ON status) were observed compared with HC or patients who were MOG-IgG seropositive using the 5 mm diameter macular data (table 2, figure 2). No significant changes were observed when the OPL and ONL values were analysed as the combined OPNL. Previous studies described ORL thinning only in the foveal and parafoveal area as a sign of AQP4-IgG-induced Müller cell damage.[8 11] We therefore repeated our analyses in both 3 mm and the 1 mm diameter volumes around the fovea, but these narrower volumes showed again no relevant OPL or ONL thinning in patients who were AQP4-IgG seropositive compared with HC or patients who were MOG-IgG seropositive (see online supplemental data). Additionally, while these previous studies reported changes in the inner segment layer of the photoreceptors, this was not seen in our study.[8 11]

After a previous description[11] of ORL changes in patients who were AQP4-IgG seropositive with a history of ON, we also examined ORL differences separately in eyes with a history of ON. AQP4-IgG seropositive eyes with a history of ON (AQP4-ON) did not display any thinning of ONL and OPL compared with patients without a history of ON (AQP4-NON) or HC, despite severe neuroaxonal loss measured by pRNFL and GCIP layer (table 3, figure 3). Comparing patients who were AQP4-IgG and MOG-IgG seropositive, both groups had a comparable neuroaxonal loss (pRNFL, GCIP)—in the whole group as well as in respect of ON and non-ON eyes (table 2, figure 2). AQP4-ON (B=−1.54, SE=0.69 µm, p=0.027) as well as MOG-ON (B=−2.51, SE=0.87 µm, p=0.004) showed an OPL thinning in the fovea (1 mm diameter) compared with HC, but no difference was observed between AQP4-ON and MOG-ON (p=0.100). Also, no significant correlation between ethnicity and current therapies on outer retinal thickness was found (data not shown).

DISCUSSION

Our study suggests that neither macular OPL nor ONL loss occurs in AQP4-IgG seropositive NMOSD, regardless of ON phenotype, as compared with HC and patients who were MOG-IgG seropositive. The MOG-IgG cohort presented a unique opportunity to contrast our AQP4-IgG seropositive cohort with a highly relevant comparator group, which most likely has no astrocytopathy-component.[28]

Our results differ from those published by You *et al* in 2019[8] and Filippatou et al in 2020.[11] In both studies, thinning was observed in the ONL and the inner segment of the photoreceptor layers. In the case of You *et al*, who utilised Spectralis SD-OCT devices for the image acquisition, foveal thinning was observed along with a reduction in b-wave amplitudes in full-field electroretinography (ERG) suggestive of Müller cell dysfunction.[8] Filippatou *et al*, who employed Cirrus-SD-OCT for the image acquisition, also described thinning of the fovea in the 5 mm diameter macular area around the fovea.[11] Both studies suggested the ORL changes to be caused by a primary retinal astrocytopathy with AQP4-IgG associated glial dysfunction in Müller cells.[29] These pathological responses could account for the associated thinning observed in the ONL in these studies. However, other exogenous factors

Table 3 OCT results in patients who were AQP4-IgG seropositive stratified by history of on (Spectralis devices only)

	AQP4-ON	AQP4-NON	MOG-ON	MOG-NON	AQP4-ON vs AQP4-NON			AQP4-ON vs HC			AQP-NON vs HC			AQP4-ON vs MOG-ON			AQP4-NON vs MOG-NON		
Number of eyes	232	85	43	12	B	SE	P	B	SE	P	B	SE	P	B	SE	P	B	SE	P
pRNFL in µm (mean±SD)	72.84±24.47	96.09±12.99	68.03±22.95	95.33±7.32	−25.18	3.93	<0.001	−29.56	3.57	<0.001	−9.29	5.15	0.07	2.63	6.12	0.667	6.03	5.44	0.275
GCIP in µm (mean±SD)	62.94±12.73	77.11±7.56	63.45±11.96	75.88±6.01	−14.74	2.06	<0.001	−19.60	1.49	<0.001	−0.50	1.48	0.735	−4.00	3.20	0.215	3.22	3.36	0.344
OPL in µm (mean±SD)	25.06±2.01	24.71±1.79	25.28±2.08	24.45±1.55	0.26	0.38	0.498	0.34	0.26	0.184	0.09	0.38	0.804	0.34	0.51	0.509	−0.12	0.96	0.899
ONL in µm (mean±SD)	62.53±7.45	63.14±6.62	66.09±8.08	59.76±4.58	−0.19	1.49	0.901	−0.18	0.92	0.847	−0.82	1.40	0.560	−2.84	2.12	0.183	2.76	3.58	0.446
OPNL in µm (mean±SD)	87.58±7.67	87.85±6.78	84.21±5.68	91.37±8.69	0.00	1.53	0.879	0.27	0.95	0.775	0.37	1.42	0.794	−2.51	2.21	0.259	2.67	3.51	0.450
PR in µm (mean±SD)	80.89±2.93	79.80±2.94	82.01±3.45	79.62±3.58	0.75	0.57	0.187	−0.19	0.36	0.595	−1.08	0.59	0.071	−0.58	0.77	0.454	1.36	1.44	0.348

AQP4, aquaporin-4 ; B, estimate; GCIP, ganglion cell and inner plexiform layer; HC, healthy control; MOG, myelin-oligodendrocyte-glycoprotein; NON, non-optic neuritis; ON, optic neuritis; ONL, outer nuclear layer; OPL, outer plexiform layer; OPNL, outer plexiform and nuclear layer; PR, photoreceptor layer; pRNFL, peripapillary retinal nerve fibre layer.

cannot be ruled out as contributory, such as cohort composition and study methodologies.

On a cohort level, our population is larger (197 patients who were AQP4-IgG seropositive vs 22 and 51 by You *et al* and Filippatou *et al*, respectively)[8 11] and more diverse than prior studies, which minimises potential type I errors. While You *et al* did not specify the ethnic composition of their cohort, the cohort in Filippatou *et al* had a relatively even distribution between Caucasian Americans (43%) and African Americans (53%) with a minor subset of Asian Americans (4%)—describing a pronounced ONL thinning in African Americans. African American patients with multiple sclerosis (MS) are also known to suffer from faster and often more aggressive disease course in general, which could also be true for other neuroinflammatory diseases like NMOSD.[30 31] Our AQP4-IgG seropositive cohort included an ethnically diverse dataset acquired worldwide with a lower African American patient composition (5.6%), which might have contributed to the less profound foveal ONL changes.[11 32]

Recently, it has been hypothesised that the neuroplastic characteristics of the INL may act as a barrier to retrograde (but not anterograde) trans-synaptic axonal degeneration—rectified to the ORLs—in patients with MS following ON.[33] This limited neuroplastic ability is hypothesised to rest with the bipolar, amacrine and horizontal cells, which feed into the synaptic tree at the level of the INL, and raises questions as to whether such protective mechanisms may also play a limited part in NMOSD and whether it remains so as we age.[33] The average age of participants in the two other studies were relatively older (mean age for both being 47 years), whereas for our AQP4-IgG cohort it was 42 years. Previously reported studies concerning cohorts of similar demographic distribution to ours reported no significant correlation between age and retinal thickness.[34 35] However, age-related changes in the retina cannot be ruled out and ORLs may be more susceptible to change with increasing age and/or disease duration. It is well-known that the plasticity of the CNS markedly reduces over time, and as a corollary, the regenerative properties of the INL may also be affected thereby diminishing its protective effects in reducing retrograde (trans-synaptic) axonal degeneration.[36] The retina is also a vascularised organ, particularly at the interface between inner and outer retina, where the deep vascular plexus intercepts the boundary between the INL and OPL.[37] Should the blood–retina barrier be compromised in the boundary between the INL and OPL, it is conceivable that the protective abilities of the INL may be circumvented and thereby mediating glial dysfunction in the Müller cells. This may have been what was observed in the OPL from the 1 mm AQP4-ON and MOG-ON cohort given the relative location of the OPL to the INL. To that end, while disease duration did not reveal to any correlates with OPL (p=0.805) or ONL (p=0.835) values, we cannot exclude time-dependent effects in a cross-sectional analysis. We believe that this area warrants more research to quantify if (1) age is a factor, (2) ON damages the barrier function and (3) the INL does indeed play a role as a dam to retrograde axonal degeneration in NMOSD.

A strength of our study rests on its cohort size and composition, which mirrors that of a global population. This result derives from a consortium of expert NMOSD researchers enabling the enrolment of participants through a multicentre strategy. This approach was designed to overcome many of the earlier NMOSD study limitations, for example small and homogeneous sample populations. Additionally, the use of differing OCT devices compounds complexities in OCT comparisons and a high degree of caution is needed in order to rely on differing platforms interchangeably.[38] Thus, our study focuses on use of

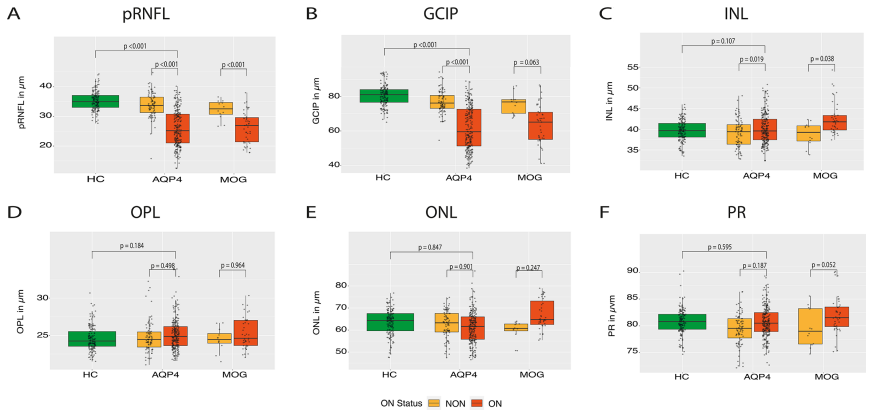

Figure 3 OCT results stratified by ON status (tested with Spectralis devices): boxplots of mean OCT values with individual eyes (jitter) in HC (left, green), AQP4-IgG cohort (middle) and MOG-IgG cohort (right). Seropositive patients with a history of ON are highlighted with light yellow and seropositive patients without a history of ON are highlighted in orange. (A) pRNFL; (B) GCIP; (C) INL; (D) OPL; (E) ONL; and (F) PR. AQP4, aquaporin-4; GCIP, ganglion cell and inner plexiform layer; INL, inner nuclear layer; MOG, myelin-oligodendrocyte-glycoprotein; OCT, optical coherence tomography; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptive layer; pRNFL, peripapillary retinal nerve fibre layer.

three widely available OCT devices, and obtained confirmatory results with each of them; of these, two were also employed respectively in the studies by You *et al*[8] and Filippatou *et al*.[11]

Limitations of the current study should also be considered. First, the HCs and patients with MOGAD were not matched, which makes it difficult to rule out age-related and gender-related affects. Notably, retinal thickness decreases with age and males generally exhibit higher GCIP and RT.[25] Also, no ERG or functional visual pathway assessments were conducted, which could have potentially shown more subtle functional impairment of ORLs without associated tissue loss. Outer retinal studies are additionally complicated by Henle Fibre morphologies as OCT beam placement plays a major role in how this layer is depicted; the high level of irregularity and variability in these morphologies add a level of subjectiveness in the quantification and correction of outer layer segmentation and analyses.[39] Finally, Cirrus and Topcon measurements could not be utilised as confirmatory cohorts as there lacked sufficient HCs examined with these devices. Nonetheless, the current findings provide insights into relationships between retinal layer changes and axonal damage that have not previously been recognised; as no ORL changes can be observed on account of a primary astrocytopathy in NMOSD, it potentially alleviates the burden of monitoring the ORLs when tracking disease progression and reinforces the need to focus primarily on the inner layers, particularly the RNFL and the GCIP layer.

CONCLUSION

Our results show no evidence of macular ORL changes as a major component of retinal damage in patients who were seropositive AQP4-IgG NMOSD and patients with MOGAD. Further studies will be necessary to clarify (1) if OPL and ONL are damaged in late disease stages due to retrograde trans-synaptic axonal degeneration across the damaged INL barrier and (2) if outer retinal dysfunction without a measurable structural correlate occurs. Longitudinal studies could help quantify changes in the ORLs alongside disease progression.

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Conference abstracts

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