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Transorbital sonography reveals retrobulbar optic nerve neuroaxonal changes in multiple sclerosis

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ABSTRACT

Background: The optic nerve (ON), as an extension of the central nervous system, reflects multiple sclerosis (MS) pathological processes. Transorbital sonography (TOS) is emerging as a novel technique to detect ON changes. Our aim was to investigate retrobulbar ON morphometric changes in MS and their relationship with prior history of optic neuritis.

Methods: 122 MS subjects were enrolled, 68 with and 54 without history of optic neuritis. ON diameters without and with sheath were measured at 3 mm (OND3 and ONSD3) and 5 mm (ONSD5) posterior to the optic disc; myelination index (MI=ONSD3/ONSD5) was calculated. To account for intrasubject dependencies, linear mixed models (LMMs) for repeated measures were employed, adjusting for age at onset and body mass index.

Results: ONs with history of inflammation (I-ON) exhibited significantly reduced diameters versus companion nerves in both measurements with and without sheath (OND3: $p = 0.002$; ONSD3: $p = 0.010$; ONSD5: $p = 0.046$). All ONs diameters progressively decreased with increasing neuritis frequency, demonstrating a dose-response effect. Significant measurement reductions in OND and ONSD3 were observed in I-ON of patients with Expanded Disability Status Scale >3, whereas no significant differences emerged for progression index (PI) > 0.20 among ONs with and without history of optic neuritis.

Conclusions: TOS reliably captures ON morphometric changes related to inflammatory history and chronic neuroaxonal injury in MS, supporting its role as a promising biomarker of disability burden and progression independent of relapse activity (PIRA).

1. Introduction

Retrobulbar optic neuritis (RBON) is a common manifestation of multiple sclerosis (MS), a demyelinating and degenerative chronic immune-mediated disease of the central nervous system (CNS). In MS RBON occurs in 25–35% cases at clinical onset and in 70% during the disease course. Fifty-per-cent of the subjects with a first episode of RBON feature a 15-year risk of conversion to MS [1–3]. The 2024 revision of McDonald diagnostic criteria for MS [4] establishes, among others, the cardinal role of the optic nerve (ON) as a fifth topography for dissemination in space, enhancing sensitivity for MS diagnosis [4,5].

The ON originates from the diencephalon as an extroflection of the CNS and, as such, shares brain pathological processes, including inflammation, demyelination and neurodegeneration [6]. There is growing interest on ON multiparametric characterization to detect early brain demyelination and axonal degeneration, and to predict brain biological changes during the disease course. Magnetic resonance imaging of the ON, optical coherence tomography (OCT) and visual evoked potentials (VEPs) are the reference standard to monitor progression in MS. However, these techniques do not concomitantly capture morphological, functional and dynamic changes across ON different retrobulbar portions, at various disease stages and with high spatial resolution

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[3,5,7].

Transorbital sonography (TOS) is non-invasive and easy-to-perform imaging modality that enables the assessment of retrobulbar ON morphometric changes, by measuring nerve diameters with or without its sheath [8]. TOS has also proven to be useful and sensitive to intracranial pressure changes, such as in idiopathic intracranial hypertension [9], and features good reliability as compared to MRI [10].

Evidence and robustness on the use of TOS in CNS demyelination shows conflicting results [8,11–15], due to substantial methodological heterogeneity across studies, including measurement protocols, small sample sizes, statistical approaches, and a consequent overall high risk of bias.

In a population of MS patients [16], we used TOS to detect morphological changes of the retrobulbar diameters in ONs with vs. without past diagnosis of RBON and attempted to minimize the risk of bias through ad hoc statistical approaches.

2. Methods

2.1. Study population

Optic nerves from a cohort of 122 subjects with a diagnosis of MS [16], consecutively referred to the MS Center, University Neurology Unit, University Hospital, Ferrara, northern Italy, and with (I-ON) vs. no (NI-ON) past diagnosis of RBON or vs. companion nerves (C-ON) with no diagnosis of RBON, were studied with TOS. RBON was diagnosed by neurologists and ophthalmologists based on conventional clinical presentation and supportive instrumental findings (OCT, VEPs, MRI) [17]. Acute RBON at study time was an exclusion criterion for study enrollment. A sub-analysis was conducted on forty ($n = 40$) ONs from a small sample of control subjects by convenience with no previous diagnosis nor clinical history of either RBON, MS nor other ON disease (Ctrl-ON).

2.2. Optic nerve measurements

In its retrobulbar sections, the ON is enveloped by a meningeal sheath comprising the dura mater, the arachnoid mater, the subarachnoid space which consists of a rich and complex system of septa, trabeculae and pillars, and pia mater (Fig. 1A) [18]. TOS was used to measure the nerve diameter at 3 mm without (OND3) and with its sheath (ONSD3) and at 5 mm with its sheath (ONSD5), respectively, from the optic disc. The so-called 'myelination index' (MI) was also computed, as the ONSD3/ONSD5 ratio [12].

2.3. Transorbital sonography examination

B-mode TOS was performed as an off-label use of MyLab X90 ultrasound system (US) (Esaote, S.p.A., Genoa, Italy). The US diagnostic imaging device was used combined with a multifrequency 3–11 MHz linear probe LA332. Patients laid in supine position, with the head tilted forward of 30°, and were asked to keep their eyes closed, maintaining their gaze oriented straight ahead as if they were staring at the ceiling. Axial images of the ON were acquired based on the axial plan of the nerve, avoiding beam artifacts and reaching a symmetric visualization of the ON sheaths also in depth. Subjects were asked to move their eyes with the purpose of obtaining the best axial visualization and perspective of the ON.

The examination was carried out by a neurologist with expertise in neuro- and transorbital sonography (N.M.) who was blind to the ON condition. The "*as low as reasonably achievable*" (ALARA) principle was

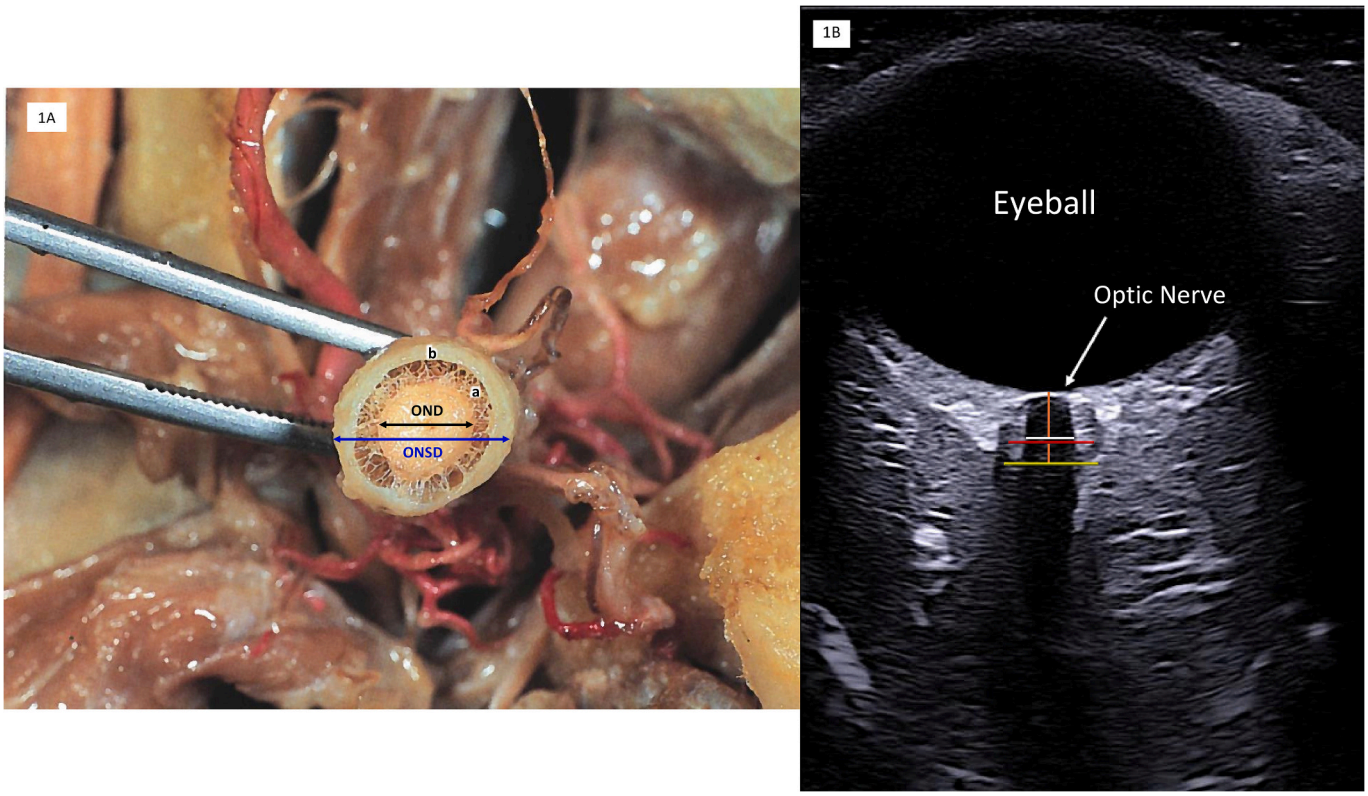


Fig. 1. (A) Surgical cross-sectional anatomy of optic nerve and its sheath [*With permission and modified from American Academy of Ophthalmology, 2021*]; (B) B-Mode axial view imaging of optic nerve with ultrasonographic nerve measurement protocol: OND at 3 mm from the optic disc (OND3): white line; ONSD at 3 mm from the optic disc (ONSD3): red line; ONSD at 5 mm from the optic disc (ONSD5): yellow line.
OND = optic nerve diameter; ONSD = optic nerve diameter with its meningeal sheath; a = subarachnoid space; b = dura mater.
Caption: Anatomical–sonographic correlation of the retrobulbar optic nerve and transorbital sonography imaging protocol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

applied by reducing mechanical index to 0.23, in line with the American Institute of Ultrasound in Medicine (AIUM) indications for ocular safety [19]. Particular care was used to minimize subject's eye exposure to color Doppler, in line with the European Academy of Neurology Scientific Panel of Neurosonology, the European Society of Neurosonology and Cerebral Hemodynamics, and the European Reference Centers in Neurosonology (EAN SPN/ESNCH/ErcNsono Neuro-POCUS) Working Group for Orbital Sonography (MI ≤ 0.2) [20]. Shear wave elastography (SWE), i.e., acoustic radiation force impulse, requiring a long-duration and high-energy push pulse was not used in this study. No TOS-related adverse events nor undesirable side effects were registered in participants during the conduction of this study. After optimizing resolution and imaging quality, B-mode images of both optic nerves were recorded (3–5 images for each side).

The ON and its pia mater lining are visualized as hypoechoic at TOS while the surrounding subarachnoid space and dura mater appear as a stripped hyperechoic band. (Fig. 1b) [13,21].

2.4. Statistical analysis

MS patients' demographic and clinical characteristics were reported as means and standard deviations or as median and range, and as frequency and percentages for continuous and categorical variables, respectively. Comparisons between groups were performed with *Student-t-test* for continuous variables and Chi-square test or Fisher exact test for categorical variables.

Statistical comparisons of OND3, ONSD3, ONSD5 and MI between I-ON vs. C-ON and I-ON vs NI-ON were performed using linear mixed models (LMMs) for repeated measures which allowed to adjust for both intrasubject nerve diameters, and for covariates assigned to intra-subjects' nerves in potential different status (eg., I-ON and NI-ON in the same subject). The ON side was therefore used as repeated measure and the subject ID for random effect; OND3, ONSD3, ONSD5 and the MI were the independent variables and the ON status (I-ON vs. C-ON, or vs. NI-ON) the fixed factor. As age at MS onset and body mass index (BMI) significantly differed by ON diameters already among NI-ON (reference), the results from LMM analysis were also reported after adjusting for these covariates when appropriate. Stratification by sex and Expanded Disability Status Scale (EDSS) [22] at MS clinical onset is presented, as well as by progression index (PI), ie., the delta between two EDSS scores at two time points (T0 and T1), divided by the T1-T0 time lag in years; PI greater than 0.20 indicated disease progression [23]. EDSS scores had been assigned to subjects by other MS clinicians and prior to the study time. Results from LMM analysis were reported as estimated marginal means and 95% confidence intervals (95%Cis).

We also present results from a LMM subanalysis comparing ON diameters of MS patients (I-ON and NI-ON) with those from a small convenience sample of forty control subjects' optic nerves (Ctrl-ON).

The IBM Statistical Package for Social Sciences (SPSS) v30 was used for the analyses.

2.5. Ethical issues

The study was approved by the local Committee of Bioethics "Comitato Etico di Area Vasta Emilia Centro della Regione Emilia-Romagna (CE-AVEC)", Prot. N. 68/2023/Oss/AOUFe, Jan. 19th, 2023. All procedures performed were conducted in accordance with the ethical standards of the research committee and with the 1964 Declaration of Helsinki and its later amendments. Informed consent for the TOS study was obtained from each participant.

3. Results

3.1. Descriptive statistics

The distribution of main demographic and clinical characteristics of the MS study population by past ($N = 68$) vs. no ($N = 54$) diagnosis of optic neuritis is reported in Table 1.

Among MS patients with no past diagnosis of RBON (reference group of patients to the purpose of this study), sheathed diameters were greater in men: 5.090 vs 4.768 mm ($p = 0.001$) for ONSD3, and 5.521 vs 5.035 mm for ONSD5 ($p = 0.0001$). MI was smaller in men ($p = 0.005$) (Table 2). ONSD5 was greater in individuals aged 26 years and above as compared to younger ones ($p = 0.015$). Nerves were larger among overweighted than normal weighted MS patients for ONSD3 (5.015 vs. 4.791 mm, $p = 0.023$) and ONSD5 (5.459 vs. 5.063 mm, $p = 0.001$), while MI was smaller in the first group ($p = 0.011$). No differences between the two groups by smoking habit were observed for all diameters.

In the main LMM analysis all diameters from optic nerves with documented past history of RBON (I-ON) were smaller than those from the contralateral unaffected fellow nerves (C-ON) (Table 3, Model 1; Fig. 2). Such difference was statistically significant even after adjusting

Table 1
Distribution of main demographic and clinical characteristics of the MS study population by past vs. no diagnosis of optic neuritis.

Characteristics	MS patients with no past diagnosis of optic neuritis $N = 54$	MS patients with past diagnosis of optic neuritis $N = 68$	p
Sex, N (%)			
Men	19 (35.2)	17 (25.0)	*0.221*
Women	35 (64.8)	51 (75.0)	
Age at study time, mean (SD), years	47.7 (12.7)	44.3 (11.9)	*0.131*
Age at MS clinical onset, mean (SD), years	34.1 (11.2)	30.1 (8.7)	***0.027***
MS duration from clinical onset, mean (SD), years	13.6 (11.7)	14.2 (9.5)	*0.739*
MS course at onset, N (%)			
Relapsing-remitting	32 (59.3)	65 (95.6)	***0.000001***[a]
Progressive	22 (40.7)	3 (4.4)	
Progression index, N (%)			
Decreased, stable (≤0.20)	45 (88.2)	61 (92.4)	*0.441*
Increased (>0.20)	6 (11.8)	5 (7.6)	
EDSS at MS clinical onset, median (range)	2.0 (1.0–6.0)	1.0 (0–3.0)	***0.001***
EDSS at study time, median (range)	2.0 (1.0–8.0)	2.0 (0–6.0)	***0.031***
Episodes of optic neuritis, N (%)			
1 episode	–	53 (77.9)	–
2 episodes	–	12 (17.6)	–
3+ episodes	–	3 (4.4)	–
Age at first episode of optic neuritis, mean (SD), years	–	31.8 (9.2)	–
BMI, mean (SD)	25.1 (4.6)	24.0 (5.0)	*0.211*
Smoking habit, N (%)			
No	37 (68.5)	46 (67.6)	*0.918*
Yes	17 (31.5)	22 (32.4)	
Arterial hypertension, N (%)			
No	42 (79.2)	55 (80.9)	*0.823*
Yes	11 (20.8)	13 (19.1)	
Hypercholesterolemia, N (%)			
No	40 (75.5)	58 (85.3)	*0.172*
Yes	13 (24.5)	10 (14.7)	
Diabetes mellitus, N (%)			
No	52 (98.1)	66 (98.5)	*0.867*
Yes	1 (1.9)	1 (1.5)	
Oligoclonal bands, N (%)			
<2	6 (19.4)	18 (43.9)	***0.043***[a]
2+	25 (80.6)	23 (56.1)	

SD = standard deviation.
BMI = body mass index; EDSS = Expanded Disability Status Scale[22]; MRI = magnetic resonance imaging; Progression Index (PI): PI>0.20 indicates disease progression (from clinical onset to study time)[23].
[a] Fisher test.

Table 2
Diameters (mm) and myelination index measured with transorbital sonography of optic nerves of MS patients with no diagnosis of RBON and by main covariate: estimated marginal means (95% confidence interval).

	OND3	*p*	ONSD3	*p*	ONSD5	*p*	MI	*p*
Sex								
Male	3.060 (2.978, 3.143)	*0.677*	5.090 (4.930, 5.249)	***0.001***	5.521 (5.325, 5.717)	***0.0001***	0.927 (0.915, 0.940)	***0.005***
Female	3.082 (3.023, 3.140)		4.768 (4.657, 4.880)		5.035 (4.896, 5.173)		0.950 (0.941, 0.959)	
Age at clinical onset								
<26 years	3.071 (3.009, 3.133)	*0.864*	4.801 (4.680, 4.922)	*0.063*	5.078 (4.926, 5.299)	***0.015***	0.948 (0.938, 0.957)	*0.083*
26+ years	3.080 (3.005, 3.154)		4.981 (4.835, 5.127)		5.374 (5.191, 5.557)		0.934 (0.923, 0.946)	
BMI								
Normal	3.080 (3.019, 3.140)	*0.680*	4.791 (4.672, 4.909)	***0.023***	5.063 (4.194, 5.211)	***0.001***	0.947 (0.938, 0.956)	***0.011***
Overweight	3.059 (2.982, 3.137)		5.015 (4.863, 5.168)		5.459 (5.270, 5.649)		0.928 (0.917, 0.940)	
Smoking habit								
Never smoker	3.093 (3.036, 3.151)	*0.263*	4.917 (4.804, 5.031)	*0.185*	5.234 (5.090, 5.377)	*0.384*	0.945 (0.936, 0.954)	*0.367*
Ever smoker	3.035 (2.951, 3.119)		4.782 (4.616, 4.948)		5.121 (4.912, 5.331)		0.937 (0.924, 0.951)	

OND3 = optic nerve diameter at 3 mm from optic disc; ONSD3 = optic nerve sheath diameter at 3 mm from optic disc; ONSD5 = optic nerve sheath diameter at 5 mm from optic disc; MI = myelination index.
Linear Mixed Models for repeated measures (see Methods): estimated marginal means (95%CI) are reported.

for age at MS clinical onset and BMI (Table 3, Model 2). The adjusted estimated marginal means (95%CI) of I-ON vs. C-ON were 2.988 mm (2.918, 3.050) vs. 3.170 (3.082, 3.257) for OND3 ($p = 0.002$), 4.709 mm (4.588, 4.829) vs. 4.964 (4.815, 5.113) for ONSD3 ($p = 0.010$), and 5.021 mm (4.873, 5.170) vs 5.261 (5.080, 5.442) for ONSD5 ($p = 0.046$), respectively (Table 3). No difference was observed for MI in either analysis.

All diameters from optic nerves with documented past history of RBON (I-ON) were also significantly smaller than those of nerves with no diagnosis of RBON (NI-ON) (Table 3, Model 1). This difference remained after adjusting for age at MS clinical onset and BMI but lost statistical significance (Table 3, Model 2).

The same crude and adjusted analysis stratified by sex yielded significant differences between I-ON vs. C-ON only among women: 2.956 (2.872, 3.040) vs. 3.147 mm (3.039, 3.255) for OND ($p = 0.007$), 4.848 (4.676, 5.019) vs. 4.576 mm (4.42, 4.709) for ONSD3 ($p = 0.015$), 4.851 (4.692, 5.009) vs. 5.113 mm (4.692, 5.009) for ONSD5 ($p = 0.046$), while no difference was observed for MI, 0.947 (0.937, 0.957) vs 0.947 (0.934, 0.961) ($p = 0.967$).

In order to explore the relation between diameters of optic nerves with vs. no documented history of RBON and disability measures, we stratified the analysis by EDSS scores at study time (up to 2.5, and greater than 2.5) and by progression index (up to 0.20 and greater than 0.20) (Table 4).

We report ON diameters by number of RBON episodes and by sex (Table 5).

A dose response effect was observed in the nerve study population with diameters significantly decreasing with increased number of episodes for OND, ONSD3 and ONSD5 ($p = 0.034$, $p = 0.019$, $p = 0.042$, respectively). Such difference was also observed after stratification by sex, but was only statistically significant in women and for OND3 ($p = 0.023$) and ONSD3 ($p = 0.043$) (Table 5).

4. Discussion

The morphologic study of the ON retrobulbar portion is pivotal in providing insights into the biological processes of CNS demyelination and atrophy in MS. In light of the recently updated diagnostic criteria for MS [4], non-invasive and easy-to-perform multiparametric approaches including TOS are sought to predict progression at an early disease stage.

ONs of patients with MS are reported to be smaller than in controls [11,12,14,24]. In a sub-analysis, we compared diameters of both MS NI-ON and I-ON with those of forty nerves from a small convenience control sample and found evidence in line with literature (Table 6).

In the present study, however, we were primarily interested in investigating the ON status in chronic MS. We therefore compared diameters of ONs with documented past RBON vs those with no such exposure (our study reference) in MS patients. Optic nerves with past diagnosis of neuritis (I-ON) featured decreased diameters without and with sheath at 3 and 5 mm from optic disc as compared to both apparently healthy companion nerves (Fig. 2) and NI-ON. After adjusting for potential confounders, such as age and BMI, the difference between I-ON and NI-ON lost significance, likely in relation to a diffuse neuroaxonal injury, chronic active inflammation and progression irrespective of relapse activity (ie., PIRA) [25,26]. Interestingly, C-ON diameters were significantly larger than I-ON.

In our cohort, history of optic neuritis was significantly associated to relapsing-remitting MS course and to younger age at MS clinical onset. Subjects with prior documented RBON showed smaller median EDSS at onset and study time. Subjects' overweight was associated to larger ONSD3 and ONSD5 compared to normal BMI, suggesting a direct effect of body mass on sheathed vs. non sheathed diameters. Stratification by sex revealed a greater impact of a previous history of RBON in women as compared to men, and especially for OND3 and ONSD3, with thinner diameters in female I-ON vs. C-ON or NI-ON, also after adjusting for confounders.

Schroeder and coll., found reduced OND and ONSD in patients with previous history of RBON, but no adjustment for potential confounders or effect modifiers was performed [8]. Carcelen-Gadea and coll. observed reduced ON diameters after a 3-year follow-up compared to controls, and a tendency for a thinner ON in subjects with history of optic neuritis at baseline [27]. However, this analysis did not consider potential confounding, and whether the sheath was included in the diameter measurement was not specified [27]. Conversely, Carraro et al. did not find significant differences between ON with and without history of neuritis [14], but the ON of MS patients was thinner than in controls, consistently with other authors' findings [11,14,24,28,29]. De Masi et al. observed similar differences between MS patients and controls [12], while Koraysha and colleagues did not detect statistical differences for OND3 nor ONSD3 between MS patients and controls [13].

The 'myelination index' (MI) is the ratio between ONDS3 over ONSD5 [12]. In normal ONs, fibers show greater myelination at 5 mm from optic disc and the MI is therefore smaller than 1. Increased MI reveals changes in the ON morphology, with either increased ONSD3 or decreased ONSD5. However, whether MI changes truly reflect changes in myelination or rather in axonal or sheath diameters in relation to past inflammation remains to be elucidated. MI did not significantly differ between I-ON vs NI-ON and vs C-ON, potentially corroborating a biological process of chronic active inflammation. This is in contrast with the work by De Masi and colleagues, where a reduced MI was reported in MS subjects compared to controls [12]. Similar findings are reported for a small sample by Orabi and coll. [30]. Our findings may be attributed to a more sophisticated statistical approach employing LMM and larger MS population.

Table 3
Diameters (mm) and myelination index measured with transorbital sonography of optic nerves with (I-ON) vs. no (NI-ON[1]) past diagnosis of optic neuritis: estimated marginal means (95% confidence interval).

	Model 1								Model 2							
ON status	**OND3 (mm)**	***p***	**ONSD3 (mm)**	***p***	**ONSD5 (mm)**	***p***	**MI**	***p***	**OND3 (mm)**	***p***	**ONSD3 (mm)**	***p***	**ONSD5 (mm)**	***p***	**MI**	***p***
Total									**Total**							
C-ON	3.183	***<0.001***	4.993	***0.004***	5.293	***0.016***	0.945	*0.961*	3.170	***0.002***	4.964	***0.010***	5.261	***0.046***	0.944	*0.918*
	(3.094, 3.272)		(4.831, 5.155)		(5.096, 5.490)		(0.933, 0.957)		(3.082, 3.257)		(4.815, 5.113)		(5.080, 5.442)		(0.932, 0.955)	
I-ON	2.973		4.688		4.981		0.945		2.988		4.709		5.021		0.943	
	(2.903, 3.043)		(4.560, 4.815)		(4.824, 5.139)		(0.936, 0.955)		(2.918, 3.050)		(4.588, 4.829)		(4.873, 5.170)		(0.933, 0.952)	
NI-ON	3.074	***0.018***	4.874	***0.022***	5.196	***0.034***	0.942	*0.614*	3.067	*0.064*	4.852	*0.147*	5.177	*0.239*	0.941	*0.961*
	(3.025, 3.124)		(4.780, 4.968)		(5.080, 5.312)		(0.935, 0.949)		(3.017, 3.117)		(4.761, 4.942)		(5.067, 5.287)		(0.933, 0.948)	
I-ON	2.973		4.687		4.980		0.945		2.986		4.736		5.062		0.941	
	(2.905, 3.041)		(4.558, 4.816)		(4.818, 5.142)		(0.935, 0.956)		(2.916, 3.056)		(4.609, 4.863)		(4.905, 5.219)		(0.931, 0.951)	
Men									**Men**							
C-ON	3.243	*0.114*	5.294	*0.313*	5.715	*0.406*	0.934	*0.826*	3.253	*0.058*	5.289	*0.349*	5.679	*0.552*	0.936	*0.660*
	(3.081, 3.404)		(4.971, 5.618)		(5.293, 6.138)		(0.908, 0.960)		(3.107, 3.399)		(4.961, 5.616)		(5.254, 6.105)		(0.910, 0.962)	
I-ON	3.073		5.082		5.480		0.930		3.066		5.088		5.510		0.929	
	(2.933, 3.214)		(4.808, 5.357)		(5.093, 5.867)		(0.907, 0.954)		(2.939, 3.194)		(4.807, 5.369)		(5.122, 5.898)		(0.905, 0.952)	
NI-ON	3.056	*0.833*	5.090	*0.949*	5.518	*0.873*	0.926	*0.732*	3.067	*0.064*	5.095	*0.872*	5.523	*0.808*	0.926	*0.705*
	(2.973, 3.139)		(4.913, 5.266)		(5.299, 5.736)		(0.914, 0.938)		(3.017, 3.117)		(4.917, 5.274)		(5.304, 5.743)		(0.914, 0.938)	
I-ON	3.073		5.079		5.484		0.930		2.986		5.067		5.470		0.931	
	(2.937, 3.209)		(4.788, 5.369)		(5.117, 5.850)		(0.910, 0.951)		(2.916, 3.056)		(4.769, 4.364)		(5.097, 5.843)		(0.910, 0.951)	
Women									**Women**							
C-ON	3.159	***0.002***	4.858	***0.014***	5.103	*0.050*	0.950	*0.992*	3.147	***0.007***	4.848	***0.015***	5.113	***0.046***	0.947	*0.967*
	(3.051, 3.267)		(4.682, 5.035)		(4.901, 5.306)		(0.937, 0.963)		(3.039, 3.255)		(4.676, 5.019)		(4.692, 5.009)		(0.934, 0.961)	
I-ON	2.942		4.578		4.848		0.950		2.956		4.576		4.851		0.947	
	(2.861, 3.024)		(4.445, 4.712)		(4.693, 5.002)		(0.940, 0.960)		(2.872, 3.040)		(4.42, 4.709)		(4.692, 5.009)		(0.937, 0.957)	
NI-ON	3.082	***0.007***	4.765	***0.030***	5.026	*0.093*	0.950	*0.980*	3.072	***0.028***	4.743	*0.089*	5.020	*0.211*	0.947	*0.925*
	(3.020, 3.143)		(4.659, 4.870)		(4.889, 5.153)		(0.941, 0.959)		(3.010, 3.133)		(4.641, 4.845)		(4.897, 5.144)		(0.938, 0.955)	
I-ON	2.943		4.575		4.849		0.950		2.957		4.597		4.890		0.946	
	(2.864, 3.022)		(4.441, 4.710)		(4.685, 5.013)		(0.938, 0.961)		(2.877, 3.037)		(4.463, 4.731)		(4.726, 5.053)		(0.934, 0.958)	

OND3 = optic nerve diameter at 3 mm from optic disc; ONSD3 = optic nerve sheath diameter at 3 mm from optic disc; ONSD5 = optic nerve sheath diameter at 5 mm from optic disc; MI = myelination index.
Model 1: Linear Mixed Models for repeated measures (see Methods): estimated marginal means (95%CI) are reported.
Model 2: Linear Mixed Models for repeated measures (see Methods): estimated marginal means (95%CI) are reported after adjustment for age at MS clinical onset, BMI.

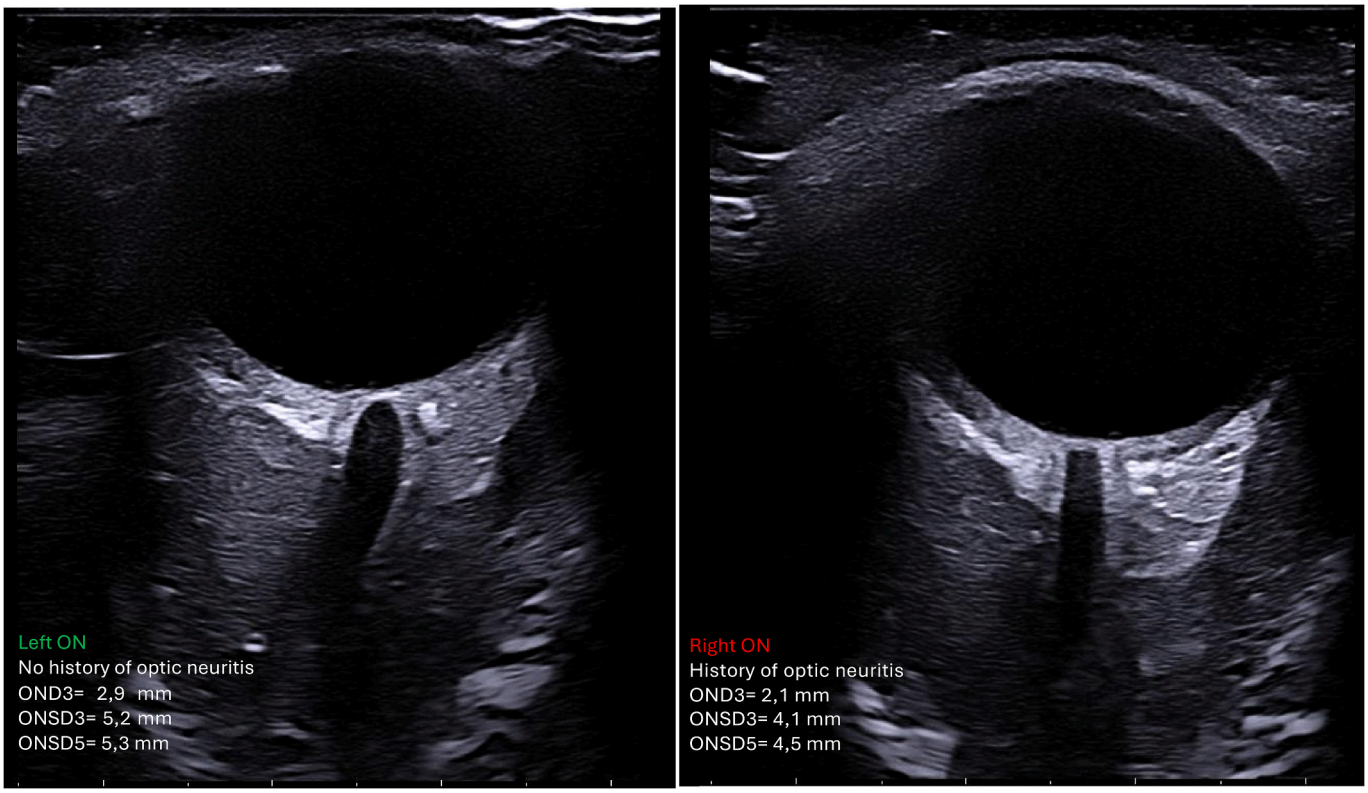


Fig. 2. B-Mode TOS imaging of a patient with MS and prior clinical history of right optic neuritis. Marked asymmetry in optic nerves morphology is evident, with reduced diameters of the affected nerve at different retrobulbar levels, consistent with overt post-inflammatory atrophy.
TOS = transorbital sonography; ON = optic nerve; OND3 = optic nerve diameter at 3 mm from optic disc; ONSD3 = optic nerve sheath diameter at 3 mm from optic disc; ONSD5 = optic nerve sheath diameter at 5 mm from optic disc.
Caption: Optic nerve atrophy in MS patient with previous history of right retrobulbar optic neuritis: transorbital sonography assessment.

Table 4
Diameters (mm) and myelination index measured with transorbital sonography of optic nerves with (I-ON) vs. no (NI-ON) past diagnosis of optic neuritis and by outcomes of disability or progression: estimated marginal means (95% confidence interval).

	OND3	*p*	ONSD3	*p*	ONSD5	*p*	MI	*p*
EDSS at MS clinical onset								
0–2.5								
NI-ON	3.039 (2.976, 3.102)	*0.225*	4.770 (4.655, 4.886)	*0.480*	5.084 (4.947, 5.221)	*0.607*	0.940 (0.933, 0.948)	*0.840*
I-ON	2.973 (2.886, 3.059)		4.700 (4.542, 4.859)		5.023 (4.834, 5.213)		0.939 (0.929, 0.949)	
3.0+								
NI-ON	3.127 (3.046, 3.208)	***0.048***	5.027 (4.879, 5.174)	***0.011***	5.329 (5.131, 5.528)	*0.059*	0.946 (0.931, 0.962)	*0.707*
I-ON	2.983 (2.869, 3.097)		4.688 (4.481, 4.895)		4.993 (4.712, 5.275)		0.951 (0.930, 0.973)	
Progression Index								
Improvement, stable								
NI-ON	3.083 (3.030, 3.136)	***0.039***	4.841 (4.743, 4.939)	*0.105*	5.135 (5.014, 5.256)	*0.199*	0.946 (0.938, 0.954)	*0.960*
I-ON	2.989 (2.918, 3.060)		4.705 (4.574, 4.835)		5.001 (4.839, 5.164)		0.947 (0.935, 0.957)	
Progression								
NI-ON	2.950 (2.800, 3.099)	*0.127*	4.759 (4.453, 4.966)	*0.470*	5.163 (4.923, 5.403)	*0.262*	0.922 (0.906, 0.938)	*0.223*
I-ON	2.710 (2.434, 2.986)		4.604 (4.217, 4.991)		4.886 (4.445, 5.327)		0.942 (0.913, 0.972)	

OND3 = optic nerve diameter at 3 mm from optic disc; ONSD3 = optic nerve sheath diameter at 3 mm from optic disc; ONSD5 = optic nerve sheath diameter at 5 mm from optic disc; MI = myelination index;
EDSS = Expanded Disability Status Scale[22]; Progression Index (PI): PI>0.20 indicates disease progression (from clinical onset to study time)[23].
Linear Mixed Models for repeated measures (see Methods): estimated marginal means (95%CI) are reported after adjustment for age at MS disease onset;

According to the stratification by EDSS at MS clinical onset, a clear correlation between disease progression and disability and the extent of neuroaxonal loss was found, expressed by a statistically significant decrease of OND3 and ONSD3 in nerves with previous history of RBON (I-ON) of patients with EDSS >3. This association remains robust after adjusting for age at MS onset and underscores the relationship between clinical disability and increased ON neuroaxonal injury, expressed as structural damage detectable with TOS, particularly evident in eyes with history of inflammation.

An inverse correlation between ON diameters and EDSS has been reported in literature [11–13,28], although with conflicting positions [24,27,29]. Schroeder et al. observed an inverse correlation between TOS measures and disease duration but not with EDSS, and the effect of age in this correlation could not be ruled out.

For PI<0.20 a detectable significant difference was observed with I-ON being smaller than NI-ON, especially in OND. However, such differences were mitigated for PI>0.20, highlighting the potential role of chronic inflammatory activity or PIRA.

Table 5
Diameters (mm) and myelination index measured with TOS by number of past diagnosed episodes and by sex: estimated marginal means (95% confidence interval).

N. of RBON	OND3 (mm)	ONSD3 (mm)	ONSD5 (mm)	MI
Total				
No RBON	3.072 (3.023, 3.122)	4.873 (4.779, 4.966)	5.206 (5.090, 5.321)	0.940 (0.933, 0.947)
1 RBON	3.007 (2.924, 3.089)	4.770 (4.614, 4.926)	5.088 (4.893, 5.283)	0.943 (0.931, 0.955)
2+ RBON	2.900 (2.770, 3.029)	4.505 (4.262, 4.749)	4.803 (4.501, 5.104)	0.939 (0.921, 0.958)
p	***0.034***	***0.019***	***0.042***	*0.875*
Men				
No RBON	3.050 (2.972, 3.137)	5.092 (4.914, 5.269)	5.519 (5.300, 5.737)	0.926 (0.914, 0.938)
1 RBON	3.090 (2.947, 3.233)	5.072 (4.761, 5.383)	5.478 (5.088, 5.868)	0.930 (0.909, 0.952)
2+ RBON	2.936 (2.507, 3.364)	5.116 (4.193, 6.039)	5.526 (4.386, 6.667)	0.929 (0.866, 0.992)
p	*0.770*	*0.992*	*0.983*	*0.942*
Women				
No RBON	3.079 (3.016, 3.141)	4.758 (5.652, 4.863)	5.037 (4.909, 5.165)	0.947 (0.938, 0.955)
1 RBON	2.979 (2.877, 3.081)	4.645 (4.472, 4.818)	4.938 (4.727, 5.150)	0.949 (0.935, 0.964)
2+ RBON	2.884 (2.745, 3.023)	4.437 (4.201, 4.673)	4.719 (4.431, 5.007)	0.941 (0.921, 0.961)
p	***0.023***	***0.043***	*0.130*	*0.816*

OND3 = optic nerve diameter at 3 mm from optic disc; ONSD3 = optic nerve sheath diameter at 3 mm from optic disc; ONSD5 = optic nerve sheath diameter at 5 mm from optic disc; MI = myelination index.
Linear Mixed Models for repeated measures (see Methods): estimated marginal means (95%CI) are reported adjusted for BMI.

Table 6
Diameters and myelination index of optic nerves with (I-ON) and without (NI-ON) diagnosis of past optic neuritis vs. optic nerves from control subjects (Ctrl-ON).

	OND3 (mm)	*p*	ONSD3 (mm)	*p*	ONSD5 (mm)	*p*	MI	*p*
Ctrl-ON	2.938 (2.836, 3.040)	***0.015***	5.020 (4.832, 5.208)	***0.009***	5.460 (5.231, 5.690)	***0.004***	0.921 (0.907, 0.936)	***0.039***
NI-ON	3.073 (3.024, 3.121)		4.866 (4.776, 4.956)		5.193 (5.083, 5.302)		0.941 (0.934, 0.947)	
I-ON	2.976 (2.908, 3.044)		4.688 (4.562, 4.813)		4.994 (4.840, 5.148)		0.943 (0.933, 0.952)	

ON = optic nerve; C-NO = control subjects; I-ON = history of optic neuritis; NI-ON = no clinical history of optic neuritis.
OND3 = optic nerve diameter at 3 mm from optic disc; ONSD3 = optic nerve sheath diameter at 3 mm from optic disc; ONSD5 = optic nerve sheath diameter at 5 mm from optic disc; MI = myelination index.
Linear Mixed Models for repeated measures (see Methods): estimated marginal means (95%CI) are reported after adjusting for BMI.

Remarkably, TOS could capture a dose-response effect with ON diameters decreasing with increased number of past inflammatory episodes. Such finding increases the reliability of TOS in detecting optic nerve atrophy as a potential proxy of progressive neurodegeneration [8]. This pattern remained evident in women after stratification by sex, consistently with overall ON findings.

Taken together, these findings may be ascribed to a pathophysiological mechanism of chronic retrobulbar axonal degeneration and depletion, more prominent in ON with history of inflammation, but also detectable in apparently clinically unaffected optic nerves compared to healthy controls, as in brain 'normal appearing white matter' and biological expression of chronic active neuroinflammation, underscoring subclinical injury even in absence of prior focal inflammation or overt optic neuritis, conforming to OCT studies [31,32].

To the best of our knowledge, the present study provides the most extensive ultrasonographic investigation of the retrobulbar ON in MS population to date.

When scrutinizing the literature, concern is raised upon the dissimilar ultrasonographic protocols adopted and the subsequent heterogeneity across studies. Current scientific evidence is rather discordant concerning diameter measurements, i.e., how distant from the optic disc ON should be measured. De Masi et al. explored both ONSD at 3 and 5 mm from the papilla while others only measured OND3, ONSD3 or both [8,11,12,27–29], or where 'ON was best recognizable' or again within 10 mm from the papilla [14,15]. We explored ON diameter without and with sheath (OND, ONSD) at 3 mm from optic disc, extending the measures to a more retrobulbar segment, i.e., at 5 mm.

Furthermore, with respect to the statistical approach, only few authors considered adjusting for potential confounders, such as demographic [24,33], lifestyle factors or MS clinical variables [13] when comparing ON diameters across groups. Moreover, previous investigations have primarily relied on conventional statistical methods, such as univariate analysis [11,12,14,28,33] or one-eye only analysis [34], assuming independence between groups but in fact not considering actual intra-subject and inter-eye correlations of ON measurements. As such measurements are inherently *not* independent, we used LMMs to account for such dependency and overcome potential inference biases [35,36]. We aimed to enhance statistical accuracy by incorporating rigorous adjustments for biological and clinical confounders both in patients and control groups. With LMM we used a more rigorous statistical approach which allowed for minimalizing within-patient inter-eye dependencies (repeated measures) as well as including random effect. Furthermore, and for the first time to the best of our knowledge, we considered adjustment for potentially relevant confounders, such as age and BMI. This analytical approach, combined with stratification by disability burden (EDSS), enabled a more refined identification of MS-specific subgroups in which TOS metrics more reliably predict disease-related atrophy.

To date, only studies by Antal et al. [24] and Tabatabaee et al. [33] partially attempted to address these limitations by using LMM and adjusting for age respectively, however, in small cohorts and with no correction for repeated measurements or for multiple comparisons.

In our study some limitations should be considered.

The ascertainment of optic neuritis was retrospective, but diagnoses were documented in medical records and according to conventional criteria. The cross-sectional nature of the study may have hampered prognostic implications and predictive deductions, however, a dose-response effect on the changes of the ON diameters according to the number of past subsequent optic neuritides still captured the progressive biological changes due to chronic active inflammation.

No formal intra- nor inter-observer reproducibility was assessed; however, all examinations were performed by the same experienced operator using a standardized acquisition protocol in order to minimize measurement variability.

Contemporaneous OCT and MRI data were not systematically available for the present cohort at the time of TOS study, and no

correlation with TOS-derived metrics could therefore be included in the study. Although ON-MRI data may have provided additional morphological information about the ON with no clinical history of optic neuritis, TOS data were collected prior to the publication of the 2024 revised MS diagnostic criteria, and orbital MRI was not routinely performed. Furthermore, a correlation with OCT and retinal markers of neuroaxonal damage could be searched, but no contemporaneous OCT data to the US study were available. In the absence of longitudinal OCT and TOS measurements collection, a correlation with available OCT acquired at the time of acute RBON not only would not be informative, but even misleading. Nevertheless, as optic neuritis affects the posterior (retrobulbar) portion of the nerve, OCT and TOS study address two different anatomical regions of the visual system and the search for a correlation may not only lead to speculative findings, but, if significantly present, would however support the more cost-effective use of TOS-metrics during the chronic stage of the disease.

5. Conclusion

TOS is a non-invasive, real-time, dynamic, high-resolution and easy-to-perform reliable method for detecting ON morphometric changes in MS. TOS-derived metrics, combined with rigorous and appropriate statistical modeling, prove effective in identifying reduced ON diameters reflecting atrophy related to prior RBON, and may represent a promising accessible neuroimaging biomarker predicting disease accrual and chronic inflammatory neuroaxonal injury in MS.

CRediT authorship contribution statement

N. Merli: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **C. Ferri:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation. **M. Copetti:** Writing – review & editing, Validation, Supervision, Software, Methodology, Formal analysis, Data curation. **V. Inchingolo:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **G. Malferrari:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **M. Paciaroni:** Writing – review & editing, Validation, Supervision, Methodology. **M. Pugliatti:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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Data availability

The datasets generated and/or analyzed during the current study are not publicly available due to institutional and ethical restrictions regarding sensitive patient data and clinical information.

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