1. INTRODUCTION

Neuro-ophthalmological disorders are a heterogeneous group of diseases caused by cerebral or systemic abnormalities that can affect visual function. It is well known that visual impairment can be a manifestation of neurodegenerative diseases, where inflammation and axonal degeneration contribute to neurological damage leading to clinical disability. Several degenerative and inflammatory disorders of the nervous system are associated with visual or ocular motor disturbances. The retina and optic nerve share the embryological origin, anatomical structures, and connections with the central nervous system (CNS). Therefore, the ophthalmic structures are ideal for studying processes of neurodegeneration, neuroprotection, and potentially even neuro-restoration. They represent an accessible *"window"* where nervous tissue and retinal and optic nerve circulation can be directly evaluated, allowing the study of correlations and consequences of subclinical pathology in the brain. In particular, retinal imaging enables us to investigate a specific compartment of the CNS that is targeted by the disease process. Relevant diseases such as multiple sclerosis (MS), neuromyelitis optica (NMO), Alzheimer's disease, and Parkinson's disease have a strong impact on the quality of life of affected patients, and an early diagnosis and prevention of worse prognosis could be beneficial (1,2).

1.1. Multiple sclerosis

Multiple sclerosis (MS) is characterized by both inflammation and axonal degeneration. One of the most frequent presenting symptoms is optic neuritis (ON), which occurs as the first symptom in about 20% of patients. However, disturbances affecting the optic pathways also develop in those who have never had episodes of optic neuritis (ON), as demonstrated by post-mortem studies, which have found anomalies in about 90% of patients (3,4). The anterior visual pathways are made up of retinal ganglion cells, the somas of which are located in the ganglion cell layer (GCL). Their axons form the retinal

nerve fiber layer (RNFL) and are unmyelinated until they leave the eye (figure 1). The axons that form the optic nerve reach the level of the optic chiasm where the nasal fibers decussate, and most of the fibers synapse with the lateral geniculate nucleus (5-7).

Damaged and/or demyelinated optic nerve axons are thought to undergo retrograde degeneration and because these axons arise from the retinal nerve fiber layer (RNFL), this fiber layer can develop neuronal atrophy. In turn, the ganglion cell neurons from which come these axons degenerate correspondingly. Optical coherence tomography (OCT) enables high-resolution quantification of retinal structures and demonstrates thinning of the peripapillary RNFL in MS eyes with and without a history of optic neuritis (8,9).

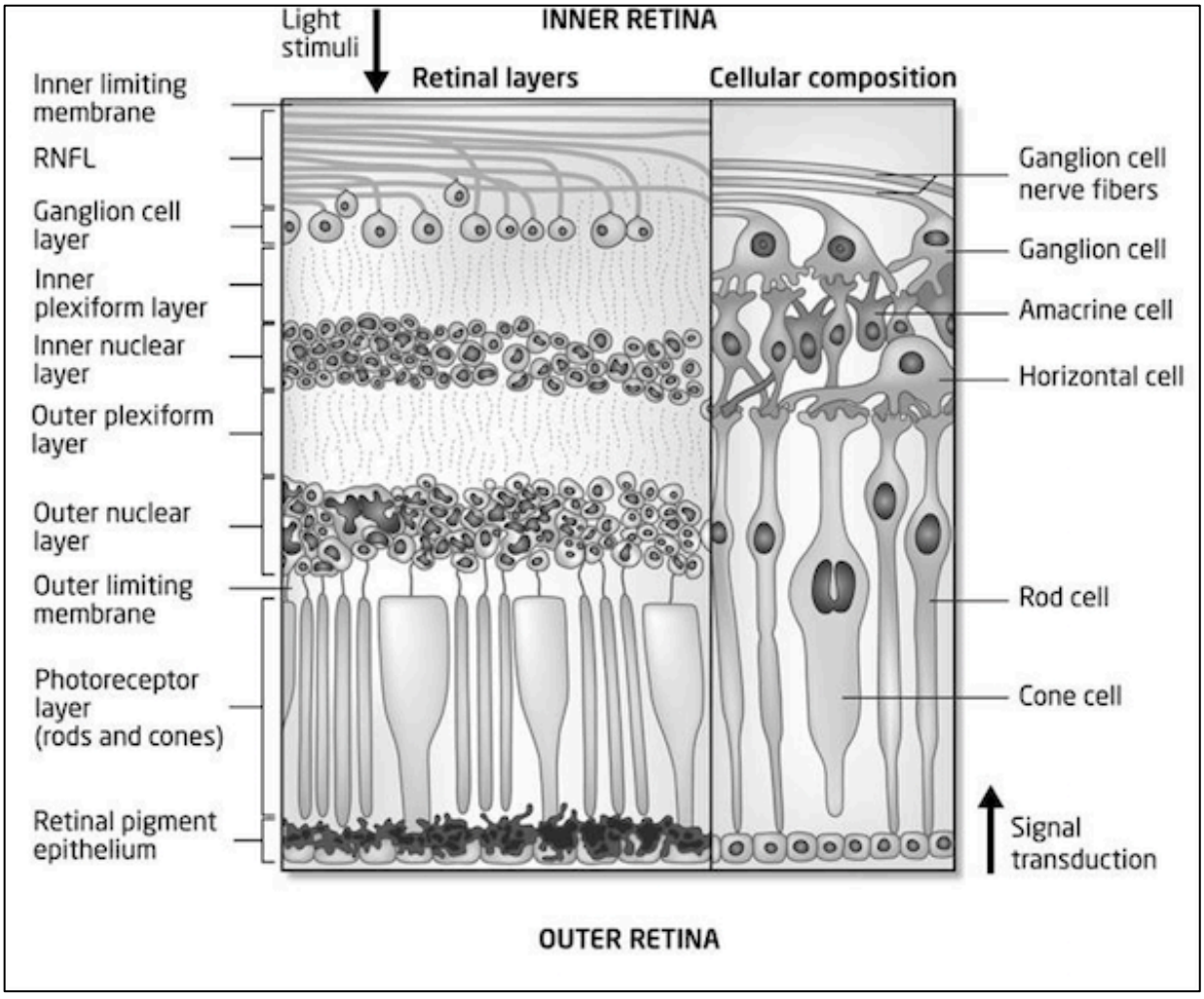

Figure 1. Image of the retinal layers and cellular compositions. The retinal nerve fiber layer (RNFL) is composed of axons of the ganglion cells.

Patients with optic neuritis experience a significant reduction in GCL and RNFL, and the importance of performing OCT objectively, demonstrate this cell loss. Thanks to these ophthalmological tools, it has been demonstrated in various studies that even MS patients who never had optic neuritis could still present a significant loss of GCL and optic nerve fibers.

As demonstrated by Ratchford et al., MS patients with clinical and/or radiologic non-ocular disease activity, particularly early in the disease course, exhibited accelerated GCL thinning. These findings suggested that retinal changes in MS reflect global CNS processes, and that OCT-derived GCL thickness measures may have utility as an outcome measure for assessing neuroprotective agents, particularly in early and active MS (10,11).

The diagnosis of MS often is not clear and relies on the use of combined clinical and magnetic resonance imaging (MRI) findings, which is an expensive and time-consuming method. Therefore, the introduction of low-cost sensitive biomarkers that can be used in the early diagnosis and monitoring of MS remains very important and indispensable for the follow-up of these patients, especially for monitoring the response to the therapies.

The introduction of two commonly used ophthalmological imaging techiniques, optical coherence tomography (OCT) and, more recently, OCT with angiography (OCT-A) has revolutionized our understanding of the pathophysiological mechanisms underlying MS. Retinal imaging devices are primarly used for the diagnosis and for monitoring the response to treatment of retinal diseases, such as age-related or inherited maculopathies, retinal vascular disorders and degenerative diseases such as glaucoma. Given that the retina is an extension of the brain, its use has expanded in the study of neurological and neuro-ophthalmological conditions (12). A wide variety of conditions belonging to the neuro-ophthalmological and neurological chapter can be non-invasively investigated thanks to a detailed ophthalmological imaging and clinical assessment.

Indeed, modern neuro-ophthalmology takes advantages on neuroimaging to reach a diagnosis. New high-resolution diagnostic tools such as enhanced-dept OCT and OCT angiography have been developed and recently introduced in ophthalmological clinical practice in the last years. They revolutionized the era of early diagnosis at a cellular level of retinal diseases and glaucoma. Therefore they may provide also a more objective and precise measures, quantitative assessment of biomarkers in order to reach an early diagnosis, characterization and prognosis of neuro-ophthalmological disorders (13).

1.2. Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is a powerful tool for detecting and monitoring the progression of MS. It can detect changes in the brain and spinal cord that are characteristic of MS, such as old lesions, active inflammation and atrophy. MRI can also help to distinguish between different subtypes of MS, such as relapsing-remitting MS (RRMS) and progressive MS.

The 2017 revised McDonald criteria (12,13) have been instrumental in enabling earlier diagnosis of MS and initiating disease-modifying treatments, which can improve patient outcomes. However, these criteria have limitations, such as imperfect sensitivity and specificity, which can lead to misdiagnosis in some cases. Misdiagnosis is a prevalent problem in MS, as symptoms and MRI findings can be similar to other neurological conditions.

Therefore, more accurate and pathologically specific MRI criteria are still needed to rule out other disorders that can mimic MS, especially for atypical cases with unclear MRI changes. Several studies have proposed new neuro-radiological indicators to differentiate between typical and atypical MRI signs in patients with an unclear diagnosis of MS (14-17). These indicators include measures of lesion topography, lesion shape, and lesion characteristics, such as the presence of gadolinium enhancement or restricted diffusion. These indicators can help to improve the accuracy of MS diagnosis and reduce the risk of misdiagnosis.

In addition, MRI is also important for monitoring the response to therapies in MS patients. It can provide information on disease progression and the effectiveness of treatments, allowing for adjustments to be made as necessary.
Overall, MRI plays a fundamental role in the diagnosis and management of MS, and continued research in this area is critical for improving patient outcomes.

1.3. The Central Vein Sign

The central vein sign (CVS) is a radiological finding observed in magnetic resonance imaging (MRI) scans using specialized gradient-echo MRI sequences of the brain in patients with demyelinating disorders such as multiple sclerosis (MS) (18).
The CVS refers to the appearance of a dark central vein or hypointense signal within the lesion on T2-weighted or T2*-weighted MRI sequences. The central vein sign is thought to reflect the presence of pathological feature in demyelinating lesions, known as the "perivenular" lesion (PVL) pathology. This refers to the infiltration of immune cells around the veins in the central nervous system, resulting in inflammation, demyelination and axonal damage. On MRI, the central vein sign appears as a linear or serpentine hypointense structure in the center of the lesion, surrounded by a hyperintense signal. The vein may appear as a bright signal on T1-weighted MRI, reflecting the presence of blood products. The central vein sign has been proposed as a useful diagnostic tool for MS, as it is more specific for MS lesions compared to other white matter abnormalities. It has also been associated with a more severe disease course and poorer outcomes in MS patients; infact, it has been shown to be a sensitive and specific predictor of future lesion activity and clinical disability.
The presence of the CVS is associated with a higher likelihood of gadolinium enhancement, a marker of active inflammation, and with a greater risk of developing new or enlarging lesions (19). The CVS is thought to be a marker of inflammation and blood-brain barrier disruption within the lesion.

Several studies have demonstrated how this promising imaging biomarker can differentiate MS from other disorders including migraine, primary and secondary CNS vasculitis, cerebral small vessel disease and Susac syndrome, since similar white matter lesions are present in all of these disorders on MRI (18-23).

The central vein sign (CVS) has recently been proposed as a novel MRI biomarker to improve the accuracy of MS diagnosis and the use of the CVS as a diagnostic tool is still being investigated and more research is needed to fully understand its clinical utility.

Recent guidelines from the Magnetic Resonance Imaging in MS (MAGNIMS) group and the Consortium of MS Centers (CMSC) task force have recognized the potential of CVS and its dedicated MRI acquisitions for the differential diagnosis of MS, although for the moment this diagnostic criterion still need to be strengthened in order to be included in the official diagnostic criteria for the diagnosis of MS (24,25).

According to this important new MRI finding, some radiological features for the definition of central vein sign are proposed, as described by Sati et al (26).

A central vein exhibits the following properties on T2*-weighted images (Figure 2):

- Appears as a thin hypointense line or small hypointense dot
- Can be visualized in at least two perpendicular MRI planes, and appears as a thin line in at least one plane
- Has a small apparent diameter
- Runs partially or entirely through the lesion
- Is positioned centrally in the lesion (that is, located approximately equidistant from the lesion's edges and passing through the edge at no more than two places), regardless of the lesion's shape

Exclusion criteria for lesions:

- Lesion is <3mm in diameter in any plane
- Lesion merges with another lesion (confluent lesions)
- Lesion has multiple distinct veins
- Lesion is poorly visible (owing to motion or other MRI-related artefacts)

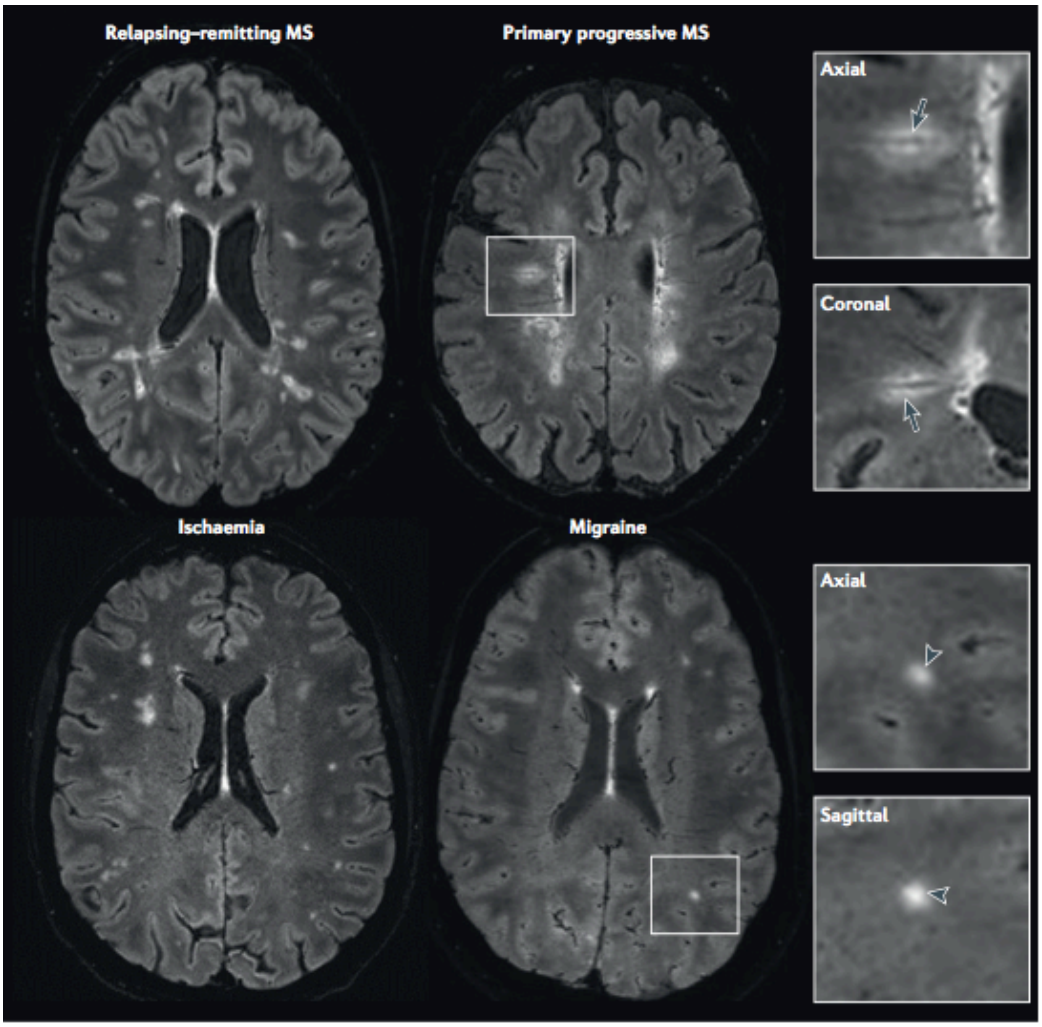

Figure 2. Perivenous distribution of multiple sclerosis lesions. 3T FLAIR images from four patients with different neurological conditions. In the patients with relapsing–remitting or primary progressive multiple sclerosis (MS), a central vessel is visible in most hyperintense lesions. The dark veins are located centrally in the lesion and can be visualized in at least two perpendicular planes (arrows in magnified boxes). On the other hand, a central vein is absent from most of the lesions (arrowheads in magnified boxes) in the patient with migraine and the patient with ischaemic small vessel disease.

Although the CVS is not specific for MS, as it can also be seen in other inflammatory or infectious conditions affecting the central nervous system, its presence in demyelinating lesions can help to distinguish them from non-demyelinating lesions and aid in the diagnosis of MS. Moreover, the percentage of white matter lesions with CVS is considerably higher in patients with MS compared to other inflammatory or non-inflammatory neurological diseases. Thus, CVS allows for the accurate identification of true-MS patients by evaluating the frequency of CVS over the total number of white matter lesions (i.e., ≥40% or ≥50% according to different studies by Maggi et al. and Suthiphosuwan et al) (19,28).

In a prospective multicenter study Maggi et al. (27) recruited 51 patients with suspected MS who had clinical, radiological or laboratory *"red flags"* (i.e. features atypical for MS). Patients underwent 3T fluid-attenuated inversion recovery (FLAIR*) magnetic resonance imaging (MRI) for CVS assessment. After the diagnostic work-up, the expert physicians unaware of the CVS assessment results, arrived at a clinical diagnosis. In the study the authors evaluated the value of CVS for prospectively predicting a diagnosis of MS and they found that, using a 40% perivenular lesion threshold, the CVS could predict an MS diagnosis with 97% accuracy and a 96% positive/100% negative predictive value.

These results are particularly relevant considering that the specificity of the current diagnostic imaging criteria for MS is limited and that the prevalence of MS misdiagnosis is high in clinical practice (29). Infact, the 2017 McDonald criteria were created to facilitate an earlier diagnosis in patients presenting with typical features for MS.

But when McDonald criteria, using the disease dissemination in space (DIS) and disease dissemination in time (DIT) are not fulffiled, in the context of a clinical presentation compatible with inflammatory demyelination, the evaluation of the perivenular lesions and its frequency is of great added value. Indeed, in the study of Maggi et al. the frequency of perivenular lesions was significantly lower in these atypical patients compared with those who received a diagnosis of MS (19).

Nevertheless, this promising data need to be further studied, and it could be important to focus on the sign of the central vein as an important research topic in the neurological

field. Greater knowledge of CVS could help to characterize patients with atypical features of demyelinating disease with more certainty and accuracy.

2. OPHTHALMOLOGICAL IMAGING

2.1. Optical Coherence Tomography

Optical coherence tomography (OCT) was first introduced in 1991 and it is a non-invasive imaging technique that uses light waves to capture high-resolution, three-dimensional images of biological tissues. OCT is similar to ultrasound, but instead of sound waves, it uses light waves to create images.

In OCT, a light source is directed onto the tissue of interest, and the light is scattered and reflected back to a detector. The time delay and intensity of the reflected light are measured, and a computer algorithm uses this information to construct a three-dimensional image of the tissue.

OCT has many applications in ophthalmological imaging, where it is used to identify and monitor retinal diseases such as age-related macular degeneration and glaucoma.

Overall, OCT is a powerful tool that allows physicians to visualize the internal structure of tissues and diagnose diseases in early stages.

The two most commonly studied layers in multiple sclerosis (MS) are the retinal nerve fiber layer (RNFL) and the complex of ganglion cell layer and inner plexiform layer (GC-IPL). Studies have shown that the thickness of both the RNFL and GC-IPL are reduced in MS patients, and that this reduction is inversely related to the duration of the disease (10; 30-32). Despite several theories, the underlying pathophysiological mechanisms for these findings remain unclear (33).

Retrograde degeneration leading to axonal loss is the most probable pathological mechanism causing RNFL and GC-IPL thinning. However, in patients with no history of optic neuritis (ON), these findings could be due to primary degeneration caused by the

disease itself or even to retrograde degeneration after one or more subclinical episodes of ON (34).

The application of OCT in clinical practice may provide new and potentially important insights into cerebrovascular neurodegenerative processes in addition to what is currently possible with neuro-imaging.

Since MRI is the most common imaging modality used in the diagnosis of MS, recently many studies evaluated the application and integration of new ophthalmic non-invasive imaging techniques in neurodegenerative diseases, focusing on the neurological relevant findings with those of OCT (35–37).

The application of optical coherence tomography in multiple sclerosis, neuromyelitis optica spectrum disease (NMOSD), Alzheimer's disease, and Parkinson's disease is actually being studied. Various OCT technologies such as spectral domain OCT (SD-OCT), enhanced-depth imaging OCT (EDI-OCT) and *en face* swept-source OCT (SS-OCT) have helped us to study in-vivo changes in the retina, choroid, and optic nerve. The new high-resolution OCT devices correct eye movement, reducing retinal slip and image quality degradation, allowing its easier use in patients with neurological disorders.

Loss of retinal nerve fiber layer (RNFL) and ganglion cell-inner plexiform layer (GC-IPL) thickness correlates with clinical and paraclinical parameters such as biomarkers, visual function, disability and magnetic resonance imaging in MS. Some studies indicate that OCT parameters may be able to predict disability progression and visual function in MS. It has widely been demonstrated that the average RNFL thickness and macular volume (a reflection of retinal ganglion cell neuronal integrity) were significantly reduced in many CNS diseases (38-40).

In diseases such as MS and Neuromyelitis Optica (NMO), longitudinal studies have demonstrated that thinning of the RNFL occurs over time and that occult optic neuropathy occurs in most eyes, even in those not affected by acute onset optic neuritis (typical onset of optic neuritis). Moreover, patients with NMO usually have a greater degree of RNFL loss. These findings also correlated with more severe visual loss in the NMO group. Furthermore, NMO eyes have a more diffuse RNFL loss compared to the

temporal (papillomacular bundle) nerve fiber loss that predominates in optic neuritis (ON) in the setting of relapsing remitting MS (41).

It is important to emphasize that the papillomacular bundle is composed of nerve fibers which carry visual impulses from the macula to the optic nerve and consequently, to the brain. Its clinical importance is due to the fact that demage to this structure results in a loss of central vision, with the developement of cecocentral scotoma. The cecocentral scotoma is typical of ON onset, but it can also be found in other different ocular pathologies, such as glaucoma, vascular disorders of the retina and optic nerve head, toxic optic neuropathies and mitocondrial diseases.

In addition, optical coherence tomography (OCT) has been used to investigate the effectiveness of various therapeutic options. A study by Pul et al. in 2016 did not find a significant correlation between treatment with interferon beta (IFNβ-1b) and thinning of the retinal nerve fiber layer (RNFL) (42). However, in a subsequent study by Button et al., the effect of different treatments including glatiramer acetate, natalizumab, and interferon-β-1a on the thinning of the ganglion cell-inner plexiform layer (GC-IPL) was compared. The natalizumab-treated group exhibited a significantly lower rate of GC-IPL reduction compared to the other two treatment groups (43).

As listed, there are many fields still under investigation regarding the function of OCT in the evaluation of patients with CNS demyelinating disease. Further investigations are still needed to determine useful parameters for characterization of patients with central nervous system pathologies.

2.2. OCT-Angiography

Recent development of OCT-based angiography (OCTA) has started to shed some new light on cerebral hemodynamics in neuroscience. OCT angiography is a non-invasive, dye-less imaging modality which can visualize the blood movement within retinal vessel and can visualize different retinal capillary plexuses, layer by layer. In the normal eye, a dense microvascular network is also seen on the OCTA around the optic nerve head, which is a more superficial capillary network, called the radial peripapillary capillary plexus (RPCP). Using OCTA, in addition to superficial retinal vessels, we can also study the changes in deep retinal vessels and choriocapillary layer. Presently, only a few studies have been published about the usefulness of this device in relation to MS. The study of blood flow in retinal microcirculation has already been investigated in MS, demonstrating the role of hypoxia in these patients. OCTA is able to document the retinal and optic nerve microvascular changes and could be important to better understand the pathophysiology of some neurological disorders such MS and other vascular central nervous system (CNS) diseases. All current data suggest reduced vessel density (VD) in the macular and peripapillary areas in MS patients (44-52). Indeed, in a large study by Murphy et al., OCTA parameters were correlated with visual function and disability status of MS patients (48); they also showed a significant reduction in superficial vascular plexus density in eyes with MS but not in the deep vascular plexus. However, one study found a significant reduction in deep vascular plexus in MS compared to controls (49), while another study found a reduction only in MS patients with a history of ON, compared with those with no history of ON (50). Interestingly, superficial vascular plexus thinning and foveal avascular zone (FAZ) enlargement appear to progress rapidly after an episode of optic neuritis and these data suggest a correlation with a persistent visual impairment after inflammation of the optic nerve (53,54).

As described by Spaide et al, the foveal avascular zone (FAZ) represents an area without capillaries at the center of the fovea and can show considerable variation in

dimension even among normal eyes. The foveal capillaries terminate as they approach the center of the fovea. The superficial vascular plexus, which surrounds the ganglion cell layer, terminates slightly further from the center of the fovea compared to the deep vascular layer (55).

Research has also focused on measuring the vessel density around the optic nerve, with data suggesting a significant reduction in MS (56,57). The most severely affected optic nerve quadrant remains ambiguous, with one study suggesting the inferior and nasal quadrant (51), while another study indicates the temporal quadrant.

Promising results have recently emerged regarding the use of OCT-A for differentiating MS from NMOSD. In a study by Tiftikcioglu et al., peripapillary VD in monophasic seronegative NMOSD+ON eyes was significantly lower than monophasic RRMS+ON eyes ($p = 0.030$), which was no different from controls. FAZ area was smaller in unaffected eyes in NMOSD than RRMS and controls (58). Moreover, another study suggested that the best OCTA discriminant between NMOSD and MS was the VD reduction pattern, that is, the inferior to nasal (I/N) and I/T ratios for ON eyes and S/T and N/T ratios for non-ON eyes (59).

Additionally, OCTA has been used as a marker for disability (50,51). Higher levels of disability, as measured by the expanded disability status scale (EDSS), are related to a lower VD of the macular superficial vascular plexus and low-contrast visual acuity in MS. It is worth mentioning that one study associated higher choriocapillary VD with ongoing inflammatory disease activity. Finally, Jiang et al. demonstrated lower volumetric VD (VVD), a newly developed metric system, in the retinal vascular network and DVP in MS patients with ON, and found a positive correlation between VVD of the macular SVP and EDSS and disease duration (52).

OCTA parameters, along with OCT, may help differentiate the two conditions, as NMOSD patients tend to have lower peripapillary RNFL and GC-IPL thickness, whole vessel density (VD), and perfusion density areas than those with MS (60).

Studies have also identified OCTA as a potential tool for monitoring disease progression. Recent data suggest that marked vascular loss in the superficial vascular

plexus and deep vascular plexus may be observed in people with an initial demyelinating event, although no changes might occur in the RNFL, GC-IPL thickness, or clinical and radiological examinations (61).

All these observations underline the importance of OCTA as an important and promising tool to study in depth the characteristics of patients affected by neurological pathologies. The microvascular density analysis using the OCTA technique can allow to study the retinal and optic disc capillary network and to evaluate the vascular involvement in various neurological disorders in a more objective way, as well as being able to help in differential diagnosis in challenging cases (62-67).

3. PURPOSE OF THE STUDY

The aim of our project was to evaluate the potential role of high-resolution retinal imaging techniques to provide biomarkers for the diagnosis and monitoring of neurological disorders, in particular in inflammatory-demielinating neurological diseases. Specifically, we aimed to assess changes in retinal nerve fiber layer (RNFL) and macular ganglion cell thickness, using structural OCT, as well as alterations in vascular retinal flow detected using OCTA, in patients affected by clinically and radiologically typical MS and those MS patients presenting with “red flags” as an atypical sign for MS (MS-plus group). We also aimed to correlate these imaging parameters with neurological and neuro-radiological markers, particularly the presence and percentage of the "central vein sign" as evaluated by a neurologist using focused MRI sequences.

To achieve this, we used structural B-scan OCT and OCT angiography to non-invasively evaluate qualitative and quantitative alterations in retinal layers and retinal and peripapillary vascular flow. The ultimate aim was to aid in the differential diagnosis between typical MS and possible alternative diagnosis of MS, to identify early biomarkers, and to monitor treatment response in patients affected by neuro-ophthalmological inflammatory diseases.

3.1. MS-plus and definition of "red flags"

MS plus (MS+): patient with diagnosis of MS according to 2001 McDonald Criteria (12,13) with $\geq$ 1 clinical, laboratory and/or MRI red flag, suggestive but not sufficient to allow a well-defined diagnosis to MS.

Red flag definition: the term "red flag" will be hereafter referred to a specific list of laboratory (CSF and serum), clinical and neuroradiological items supporting a possible alterative diagnosis to MS. The most relevant red flags were included in a study-specific form used for MS-plus patients enrolment (Table 1). The list was elaborated after a revision and summary of the main available literature data (68-72).

Table 1. Specific list of red flags adopted for MS+ patients enrolment.

APPENDIX 1: Study specific form for detection of Red flags

CLINICAL RED FLAGS:

- ☐ Synchronous bilateral retrobulbar optic neuritis
- ☐ Positive anamnesis of Retinopathy (e.g. retinitis pigmentosa, retinal vasculitis, not diabetic or hypertensive) or Uveitis.
- ☐ Systemic symptoms suggestive of autoimmune/rheumatologic disease (i.e. diffuse arthritis, recurrent oral/genital ulcers, livedo reticularis, malar rash, xerostomia, xerophthalmia, myalgias, recurrent clinical relevant gastrointestinal disturbances, Raynaud phenomenon etc)
- ☐ Patent foramen oval with a documented medium-severe shunt entity
- ☐ Onset before 14 year or after 55 years
- ☐ Recurrent abortivity and/or arteriovenous thromboembolism
- ☐ Iperacute onset and/or fulminant course
- ☐ Systemic comorbidity that suggests a concurrent genetically determined syndromic state (e.g., various associations between early cataract, juvenile diabetes, hepatopathies or nephropathies, etc.)
- ☐ No clinical response to corticosteroids within 30 days from symptoms/relapse onset
- ☐ Concomitant myopathy and/or neuropathy
- ☐ Complete or fluctuating paralysis of the gaze
- ☐ Stereotyped and monomorphic attacks
- ☐ Postrema or diencephalic area syndrome

Within 12 months from "MS" symptoms onset:

- ☐ New onset relevant psychiatric symptoms as psychosis, behavioural and personality changes (depression excluded)+;
- ☐ New onset atypical, severe, persistent, drugs resistant headache
- ☐ Persistent (≥3months) flu of unknown origin or recurrent/periodic, generalized malaise, constitutional symptoms (flu-like)
- ☐ New onset epilepsy
- ☐ New onset neurosensory hypoacusia
- ☐ Encephalopathy (lethargy, mental confusion, slowing down, etc.)
- ☐ Concomitant cranial nerves multineuropathy (excluding II and VII) or polyradiculonevritis

MRI RED FLAGS

1.Atypical lesion distribution/features

- ☐ Predominance of small lesions (<3 mm)
- ☐ Sparing: corpus callosum, U-shaped iuxtacortical fibers, iuxtaventricular areas, i.e no "Dawson's fingers".
- ☐ Predominance of cortical lesions or deep grey matter involvement
- ☐ exclusive and widespread involvement of the anterior and inferior anterior temporal lobe pole
- ☐ Symmetric White Matter Lesions (WML)
- ☐ Medullary cone involvement
- ☐ Longitudinally extended transverse myelitis
- ☐ Predominant brainstem central lesions
- ☐ Brain WML necklace distribution

2.Enhancement

- ☐ Synchronous and widespread WML enhancement (e.g. ADEM like)
- ☐ Persistent enhancement of the same lesion;
- ☐ Atypical enhancement pattern;
- ☐ Punctate pontine (CLIPPERS like salt-and-pepper) or punctate supratentorial (e.g. sarcoidosis)
- ☐ Enhancement of multiple cranial nerves/spinal nerve roots
- ☐ Leptomeningeal or pachimeningeal enhancement

3. Atypical lesions

- ☐ Voluminous and/or with mass effect
- ☐ With poorly defined margins, marbled, cottoned
- ☐ Edematous, with mass effect at brainstem level
- ☐ T1 hyperintensity of thalamic pulvinar
- ☐ T2 hyperintensity of dentate nuclei
- ☐ Diffuse calcifications at CT/MRi scans

4. Small vessel disease and cerebrovascular disease markers

- ☐ Lacunae
- ☐ Status cribrosus
- ☐ Microbleeds
- ☐ Leukoaraiosis
- ☐ Cerebral infarcts, macrohaemorrhages
- ☐ Cortical hemosiderosis

LABORATORY RED FLAGS

1.Positivity to Persistently elevated systemic inflammatory/disimmune markers

- ☐ ANA (antinuclear antibodies) >1:160 or 1:160 persistent
- ☐ Anti ds-DNA antibodies
- ☐ ENA (Extractable Nuclear Antibodies)
- ☐ Antiphospholipid antibodies (anti-cardiolipin, anti-phosphatidylserine, anti β2GPI)
- ☐ Anti citrullinated cyclic peptide (anti-CCP)
- ☐ ANCA (Anti-Neutrophil Cytoplasmic Antibody;
- ☐ Anti endomysial, and/or anti deamidated gliadine, and/or anti transglutaminase antibodies
- ☐ C-reactive protein
- ☐ Erythrocyte sedimentation rate
- ☐ Sieroamyloid-A
- ☐ Rheumatoid factor IgG/IgM
- ☐ Circulating immune complexes
- ☐ C3 and/or C4 complement fractions reduction, hypogammaglobulinemia

4. ☐ Increased levels of ACE (angiotensin converting enzyme) or/and chitotriosidase

5. ☐ Thrombocytopenia, leukopenia, anaemia of possible autoimmune origin

6. Cerebrospinal fluid (CSF) analysis

- ☐ Absence or different from pattern II intrathecal IgG oligoclonal production
- ☐ CSF leukocytes > 50 cell/mm3
- ☐ CSF proteins > 0.9 g/L
- ☐ CSF Glucose alteration

4. MATERIALS AND METHODS

Patients referring from Neurology Department to our Eye Clinic (AOU Careggi, Florence, Italy) from January 2020 until to December 2022 were evaluated using high-resolution retinal imaging techniques. Diagnosis of multiple sclerosis (MS) was made according to the 2001 McDonald Criteria (73).

We included patients with relapsing remitting MS (RRMS), with typical radiological and clinical features for MS. Patients with disease duration ≥ 5 years were considered.

We included MS-plus patients defined by the following inclusion criteria: presence of demyelinating syndrome with dissemination in space (DIS) and dissemination in time (DIT), according to 2001 McDonald criteria; cerebrospinal fluid (CSF) examination at diagnosis and at least one "red flag" suggesting a better explanation of the disease.

Patients with primary progressive MS (PPMS), secondary progressive MS (SPMS), seropositivity for myelin oligodendrocyte glycoprotein IgG (MOG-IgG) or seropositivity for aquaporin-4 IgG (AQP4-IgG) were escluded. Patients with contraindication to MRI scanning or gadolinium administration were excluded.

Additional exclusion criteria included relevant known ophthalmological co-morbidities for example glaucoma, macular degeneration, history of vascular retinal diseases, ocular surgery or prior ocular trauma.

This study adhered to the tenets of the current version of the Declaration of Helsinki (52nd WMA General Assembly, Edinburgh, Scotland, October 2000), and written informed consent was obtained from all patients prior to participation in the study. Approval from The Institutional Review Board/Ethics Committee was obtained.

All patients underwent a neurological evaluation and a magnetic resonance imaging (MRI) using T2 FLAIR (fluid attenuated inversion recovery) sequences and an accurate MRI assessment was performed by an experienced neurologist, evaluating the white matter lesions (WML) of central nervous system and dividing patients into groups based on the number of perivenular lesions (PVLs) characterized by the central vein sign.

As already mentioned, the central vein sign (CVS) is a radiological finding observed on

MRI in MS lesions. It refers to the appearance of a dark central vein or hypointense signal within the lesion on T2-weighted or T2*-weighted MRI sequences.

The central vein in MRI appeared as a thin hypointense line or small hypointense dot and was visualized in at least two perpendicular MRI planes, appearing as a thin line in at least one plane running partially or entirely through the lesion and positioned centrally in the lesion.

According to the characteristics of the white matter lesions in MRI, MS-plus patients were divided into two subgroups, group 1 and 2, respectively if the central vein sign was present in more or less than 50% of the total number of white matter lesions (group 1 > 50% PVLs, group 2 < 50% PVLs).

All patients underwent a baseline ophthalmic examination including medical and ocular history, family medical history, measurement of best-corrected visual acuity (BCVA) using the Early Treatment for Diabetic Retinopathy Study (ETDRS) chart and converted into a logarithm of the minimum angle of resolution (LogMAR) for statistical evaluation. Slit lamp examination of the anterior and posterior segments, measurement of intraocular pressure, dilated fundus examination, B-scan OCT and OCT Angiography were carried-out in all patients. The RS-3000 Advance 2 spectral domain OCT (NIDEK Co. Ltd., Gamagori, Japan) was used to acquire structural OCT and OCTA in all eyes. This device uses an 880 nm wavelength and with a scanning speed of 53,000 A-Scans/sec. A 3 mm X 3 mm (256x256 scan points) scanning pattern was performed in all eyes. All scans were centered on the fovea and on the optic nerve based on the live scanning laser ophthalmoscopy (SLO) image.

All B-scans were performed 4 times and averaged for higher sensitivity. A real time SLO based active eye tracker was used to compensate for eye movement during image acquisition. In all cases the SLO image was captured prior to OCT-A analysis. Low-quality OCTA images, severe artifacts due to poor fixation or cases of failed automatic layer segmentation were excluded from analysis. Images were reviewed by two investigators (CL and DB) for segmentation accuracy.

The default RS-3000 Advance 2 AngioScan software (NIDEK Co. Ltd., Gamagori,

Japan) as been used (%) to evaluate the vessel density, defined as the percentage of the total area occupied by vessels. In addition, foveal avascular zone (FAZ) area, perimeter and circularity (an index that is equal to 1 when the FAZ shape is a circle) were automatically calculated by the in-built software.

Structural B-scan OCT measurements included:

- Central foveal thickness (CFT) (μm) (Fig.3)
- RNFL quadrant analyses (superior, inferior temporal, nasal) (μm) (Fig.4)
- Ganglion Cell Complex superior sector (μm) (Fig.3)
- Ganglion Cell Complex inferior sector (μm) (Fig.3)

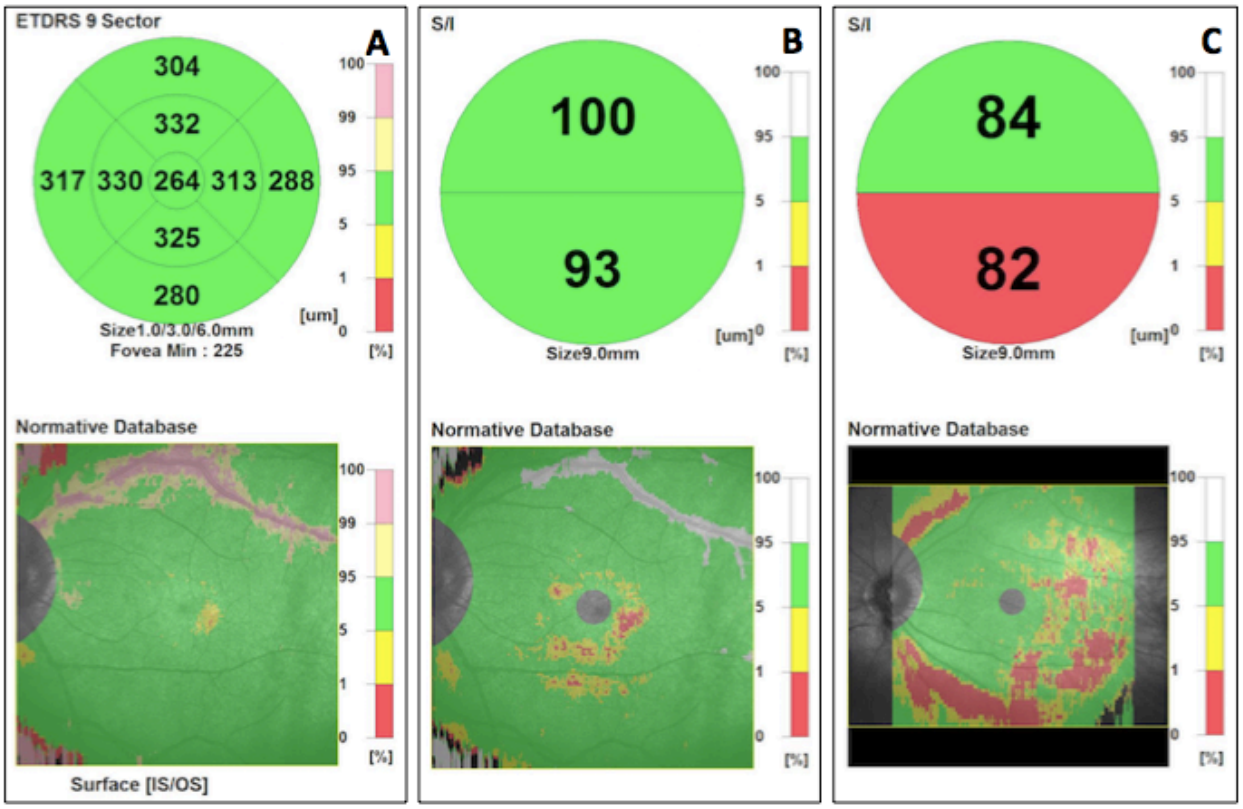

Figure 3. Automatic analysis of the macular region using structural OCT. A) Central foveal thickness (264 micron) detected automatically with the *thickness map* analysis. B) Ganglion cell complex (GCC) using the *thickness map* analysis in "glaucoma" section. The GCC were evaluated both in the superior and inferior sectors and measured in μm, and differentiated in color scale from green (normal thickness) to red color (reduction of thickness). C) GCC analysis of a MS-plus patient, revealing a reduction of the mean thickness in the inferior sector, highlighted in red.

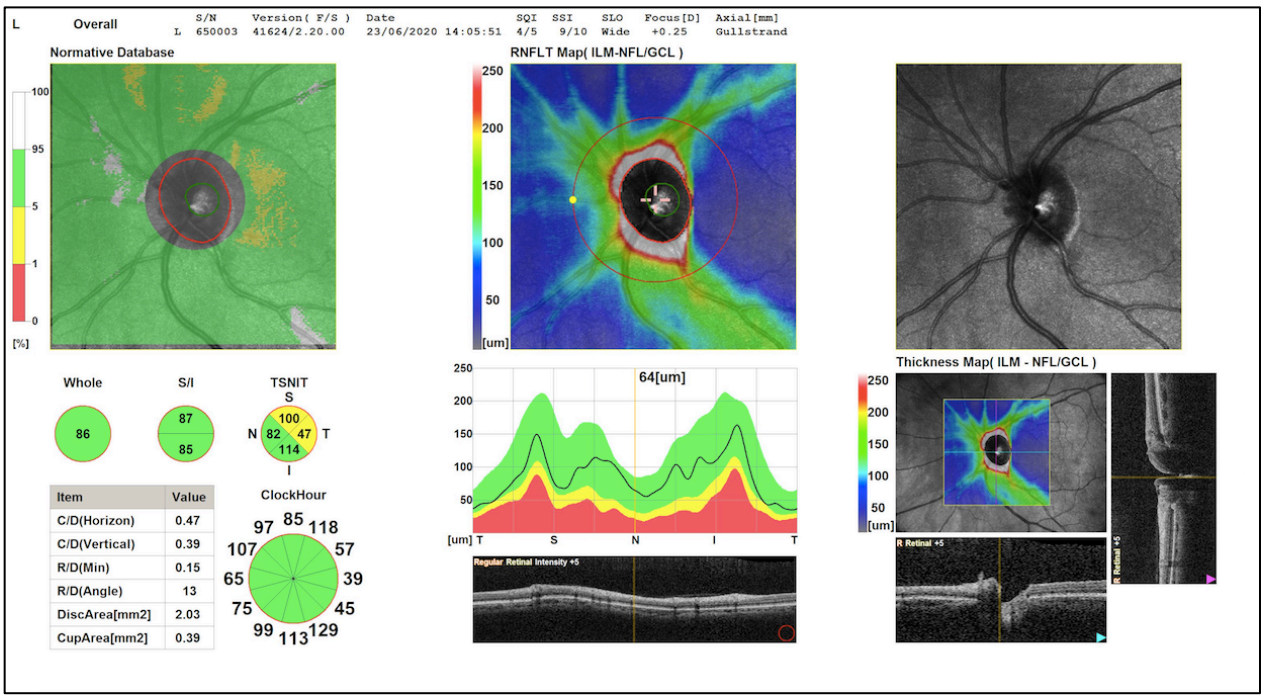

Figure 4. Evaluation of retinal nerve fiber layer (RNFL) using OCT. The four different optic disc sectors superior (S), inferior (I), temporal (T) and nasal (N) were automatically evaluated by the instrument and reported in numeric parameter (μm) and color scale (green to red).

Using OCT-Angiography, the following quantitative parameters were evaluated (Fig.5-7):

- Foveal avascular zone (FAZ) area (mm^2);
- FAZ perimeter (mm);
- FAZ circularity;
- Superficial capillary plexus (SCP) vessel density;
- Deep capillary plexus (SCP) vessel density;
- Optic nerve whole vessel density: evaluation of vessel density from internal limiting membrane (ILM) to retinal pigment epithelium/Bruch membrane (RPE/BM);
- Optic disc radial peripapillary capillary (RPC) plexus: evaluation of vessel density from ILM to nerve fiber layer/ganglion cell layer (NFL/GCL);
- Optic disc lamina cribrosa vessel density

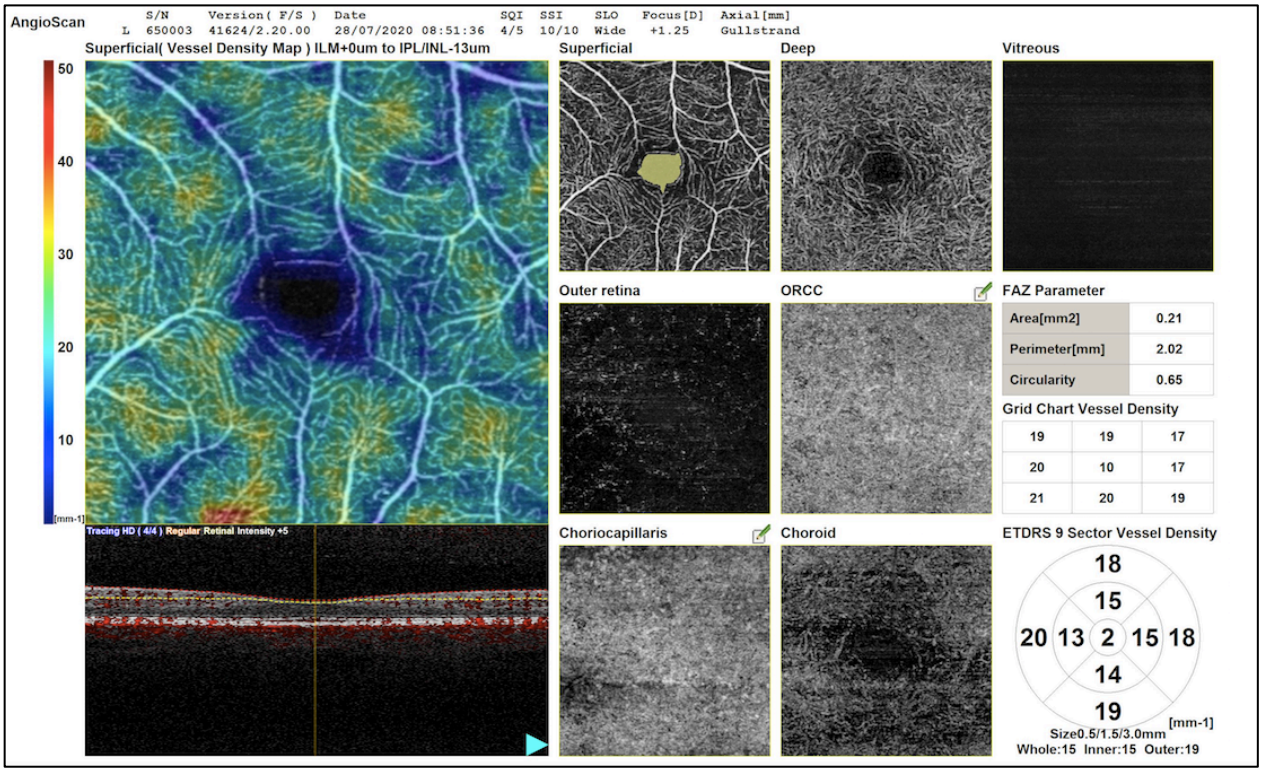

Figure 5. OCTA of macular region of a patient affected by MS. The automatically calculated parameters are visible on the right. We evaluated FAZ parameters (area, perimeter and circularity) and vessel density parameters (whole, inner and outer), both for superficial and deep capillary plexus (SCP and DCP).

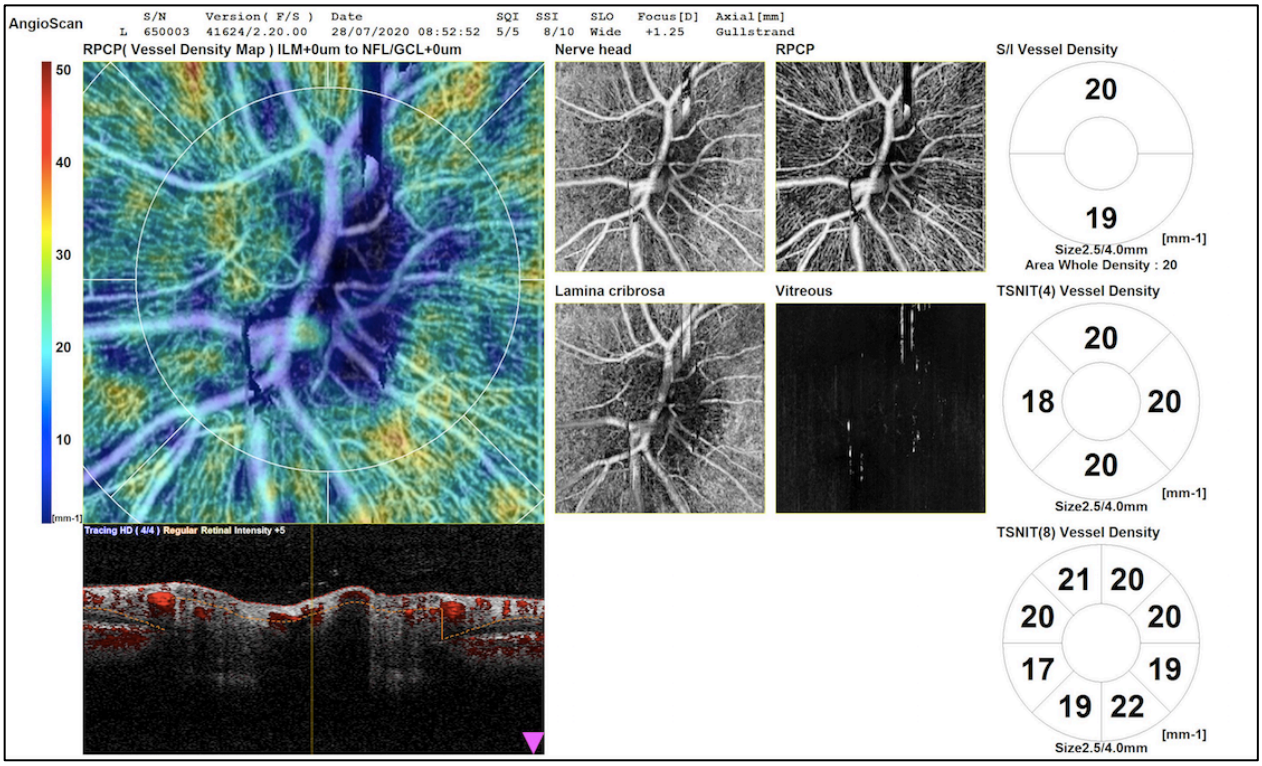

Figure 6. OCTA of optic nerve head in a patient affected by MS. The automatically calculated parameters are visible on the right. We evaluated *optic nerve head* vessel density, *radial peripapillary capillary plexus* (RPCP) and *lamina cribrosa* vessel density. We used the *area whole density* value (on top right) for each segmentation.

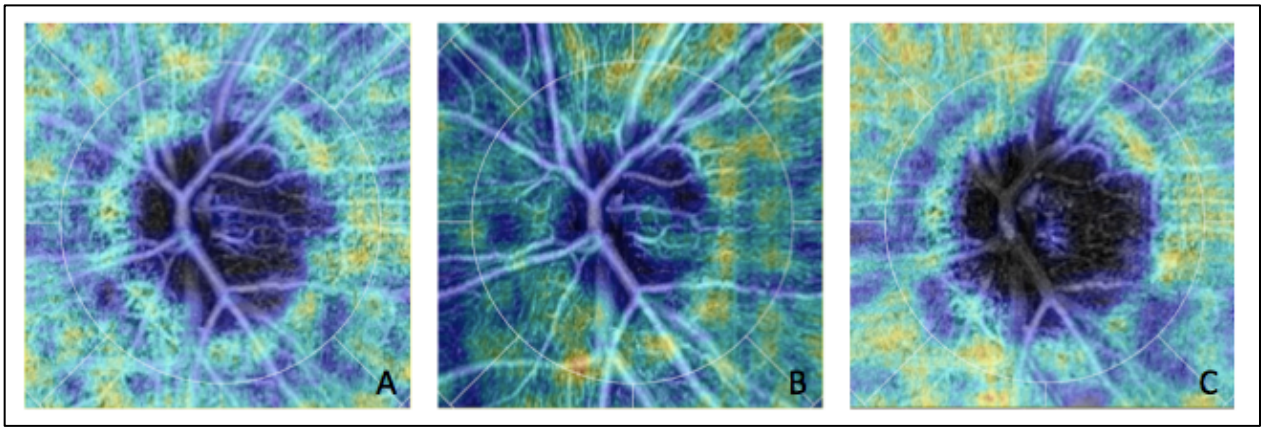

Figure 7. Qualitative OCTA images of different vascular layers of the optic nerve.
A) Optic nerve whole vessel density scan: evaluation of vessel density from internal limiting membrane (ILM) to retinal pigment epithelium/Bruch membrane (RPE/BM); B) radial peripapillary capillary plexus (RPCP): evaluation of vessel density from ILM to nerve fiber layer/ganglion cell layer (NFL/GCL); C) optic disc lamina cribrosa vessel density.

Vessel densities of the superficial capillary plexus (SCP) and deep capillary plexus (DCP) were automatically calculated by the software on OCTA 3 x 3 mm volume scans in the fovea and in the inner and outer rings. Different retinal layers were evaluated with the automatic stratification of the OCT instrument, both for macular area and the optic disc area (Figure 8).

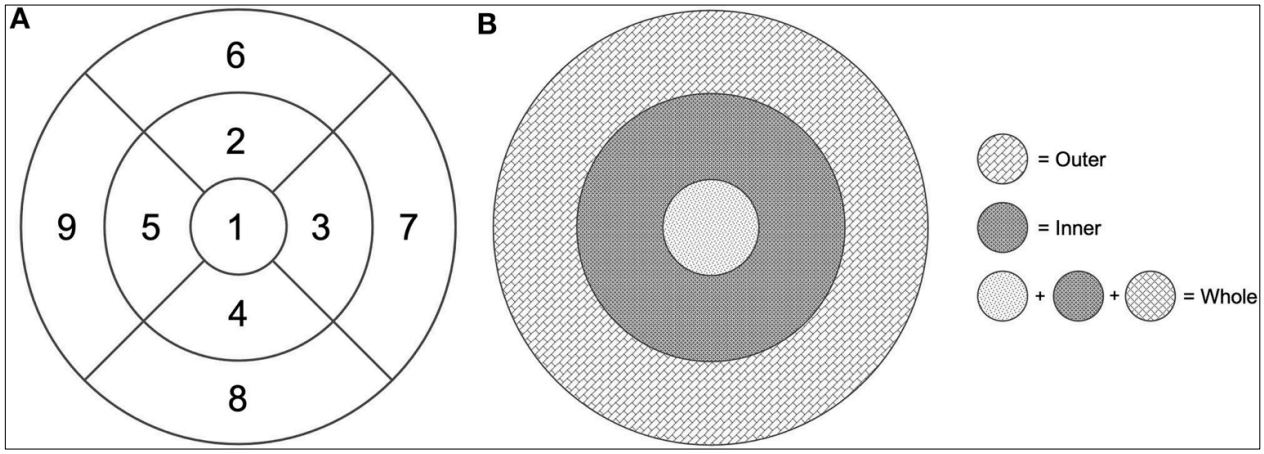

Figure 8. The ETDRS-based vessel density [%], with division of the macular area into the nine ETDRS subfields (on the left). On the right, a scheme showing the rings centered around the fovea. The fovea is defined as the area within the central 1-mm ring of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid. The surrounding ring with an inner diameter of 1 mm and an outer diameter of 3 mm is considered as the inner ring. The ring with an inner diameter of 3 mm and an outer diameter of 6 mm is considered as the outer ring. The whole ring includes the fovea and the inner and outer rings.

Sample size:

We recruited the following patients referred by the Departments of Neurology to Eye Clinic (Careggi Teaching Hospital, Florence):

- MS group: patients affected by typical form of MS according to the 2001 McDonald Criteria.

- MS-plus group: patients affected by MS with clinical/radiological or laboratory “red flags”.

5. RESULTS

In our study we included:

- **MS group *(group 0)*:** 12 patients affected by typical form of MS (6 males, 7 females). The mean age was 53.7 (SD: 9.6) years. We evaluated 24 eyes of the 12 patients affected by MS. Eight patients had a history of optic neuritis in one eye and two patients have had optic neuritis in both eyes (total of 12 eyes).
- **MS-plus group:** 24 patients affected by MS with clinical/radiological or laboratory red flags. Regarding MS-plus group we evaluated 48 eyes of 24 patients. Twelve patients of this group had a history of monolateral optic neuritis (total of 12 eyes).

Out of the MS-plus group, 12 patients presented more than 50% CVS in white matter lesions in MRI scans ***(Group 1)*** and 12 patients presented less than 50% CVS in white matter lesions in MRI scan ***(Group 2).*** Mean age of Group 1 was 51.6 (SD: 12.3) years (5 males, 7 females) and mean age of Group 2 was 52.8 (SD: 8.1) years (12 females). Twelve patients of these groups had a history of monolateral optic neuritis (total of 12 eyes): 8 patients in group 1 and 4 patients in group 2.

The different parameters were compared between the 3 groups and statistical analysis was performed with STATA software version 17.1 (StataCorp, College Station, TX, USA). Descriptive statistics such as mean values and standard deviations were calculated for all quantitive variables. Between-group comparisons were performed with linear mixed models accounting for within-patient random effects. A *P* value less than 0.05 was considered statistically significant.

Table 2 presents mean and standard deviation (SD) for all parameters across the three groups, with p-values for subgroup differences and linear trend (only p-values for linear trend are presented in the following text).

The mean value of central foveal thickness (CFT) was 270 μm in group 0, 267 μm in group 1 and 277 μm in group 2: the difference among the groups was not statistically significant ($p = 0.536$).

Regarding the retinal nerve fiber layer (RNFL) thickness we evaluated the different values of the 4 quadrants (superior, inferior, temporal and nasal) using automated scans of structural OCT. The mean value of superior RNFL thickness was 108 μm in the group 0, 106 μm in group 1 and 110 μm in group 2; the mean value of inferior RNFL quadrant was 112 μm in the group 0, 115 μm in group 1 and 118 μm in group 2; the mean value of temporal RNFL quadrant was 56 μm in group 0, 67 μm in group 1 and 74 μm in group 2; the mean value of nasal RNFL quadrant was 62 μm in group 0, 67 μm in group 1 and 69 μm in group 2. We did not find any statistically significant differences about the superior, inferior and nasal RNFL quadrants among the three groups ($p = 0.536$, $p = 0.793$ and $p = 0.226$ respectively). Conversely, a stastistically significant difference was found ($p < 0.001$) regarding the temporal RNFL thickness, with a significant reduction of the RNFL thickness in group 0 (MS) compared to group 1 and 2.

The mean value of ganglion cell complex (GCC) in the superior sector was 89 μm in group 0, 86 μm in group 1 and 93 μm in group 2. The GCC in the inferior sector showed a mean value of 93 μm in group 0, 88 μm in group 1 and 95 μm for the group 2. We found no significant differences between the three groups ($p = 0.446$ for superior sector and $p = 0.594$ for inferior sector) (Table 2).

Structural OCT	MS group *Group 0*	MS Plus >50% *Group 1*	MS Plus <50% *Group 2*	p-value trend	p-value groups
Mean					
CFT	270	267	277	0,536	0,604
RNFL superior quadrant	108	106	110	0,793	0,872
RNFL inferior quadrant	112	115	118	0,479	0,778
RNFL temporal quadrant	**56**	**67**	**74**	**<0.001**	**<0.001**
RNFL nasal quadrant	62	65	69	0,226	0,469
GCC superior sector	89	86	93	0,446	0,330
GCC inferior sector	93	88	95	0,594	0,275
Standard deviation					
CFT	19	14	17		
RNFL superior quadrant	22	18	23		
RNFL inferior quadrant	24	18	20		
RNFL temporal quadrant	13	15	13		
RNFL nasal quadrant	14	16	15		
GCC superior sector	12	11	10		
GCC inferior sector	16	12	10		

Table 2. CFT (central foveal thickness), RNFL (retinal nerve fiber layer), GCC (ganglion cell complex).

The mean value of foveal avascular zone (FAZ) area was respectively 0.26 mm^2, 0.23 mm^2 and 0.25 mm^2 in group 0, group 1 and group 2 with no significant differences among the three groups (p = 0.812).

FAZ perimeter showed a mean value of 2.6 mm in group 0, 2.3 mm in group 1 and 2.6 mm in group 2 (p = 0.752), without statistically significant differences. The mean values of FAZ circularity were 0.46 in the group 0, 0.52 in group 1 and 0.48 in group 2 (p = 0.631).

The evaluation of whole, inner and outer rings of superficial capillary plexus (SCP) vessel density (VD) did not reveal statistically significant differences between the three groups (p = 0.154, p = 0.111 and p = 0.213 respectively).

Regarding the evaluation of deep capillary plexus (DCP) vessel density, the mean value of DCP whole VD was 17 in group 0, 15 in group 1 and 13 in group 2, ($p = 0.050$); the mean value of DCP inner VD was 18 in group 0, 15 in group 1 and 14 in group 2 ($p = 0.068$). Consequently, the vessel density of whole DCP showed to be reduced more in the MS-plus group with < 50% PVLs (group 2) compared to the MS-plus group > 50% PVLS (group 1) and in MS group (group 0). The vessel density of inner DCP demonstrated a borderline trend of reduction in the MS-plus group 2 compared to the other groups.

The mean value of outer DCP vessel density was 20 in the group 0, 17 in group 1 and 15 in group 2, with a statistically significant difference ($p < 0.05$). The vessel density of outer DCP was inferior in the MS-plus group with <50% PVLs (group 2) compared to the MS-plus group > 50% PVLs (group 1) and in the MS group (group 0).

The optic disc whole density had a mean value of 19 in group 0, 18 in group 1 and 18 in group 2 ($p < 0.05$), with a reduced density in the MS-plus group 2 compared to the other groups.

The optic disc radial peripapillary capillary plexus (RPCP) whole density and optic disc lamina cribrosa density did not show any statistically differences between the groups with a p-value of 0.331 and 0.998 (mean value of optic disc RPCP whole density was 18 in group 0, 18 in group 1 and 18 in group 2; mean value of optic disc lamina cribrosa density was 19 in group 0, 20 in group 1 and 19 in group 2).

The mean OCTA parameters and standard deviations are listed in Table 3.

OCT-Angiography	MS group *Group 0*	MS Plus >50% *Group 1*	MS Plus <50% *Group 2*	p-value trend	p-value groups
Mean					
FAZ area	0,26	0,23	0,25	0,812	0,66
FAZ perimeter	2,6	2,3	2,6	0,752	0,184
FAZ circularity	0,46	0,52	0,48	0,631	0,39
OCTA SCP whole	17	17	15	0,154	0,317
OCTA SCP inner	18	17	15	0,111	0,262
OCTA SCP outer	19	18	17	0,213	0,411
OCTA DCP whole	**17**	**15**	**13**	**0,051**	**0,148**
OCTA DCP inner	18	15	14	0,068	0,189
OCTA DCP outer	**20**	**17**	**15**	**0,029**	**0,09**
Optic disc whole density	**19**	**18**	**18**	**0,029**	**0,063**
Optic disc RPCP whole density	18	18	18	0,331	0,459
Optic disc lamina cribrosa	19	20	19	0,998	0,085
Standard deviation					
FAZ area	0,094	0,09	0,042		
FAZ perimeter	0,49	0,44	0,33		
FAZ circularity	0,084	0,14	0,098		
OCTA SCP whole	2,2	3	4,3		
OCTA SCP inner	2,3	3,3	4,6		
OCTA SCP outer	2,5	2,6	4,6		
OCTA DCP whole	3,6	5,4	7,4		
OCTA DCP inner	3,7	6,2	7,9		
OCTA DCP outer	4	5,6	7,8		
Optic disc whole density	1,1	1	1,1		
Optic disc RPCP whole density	1,4	1,7	1,7		
Optic disc lamina cribrosa	1,3	1,3	1,5		

Table 3. FAZ (foveal avascular zone), OCTA (optical coherenze tomography angiography), SPC (superficial capillary plexus), DCP (deep capillary plexus), radial peripapillary capillary plexus (RPCP).

The statistically significant parameters are reported in graphics in the Figure 9.

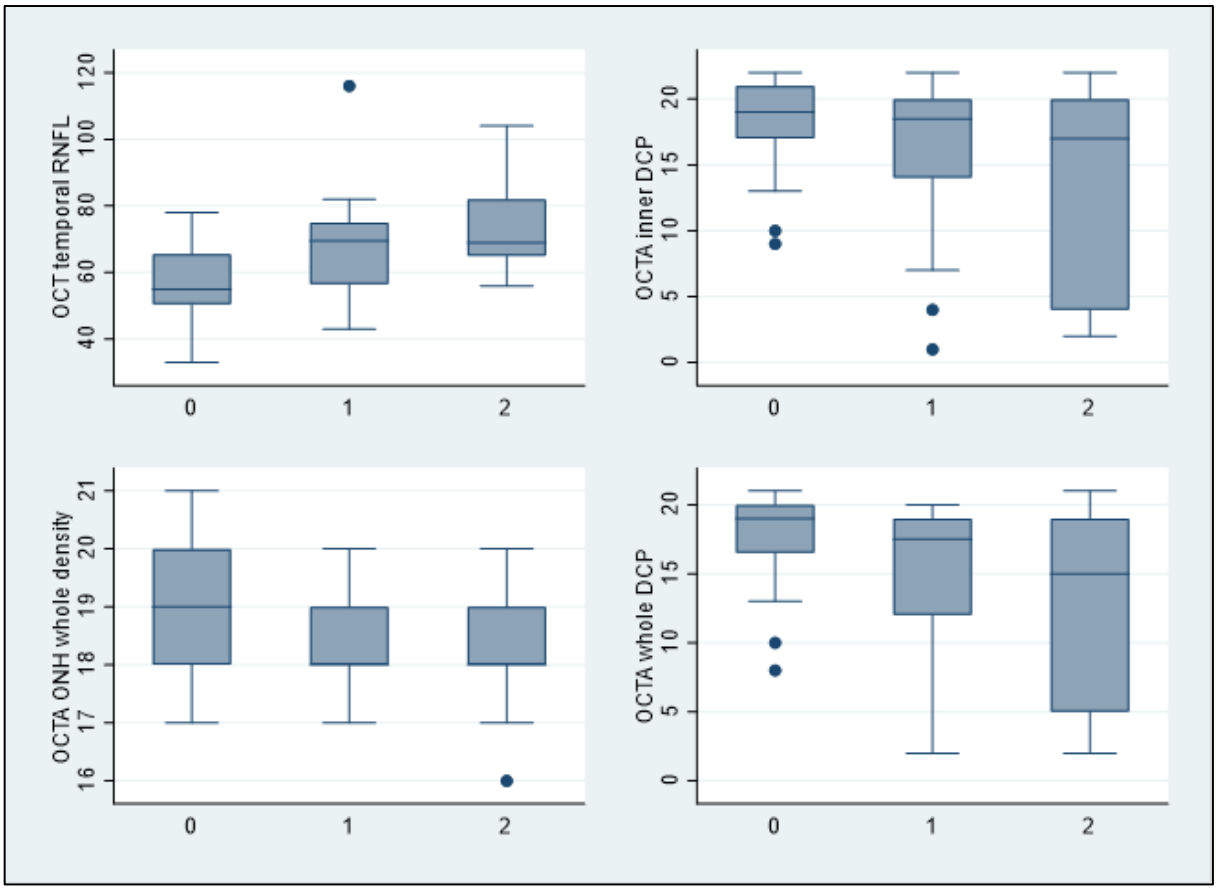

Figure 9. Box-plots of relevant parameters across MS subgroups (0: typical, 1: MS-plus >50%; 2: MS-plus <50%). OCT (optical coherence tomography), RNFL (retinal nerve fiber layer), OCTA (optical coherence tomography angiography), DCP (deep capillary plexus).

As expected, we found that optic neuritis (ON) was more common in typical MS patients (50% eyes), as compared to MS-plus > 50% (33.3%) and MS-plus < 50% (16.7%) (p = 0.050) (Table 4).

Optic neuritis	MS group *Group 0*	MS Plus >50% *Group 1*	MS Plus <50% *Group 2*	
No, *n (%)*	12 (50%)	16 (66.6%)	20 (83.3%)	48 (66.6%)
Yes, *n (%)*	12 (50%)	8 (33.3%)	4 (16.7%)	24 (33.3%)
Total eyes, *n*	24	24	24	72

Table 4. Patients with a history of ON in the three different groups."

We found having known ON episodes were marginally associated with temporal RNFL thickness (p = 0.085), and no other association were observed with other OCT or OCTA parameters. Such non-significant association was even weaker with multivariate regression, once MS subgroups were considered, which can be explained by the fact that the actual optic nerve damage was more common in typical MS subgroup.

6. DISCUSSION

Optical Coherence Tomography (OCT) and OCT Angiography are advanced imaging techniques that play a crucial role in non-invasively visualizing the microvascular structures of the retina and peripapillary region. These technologies have proven to be valuable tools in studying and understanding various medical conditions, including multiple sclerosis (MS) (74,75).

OCT is a non-invasive imaging method that employs low-coherence interferometry to capture high-resolution, cross-sectional images of biological tissues. It provides detailed structural information about the layers of the retina, allowing clinicians to assess the thickness of retinal layers and identify abnormalities. OCT Angiography, an extension of traditional OCT, adds a vascular component to the imaging process. By detecting motion contrast between blood flow and static tissue, OCT Angiography enables visualization of the retinal and peripapillary microvascular networks. This allows for a comprehensive assessment of blood vessel density, perfusion, and alterations in the microvasculature. In the context of multiple sclerosis, OCT and OCT Angiography have emerged as valuable tools for studying the effects of the disease on the visual system. MS is a chronic inflammatory demyelinating disorder of the central nervous system, and it can manifest with various neurological symptoms, including visual impairment (76).

In this study we evaluated the potential role of high-resolution retinal imaging techniques to assess changes in retinal nerve fiber layer (RNFL) and macular ganglion cell thickness, using structural OCT, as well as alterations in vascular retinal flow detected using OCTA, in patients affected by MS and those with MS presenting atypical “red flags”, which are clinical/radiological or laboratory signs can could be associated to other pathologies (MS-plus patients). We also aimed to correlate these imaging parameters with neurological and neuro-radiological markers, particularly the presence and percentage of the "central vein sign", as evaluated by a neurologist using focused MRI sequences an localizing the presence of perivenular lesions (PVLs).

We used structural B-scan OCT and OCTA to non-invasively evaluate qualitative and quantitative alterations in retinal layers and retinal and peripapillary vascular flow, to identify differences between typical and atypical MS forms.

Evaluation of structural OCT of macula and optic nerve head parameters

Structural optical coherence tomography (OCT) is a non-invasive imaging technique that can be used to assess the structure of the retina and optic nerve. In multiple sclerosis (MS), OCT can be used to measure the thickness of the retinal nerve fiber layer (RNFL) and the ganglion cell complex (GCC), both of which have been shown to be useful in the diagnosis and management of MS.

RNFL thickness is a reliable indicator of axonal loss in MS, and studies have shown that it is reduced in MS patients compared to healthy individuals. RNFL thinning can be detected by OCT even in the early stages of MS, before other clinical symptoms appear. Monitoring changes in RNFL thickness over time can be used to assess disease progression and the effectiveness of treatments.

In our study, a statistically significant difference of temporal RNFL (tRNFL) thickness was found ($p < 0.001$) among the different groups of patients, with a significant reduction of the tRNFL thickness in group 0 (MS) compared to group 1 and 2 (MS-plus patients presenting > 50% PVLs and MS-plus patients presenting < 50% PVLs, respectively).

Our results are in agreement with previous studies that have suggested that tRNFL thickness is reduced in typical MS compared with forms of MS exhibiting clinical signs of alternative diagnosis. We did not correlate these parameters with healthy controls, but it consideration may be an additional finding, which enhances the suspicion of alternative diagnosis in patients with lower number of white matter lesion presenting the central vein sign (CVS). In fact, the comparison between the 3 groups showed that the tRNFL was significantly reduced in group 0, compared to the SM-plus groups, and that the average thickness of the RNFL progressively increases from the MS group towards the groups with ever fewer PVLs presenting on MRI.

There are many studies that have demonstrated a correlation between RNFL thickness and multiple sclerosis (MS).

A study determined the thickness of the RNFL in MS patients with or without previous optic neuritis (ON) and in patients with ON evaluated the relationship between the structural damage and functional alterations in visual acuity (VA) and visual field (VF). OCT thickness in the unaffected eye was significantly thicker in ON patients than in the other groups, however there were no differences among the affected eyes. There were significant differences in VA and VF among the non-affected eyes ($p = 0.007$), but not among the affected eyes ($p = 0.878$). All MS patients showed axonal damage in both optic nerves, greater in patients with previous ON. Axonal damage was detected early, so Authors hypothesized that OCT could be used as a structural biomarker for MS, also in early stages (77).

A systematic review and meta-analysis of studies that used time domain optical coherence tomography (TD-OCT) to assess RNFL thickness in MS patients found that RNFL thickness was significantly reduced in MS patients compared to healthy controls, and that RNFL thinning was associated with disease duration and disability. The estimated RNFL thinning in patients with MS was greater than the extent expected in normal ageing, probably because of retrograde trans-synaptic degeneration and progressive loss of retinal ganglion cells, in addition to the more pronounced thinning caused by optic neuritis if present (9).

A recent study found that both peripapillary and macular OCT measurements could differentiate all MS subtypes from healthy controls and correlate with the disease gravity. The Symbol Digit Modalities Test (SDMT) score was significantly associated with reduced thickness of all OCT measures, mostly in average peripapillary RNFL and temporal RNFL. The Expanded Disability Status Scale (EDSS) score was significantly associated with reduced inner retinal layer thickness. The largest reduction was seen in temporal RNFL (tRNFL) (-1.52 µm, $p < 0.001$) and inner GCC (–1.78 µm, $p < 0.001$), showing that tRNFL was highly sensitive and associated with level of both cognitive and physical disability (78).

Saidha et al. (2013) investigated the relationship between RNFL thickness and global central nervous system (CNS) pathology in MS patients using OCT and magnetic resonance imaging (MRI). They found that RNFL thickness was significantly correlated with measures of brain atrophy and lesion burden, indicating that RNFL thinning reflects overall CNS damage in MS. OCT measures appear to correlate with intracranial volume in patients with MS and healthy control subjects, an important unexpected factor unaccounted in prior studies examining the relationships between peripapillary retinal nerve fiber layer thickness and brain substructure volumes (79).

Syc et al. (2012) used OCT to assess RNFL thickness in patients with acute optic neuritis, which is often an early symptom of MS. They found that RNFL thickness was significantly reduced in patients who went on to develop MS compared to those who did not, suggesting that RNFL thinning may be an early marker of MS (80).

Villoslada et al. (2008) used OCT to assess RNFL thickness in patients with different types of MS, including typical and atypical forms. They found that tRNFL thickness was significantly reduced in typical MS compared to atypical MS and healthy controls. They also found a significant association between color vision and RNFL thickness in MS patients (81).

Merle et al. (2015) conducted a study in which they measured RNFL thickness using OCT in patients with different types of MS, including relapsing-remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS). They found that temporal RNFL thickness was significantly reduced in RRMS compared to PPMS and SPMS (82).

Recently, Gundogan et al found among 39 MS cases without ocular antecedents a significant reduction of thickness of RNFL only in the temporal quadrant (83).

Jindahra et al. (2009) used OCT to assess RNFL thickness in patients with MS, including those with typical and atypical forms. They found that temporal RNFL thickness was significantly reduced in patients with typical MS compared to those with atypical MS. Additionally, they identified retrograde trans-synaptic retinal ganglion cell loss in MS patients using OCT (84).

While these studies suggest that there may be a statistically significant reduction in temporal RNFL thickness in typical MS compared to atypical forms, it is important to note that the results are not consistent across all studies and further research is needed to fully understand the relationship between RNFL thickness and different types of MS.

Klistorner and coworkers showed that the largest thinning was seen in the temporal peripapillary RNFL when comparing MS eyes and controls. Significant temporal RNFL (tRNFL) thickness reduction was observed over the 3-years follow-up period at a relatively constant rate (1.02 μm/year). Thinning of tRNFL fibers was more prominent in younger patients and tRNFL loss was associated with new MRI lesions There was significantly greater tRNFL thinning in patients with new lesions activity (85). This study supports the notion that retrograde transneuronal degeneration might play a role in progressive retinal nerve fiber layer loss. In addition, the results of the study also indicated that the disease-related neurodegenerative retinal changes start much earlier than the clinical diagnosis of MS (85).

These results are in line with our study. A preferential loss of axons in the temporal quadrant of the RNFL may be due to the fact that this region allows central vision and consists primarily of small parvocellular axons, which are likely more susceptible to damage in MS than larger magnocellular axons (86).

Another important parameter that we have studied with OCT was the ganglion cell complex (GCC) of the macular area.

The GCC is a composite measurement of the combined thickness of the ganglion cell layer, inner plexiform layer, and the inner nuclear layer of the retina. GCC thickness can be automatically measured using OCT.

To measure GCC thickness using OCT, a special scanning protocol is used to capture high-resolution cross-sectional images of the retina. The images are then analyzed using specialized software that can calculate the thickness of the GCC layer. GCC thickness measurements can provide information about the health and function of the ganglion cells in the retina.

In patients with multiple sclerosis (MS), GCC thickness has been shown to correlate with

disease severity and visual function. Reduction in GCC thickness has been found in MS patients with a history of optic neuritis, a common early symptom of MS. Therefore, measuring GCC thickness using OCT can be a useful tool for monitoring the progression of MS and assessing the effectiveness of treatments aimed at preserving visual function. In addition, OCT can also be used to assess other structural changes in the retina and optic nerve that occur in MS, such as macular volume and thickness.

These measurements may provide important informations about disease progression and treatment efficacy, and could be used to guide clinical decision-making in MS patients. A study by Pietroboni et al. compared newly diagnosed MS patients to controls. They found that macular ganglion cell layer (mGCL), macular inner plexiform layer (mIPL), and macular ganglion cell-inner plexiform layer (mGCIPL) thickness were significantly reduced ($p = 0.0003$, $p = 0.0049$, and $p = 0.0007$, respectively). Peripapillary RNFL (pRNFL) and temporal pRNFL (T-pRNFL) did not show any significant changes, although there was a trend toward T-pRNFL thinning ($p = 0.0254$). They demonstrated that macular ganglion cells involvement occurred earlier in newly diagnosed MS patients, before the onset of RNFL thickness reduction. These retinal changes showed a significant association with cortical regions that are known to be important for visuospatial performance (87).

Interestingly, in another recent study by Pietroboni et al. it was demonstrated that a loss of macular ganglion cells was not accompanied by a pRNFL thinning in newly diagnosed relapsing-remittins MS (RRMS) patients without previous history of ON, compared with healthy controls. They concluded that this finding suggests that the retinal damage might begin in the macular ganglion cell layer and is then spread to the axons (88).

In our cohort the evaluation of ganglion cell complex (GCC) in the superior sector and in the inferior sector showed no statistically significant differences among the three groups ($p = 0.446$ for superior and $p = 0.594$ for inferior sector).

This is probably due to the fact that we did not compare patients to healthy controls, and that our study cohort was quite small. We know that ganglion cell loss may precede the damage of RNFL of optic nerve head, and that GCC loss represents a very sensitive

parameter of CNS damage. Thus, the finding of non-significant differences between the groups may mean that in the different subgroups there is no significant sparing of this cell layer, as it could be impaired in the majority of patients presenting with neurological pathology.

OCTA analysis and evaluation of vessel density changes

OCT angiography is a non-invasive imaging technique that can provide high-resolution visualization of the retinal and peripapillary microvascular networks. Some studied have proposed that OCT angiography could provide some potential biomarker useful for multiple sclerosis (MS).

In typical MS forms, OCT angiography has been shown to be a useful tool for assessing retinal perfusion abnormalities, which are thought to be related to the pathological changes in the central nervous system. Several studies have reported a decrease in retinal vessel density and perfusion in MS patients, which may reflect the axonal loss and neurodegeneration in the disease. Additionally, OCT angiography can detect changes in the macular perfusion that are associated with visual function impairment in MS patients, providing a potential biomarker for monitoring the disease progression.

Previous studies employing OCTA have shown that retinal vascular plexus densities are reduced in MS, in particular within the superficial capillary plexus (SCP), which mainly supplies the ganglion cell layer. The greatest reductions in SCP density are detectable in eyes with a history of optic neuritis (ON). Murphy et al (89) found that the SCP density is largely reducted in MS patients with a history of ON, than in those without history of ON. Additionally, they found significant relationship between inter-eye differences in superficial vascular plexus and visual function measures in MS-ON patients (after the acute phase of ON), and similar relationship were also identified between inter-eye differences in GC-IPL thickness and visual function and contrast sensitivity (49, 57).

In other central nervous system (CNS) diseases, such as neuromyelitis optica spectrum disorder (NMOSD), OCT angiography has also demonstrated its usefulness in the evaluation of the retinal vasculature.

Several studies have investigated the potential correlation between OCTA and neuromyelitis optica spectrum disorders (NMOSD). For example, a study published in the 2021 by Rogaczewska found that retinal vessel density, as measured by OCTA, was significantly reduced in patients with NMOSD compared to healthy controls, suggesting that the vasculopathy could be the primary process in NMOSD patients (90).

NMOSD is a rare autoimmune disorder that predominantly affects the optic nerves and spinal cord, leading to severe visual impairment and disability. OCT angiography could detect changes in retinal perfusion and vascular abnormalities in NMOSD patients, and may provide insights into the pathophysiology of the disease as a non-invasive marker for monitoring treatment response.

Unlike other studies, we performed a comparative analysis between typical MS and patiens with MS presenting atypical “red flags” suggestive for alternative diagnosis, and we correlated the OCT and OCTA parameters in relation to the percentage of perivenular lesions (PVLs) in the white matter.

Interestingly, in our sample we found that the deep capillary plexus (DCP) was reduced in the MS-plus group with < 50% PVLs (group 2) both for the whole and outer vessel densities, compared to the MS-plus group > 50% PVLs (group 1) and the MS group (group 0). The vessel density of inner DCP demonstrated a borderline trend of reduction in the MS-plus group 2 compared to the other groups (Figure 10).

This data are interesting findings, since in previous studies emerged that especially the superficial capillary plexus (SCP) was the one most affected by the vessel density reduction in MS. In particular, the reduction of DCP vessel density in MS-plus patients, suggest an impaired retinal and peripapillary blood flow, and consequently we could hypothesize a different pathogenesis for atypical MS.

We also found a significant reduction on ONH vessel density for whole vessel density and RPCP vessel density in group 2, compared to group 0 and 1, reflecting an higher involvement of microvascular structures in MS-plus patients < 50% PVLs (Figure 11).

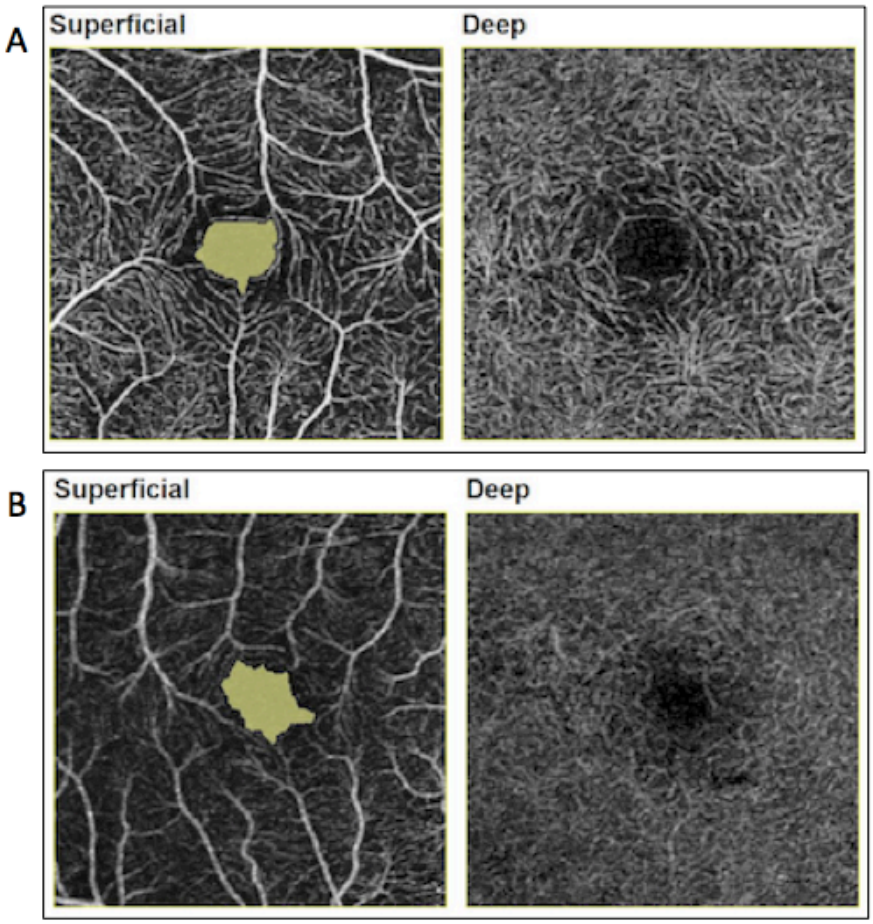

Figure 10. Automated FAZ detection on the superficial capillary plexus (SCP). Qualitative evaluation of macular vessel density, which revealed a lower vessel density both of superficial capillary plexus (SCP) and deep capillary plexus (DCP) in a MS-plus patient (B) compared to MS patient (A). In our cohort we only found a statistically significant reduction in DCP vessel density in group 2 (MS-plus < 50% PVLs).

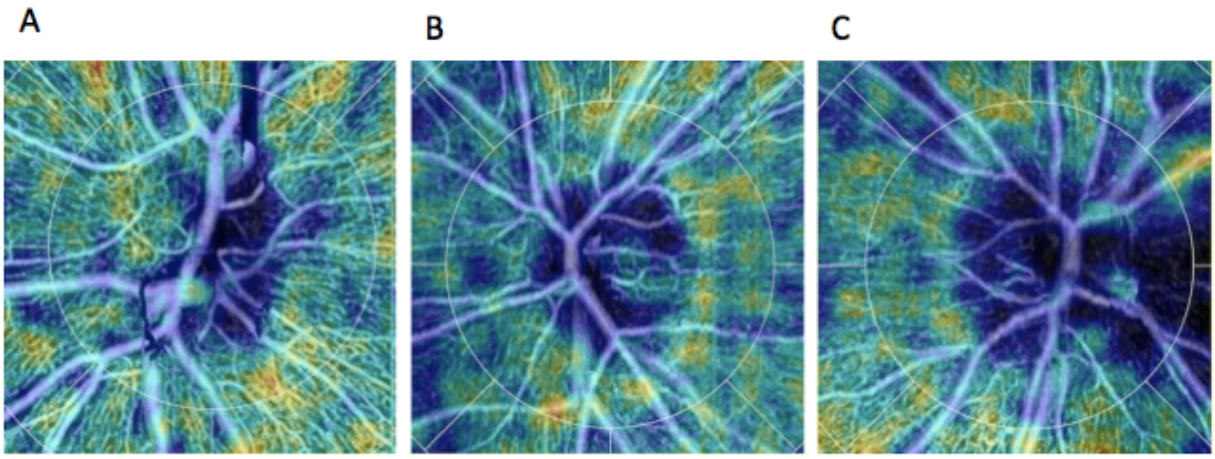

Figure 11. OCTA perfusion images of the optic nerve head of the radial peripapillary capillary plexus (RPCP). Vessel density in three different patients with a history of optic neuritis. We can see a vessel rarefaction from image A (group MS) to image B and C (group MS-plus > 50% perivenular lesions and group MS-plus < 50% perivenular lesions, respectively).

Overall, OCTA technique can be used to detect retinal and optic nerve vessel density changes in patients with MS, and could be a value-added tool for helping in challenging diagnosis. However, further studies are needed to confirm these findings and to investigate the potential use of OCTA and its implications in clinical practice.

Central vein sign and correlation with OCT and OCTA findings

The central vein sign (CVS) is a radiological finding that is observed on magnetic resonance imaging (MRI) scans of the brain and spinal cord in patients with multiple sclerosis (MS). It refers to the presence of dark signal voids within the central veins of brain lesions and it is thought to be associated with a condition known as chronic cerebrospinal venous insufficiency (CCSVI).

The concept of CCSVI and its relationship with MS remains controversial and unproven. Some studies have reported a higher prevalence of the CVS in MS patients compared to healthy controls, while others have failed to find a significant association. Moreover, the

imaging technique used to detect the CVS is not standardized, and there are concerns about its reliability and reproducibility.

As a result, the usefulness of the CVS as a biomarker of uncertain MS is currently unclear. While it is an interesting concept that could potentially provide insights into the underlying pathophysiology of MS, more research is needed to fully understand its significance and its potential value in clinical practice.

A large multicenter and cross-sectional study including 606 patients evaluated the sensitivity and specificity of various central vein sign lesion criteria for differentiating MS from non-MS conditions using 3T brain MRI with various commonly used pulse sequences. The authors found that the use of the central vein sign at 3T MRI protocol had a high specificity and a moderate sensitivity in differentiating MS from not MS (sensitivity 68.1%, specificity 82.9%) (91).

There is some research investigating the relationship between central venous sign (CVS) as a potential biomarker of uncertain MS and OCT angiography. One study published in 2018 found that MS patients with the CVS on MRI scans had decreased retinal perfusion on OCT angiography compared to MS patients without the CVS. The authors of the study suggested that this may be due to impaired venous drainage in the retina, similar to the proposed mechanism of CCSVI in the brain and spinal cord (92).

However, this study was small and further research is needed to confirm these findings and explore the potential utility of OCT angiography as a biomarker for MS. Additionally, the relationship between the CVS and OCT angiography may not be specific to uncertain MS and may also be observed in other MS subtypes.

In our cohort we defined different groups of patients based on the presence of typical manifestation of MS and, on the other hand, atypical “red flags” suggestive of alternative diagnosis.

Moreover, we divided patients with “red flags” into 2 subgroups in correlation with a high or low number of perivenular lesions (PVLs) detectable on MRI scan. We used a cut-off of 50% of number of lesions with CVS.

Interestingly, we found that, in patients with a low number of PVLs, there was a rarefaction of deep capillary plexus (DCP) vessel density of macular region and a lower vessel density of optic nerve head (ONH), compared with MS group. This is a promising finding, because it could be strongly associated with the hypothesis of a differential etiology of patients with atypical clinical/radiologic sign for MS, primarly due to microvascular changes.

Overall, while the CVS and OCT angiography are both interesting potential biomarkers for MS, further research is needed to fully understand their significance and their potential value. The diagnosis and management of MS remains primarily based on clinical assessment and imaging, and no single biomarker has yet been identified that can reliably diagnose or monitor MS on its own.

The diagnosis of MS is based on a combination of clinical symptoms, neurological examination findings, and imaging studies such as MRI. Other biomarkers, such as levels of certain proteins in the cerebrospinal fluid, have also been studied as potential indicators of disease activity and progression.

In our study we found that OCT and OCTA retinal and ONH changes support the proposed classification of MS in typical forms, with mainly perivenular lesions characterized by CVS, and ONH axonal damage, as compared to MS patients with atypical red-flags suggestive of primary microvascular damage. This is highlighted by the RNFL thinning in typical MS and by the reduction DCP vascular density in MS-plus with red flags.

The proposed subgroup classification is more associated with RNFL thickness than with the diagnosis of previous optic neuritis, which is underestimated by clinic and history.

This observation could reveal the fact that in MS-plus with < 50% PVLs (group 2), probably the microvascular reduction of the deep capillary plexus is related to the disease pathophysiology, and that the presence of ON does not significantly influence the lower vessel density (VD). In fact, the presence of red flags in these patients, together with a significant reduction of retinal and optic nerve head VD, suggest that the

inflammatory component of the central nervous system (CNS) is not the predominant etiological cause of multiple sclerosis.
Therefore, an alternative diagnosis to multiple sclerosis should be considered both in patients presenting red flags and in those with a low number perivenular lesions.

7. CONCLUSION

Our project evaluated non-invasive innovative retinal and optic nerve imaging techniques as a potential source of biomarkers of CNS diseases to be considered as surrogate endpoints of disease, providing a valuable tool for an early diagnosis, for monitoring progression of disease and the response to different treatments.
This study presents some limitations. First, our cohort is quite small because challenging patients presenting with symptoms suggestive of MS and red flags for diagnosis are rare. Second, the study has been conducted in a single-center investigation. A definitive diagnosis is often difficult to obtain and, in the absence of a highly specific biomarker for MS, depends on the opinion of MS experts and therefore can be variable. For this reason, it is important to find a specific objective sign that, thanks to the use of multimodal imaging, allows an early and safe identification of patients with atypical signs of the disease.
Despite the promising results, the use of OCT and OCTA in MS and atypical MS forms is still in its early stages, and further research is needed to fully understand their clinical relevance and utility. The standardization of OCTA protocols and the development of quantitative measures are necessary to establish the diagnostic and prognostic value of this technique in MS. Additionally, longitudinal studies are needed to determine the ability of OCTA to predict disease progression and response to treatment.
Using OCTA, could be possible to explore the timing of vascular changes relative to structural atrophy and may help answer important questions about the role of hypoperfusion in the pathophysiology of neuroinflammatory disease. So, qualitative

characteristics of retinal microvasculature may help discriminate between different neuroinflammatory disorders.

After all, the retina represents the only district of our body in which it is possible to directly visualize arterial, venous and capillary vascular structures, as well as nervous structures: thanks to this, the retina has been defined by many Authors as a “window ” on central nervous system, as well as on the vascular system.

For this reason, the study of retinal and optic nerve structures can provide indirect information on the systemic and cerebral vascularization and can allow us to examine the impact of various systemic diseases on the microcirculation.

In conclusion, OCT and OCTA are a promising non-invasive techniques for evaluating the retinal vasculature in MS and atypical MS forms. They have the potential role to provide insights into the underlying pathological processes and serve as a biomarker for disease monitoring.

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