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REVIEW

Determinants and Biomarkers of Progression Independent of Relapses in Multiple Sclerosis

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Clinical, pathological, and imaging evidence in multiple sclerosis (MS) suggests that a smoldering inflammatory activity is present from the earliest stages of the disease and underlies the progression of disability, which proceeds relentlessly and independently of clinical and radiological relapses (PIRA). The complex system of pathological events driving "chronic" worsening is likely linked with the early accumulation of compartmentalized inflammation within the central nervous system as well as insufficient repair phenomena and mitochondrial failure. These mechanisms are partially lesion-independent and differ from those causing clinical relapses and the formation of new focal demyelinating lesions; they lead to neuroaxonal dysfunction and death, myelin loss, glia alterations, and finally, a neuronal network dysfunction outweighing central nervous system (CNS) compensatory mechanisms. This review aims to provide an overview of the state of the art of neuropathological, immunological, and imaging knowledge about the mechanisms underlying the smoldering disease activity, focusing on possible early biomarkers and their translation into clinical practice.

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The Rationale of the Review

The heterogeneous and largely unpredictable clinical outcome of multiple sclerosis (MS) has puzzled physicians for decades. Most patients (~85%) present with a relapsing–remitting (RR) course, characterized by acute attacks followed by complete or partial recovery, and eventually experience progressive accumulation of irreversible disability[1] in the later stage of the disease (secondary progressive [SP] MS). While this phenotypic distinction enhances homogeneity in clinical trials and allows standardized communication among clinicians, emerging evidence suggests a biological continuum in which pathological mechanisms underlying relapses and progression coexist from the early stage of the disease.[2–5] Despite the successful therapeutic suppression of relapses and magnetic resonance imaging (MRI) active inflammatory lesions (Fig 2A–D), MS patients often report a gradual but progressive worsening of physical and cognitive functions. This discrepancy indicates an ongoing "smoldering" biological activity, albeit challenging to detect, which is commonly referred to as progression independent of relapsing activity (PIRA)[2] or silent progression,[6] and is distinct from relapse-associated worsening (RAW).

In the pooled analysis of the OPERA trials, in both the ocrelizumab and interferon-treated groups, PIRA events accounted for disability accumulation in a substantial proportion (>80%) of RRMS patients despite their relatively short disease duration (mean six years).[2] A large pooled clinical trials dataset[3] and recent data from a real-world observational cohort[5] confirmed this finding. Among patients from the Italian MS registry experiencing disease worsening, PIRA was more commonly reported than RAW since the second year after the disease onset. Furthermore, its frequency proportionally increased with the disease duration.[5] Notably, patients free of ongoing inflammatory attacks can also experience significant deterioration of their cognitive function from the earliest disease stages,[7,8] which accumulates silently for several years.[9] However, PIRA has historically referred to progression independent of clinical relapses but inadequately captures subclinical disease activity accounting for disease worsening. Indeed, the emerging definition of PIRA includes new asymptomatic brain and spinal cord lesions PIRMA (progression independent of relapse and MRI activity), thus aligning to outcomes measures used in MRI research and trials.

The effective suppression of acute inflammatory parameters (relapses and active MRI lesions) might falsely indicate disease control while underlying biological mechanisms leading to a subtle deterioration remain clinically undetected. Understanding mechanisms and identifying biomarkers of such silent deterioration remain essential unmet needs.

Pathological Substrates

Immunopathologically, it is plausible to hypothesize that the smoldering MS activity is characterized by a continuous interplay between intrathecal (cerebrospinal fluid [CSF] and meninges) and parenchymal chronic processes of inflammation and neurodegeneration, which associate glial activation and neuro-axonal dysfunction/loss with additional age-related pathological mechanisms.[10–14]

A complex interaction among different immunopathogenic and neurodegenerative processes (Fig 1) possibly underlines the disease progression and includes: (1) acute blood-brain barrier breakdown/brain and spinal cord atrophy; (2) perivascular/meningeal inflammation; (3) acute focal white matter (WM) lesions/ diffuse gray matter (GM) lesions; (4) acute glia priming/chronic microglia activation; (5) mitochondrial damage/impaired energy production; (6) loss of trophic support/ remyelination failure.

Several potential underlying mechanisms for PIRA[15] have been suggested in the past decade including the formation of meningeal lymphoid-aggregates with the associated subpial cortical demyelination and the consequent accumulation of neuronal damage and cortical atrophy, the accumulation of chronic active lesions (CAL, also termed slowly expanding, smoldering or mixed active/inactive lesions); and the remyelination failure. However, their exact extent, involvement, timing, and role still need to be fully understood. Possible concomitant key drivers of PIRA could also be (1) the slowly accumulating WM and GM demyelination, possibly linked to the presence of perivascular, meningeal, and choroid plexus inflammatory cell infiltrates; (2) persistent microglial activation (driven by degenerating cells and inflammation) contributing to neuro-axonal energy failure through oxidative stress mechanisms; and (3) ongoing retrograde and anterograde axonal injury.

Previous histological and more recent combined imaging-neuropathological studies highlighted heterogeneous immunopathological patterns of active demyelination among patients, although lesions' homogeneity may also occur within individual patients,[16–18] possibly converging into a final common pathway related to chronic neuronal-axonal damage and disease progression.

Chronic Active Lesions and Iron-Related Changes

Pathologically, CAL are characterized by a rim of activated myeloid phagocytes (microglia and macrophages) with occasional myelin degradation products as a sign of continuous low-level myelin destruction. These lesions differ from chronic inactive lesions in the number of myeloid phagocytes at their rims but a similar number of CD3+ T cells, which remain very sparse, suggesting a

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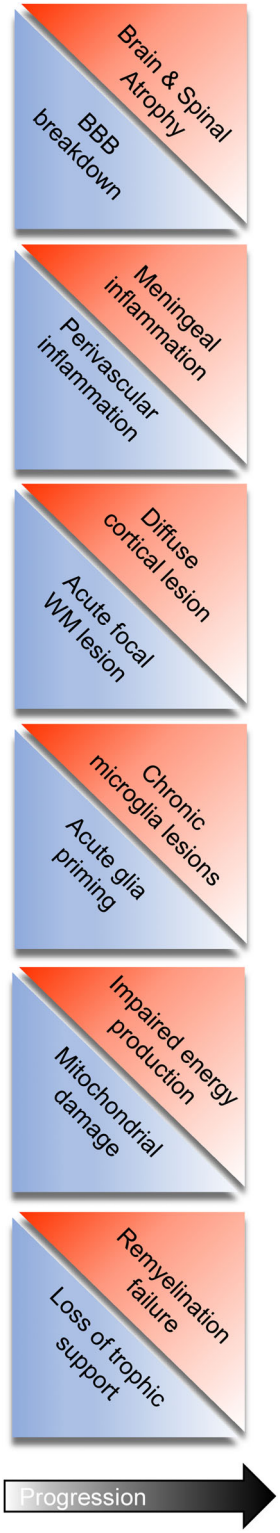


Figure 1: Diagram representing different parameters and features that may change during disease progression and contribute to progression independent of relapse: (A) acute blood-brain barrier breakdown/brain and spinal cord atrophy; (B) perivascular/meningeal inflammation; (C) acute focal white matter (WM) lesions/ diffuse gray matter (GM) lesions; (D) acute glia priming/chronic microglia activation; (E) mitochondrial damage/impaired energy production; (F) loss of trophic support/ remyelination failure. [Color figure can be viewed at www.annalsofneurology.org]

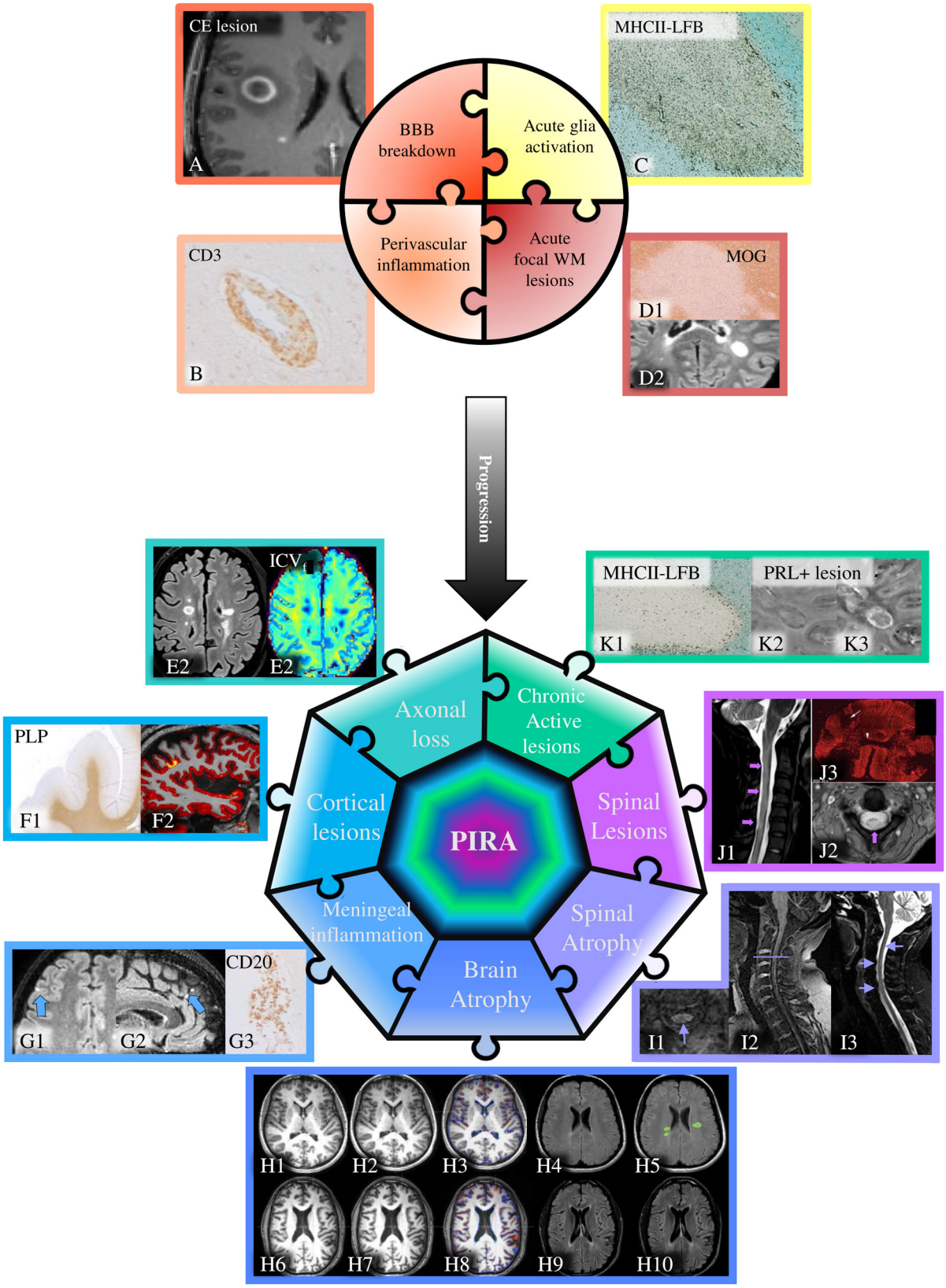
crucial role of microglia and macrophages in progressive lesion activity.[19] In ~45% of pathologically detected CAL, phagocytes accumulate iron and frequently adopt a senescent morphology (Fig 2K1).[20–22] The myeloid cells at the rim have an activated phenotype with expression of proinflammatory p22phox, CD40, CD86, and inducible nitric oxide synthase (iNOS), C1QA, as well as a lack of anti-inflammatory CD206 expression and loss of the quiescence marker P2ry12.[20,23–25] Conversely, the putatively anti-inflammatory marker CD163 is expressed on the myeloid cells rims.[24–27] In line with the pro-inflammatory activation, an increased number of APP+ axonal spheroids is found at the slowly expanding rims,[28–30] which indicates axonal degeneration, as confirmed by elevated neurofilament light chain serum levels found among patients with a large number of MRI-detected iron rim lesions.[30,31] The microglia and macrophages at the iron rims additionally express the activation marker translocator protein (TSPO), which can be detected by positron emission tomography (PET) imaging using a TSPO-specific tracer in-vivo.[32] In addition to contributing to the axonal destruction, the inflammatory environment at the slowly expanding rim, including secreted mediators from T cells, particularly IFNg,[33] appears responsible for the remyelination failure in this type of lesion.[20,26] Notably, the proportion of slowly expanding but not classically active lesions strongly correlated with disease severity in a large study on 182 autopsy MS cases.[34]

Grey Matter Demyelination and Meningeal Inflammation (Including Spinal Cord and Deep grey matter)

CAL are also seen in the cortical GM, deep GM, and the spinal cord.[35] These can be more extensive than WM lesions and frequently appear to follow an outside-in gradient, typified by the subpial lesions of the neocortical GM (Fig 1F1,F2).[36,37] While most GM lesions in the cerebral cortex follow a subpial pattern (so-called type III), intracortical perivascular lesions (type II) and lesions that extend into both the GM and WM (leukocortical type I) are also frequently observed, although in smaller numbers than subpial lesions.[38–40] Unlike WM lesions, minimal parenchymal or perivascular immune cell infiltration is associated with most cortical GM lesions,[38,39] leading to the view that they are unlikely to be linked to acute inflammatory events. Significant GM demyelination can also be seen in deeper GM regions, including the thalamus[41,42] and cerebellum.[43,44] In the spinal cord, the extent of demyelination in the GM is generally more severe than that observed in the WM.[45]

In contrast to the lack of association between GM parenchymal and perivascular infiltrates and GM demyelination,

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there is a significant relationship between demyelination and the degree of immune cell infiltration in the overlying meninges (Fig 2F1,F2,G1,G2).[10,40,46–49] The presence of large immune cell aggregates of both T and B cells, together with the development of tertiary lymphoid structures in some cases,[40,48] is suggested to give rise to an inflammatory and cytotoxic CSF that can both chronically activate cells within the CNS parenchyma and also provide cytotoxic mediators that can diffuse into the underlying tissues.[50,51] The degree of immune cell infiltration in the meninges is also associated with increased WM lesion number and activity, particularly in the spinal cord,[45,52] but also in the subcortical WM,[53] suggesting a similar mechanism of compartmentalized inflammation-induced pathology in both the WM and GM. Thus, there is substantial evidence for a progressive but slow accumulation of demyelinating and neurodegenerative pathology that is not related to clinical inflammatory relapses and may drive the accumulating disability throughout the disease course of MS.

Focus on Surface-in Gradient of Neuro-glia Abnormalities. In progressive MS, the meningeal inflammation is associated with a substantial "surface-in" gradient of neuronal and axonal loss in subpial cortical GM lesions[54] and subependymal thalamic lesions.[41] Even in the absence of demyelination, a substantial graded neuroaxonal damage can be observed, albeit always associated with a concomitant gradient of astrocyte and oligodendrocyte loss, microglia activation, high levels of meningeal inflammation, and with faster and more severe disease progression.[37,41,54,55] Similarly, the surface-in gradient of MS thalamic damage is correlated to a specific CSF protein profile, including (1) a high level of neurodegeneration markers, such as neurofilaments light chain (NfL); (2) molecules related to both innate immune activity and lymphoid neogenesis (CCL19, CXCL10, CXCL13); and (3) major inflammatory factors (including sTNFR1, fibrinogen, IFN-γ, IL2 and IL10). These data strongly suggest a key role of intrathecal inflammation in the slow build-up of diffuse GM pathology and support the use of CSF profile as a biomarker signature of progressive GM pathology. A similar gradient of brain abnormalities is found at early disease phases, even in pediatric MS.[37,56–58] In addition, these data suggest a link between inflammatory/cytotoxic CSF and accumulating neuronal loss, also reproduced in animal models in which the levels of neurotoxic pro-inflammatory cytokines, such as TNF and lymphotoxin-alpha, are chronically elevated in the meningeal space.[37,41,51,55,59]

Neuronal and Synaptic Damage in the Cortex and Deep Grey Matter

Neuroaxonal loss, rather than demyelination, is likely to be one of the main determinants of GM atrophy,[60–62] and is strongly associated with disability progression. The neuroaxonal loss has been described to a variable extent in all GM structures, including the cerebral cortex (19%–40%),[54,60,63–67] cerebellar cortex (29%),[43] hippocampus (27%),[68] thalamus (30%–35%),[69,70] and spinal cord (36–75%).[71–74] Neuroaxonal loss is probably related to lesion-dependent and lesion-independent mechanisms. Indeed, retrograde neuronal degeneration can occur because of focal WM damage.[46] In the GM, reduced

Figure 2: Diagram representing the parameters and features that may contribute to the different MS phases and progression independent of relapse: (A) Contrast enhancing lesion with blood—brain barrier (BBB) breakdown; (B) CD3+ T cell infiltrating perivascular space; (C) active multiple sclerosis (MS) lesions characterized by elevated presence of MHC class II+ activated microglia/macrophages; (D) focal white matter lesions detected neuropathologically by myelin oligodendrocyte glycoprotein immunostaining (D1) and using fluid attenuated iversion recovery (FLAIR) MRI images (D2); (E) T2 hyperintense white matter lesions visible on T2-FLAIR sequence (E1) characterized by a lower intracellular volume fraction (ICV_f), E2, (derived from neurite orientation dispersion and density imaging, NODDI, and representing the space bounded within membranes processes typically considered a measure of axons and dendrites contribution) compared to normal appearing white matter suggesting substantial intralesional axonal loss; (F) cortical lesions visible as extensive areas of demyelination by using myelin proteolipid protein (PLP) immunostaining (F1) or appearing as hyperintensities in double inversion recovery sequences overlayed to the structural T1w sequence (F2) to assess the extension in the cortex; (G) meningeal inflammation as represented by leptomeningeal enhancement in post-gadolinium FLAIR acquisitions (G1-G2) and meningeal infiltrate enriched in CD3+ T cells (G3); (H) brain volume loss independent of disease activity with images referring to 2 patients (subject 1 images H1 to H5; subject 2 images from H6 to H10) with the same atrophy rates over 2 years (∼ −1.1% per year) and different focal activity, H1/H6 T1w at baseline, H2/H7 T1w after 24 months, H3/H4 regions where edge displacement is most evident as measured by the SIENA method, H4/H9 shows the relative FLAIR images at baseline and H5/H10 at M24 where the first subject is active (green border/light blue body indicating new lesions) the second shows no changes in lesion load over the 2 years; (I) spinal cord progressive damage as represented in an axial (I1) and sagittal (I2) 3 T 3D MP2RAGE and in a 3 T 3D STIR (I3) [courtesy of Dr. Daniel Reich NINDS, NIH]; (J) diffuse spinal cord lesions as represented in a sagittal STIR (J1), axial multi-echo GRE (J2) and myelin oligodendrocyte glycoprotein immunostaining (J3) [Reali et al., J Neuropath 2020]; (K) chronic active lesion characterized by MHCII+ activated microglia accumulation at the lesion edge of luxol fast blue stained white matter (WM) lesion (K1) and paramagnetic rim lesions detected using MRI filtered phase (K2) and Quantitative Susceptibility Mapping (K3). [Color figure can be viewed at www.annalsofneurology.org]

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neuronal density has often been more pronounced in cortical lesions, particularly those close to pia/CSF boundaries,[43,63,64,70,73] indicating a causal relationship between GM demyelination and neuronal loss. In addition, specific neuronal subpopulations may be more susceptible to MS-related damage. Recent studies have suggested a selective vulnerability of inhibitory neuronal networks with a preferential loss of inhibitory interneurons in the MS cortex, potentially altering normal brain network functioning.[75]

Similarly, the diffuse synaptic loss observed in MS brains is probably related to lesion-dependent and lesion-independent mechanisms. Data from animal models and post-mortem MS brains suggest that acute synaptic loss is associated with inflammation, which resolves and is followed by synaptic re-organization.[76–83] Synapses are probably targeted by complement factors C1q and C3 and removed by activated microglia in close contact with neurons.[77,81–85] In animal models, inflammatory cytokines (such as TNFα, IL-1, IFNγ) have been shown to induce synaptic alterations[79,80,86] and could contribute to synaptopathy among MS patients. Beyond structural synaptic damage, inflammation in the CNS can lead to abnormalities in the ability of synapses to express forms of long-term plasticity, such as long-term potentiation, contributing to brain network failure and disconnection.

Lower synaptic density has been reported in GM lesions compared to the normal-appearing GM (NAGM),[64,74,76,87] suggesting that focal demyelination enhances long-term synaptic loss. However, the synaptic loss appears to be relatively independent of focal demyelination[76,77,88] and was found to be unrelated to the extent of the neuroaxonal loss,[76,77,88] suggesting the coexistence of a diffuse primary synaptic pathology, possibly driven by the chronic inflammation present in the GM and meninges.

Role of Chronic Oxidative Injury/Mitochondrial Damage and Energy Deficit

The occurrence of an "energetic crisis" is widely recognized as one of the key pathological mechanisms underlying the dysfunction of the neuro-axonal unit.[89] Demyelinated axons require more energy to operate and guarantee conduction, ultimately increasing the metabolic demand.[90] During demyelination, the mitochondria within the MS plaques move from the neuronal cell body to the axon, increasing the axonal mitochondrial content.[91] Such a homeostatic response cannot fully and persistently compensate for the increased axonal energy demand. Nuclear-encoded mitochondrial genes and the functional activities of mitochondrial respiratory chain complexes I and III, are decreased in the motor cortex of severely disabled MS patients,[92] and multiple deletions of mitochondrial DNA (mtDNA) have been histologically demonstrated throughout the GM in progressive MS, with respiratory-deficient neurons harboring high levels of clonally expanded mtDNA deletions at the single-cell level.[93] In demyelinated axons predominantly located at the edge of chronic active lesions, reduced levels of complex IV activity have been shown,[94] together with an inverse correlation between complex IV activity and macrophage/microglial density, suggesting that soluble products released by such cells might be responsible for the observed functional impairment.[94,95] Reactive oxygen and nitrogen species (ROS and RNS) deriving from innate immune cells, when excessively liberated, can trigger mitochondrial pathology and initiate a process of focal axonal swelling, determining axon fragmentation.[96] Further experimental evidence of the contribution of mitochondrial and energy deficits comes from fluid biomarker studies. Patients with progressive MS display increased CSF levels of mtDNA,[97] while CSF lactate levels correlate with disease-worsening and axonal damage markers, such as neurofilament light protein.[98] It is worth noting that not only a condition of "virtual" hypoxia due to an increased energy demand but also the presence of "true" tissue hypoxia (i.e., oxygen deficiency) has been hypothesized to occur in MS.[89] Taken together, evidence suggests the vicious "hypoxia-inflammation" cycle as a potential target[99] for neuroprotective strategies aiming at restoring the neuronal mitochondrial activity and ultimately preventing the disease progression.[100]

Alterations in Normal Appearing White and Grey Matter

The normal-appearing tissue of the WM (NAWM) and GM is affected by axonal injury (both intrinsic and extrinsic to the tissues), gliosis, widespread diffuse microglial activation, and disrupted myelin, which displays altered biochemical and biophysical qualities.[101–105] Normal composition of mature myelin is essential for oligodendrocyte and axonal health, myelin ensheathment, and placement and organization of the nodes of Ranvier.[106] Widespread changes at the node of Ranvier, the flanking paranodal assembly, and the juxtaparanode, are seen near and far from the MS lesion.[107–109] Notably, activated microglia are associated with nodal disruption, where attenuating microglial activation with minocycline reduces nodal and paranodal damage and blocking glutamate, which is excessively generated by activated microglia, abrogates this change.[107–109] These changes in the NAWM and NAGM could contribute to clinical worsening independent of new inflammatory attacks.[90,96]

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Immuno-pathological Mechanisms

Role of Adaptive Immunity

Focus on the Role of T Cells. Organ-specific adaptive immune mechanisms against several target antigens in the periphery, CSF, and focal lesions are involved in MS pathogenesis. It is currently assumed that the pathological immunological processes start in the periphery and become chronic in the CNS compartment (outside-in hypothesis).[110] Key players of the antigen-specific immune response are autoreactive CD4+ T cells,[110] but also proinflammatory B cells at various differentiation stages[111] (for a detailed review, see[112,113] and below).

The MS-associated HLA-DR15 molecules shape a CD4+ T cell repertoire that is fully functional and protective against infections but also carries T cell receptors with cross-reactivity against CNS antigens, including myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein (MOG),[114,115] Ras guanyl-releasing protein 2 (RASGRP2),[116] GDP L-fucose synthase,[117] Prokineticin-2, reticulon-3, fatty acid binding protein 7 (FABB7), synaptosomal-associated protein 91,[118] and alpha-crystallin B (CRYAB).[119] The majority of autoreactive CD4+ T cells in the peripheral blood and CSF are proinflammatory, expressing a T helper (Th) 1 (secreting IFN-) or Th17 (secreting IL-17) or Th1* (secreting both) phenotype.[116,120–122]

Other Th cell subtypes, such as T follicular helper cells[123] and Th2 cells secreting IL-4 and IL-5, have also been identified.[124] Th1 and Th1* CD4+ T cells appear most important and engage in crosstalk and immunological synapse formation with proinflammatory B cells, which results in increased spontaneous or autoproliferation and enrichment for brain-homing T cells.[116] These T cells recognize HLA-DR15-derived self-peptides, which may play a role during thymic selection and are also presented on peripheral memory B cells,[125] but also foreign antigens from the MS-associated EBV and gut microbiota like *Akkermansia muciniphila*.[117,125] Interestingly, 1 of the abovementioned MS target antigens, RASGRP2, is expressed by the brain's proinflammatory B cells and cortical neurons.[116] Increasing evidence from the peripheral blood, the CSF, and meningeal follicles hints at a role of the cross-talk between proinflammatory memory B cells and plasmablasts on 1 side and memory CD4+ T cells with a Th1/Th1* phenotype on the other.[116,126] The chemokine receptor CXCR3 on B and Th1 T cells,[127] its ligands CXCL9, CXCL10, and CXCL13, IFN-gamma and nuclear factor kappa B signalling[128] are all involved in this interaction. CSF-infiltrating T cells may respond to a multiple of the abovementioned MS autoantigens, but also viral epitopes from EBV[129] and Torque Teno Virus (TTV),[130] and even HLA-DR15[125] and immunoglobulin-derived peptides.[130]

A role of adaptive (T cell-mediated) immune mechanisms in PIRA is indicated by T cell receptor sequencing studies that have shown clonal CD4+ and CD8+ T cell expansions in chronic active lesions[131] and demonstration that a clonally expanded RASGRP2-specific CD4+ T cell clone has also been found in the autoproliferating T cell compartment in the periphery.[116] A contribution to chronic active lesions and PIRA is further supported by a single-cell RNA sequencing study, which showed that lymphocyte activation and proinflammatory markers contribute to their immune signature.[23] Finally, a correlation exists between lymphocyte infiltrates in the meninges and WM lesions in progressive MS.[53] While proving the relevance of adaptive immune mechanisms for PIRA is challenging, the above observations indicate that they might be involved.

Focus on the Role of B Cells. The clinical efficacy of B cell depletion strategies in preventing new lesion formation[130,132] confirmed the central role of B cells in MS pathogenesis. Several pieces of evidence underscore the close interaction between T and B cells crosstalk and the importance of related cytokine pathways in the periphery and intrathecally in the CSF/meninges. Among these pathways, the expression of chemokine receptors CXCR3 and T-bet by CD4+ Th1 cells and B cells[126,133,134], the demonstration of their ligands (CXCL10 and CXCL13) and of other cytokines (LT, TNF, IFNg)[59,135,136] were largely investigated in the past decade.

B cells are mainly seen in the subarachnoid spaces in meningeal tertiary lymphoid-like structures.[10,40,48] They can also be found in the perivascular space of medium-sized veins within the plaque centre, mainly in the WM and, to a lesser extent, in the GM.[137,138] In these intracerebral niches, B cells may contribute to compartmentalized inflammation by perpetuating humoral immune response, antigen presentation, and release of proinflammatory and cytotoxic mediators.[139,140] Supernatants from cultured B cells from MS patients were toxic and induced more significant apoptosis in cultured neurons and oligodendrocytes than supernatants from cultured B cells from healthy controls.[139,140] The same antigen-experienced B cell clones were found in meningeal immune cell infiltrates, the CSF and perivascular parenchymal lesions.[141–143] In addition, it has been demonstrated that B cell clones are bidirectionally exchanged between the CNS and the periphery and that affinity maturation occurs on both sides of the blood–brain barrier.[144] More recently, a study in mouse models suggested that meningeal B cells encompass multiple stages of development and may originate in calvaria and then infiltrate and maturate in the meninges via a network of channels uncoupled from the systemic circulation.[145]

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Genetic variation in B cell activation[146] as well as the infection by lymphotropic viruses, such as the Epstein–Barr virus,[147,148] represent the prevalent hypothesis of aberrant and altered intrathecal B cell survival and activity in MS. All these findings suggest that the same stimuli might be involved in both initial B cell activation and clonal expansion events and then in the chronic invasion of different niches of the CNS.[112]

Role of Innate Immunity

Focus on Microglia. Many pro-inflammatory mechanisms have been suggested to be involved in active demyelination and neurodegeneration. Still, the production of reactive oxygen species appears particularly important. In contrast to rodent models, NADPH oxidase (Nox2) expression in human microglia and macrophages is prominent, while the iNOS expression is sparse. This is associated with a profound acute oxidative injury in neurons and glia in active lesions.[149] In the earliest descriptions of MS pathology in the late 19th century, it was already noted that active demyelination and neurodegeneration in MS lesions are closely associated with activated microglia and macrophages. A subset of microglia and macrophages at the edge of chronic active lesions contain iron and can be visualized in vivo with MRI.[20] Such an iron rim is a marker for the slow expansion of lesions, relevant for silent or overt disease progression in MS.[28]

New markers became available during the past few years, allowing us to differentiate between activated microglia and recruited myeloid cells within the lesions. They suggest most phagocytes in active MS lesions are derived from the microglia pool.[24]

Recent spatial transcriptomics or proteomics studies confirmed the findings, which had been elucidated before with conventional methodology.[23,150,151] They provide evidence of a profound heterogeneity of microglia and macrophage phenotypes, with different compositions in lesions based on variable contributions of active demyelination, inactive lesions or remyelination. Whether these different cell phenotypes reflect genuine cellular subpopulations or the transition between naïve, pro-inflammatory and anti-inflammatory cells is a question that remains unresolved. Meningeal inflammation in progressive MS patients was recently found to induce phenotypic changes in cortical microglia that are differentially associated with neurodegeneration.[82]

Focus on the Role of Complement. Complement is a key component of the innate immune system. The microglia synthesize, activate, and respond rapidly to soluble and membrane-bound products of complement activation.[151] Genetic variants in the complement genes are associated with a more severe clinical outcome,[23,152,153] and increased complement activation is reported in cohorts that experienced a worsening progressive MS course.[42,84,154,155] Complement expression is induced in cortical glia in a chronic leptomeningeal inflammation model. Complement contributes to compartmentalized inflammation by co-activating microglia and astroglia, recruiting circulating lymphocytes and macrophages, and driving synaptic, neuritic, and myelin degeneration.[23,59,156–161]

Biomarkers of Silent Disease Activity

Imaging Biomarkers

Paramagnetic Rim Lesions and Slowly Expanding Lesions

The persistent microglia/macrophage activity within the rim of CAL (Fig 2K1) and the associated axonal damage might represent 1 of the key components of the continuous, hidden, and deleterious inflammatory activity driving PIRA in MS. Over the past decade, an increasing clinical interest has been devoted to the detection of CAL as they are associated with higher disease severity and brain and spinal cord atrophy, thus representing a negative prognostic indicator for clinical worsening and disease progression.[31,162–164]

A subset of CAL can now be visualized using susceptibility-sensitive MRI sequences, that is, T2* and phase contrast, R2* (Fig 2K2,K3) or quantitative susceptibility mapping (QSM), as non-enhancing lesions with a paramagnetic rim (termed "paramagnetic rim lesions" or PRL). MRI-neuropathological studies consistently showed that the magnetic susceptibility contrast at the lesion rim is mainly related to iron accumulation within activated microglia/macrophages.[20,26,162,165–167]

While activated microglia/macrophages play a crucial role in myelin and debris clearance (a critical step for repair and remyelination), in the context of the highly inflammatory and dysregulated environment at the CAL edge, these immune cell populations are believed to be exerting a further detrimental role, as recently demonstrated by single-nucleus RNAseq phenotyping.[23]

A recent pooled-data analysis of 31 published MRI studies highlighted that PRL are present in ~50% of relapsing and ~60% of progressive patients.[168] Compared to non-PRL, PRL are more destructive lesions, as reflected by significantly longer T1 times, decreased R2*,[169] reduced myelin water fractions, and low neurite dispersion index.[166,170] In longitudinal MRI studies, PRL has been found to persist for years,[171] and a subset of them slowly expand over time.[20,162] Little is known regarding the

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long-term dynamics of PRL and their response to current DMTs, but susceptibility reduction of the rim over a median time frame of 7 years has been reported.[23,28]

A recent 4-year longitudinal study of 445 patients directly evaluated the role of PRL in PIRA: patients treated with anti-CD20 antibodies experiencing PIRA at follow-up had higher baseline PRL count.[172]

Alternative candidate biomarkers of CAL are the slowly expanding lesions (SEL) representing the subset of non-enhancing chronic lesions showing radial and linear expansion over 1–2 years. SEL are identified by applying Jacobian determinant algorithms (non-linear deformation) to registered T1-weighted and T2-FLAIR images on at least three time-points. The computed radial and linear lesion expansion has been interpreted as lesion expansion. No histopathological validation has been provided for SEL quantified by the Jacobian determinant algorithm, and pathological processes other than inflammatory demyelination might explain the imaging finding (i.e., Wallerian neurodegeneration, and microvascular comorbidities). SEL have been detected in both relapsing and progressive MS patients, and they were shown to predict disability and progression at 7 and 9 years of follow-up.[173–175] Modest effects on SEL were seen in MS patients treated by current disease-modifying therapies.[173,176–178]

The limited overlap between PRL and SEL is a matter of current debate; in a recent study,[179] PRL satisfying the mathematical definition of SEL (39% of all PRL) showed microstructural tissue degeneration tissue over 72 weeks. A possible explanation for the discrepancy between the 2 biomarkers is that a subset of SEL has low iron content and, therefore, is not visible as PRL. In addition, recent imaging studies validated that not all CALs correspond to SELs or PRLs. Still, when PRLs are co-localized with SELs, they show expansion and worsening microstructural damage over time,[179] supporting a key role of smoldering inflammation in accumulating neurodegeneration and disease progression.[179]

Brain and Grey Matter Atrophy

MRI changes in brain volume have proven clinical relevance in MS (Fig 2H1–H10) as a marker of irreversible tissue loss (i.e., brain atrophy).[180] Several studies have consistently shown that brain atrophy rates are higher in patients with MS than in healthy subjects since the earliest disease stage. Atrophy appears diffuse in the MS brain and spinal cord, particularly in subcortical and cortical regions, possibly reflecting the early neuroaxonal involvement.[181]

Compared with clinically stable patients, patients with PIRA had an increased rate of brain volume loss, mainly driven by cerebral GM loss (Fig 2H1–H10). Moreover, MS patients with PIRA had similar brain atrophy[6] and cortical thinning and deep GM volume loss rates[182] to those patients experiencing disability accumulation due to relapses. Interestingly, these studies independently suggested that atrophy rates could be partially dissociated from relapse activity and likely to reflect mechanisms leading to the silent progression of MS.

Complementary to the brain atrophy assessment, the 'brain-age' paradigm[183] utilizes the relationship between disease and healthy brain structural ageing to understand the impact of diseases. The difference between an individual's chronological age and the brain-predicted age (brain-PAD or brain age gap) has been proposed as an age-adjusted index of structural brain health. Using 3D T1-weighted MRI and machine learning algorithms, the brain-age paradigm offers the advantage of setting a subject's state in the context of a normal population with an easily interpreted figure. MS has a pronounced effect on the brain-PAD metric, indicative of poor structural brain health. Furthermore, measures of brain-predicted age increase more rapidly than usual chronological ageing in both RR and progressive MS, implying that the brain-age approach is sensitive to accelerations in MS brain atrophy.[184] Recently, brain age was associated with the worsening of the Symbol Digit Modality Test (SDMT) performance.[185]

Focal Cortical Demyelination

Substantial immunopathological (Fig 2F1) and imaging (Fig 2F2) evidence confirmed that focal demyelination occurs in MS since the early phases of the disease within both the cortical and deep GM, accumulates over time and provides a good clinical correlate in terms of disability accumulation.[10,36,54,101,186]

CLs are at least partly independent of WM acute demyelination[88,187] and usually do not show contrast enhancement.[36] The focal cortical damage is associated with a progression of physical and cognitive disability independently of relapse[186,188–190] and its occurrence at clinical onset predicts a greater risk of disability progression.[186,190–192]

A 30-year longitudinal study indicated the focal cortical demyelination as 1 of the primary substrates of disease progression, with CLs' burden alone explaining 43% of the variance of the expanded disability status scale (EDSS).[89] On the contrary, a study on "benign MS" revealed that after 15 years of the disease, those patients who still demonstrated scarce disability progression and cognitive preservation showed a remarkably lower CLs number compared to early RRMS.[192] In line with these data, the lesions of deep GM, especially the thalamic lesions, have been associated with disability progression being greater in progressive MS compared to RRMS.[193]

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Taken together, data suggest that cortical and deep GM lesions are expressions of complex "smoldering" pathological processes and contribute to the development of the PIRA.

Nevertheless, although current imaging protocols using double inversion recovery (DIR), phase sensitive inversion recovery (PSIR), or T2* weighted and magnetization-prepared rapid gradient-echo (MPRAGE) sequences at ultra-high field MRI (i.e., 7 T) have substantially improved their detection, most of the GM lesions remain undetectable in vivo.[194]

Spinal Cord Damage

Spinal cord involvement is gaining attention as a critical region for explaining the disability progression (Fig 2J1–J3, I1–I3). The prognostic relevance of isolated spinal cord lesions (SCL) for developing a progressive disease course has been shown by studying extreme disease cases. Patients with solitary demyelinating lesions involving the spinal cord or cervical-medullary/brainstem regions developed an early progressive disease course.[195] In addition, in subjects with radiological isolated syndrome (RIS), SCL increased the risk of evolving to PPMS rather than RRMS.[196] From the analysis of conventional MRI sequences, a few imaging features have been identified that can contribute to the early identification of those patients who will develop a progressive MS course or accumulate more severe disability. These include the presence of diffuse signal hyperintensities on proton-density weighted sequences (i.e., abnormal areas of intermediate signal intensity between that of focal lesions and normal-appearing spinal cord, lacking a well-demarcated border from the adjacent normal-appearing cord)[197,198] and the involvement of cord central GM (Fig 2J1–J3).[199–201] Harboring spinal cord lesions is an important prognostic factor in identifying CIS patients with a shorter time to conversion to MS,[202] more severe accumulation of clinical disability[203] and evolution to SPMS after 15 years of follow-up.[204] Using quantitative MRI techniques, such as magnetisation transfer MRI,[205] a gradient of microstructural damage in the outer surface of the cord, which might reflect the presence of subpial demyelination, was found in patients with MS and was more severe among those with a progressive phenotype. A recent 7 T study[206] found that SCL have a greater propensity to occur closer to the central canal and subpial CSF interfaces, with a different behavior according to MS clinical phenotype (more involvement of central canal in progressive MS and of the outer subpial surface in RRMS).

In addition to focal demyelinating lesions, irreversible tissue loss (i.e., atrophy) in the spinal cord can be seen among MS patients. Spinal cord atrophy (Fig 2I1–I3) occurs in all MS clinical phenotypes, is more pronounced and diffuse in progressive rather than relapsing MS patients, is associated with concurrent clinical disability and tends to progress over time.[207,208] While a significant relationship is present between cord atrophy and accumulating disability in the short-term,[209–211] it disappears at longer clinical follow-up (more than 10 years), suggesting that spinal cord atrophy may be critical for clinical deterioration in the first years of the disease. However, it might subsequently reach a ceiling effect.[212–214] A recent study with 12 years of follow-up found that RRMS patients who developed SPMS had faster cord atrophy rates (−2.19% per year) at least 4 years before conversion compared to those patients who remained RRMS (−0.88% per year).[214] A faster spinal cord atrophy rate was associated with a shorter time to silent progression and SPMS conversion, challenging the traditional dichotomy of an RRMS and subsequent SPMS phenotype.

New Promising Imaging Biomarkers to Identify the Disease Progression

The Neurite Orientation Dispersion and Density Imaging. The neurite orientation dispersion and density imaging (NODDI) is a diffusion-weighted multi-shell MRI model that provides specific measures of tissue microstructure (Fig 2E2). These include the intracellular volume fraction (ICV_f)/neurite density index (NDI), reflecting neurite density, and orientation dispersion index (ODI), quantifying neurite orientation variability and tissue coherence and complexity.[215] In a recent study, PRL showed decreased myelin water fraction but also NDI relative to lesions without a rim,[216] confirming the pathological evidence of ongoing demyelination and axonal damage in these lesions.[34,217] Significantly lower ICV_f/NDI in the NAWM[218,219] and cortex,[216,218–220] and ODI in the cortex[219,220] were found in progressive compared to RRMS patients, and they correlated with more severe clinical disability, thus confirming in vivo that a clinically relevant progressive neurite loss and a simplification of dendritic arborization may contribute to the disease severity.[219,220]

Constrained spherical deconvolution (CSD) is another diffusion-weighted multi-shell MRI model that allows to define the fiber-bundle cross-section area, a marker of axonal shrinkage/loss across a plane perpendicular to the fiber-bundle axis that may better quantify the degeneration of the WM fiber-bundles.[221,222] A significant reduction of fiber-bundle cross-section area, gradually worsening over 1 year and reflecting more severe WM tract volume loss, has been found in progressive compared to RRMS patients,[223,224] mainly involving clinically relevant WM tracts, including the cerebellar peduncles, the cortico-spinal tract, the cingulum and the corpus

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callosum. Moreover, a lower fiber-bundle cross-section area in the NAWM and in the sensorimotor WM tracts was found to be significantly correlated with more severe clinical disability[223] and with gait and motor control impairment, respectively.[225]

Soma and neurite density imaging (SANDI) is another model that may estimate not only MRI signal fractions attributed to neurites ($f_{neurite}$), but also of bodies of any CNS cell (f_{soma}).[226] Two recent studies showed that, compared to HC, MS patients were characterized by significantly lower $f_{neurite}$ both in the NAWM and GM and lower f_{soma} in ther GM.[227,228] These microstructural abnormalities were significantly more severe in progressive MS patients,[227] and they were associated with a more severe clinical disability and structural brain damage.[227,228]

Nonetheless, the feasibility of these advanced diffusion-weighted multi-shell MRI model in the clinical setting is limited. Longitudinal studies are still needed to further explore the correlation between their derived measure changes and MS progression.

Leptomeningeal Enhancement. Leptomeningeal enhancement (LME, (Fig 2E2), Fig 2G1–G3), typically visualized on delayed post-contrast FLAIR MRI, has been recently explored as a biomarker for blood-meningeal barrier opening and meningeal inflammation in MS.[10,229]

LME detection on post-contrast 7 T FLAIR is more prevalent than on 3 T FLAIR, occurs at frequencies closer to histopathologic data and allows the investigation of the relationship between meningeal inflammation and cortical pathology.[230]

Two patterns of LME have been described: "nodular", with discrete, spherical nodules at the pial surface or subarachnoid space, and "spread/fill" with the appearance of contrast spread through the local subarachnoid space.[230] In a recent meta-analysis including 1,605 MS patients, the proportion of patients with LME was higher in progressive than in the relapsing MS group (39.8% vs. 53.4%).[231] LME is associated with a more severe MS course and disease progression,[230,232–235] with the extent of cortical,[234] thalamic,[234] and hippocampal[236] lesions and with the rate of brain and cortical atrophy.[230,232,233,236] However, LME is not specific to MS, as it has also been described in aged healthy controls[230,237,238] as well as in patients with other inflammatory and infective neurologic conditions.[231,237,239–241]

Positron Emission Tomography. Using radiotracers binding to the TSPO, a mitochondrial protein upregulated in activated microglial/macrophage and astrocytes activation, several PET studies have consistently demonstrated that increased glial activation could be detected not only in active WM lesions but also, to a lesser extent, in CLs, and a subset of chronic WM lesions (possibly CAL).[32,242–249] The number and volume of lesions with positive PET TSPO uptake were higher in progressive than in RRMS patients and correlated with the development of clinical disability[242] and with WM lesions accumulation. Using 18F-DPA7142, a fluorinated second-generation TSPO radioligand, more than half of WM lesions (53%) showed a homogeneously active core, and their number was the best predictor of cortical atrophy and disability progression over 2 years.[250]

TSPO PET also revealed a more diffuse brain inflammatory component in the NAWM, thalamus and cortex.[242–249,251,252] Consistently with pathological findings,[88] such widespread glial-microglial activation was higher in patients with a more severe disability and a progressive disease course, correlated with structural brain damage,[242–249] and predicted disability progression up to 4 years later.[243]

Fluid Biomarkers

Potential Biomarkers of Neuroaxonal Degeneration

Neurofilaments are polymeric proteins constituting microtubules and microfilaments in the neuronal cytoskeleton, providing structural support for axons. They consist of 3 subunits, according to their molecular weight, named "high," "intermediate," and "light," which are liberated in the extracellular space after axonal damage. Although In MS, high CSF NfL levels were consistently and convincingly associated with gadolinium-enhancing lesions and relapses[253], data on their association PIRA are less robust. Nevertheless, several studies suggested a relationship between serum NFL and the development of progressive disease, lower total and deep GM volume, lower mean cortical thickness, and higher T2 lesion count.[254–256]

Moreover, a recently very elegant study aimed at determining whether and when NfL levels are elevated in the context of confirmed disability worsening suggested a clear relationship between the NFL levels and the risk of disability worsening not related to disease activity.[257] An NfL z score greater than 1.0 was associated with a higher risk of diagnosing confirmed disability progression not related to relapse activity in less than 24 months.[257] All these data suggested that sNfL could be a useful tool for the early identification of patients at risk of worse disease outcomes associated with relapse-associated worsening but also with the PIRA.

Similarly, the degree of cortical neurodegeneration can be detected by measuring the CSF protein levels of parvalbumin (PVALB), a calcium-binding protein

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expressed by a subset of GABAergic inhibitory cortical interneurons, classified as fast-spiking interneurons. They were found reduced in layer II of the primary motor cortex of MS patients, particularly within the NAGM.[92] Increased CSF PVALB levels in MS have been demonstrated to reflect interneuron loss, cortical atrophy, and severe cognitive decline in MS patients, both at the time of diagnosis and, increasing with progression, at the time of death.[136]

Inflammatory Biomarkers of Glia Activity and Chronic Inflammation

In addition to specific markers of neuronal damage, several inflammatory biomarkers related to glial or immune cell activity in MS have been recently analyzed, and at the moment, a wide range of new biomarkers that can predict disability progression, monitor disease activity, and assess treatment response have been proposed.

Although kappa free light chain (KFLC) index has become a useful diagnostic biomarker in MS, in a very recent retrospectively identified based on 131 patients with clinically isolated syndrome or early RRMS, the KFLC index was observed to be significantly higher in PIRA compared with non-PIRA.[258]

Serum levels of glial fibrillary acidic protein (sGFAP), a protein highly expressed by astrocytes, were associated with disease progression in RRMS,[259] and disability worsening in PP[260]; more recently, in a study including 355 patients and 259 healthy controls, patients with worsening progressive MS showed 50.9% higher sGFAP levels compared with those with stable MS suggesting that GFAP can be a prognostic biomarker for future PIRA.[261] Differently from sNFL, sGFAP does not typically elevate during acute inflammation but reflects accelerated GM brain volume loss and is associated with a higher risk of confirmed disability worsening.[261] Notably, GFAP age and sex specific normal ranges are advocated as well as a "gold standard" detection method.[260]

Increased CSF levels of activin A, a molecule expressed by activated microglia (42), which is part of the senescence-associated secretome, may indicate that MS patients enter an early inflammation process that could be at least partly responsible for the silent disease activity among MS patients older than 45 years.[55]

While IL12p40 and CHI3L1, expressed by immune cells of the myeloid lineage, have been proposed as promising CSF biomarkers of MS lesional activity,[262] increased CSF levels of CHI3L1 have been specifically observed in patients with a higher burden of chronic active lesions.[263] In addition, specific CSF protein profiles, including high protein levels of proinflammatory cytokines (TNFa, IFNg, IL6), molecules involved in lymphoid neogenesis (CXCL12, CXCL13, TNF), and B cell and plasma cell/blast activity (IL6, IL10, TNF, BAFF, APRIL, LIGHT, TWEAK), were found associated with elevated GM lesion load either in progressive post-mortem MS cases or in naive MS patients at the time of diagnosis.[135] The same CSF inflammatory pattern was proposed to predict a higher risk of disease activity and more severe cortical damage.[136] A composite biomarker study on a large and multicenter MS population could validate all the previous studies to better identify each patient's disease state since the diagnosis is needed. A new approach integrating all the serum and CSF analyses of an extensive pattern of biomarkers with demographic, clinical, imaging, cellular, metabolomics, microbiome, genomics, and proteomics data with new bioinformatics and machine learning will help to identify also patient subgroups with high risk of PIRA outcome.

Conclusions for the Clinical Practice

It is now clear that the pathological mechanisms underlying clinical progression begin early in the disease course. However, they are usually so gradual that the progression is initially unnoticed by physicians.[6,264]

The recent finding that patients with pediatric-onset are less likely to exhibit PIRA over a decade of follow-up might correlate with their protection against disability,[265] possibly for repair capacity and for the different immunopathological mechanisms.

The accuracy in PIRA detection is also challenged by concurrent relapses with variable degrees of recovery, raising the need to monitor patients with additional visits that become necessary, mainly when defining confirmed disability accumulation at 3- or 6-month follow-up.

Composite measures, including T25FW and 9HPT, should be adopted, given the notable percentage of disability accumulation detected by such measures.[2]

All the studies so far available,[2,3,5,6] clearly suggested the need for strict clinical monitoring based on cognitive evaluation, which includes measures of information processing speed (as SDMT). From the imaging point of view, longitudinal measures of whole and regional atrophy, especially of cortical GM,[6,182] and PRL are associated with PIRA, and look like promising markers for clinical practice.[162,168,186,195]

Understanding whether fluid markers could help predict the PIRA represents a novel goal of precision medicine. sNfL and sGFAP have been suggested as potential candidates,[261] with a great effort undergoing their application at a single-patient level, but they are still not applicable to predict PIRA in a clinical context.[266] CSF inflammatory markers, such as TNF, IFNγ, and molecules associated with B cell recruitment, have been not only

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related to cortical damage but also demonstrated the capability to predict subsequent EDSS accumulation and cortical thinning, with additional predictive value to commonly adopted clinical and MRI measures.[136] Similarly, CSF inflammatory markers could be adapted to predict MS treatment response better,[267] suggesting their ability to identify patients responding to specific drugs according to their mechanisms or disease endophenotypes.[135,136,268]

We believe there is an urgent need to (1) improve the awareness about PIRA in the neurological community and improve its detection in clinical practice; (2) better define the role of MRI and fluid markers to predict PIRA; and (3) validate, integrate these markers, and finally translate them into clinical practice for a precision medicine approach.

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Potential Conflicts of Interest

M.C., P.P., A.S., E.C., D.M., M.A., M.B., N.D.S., M.D.F., S.H., O.W.H., M.I., H.L., R.M., R.N., R.R., M.A.R., A.T., M.V., L.M.V., M.F., and R.M. have nothing to report.

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