PREVENTING MULTIPLE SCLEROSIS

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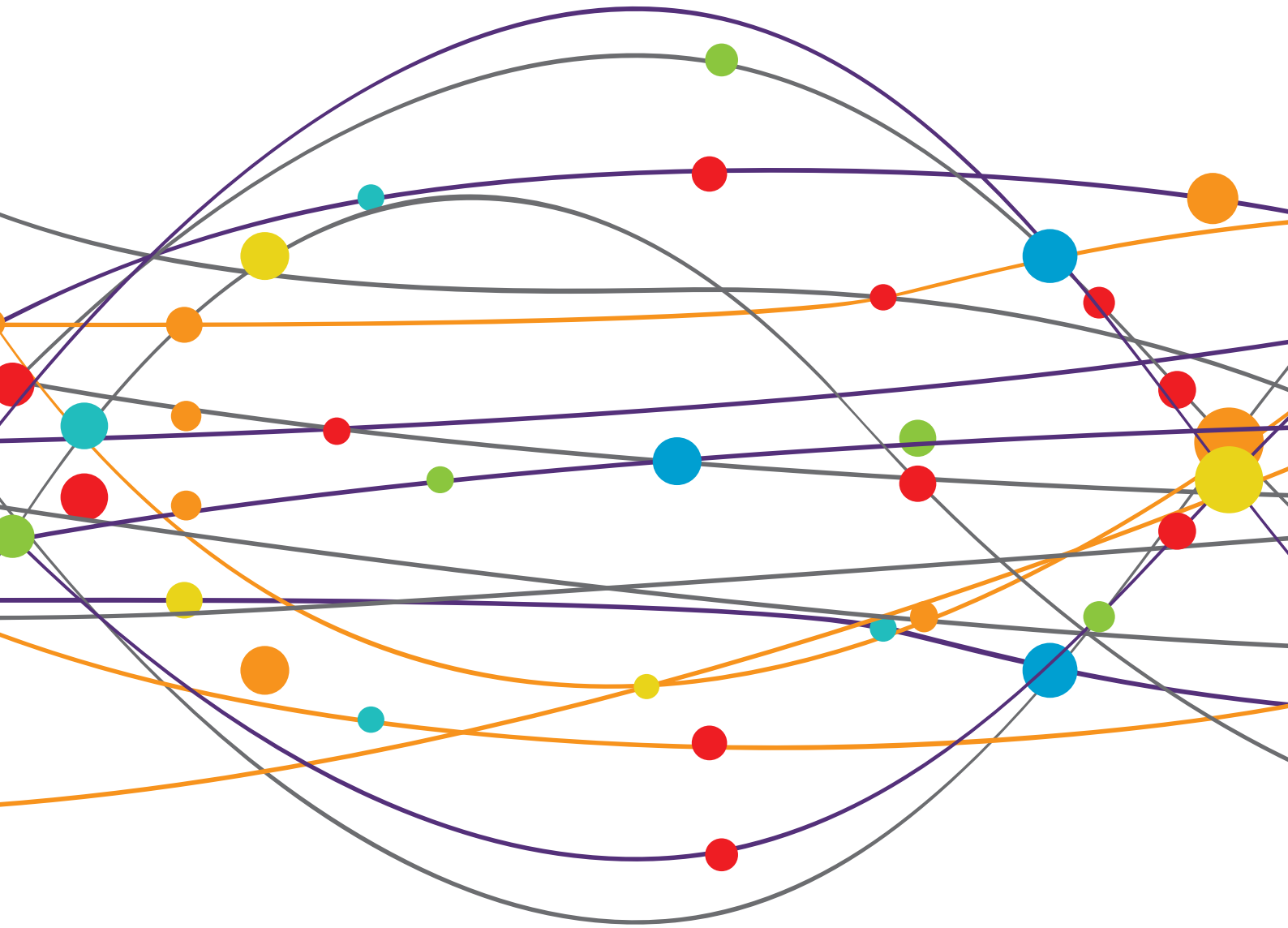

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PREVENTING MULTIPLE SCLEROSIS

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Editorial: Preventing multiple sclerosis

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Editorial on the Research Topic
Preventing Multiple Sclerosis

Multiple sclerosis (MS) prevention has been identified as a key aim across MS research. Achieving this aim is complex—MS is relatively rare, there is a long lag between many identified risk factors and clinical MS development, and many people exposed to these risk factors never develop MS. It is clear that MS has a complex pathogenic pathway with contributions from, and interactions between, genes and environment.

In this Research Topic, we present a group of papers which seek to explore opportunities and challenges around MS prevention, and how some of these may be overcome. Tremlett et al. discuss whether further exploration of the MS prodrome, which can be detected years prior to clinical MS diagnosis, could enhance prevention efforts. By identifying those at the earliest stage of disease, prior to the development of neurological disability, they argue that there needs to be a re-evaluation of the risk factor literature, such that reverse causation during the prodromal period can be considered. A greater understanding of both true risk factors, and factors acting on disease progression during the prodromal period has the potential to inform prevention and early disease modification prior to neurological symptom onset. Pediatric MS has a complex aetiological pathway, with similar environmental risk factors identified to adult onset MS. Hardy et al. argue that investigating environmental determinants in this population overcomes at least some of the challenges associated with adult onset MS, given the closer temporal association between risk factor exposure and disease onset.

One of the environmental factors consistently associated with MS development in epidemiological studies is vitamin D deficiency. However, reverse causation and lack of direct mechanistic evidence remains a concern. Haindl and Hochmeister review the evidence from animal models, particularly mouse models, which provide potential mechanistic insights around inflammatory disease, but add little to our knowledge around the impact of vitamin D on progression. They highlight limitations of such studies, including around dosage, the potential anti-inflammatory role of UV light, and differing biology between EAE and MS. Gombash et al. review whether vitamin D acts *via* immunoregulatory or direct neuroprotective mechanisms. Immunological mechanisms have been demonstrated in both animal models and human studies; vitamin D appears to have actions on both the innate and

adaptive immune systems. There is also substantial evidence that vitamin D acts as a neurosteroid, with the vitamin D reception expressed throughout the developing and mature brain.

Infection with Epstein Barr virus has been identified as a potential obligate step in MS development. Hassani et al. demonstrate, *via* a rabbit model, that primary peripheral EBV infection can lead to the virus traversing the CNS within the cells it infects. Within the CNS, B lymphocytes develop inflammatory cellular aggregates, the first direct *in vivo* evidence for the role of peripheral EBV infection in CNS pathology. Targeting EBV in order to prevent MS requires an understanding of the most appropriate means to tackle and potentially prevent infection. Kearns reviews the mathematical and biological underpinnings of gene-environment interactions with a focus on EBV, and discusses the implications for EBV-focussed prevention interventions. Vaccination, anti-virals, immunotherapies and cell-based therapies are all discussed as potential strategies. Maple et al. take this a step further, with an in depth discussion around EBV vaccination. The authors highlight the two current approaches around vaccination—either a prophylactic vaccine to prevent infection or disease, vs. a therapeutic vaccine to treat people with EBV-associated complications such as cancers. They argue that an EBV vaccine to prevent IM is the most likely strategy to be tested and adopted, but that vaccine hesitancy and high seroconversion in early childhood are important considerations in any vaccine rollout.

Smoking prevention strategies are well-established in the wider public health literature. Whilst many of these strategies focus on non-MS health consequences of smoking, such as cancer and vascular disease, the contribution of smoking to MS development should not be overlooked. Manouchehrinia et al. use a large population-based cohort to determine the population attributable risk associated with smoking, taking into account HLA type. They demonstrate that the overall attributable fraction of MS associated with smoking is 13.1%, with a higher point estimate in males (19.1%) than females (10.6%), and that approximately half of the attributable fraction due to smoking is independent of HLA-associated risk. This highlights the importance of smoking prevention and cessation efforts in terms of MS prevention.

Understanding transcriptomics may help to understand how the risk factors highlighted in this Research Topic influence disease development. Elkjaer et al. review the existing literature on transcriptomic studies within the CNS. They find support for MS as a whole brain disease, with inflammation, iron disturbances, cellular stress, and hypoxia. They show heterogeneity within MS at molecular level, contrasting with the relative clinical homogeneity. This provides insight into some of the complexities with MS prevention efforts—targeting a highly heterogenous disease is likely to require multimodal interventions.

The collection of articles within this Research Topic demonstrates that for prevention efforts to have clinically meaningful impact, identifying those at highest risk is key. One potential strategy is secondary prevention at the earliest disease stage. Amato et al. discuss predictors of evolution in those with radiologically isolated syndrome, the earliest clearly defined stage of MS. Disease modification studies are currently being performed at these earliest stages, however these are using licensed MS therapies rather than targeting modifiable risk factors.

In order to move the prevention therapeutic window earlier, Hone et al. highlight some of the challenges associated with MS risk scores, including that performance metrics fall well short of those required for a diagnostic or predictive test. Incorporating cross-ancestry portability and environmental factors are key considerations for clinical use. However, whilst we are unlikely to be able to predict MS on an individual basis in the near future, risk scores may be useful to identify high-risk populations for preventive trials, such as EBV vaccination.

This Research Topic therefore demonstrates that there is sufficient evidence to support action to trial interventions to prevent MS, with clearly defined interventions in selected high risk populations being key to success. Without the courage to set up studies to understand the impact of interventions, the need for prevention will remain unaddressed—this cannot continue given the importance of this Research Topic.

Author contributions

RD: initial drafting of manuscript and approval of final manuscript. RT and BG: critical review, input into manuscript, and approval of final manuscript. All authors contributed to the article and approved the submitted version.

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Vitamin D in Multiple Sclerosis—Lessons From Animal Studies

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Multiple sclerosis is a multifactorial disease of the central nervous system with both genetic and environmental causes. The exact disease mechanisms are still unclear. Consequently, studies of possible treatment and preventive measures cover a large setting of heterogeneous approaches. Vitamin D is one of these approaches, and in many trials the relation of vitamin D serum levels and multiple sclerosis disease risk and activity describes different effects with sometimes inconsistent findings. Animal models are substantial for the research of disease mechanisms, and many of the drugs that are currently in use in multiple sclerosis have been developed, tested, or validated *via* animal studies. Especially when clinical studies show contradicting findings, the use of standardized settings and information about the mechanistic background is necessary. For this purpose, animal models are an essential tool. There is a variety of different experimental settings and types of animal models available, each of them with own strengths but also weaknesses. This mini-review aims to overview results of vitamin D studies in different animal models and sums up the most important recent findings.

Keywords: multiple sclerosis, vitamin D, animal models-rodent, autoimmune diseases, therapy

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INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), affecting about 2.5 million people worldwide. It is an autoimmune disease with targeted myelin attack that causes demyelination (1, 2). Even though disease-modifying medications are capable to reduce disease severity, the disease continues to worsen over the patient's life span. Both genetic and environmental factors contribute to disease development, but the exact mechanisms are still not fully understood. Experimental autoimmune encephalomyelitis (EAE) in rodents is the favored model for exploring neuroinflammatory aspects of the disease, while toxin-induced demyelinating models like the cuprizone model are able to elucidate the cellular mechanism of de- and remyelination (2–5). Vitamin D (vitD), or the lack of it, is one frequently discussed environmental factor associated with MS, and its immunomodulatory ability has been widely demonstrated (6, 7). Despite numerous studies suggesting a beneficial effect of vitD intake in MS, there is still a controversy whether the supplementation can be used therapeutically (7). This work will discuss and summarize recent data from animal models on this topic. For overview, in **Table 1** and **Figure 1** the chemical and metabolic background of the vitD metabolism is summed up.

TABLE 1 | Chemical and metabolic background of vitD.

Shortcut	Explanation
vitD	In this manuscript the shortcut vitD sums up vitaminD_3 and any intermediate of vitaminD_3
vitaminD_3	Cholecalciferol (inactive)
vitaminD_2	Ergocalciferol
7-DHC	7-Dehydrocholesterol
25OHD_3	25-HydroxyvitaminD_3
1,25$(OH)_2D_3$	1,25-DihydroxyvitaminD_3, calcitriol (active)
VDR	vitD receptors

There are different chemical forms of vitD to be distinguished (in this work, the shortcut vitD sums up vitaminD3 and any intermediates). There are two sources of vitD; the majority is generated via the skin in a non-enzymatic process; the minor part is gained via food. The starting product 7-DHC is converted to Pre-vitD_3 via UV-B irradiation. This pre-vitamin isomerizes to vitD_3 in a thermo-sensible process. VitD_3 is converted to 25OHD_3 in the liver. The biologically active form of this vitamin is 1,25 $(OH)_2D_3$, generated via hydroxylases in the kidneys. This active form is able to bind to VDRs, transcription factors, present in nearly every tissue. Alternatively, 1,25 $(OH)_2D_3$ can be converted to the biologically inactive form calcitroic acid for storage (1, 8).

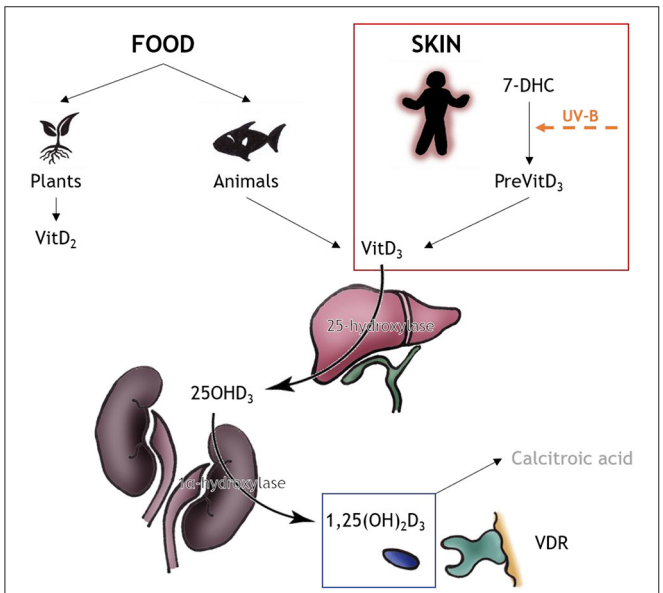

FIGURE 1 | VitD metabolism. VitD can be obtained from two sources, food (minor part) and skin (major part). The source for vitD_2 are plants and fungi, and the source of vitD_3 are animals, especially fatty fish. The major source of vitD_3 is produced nonenzymatically *via* the human skin: 7-DHC converts to PreVitD which isomerizes *via* heat to vitD_3. In the liver, vitD_3 is converted to 25OHD_3 *via* 25-hydroxylase; in the kidneys the biologically active form 1,25$(OH)_2D_3$ is produced *via* 1alpha-hydroxylase. The active product either can bind to VDR for correspondent outcome or is converted to calcitroic acid for storage. Information upon vitD metabolism obtained from (8); drawings by MT Haindl.

DISEASE PREVENTION AND RISK REDUCTION

Most studies investigating the capability of vitD to prevent MS are based on EAE findings. There is prophylactic potential of the association of myelin oligodendrocyte glycoprotein (MOG) peptide and active vitD against EAE. Vaccination with a mixture of MOG associated with vitD determines a reduction in CNS inflammation, dendritic cell maturation, clinical score, body weight loss, and production of cytokines, indicating that this association tones down the autoimmune response and prevents EAE. In other autoimmune conditions, there is a similar effect expectable (9). However, not only immunization with a MOG-vitD mixture has preventive effects. Also, very early intervention with the active form of vitD controls neuroinflammation during EAE development and was shown to decrease prevalence, clinical score, inflammation, and demyelination. Furthermore, a reduced major histocompatibility complex class II (MHCII) expression in macrophages and microglia as well as the level of oxidative stress and messenger RNA (mRNA) expression for caspase-1, interleukin (IL)-1β, and others was observed. These effects are accompanied by stabilization of blood–spinal cord barrier permeability, indicating that early intervention with vitD can control the neuroinflammatory process which is one of the hallmarks of EAE and MS (6). Conversely, low vitD levels are associated with increased risk of MS, suggesting the possibility of a gene–environment interaction in MS pathogenesis. VitD supplementation downregulates the MHCII expression in EAE. The impact of vitD on one master regulator of MHCII expression was investigated in 2020 on EAE rats. An inverse vitD dose-dependent effect on demyelination and inflammatory infiltration of the CNS, as well as downregulation of some pro-inflammatory genes, indicated an impact of vitD on pathophysiology and immune response during EAE. A modulatory effect of vitD regarding genetic variances in MS is therefore most likely probably relevant for the human disease as well (10). Additionally, vitD may reduce the MS risk in part through a mechanism involving myeloid cell vitD production and CTLA-4 upregulation in CNS-infiltrating T-cells. Humans with CTLA-4-inactivating mutations have an incompletely penetrant cellular phenotype with hyperactive effector CD4+ T-cells and a complex immune dysregulation syndrome. Another EAE study found out that CTLA-4 might act as a vitD-regulated immunological checkpoint in MS prevention (11).

INFLUENCE ON T-CELLS

The protective effect of vitD associates with decreased proliferation of CD4+ T-cells and a lower frequency of pathogenic T-helper (Th) 17 cells. Multiple pathways, critical for T-cell activation and differentiation, seem to be affected by vitD. For example, Jak/Stat, Erk/Mapk, and pi3K/Akt/mTor signaling pathway genes were downregulated upon vitD supplementation. VitD might modulate MS risk by changing myelin-reactive T-cell expression patterns as observed in EAE. Additionally, the role of vitD supplementation for prevention or treatment of autoimmune diseases in general is supported because CD4+ T-cells are driving target organ destruction in autoimmune diseases and many of the autoimmune loci are shared by multiple autoimmune diseases (7). However, the influence of vitD on T-cells seems to act not only *via* metabolic pathways but also upon dendritic cells (DCs). DCs mediate immune

response *via* their antigen presentation function, driving T-cell differentiation. VitD has the ability to induce tolerogenic DCs (VD3-DCs), increasing the negative regulatory signaling pathway programmed death 1 (PD1)/programmed death ligand 1 (PDL1). The expression of PD1 and PDL1 increases significantly after vitD treatment, enhancing the activation of this pathway. As a result, the activation of T-cells can be inhibited and the number of Tregs is increased, promoting immune tolerance (12). The induction of VD3-DCs further inhibits the infiltration of T helper type 1 (Th1) and Th17 cells into the spinal cord and increases the proportions of regulatory T-cells and regulatory B-cells in peripheral immune organs, thereby attenuating EAE (13). One dose of calcitriol plus vitD is able to reverse EAE, resulting in increased CD4+ T-cell transcripts, Helios protein, CD4+ Helios+ FoxP3+ Treg, and global DNA methylation. Calcitriol might drive a transition from CD4+ T-cell to Treg cell dominance, recycling homocysteine to methionine, reducing homocysteine toxicity, maintaining DNA methylation, and stabilizing CD4+ Helios+ FoxP3+ Treg. Structural similarity in the responsible vitD-promoters even suggests a similar regulatory mechanism for humans (14). CD4+ T-cells have a cooperative amplification loop promoting CD4+Helios+FoxP3+ Treg development, and this process is disturbed when the vitD pathway is impaired (15).

Because of the role of T-cells in MS, glucocorticoids remain the most commonly used substance in treating acute MS relapses. However, in approximately 30% of patients, a limited efficacy of glucocorticoids is reported, often in patients with low serum vitD levels. VitD increases glucocorticoid-induced apoptosis of T-cells *via* upregulation of the glucocorticoid receptor (GCR). With the help of two different EAE models with reduced or absent GCR signaling, it was demonstrated that there are synergistic effects of vitD and glucocorticoids, probably mediated through mTORc1 signaling. Severe vitD deficiency is associated with downregulation of an mTORc1 inhibitor in human T-cells. In animals with T-cell-specific depletion of mTORc1 and in animals receiving a specific mTORc1 inhibitor, the synergistic effects of vitD/glucocorticoids on GCR upregulation, T-cell apoptosis, and therapeutic efficacy in EAE failed (16).

Beside the direct influence of vitD on T-cells, also related molecules such as cytokines and chemokines can induce powerful changes. For example vitD increases the production of IL-4, IL-10, and TGF-β while decreasing IFNγ, IL-6, TNFα, and IL-17 production accompanied with a deviated balance between Th1/Th2 and Th17/Treg to Th2 and Treg under middle and high doses of vitD (17). Accordingly, vitD downregulates the expression of some Th17 cell-related cytokines, key inflammatory chemokines, and chemokine receptors in EAE considering a possible therapeutic potential of vitD in future treating MS (18).

REMYELINATION

There is also recent literature available describing the effects of vitD on remyelination. Most data are available on the cuprizone model, since it is the easiest way of studying de- and remyelination. One study found that there is a significant increase MOG and $2'$,$3'$-cyclic-nucleotide $3'$-phosphodiesterase (CNPase) expression in vitD-supplemented cuprizone-exposed mice compared to control groups. MOG is a minor component of the myelin sheath, but it has an important autoantigen link to the pathogenesis of EAE whereas the protein CNPase is one of the main proteins of myelin and its appearance seems to be one of the earliest events of oligodendrocyte differentiation and myelination. VitD may play a role in the process of remyelination by increasing MOG and CNPase expression in the cortex (19). In another study, axonal damage during de- and remyelination in the cuprizone mouse model was investigated. The authors found significantly higher neurofilament preservation in the high dose-supplemented inactive vitD group in comparison to the low dose-supplemented group. High doses of active vitD, however, given after the demyelination phase as well as during remyelination did not influence axonal regeneration, while inactive vitD, given before and during cuprizone exposure, seems to have a protective effect on axons (20). VitD might even have the ability to trigger neuronal stem cell differentiation (21).

VitD AND MS PROGRESSION

After a disease duration of about 20 years, most MS patients enter the progressive state of the disease with a steady worsening of clinical neurological symptoms. Only little data are available upon the question whether vitD could be a reasonable support during progressive MS. Some clinical studies suggest a protective role of higher vitD levels on myelin content in progressive MS and an association between a low vitD status at the beginning of MS and the early entry to the progressive disease state (22, 23). A most recent study however could not confirm these assumption—vitD levels were not associated with the severity of optical coherence tomography findings or low-contrast letter acuity in their group of progressive MS patients (24). Clinical studies may furthermore be hampered by the possibility that severely affected progressive MS patients may have limited sunlight exposure as a consequence of their disease rather than as a cause. This demonstrates the need of more mechanistic knowledge of the mode of action of vitD in progressive MS. Unfortunately, there is no animal study addressing this research question so far. More studies making use of recently established animal models of progressive MS would be most welcome to elucidate the mechanistical background of how vitD could affect this disease state (25, 26).

ISSUES AND PROBLEMS

VitD Controversy

Even though the majority of animal studies affirm a beneficial effect of vitD in experimental animal models of MS, there is also a small list of literature suggesting that vitD is not capable of positively influencing autoimmune diseases. VitD and sunlight have each been reported to protect against the development of EAE. Since exposure of ultraviolet (UV) light also causes the generation of vitD, studies investigated whether the UV-based suppression of EAE results, at least in part, from the production of vitD. One study examined UV suppression of EAE in mice

devoid of vitD receptor (VDR) and mice unable to produce 7-DHC. UV light suppression of EAE occurred in the absence of vitD production and in the absence of VDR (27). However, it is possible that the UV suppression of EAE can further be influenced by the active form of vitD. The presence of active vitD surprisingly actually counteracted the suppressive effect of UV in one study (28). Further investigations should focus on identifying the pathway responsible for the protective action of UV in EAE and presumably human MS (27). Two independent research groups have demonstrated unexpectedly that vitD deficiency blocks EAE development. In one study, the suppression of EAE is even reported as a result from hypercalcemia and not as an effect of the active form of vitD (28). Another study revealed that a NBUVB light at 311 nm is responsible for the EAE suppression, and this wavelength does not produce vitD. There are suggestions upon a mechanism of EAE suppression independent of vitD, whereas a remaining question is still whether the active form of vitD has any impact on the NBUVB suppression of EAE (28, 29). These findings emphasize the need of further mechanistic research to gain a better understanding of EAE suppression and the role of vitD and light.

VitD Supplementation: Attention Should Be Paid to Adequate Dosage

The problem of potential overdosing vitD resulting in hypercalcemia is a critical aspect to this topic. Moderate supplementation of vitD reduces the severity of subsequent EAE in mice, associated with an expansion of Tregs. Direct exposure of T-cells to vitD metabolites inhibits their activation. On the other hand, high doses of vitD (200 nmol/l) in mice result in fulminant EAE with massive CNS infiltration. This is caused by mild hypercalcemia only observed in animals receiving high, but not medium, doses of vitD (30). Because of this problem, one study investigated the therapeutic potential of Paricalcitol (Pari) on EAE, since it is a non-hypercalcemic vitD$_2$ analogue, capable of promoting anti-inflammatory activity in kidney and heart diseases. In this study, severity, apoptosis and neuropathology of EAE were reduced *via* Pari accompanied by inhibition of glial cell activation, cellular infiltration, pro-inflammatory molecules, and activation of nuclear factor κB (NF-κB). This phenomenon could further be reduced by suppressing NF-κB with its inhibitor and Pari in combination (31). On the contrary, another study found a lower production of proinflammatory cytokines and reduced inflammation only in the EAE/vitD group, not in the Pari group. The authors thus suggest, that vitD, but not Pari, has the potential to be used as a preventive therapy to control MS severity (32). Another approach to bypass the problem of vitD overdose suggests a combination of vitamin A (vitA) and vitD. The combinatory treatment with vitA and vitD using the optimal synergistic effects with low doses could be beneficial in addressing the side effects and possibly paving the way for a more efficient MS therapy. One study demonstrated a significant different cytokine gene expression profile in the treated and control groups, suggesting a benefit of this treatment approach (33).

Experimental Animal Models and Problems in Translation

Results from animal models have to be critically validated. Common EAE models reflect important aspects of MS, but one has to consider that these models are mainly based on inflammation induced by autoreactive CD4+ T-cells whereas results from clinical trials in MS indicate that CD8+ T-cells and B-lymphocytes may play an important role in MS. In EAE, the inflammatory demyelinating disease burns out when the peripheral brain antigen depot has been removed. Therefore, it is most likely that in human MS, a persistent trigger within or outside the CNS is required for chronic disease propagation (4). This emphasizes the need of a variety of carefully selected animal models to cover different aspects of different phases of MS. However, especially concerning progressive MS, data are currently scarce.

VitD and Clinical Data in MS

Most of the clinical data regarding vitD and MS focus on its ability to reduce the risk of MS development. The suggestion that low vitD serum level is one MS risk factor is nowadays mainly accepted. One important question however remains upon its actions once the disease has started. Many studies concerning with this question are unfortunately insufficiently powered, most often without a long-lasting follow-up or with methodological bias, hindering conclusive results. Nevertheless, it appears highly likely that vitD is able to decrease components of the inflammatory pathway of the disease. Of course, further scientific validation is needed; a systematic vitD supplementation of MS patients has already been recommended in clinical practice, anyway (34). In comparison to the evidence of benefit of vitD supplementation in early MS, there is little known about the role of vitD in the progressive disease phase. Even though it is well known that these patients commonly suffer from low vitD serum levels, there is still the requirement of long-term observational studies (23, 35).

DISCUSSION

Numerous animal studies attest benefits of vitD. Based on the current state of knowledge, vitD supplementation may be considered as a preventative measure for decreasing the risk for developing autoimmune diseases and potentially as adjunctive therapy (7). **Figure 2** sums up the most recent findings discussed in this work. Studies on the beneficial effect of vitD in EAE suggest that treatment with vitD before EAE induction or from peak disease is effective at reducing disease severity. This beneficial effect may be mediated at least in part through the attenuation of T-cells, reduction of axonal and neuronal loss, and support of oligodendrocyte maturation (36, 37). Some other recent studies focused on genetic variations and how vitD could intervene. So far, it has been shown that vitD promotes negative feedback regulation of Toll-like receptor (TLR) signaling in macrophages *in vitro*, which in turn ameliorates inflammation. Naturally occurring allelic differences in the Vra4locus/Mhc2ta

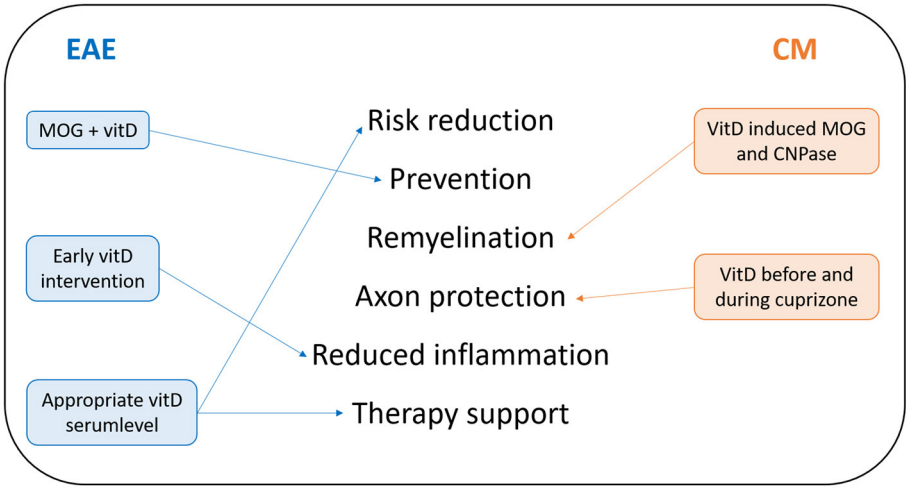

FIGURE 2 | Summary of recent findings of protective vitD effects. Most results upon vitD effects in MS-related animal models were obtained by EAE and the cuprizone model (CM). Lessons from EAE are written on the left side in blue; lessons from CM are shown in orange on the right side. In the middle of this summary, recent research findings of protective effects of vitD are listed.

could be relevant for the efficacy of vitD in modulation of MS-like neuroinflammation and potentially even in MS, but sufficient information about response to vitD within the Vra4 gene locus is still missing (10). Data from EAE allow the conclusion that vitD synthesis by activated microglial cells and macrophages in the CNS preserves neurological function by dampening the inflammatory process. VitD seems to support the CTLA-4 immunological checkpoint to prevent immune-mediated neurological damage. Additionally, the protective effect of vitD seems to involve epigenetic mechanisms (DNA methylation), which may provide a molecular basis for cellular memory that mediates long-term effects and suggests potential for future combined therapies (7). VitD and VDR are closely associated with the development of autoimmune diseases. There might be unknown factors capable of regulating VDR. It is already known that miRNAs are associated with VDRs. Even though the involvement of miRNAs in human diseases strengthened our understanding of pathogenesis, candidates for miRNAs that have the potential to control autoimmune pathomechanisms remain limited (38). Those strong regulatory molecules could become powerful tools of autoimmune disease management, if the exact mechanisms are elucidated in further studies. Beside validated findings on vitD beneficial effects, one important aspect is the reasonable application of vitD. Findings of studies regarding dose dependence of vitD suggest that vitD at moderate levels may exert a direct regulatory effect, while continuous high-dose vitD treatment could trigger MS disease activity by raising T-cell excitatory calcium (30, 39). This is indeed a very important lesson from animal studies since hypercalcemia was reported also in humans supplemented with high doses of vitD (40, 41). Monitoring the vitD level in MS patients thus is crucial to ensure positive effects of the supplementation. Another important topic is the impact of vitD on different MS treatments. Current MS treatments are found to be directly or indirectly linked to NF-κB pathways and act to adjust the immune system. MS is associated with constitutive activation of NF-κB, which results in excessive expression of related effector molecules, driving inflammation, and there is a very complex association of this factor and different cytokine patterns involved in EAE progression too (31). Pari could be one potential NF-κB blocker, and other vitD analogues might act in a similar way. Again, most of our knowledge about NF-κB is based on results from animal studies and further animal studies will be needed to investigate the mechanism further (31, 32). A similar issue concerns MS therapies working by increasing PDL1 expression. The PD1/PDL1 pathway might act as a key player in the mechanism of demyelination and autoimmune response due to its regulation of antigen-presenting cell and T-cell interaction. VD3-DCs attenuate the clinical symptoms of EAE by increasing the activation of the PD/PDL1 signaling pathway. However, the specific mechanisms of this signaling pathway still remain unsolved and further research is necessary in order to apply VD3-DCs to clinical practice (12). Translation of data from preclinical models to humans is always to be used with caution. However, it is still necessary to investigate pathophysiological mechanisms with the help of animal models. Even though most preclinical assays indicated a strong potential of vitD as a useful agent for EAE prevention or therapy, clinical trials with patients revealed mixed data (32). One possible reason is the missing knowledge in how vitD exactly acts in many different signaling pathways. Another interesting research goal is to elucidate gender aspects of vitD effects (42, 43). In general, autoimmune diseases are characterized by a significant female bias. This is also the case in MS where more females are affected (43). This sexual dimorphism in autoimmune diseases seems to be related to sex hormones, which differently affect the immune system. In general, males

show higher immunosuppression may be due to androgens, and females show a higher immunoreactivity and competence likely related to estrogens. This leads to a greater resilience to infections but also to a higher risk for developing autoimmune diseases (42, 43). Additionally, the outcome of vitD status in MS is determined by gene-by-sex interactions (44). Thus, gender and sex hormones could be included as variables when evaluating the potential power of vitD to influence autoimmune diseases (42, 43). Beside all these findings, one has to consider that there is a developmental stage-dependent efficiency of vitD to ameliorate neuroinflammation, suggesting that childhood and adolescence should be the target for the most effective preventive vitD treatment (45).

CONCLUSION

The majority of literature suggests a beneficial role of vitD at least in therapy of MS related animal models. When it comes to the translation of these findings to the human situation, the most important aspect to be considered is the right dosage, to avoid negative side effects. Nevertheless, for an effective treatment or support of MS therapies with the help of vitD and probably other vitamins, further studies are necessary. Especially if and how exactly vitD could intervene in pathophysiological mechanisms of progressive MS remains largely unsolved.

AUTHOR CONTRIBUTIONS

MH wrote the original draft and generated the figures. All authors contributed to the writing of this article, approved the submitted version, were involved in developing the plan for the article, and in reviewing and editing the manuscript.

REFERENCES

1. Wasnik S, Sharma I, Baylink DJ, Tang X. Vitamin D as a potential therapy for multiple sclerosis: where are we? *Int J Mol Sci.* (2020) 21:3102. doi: 10.3390/ijms21093102
2. Burrows DJ, McGown A, Jain SA, De Felice M, Ramesh TM, Sharrack B, et al. Animal models of multiple sclerosis: from rodents to zebrafish. *Mult Scler.* (2019) 25:306–24. doi: 10.1177/1352458518805246
3. Bjelobaba I, Begovic-Kupresanin V, Pekovic S, Lavrnja I. Animal models of multiple sclerosis: focus on experimental autoimmune encephalomyelitis. *J Neurosci Res.* (2018) 96:1021–42. doi: 10.1002/jnr.24224
4. Lassmann H, Bradl M. Multiple sclerosis: experimental models and reality. *Acta Neuropathol.* (2017) 133:223–44. doi: 10.1007/s00401-016-1631-4
5. Procaccini C, De Rosa V, Pucino V, Formisano L, Matarese G. Animal models of multiple sclerosis. *Eur J Pharmacol.* (2015) 759:182–91. doi: 10.1016/j.ejphar.2015.03.042
6. de Oliveira LRC, Mimura LAN, Fraga-Silva TFC, Ishikawa LLW, Fernandes AAH, Zorzella-Pezavento SFG, et al. Calcitriol prevents neuroinflammation and reduces blood-brain barrier disruption and local macrophage/microglia activation. *Front Pharmacol.* (2020) 11:161. doi: 10.3389/fphar.2020.00161
7. Zeitelhofer M, Adzemovic MZ, Gomez-Cabrero D, Bergman P, Hochmeister S. N'diaye M, et al. Functional genomics analysis of vitamin D effects on CD4+ T cells in vivo in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A.* (2017) 114:E1678–87. doi: 10.1073/pnas.1615783114
8. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol.* (2014) 21:319–29. doi: 10.1016/j.chembiol.2013.12.016
9. Mimura LA, Chiuso-Minicucci F, Fraga-Silva TF, Zorzella-Pezavento SF, França TG, Ishikawa LL, et al. Association of myelin peptide with vitamin D prevents autoimmune encephalomyelitis development. *Neuroscience.* (2016) 317:130–40. doi: 10.1016/j.neuroscience.2015.12.053
10. Hochmeister S, Aeinehband S, Dorris C, Berglund R, Haindl MT, Velikic V, et al. Effect of vitamin D on experimental autoimmune neuroinflammation is dependent on haplotypes comprising naturally occurring allelic variants of CIITA (*Mhc2ta*). *Front Neurol.* (2020) 11:600401. doi: 10.3389/fneur.2020.600401
11. Spanier JA, Nashold FE, Nelson CD, Praska CE, Hayes CE. Vitamin D_3-mediated resistance to a multiple sclerosis model disease depends on myeloid cell 1,25-dihydroxyvitamin D_3synthesis and correlates with increased CD_4^+ T cell CTLA-$_4$ expression. *J Neuroimmunol.* (2020) 338:577105. doi: 10.1016/j.jneuroim.2019.577105
12. Cao Q, Zheng C, Xie Z, Liu L, Zhu J, Jin T. The change of PD1, PDL1 in experimental autoimmune encephalomyelitis treated by $1,25(OH)_2D_3$. *J Neuroimmunol.* (2020) 338:577079. doi: 10.1016/j.jneuroim.2019.577079
13. Xie Z, Chen J, Zheng C, Wu J, Cheng Y, Zhu S, et al. 1,25-dihydroxyvitamin D_3 -induced dendritic cells suppress experimental autoimmune encephalomyelitis by increasing proportions of the regulatory lymphocytes and reducing T helper type 1 and type 17 cells. *Immunology.* (2017) 152:414–24. doi: 10.1111/imm.12776
14. Moore JR, Hubler SL, Nelson CD, Nashold FE, Spanier JA, Hayes CE. 1,25-Dihydroxyvitamin D_3 increases the methionine cycle, $CD4^+$ T cell DNA methylation and $Helios^+Foxp3^+$ T regulatory cells to reverse autoimmune neurodegenerative disease. *J Neuroimmunol.* (2018) 324:100–14. doi: 10.1016/j.jneuroim.2018.09.008
15. Spanier JA, Nashold FE, Mayne CG, Nelson CD, Hayes CE. Vitamin D and estrogen synergy in Vdr-expressing CD4(+) T cells is essential to induce Helios(+)FoxP3(+) T cells and prevent autoimmune demyelinating disease. *J Neuroimmunol.* (2015) 286:48–58. doi: 10.1016/j.jneuroim.2015.06.015
16. Hoepner R, Bagnoud M, Pistor M, Salmen A, Briner M, Synn H, et al. Vitamin D increases glucocorticoid efficacy via inhibition of mTORC1 in experimental models of multiple sclerosis. *Acta Neuropathol.* (2019) 138:443–56. doi: 10.1007/s00401-019-02018-8
17. Haghmorad D, Yazdanpanah E, Tavaf MJ, Zargarani S, Soltanmohammadi A, Mahmoudi MB, et al. Prevention and treatment of experimental autoimmune encephalomyelitis induced mice with 1, 25-dihydroxyvitamin D_3. *Neurol Res.* (2019) 41:943–57. doi: 10.1080/01616412.2019.1650218
18. Jafarzadeh A, Azizi SV, Arabi Z, Ahangar-Parvin R, Mohammadi-Kordkhayli M, Larussa T, et al. Vitamin D down-regulates the expression of some Th17 cell-related cytokines, key inflammatory chemokines, and chemokine receptors in experimental autoimmune encephalomyelitis. *Nutr Neurosci.* (2019) 22:725–37. doi: 10.1080/1028415X.2018.1436237
19. Mashayekhi F, Salehi Z. Administration of vitamin D3 induces CNPase and myelin oligodendrocyte glycoprotein expression in the cerebral cortex of the murine model of cuprizone-induced demyelination. *Folia Neuropathol.* (2016) 54:259–64. doi: 10.5114/fn.2016.62535
20. Nystad AE, Torkildsen Ø, Wergeland S. Effects of vitamin D on axonal damage during de- and remyelination in the cuprizone model. *J Neuroimmunol.* (2018) 321:61–5. doi: 10.1016/j.jneuroim.2018.05.016
21. Tiwari S, Lapierre J, Ojha CR, Martins K, Parira T, Dutta RK, et al. Signaling pathways and therapeutic perspectives related to environmental factors associated with multiple sclerosis. *J Neurosci Res.* (2018) 96:1831–46. doi: 10.1002/jnr.24322
22. Abbatemarco JR, Fox RJ, Li H, Ontaneda D. Vitamin D and MRI measures in progressive multiple sclerosis. *Mult Scler Relat Disord.* (2019) 35:276–82. doi: 10.1016/j.msard.2019.08.014

23. Muris AH, Rolf L, Broen K, Hupperts R, Damoiseaux J, Smolders J, et al. Low vitamin D status at diagnosis is associated with an early conversion to secondary progressive multiple sclerosis. *J Steroid Biochem Mol Biol.* (2016) 164:254–7. doi: 10.1016/j.jsbmb.2015.11.009
24. Abbatemarco JR, Fox RJ, Li H, Bermel RA, Ontaneda D. Vitamin D levels and visual system measurements in progressive multiple sclerosis: a cross-sectional study. *Int J MS Care.* (2021) 23:53–8. doi: 10.7224/1537-2073.2020-005
25. Üçal M, Haindl MT, Adzemovic MZ, Strasser J, Theisl L, Zeitelhofer M, et al. Widespread cortical demyelination of both hemispheres can be induced by injection of pro-inflammatory cytokines via an implanted catheter in the cortex of MOG-immunized rats. *Exp Neurol.* (2017) 294:32–44. doi: 10.1016/j.expneurol.2017.04.014
26. Haindl MT, Ücal M, Fazekas F, Hochmeister S. Overview of promising rat models for cortical lesion research- 2006 until now. *J Neurol Neuromed.* (2018) 3:8–12. doi: 10.29245/2572.942X/2018/5.1218
27. Irving AA, Marling SJ, Seeman J, Plum LA, DeLuca HF. UV light suppression of EAE (a mouse model of multiple sclerosis) is independent of vitamin D and its receptor. *Proc Natl Acad Sci U S A.* (2019) 116:22552–5. doi: 10.1073/pnas.1913294116
28. Wang Y, Marling SJ, Martino VM, Prahl JM, Deluca HF. The absence of 25-hydroxyvitamin D3-1α-hydroxylase potentiates the suppression of EAE in mice by ultraviolet light. *J Steroid Biochem Mol Biol.* (2016) 163:98–102. doi: 10.1016/j.jsbmb.2016.04.010
29. de Abreu DAF, El Ibrahim C, Boucraut J, Khrestchatisky M, Féron F. Severity of experimental autoimmune encephalomyelitis is unexpectedly reduced in mice born to vitamin D-deficient mothers. *J Steroid Biochem Mol Biol.* (2010) 121:250–3. doi: 10.1016/j.jsbmb.2010.03.006
30. Häusler D, Weber MS. Vitamin D supplementation in central nervous system demyelinating disease-enough is enough. *Int J Mol Sci.* (2019) 20:218. doi: 10.3390/ijms20010218
31. Zhang D, Qiao L, Fu T. Paricalcitol improves experimental autoimmune encephalomyelitis (EAE) by suppressing inflammation via NF-κB signaling. *Biomed Pharmacother.* (2020) 125:109528. doi: 10.1016/j.biopha.2019.109528
32. Mimura LAN, Fraga-Silva TFC, Oliveira LRC, Ishikawa LLW, Borim PA, Machado CM, et al. Preclinical therapy with Vitamin D3 in experimental encephalomyelitis: efficacy and comparison with paricalcitol. *Int J Mol Sci.* (2021) 22:1914. doi: 10.3390/ijms22041914
33. Parastouei K, Solaymani-Mohammadi F, Shiri-Shahsavar MR, Chahardoli R, Nasl-Khameneh AM, Zarandi MB, et al. The effect of calcitriol and all-trans retinoic acid on T-bet, IFN-γ, GATA3 and IL-4 genes expression in experimental autoimmune encephalomyelitis. *APMIS.* (2020) 128:583–92. doi: 10.1111/apm.13073
34. Pierrot-Deseilligny C, Souberbielle JC. Vitamin D and multiple sclerosis: an update. *Mult Scler Relat Disord.* (2017) 14:35–45. doi: 10.1016/j.msard.2017.03.014
35. Shoemaker TJ, Mowry EM. A review of vitamin D supplementation as disease-modifying therapy. *Mult Scler.* (2018) 24:6–11. doi: 10.1177/1352458517738131
36. Sloka S, Zhornitsky S, Silva C, Metz LM, Yong VW. 1,25-Dihydroxyvitamin D3 protects against immune-mediated killing of neurons in culture and in experimental autoimmune encephalomyelitis. *PLoS ONE.* (2015) 10:e0144084. doi: 10.1371/journal.pone.0144084
37. Shirazi HA, Rasouli J, Ciric B, Wei D, Rostami A, Zhang GX. 1,25-Dihydroxyvitamin D_3 suppressed experimental autoimmune encephalomyelitis through both immunomodulation and oligodendrocyte maturation. *Exp Mol Pathol.* (2017) 102:515–21. doi: 10.1016/j.yexmp.2017.05.015
38. Long HC, Wu R, Liu CF, Xiong FL, Xu Z, He D, et al. MiR-125a-5p regulates Vitamin D receptor expression in a mouse model of experimental autoimmune encephalomyelitis. *Neurosci Bull.* (2020) 36:110–20. doi: 10.1007/s12264-019-00418-0
39. Häusler D, Torke S, Peelen E, Bertsch T, Djukic M, Nau R, et al. High dose vitamin D exacerbates central nervous system autoimmunity by raising T-cell excitatory calcium. *Brain.* (2019) 142:2737–55. doi: 10.1093/brain/awz190
40. Malihi Z, Wu Z, Stewart AW, Lawes CM, Scragg R. Hypercalcemia, hypercalciuria, and kidney stones in long-term studies of vitamin D supplementation: a systematic review and meta-analysis. *Am J Clin Nutr.* (2016) 104:1039–51. doi: 10.3945/ajcn.116.134981
41. Marcus JF, Shalev SM, Harris CA, Goodin DS, Josephson SA. Severe hypercalcemia following vitamin d supplementation in a patient with multiple sclerosis: a note of caution. *Arch Neurol.* (2012) 69:129–32. doi: 10.1001/archneurol.2011.1199
42. Crescioli C, Minisola S. Vitamin D: autoimmunity and gender. *Curr Med Chem.* (2017) 24:2671–86. doi: 10.2174/0929867323666161220105821
43. Magyari M. Gender differences in multiple sclerosis epidemiology and treatment response. *Dan Med J.* (2016) 63:B5212.
44. Krementsov DN, Asarian L, Fang Q, McGill MM, Teuscher C. Sex-specific gene-by-Vitamin D interactions regulate susceptibility to central nervous system autoimmunity. *Front Immunol.* (2018) 9:1622. doi: 10.3389/fimmu.2018.01622
45. Adzemovic MZ, Zeitelhofer M, Hochmeister S, Gustafsson SA, Jagodic M. Efficacy of vitamin D in treating multiple sclerosis-like neuroinflammation depends on developmental stage. *Exp Neurol.* (2013) 249:39–48. doi: 10.1016/j.expneurol.2013.08.002

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Primary Peripheral Epstein-Barr Virus Infection Can Lead to CNS Infection and Neuroinflammation in a Rabbit Model: Implications for Multiple Sclerosis Pathogenesis

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Epstein-Barr virus (EBV) is a common herpesvirus associated with malignant and non-malignant conditions. An accumulating body of evidence supports a role for EBV in the pathogenesis of multiple sclerosis (MS), a demyelinating disease of the CNS. However, little is known about the details of the link between EBV and MS. One obstacle which has hindered research in this area has been the lack of a suitable animal model recapitulating natural infection in humans. We have recently shown that healthy rabbits are susceptible to EBV infection, and viral persistence in these animals mimics latent infection in humans. We used the rabbit model to investigate if peripheral EBV infection can lead to infection of the CNS and its potential consequences. We injected EBV intravenously in one group of animals, and phosphate-buffered saline (PBS) in another, with and without immunosuppression. Histopathological changes and viral dynamics were examined in peripheral blood, spleen, brain, and spinal cord, using a range of molecular and histopathology techniques. Our investigations uncovered important findings that could not be previously addressed. We showed that primary peripheral EBV infection can lead to the virus traversing the CNS. Cell associated, but not free virus in the plasma, correlated with CNS infection. The infected cells within the brain were found to be B-lymphocytes. Most notably, animals injected with EBV, but not PBS, developed inflammatory cellular aggregates in the CNS. The incidence of these aggregates increased in the immunosuppressed animals. The cellular aggregates contained compact clusters of macrophages surrounded by reactive astrocytes and dispersed B and T lymphocytes, but not myelinated nerve fibers. Moreover, studying EBV infection over a span of 28 days, revealed that the peak point for viral load in the periphery and CNS coincides with

increased occurrence of cellular aggregates in the brain. Finally, peripheral EBV infection triggered temporal changes in the expression of latent viral transcripts and cytokines in the brain. The present study provides the first direct *in vivo* evidence for the role of peripheral EBV infection in CNS pathology, and highlights a unique model to dissect viral mechanisms contributing to the development of MS.

Keywords: EBV - Epstein-Barr virus, peripheral infection, neuroinflammation, demyelination, multiple sclerosis, rabbit model, CNS infection

1 INTRODUCTION

Epstein-Barr virus (EBV) is a B cell-tropic DNA virus belonging to the *Herpesviridae* family. The virus is often acquired early in childhood and then persists asymptomatically for life. EBV spreads from one host to another through virions intermittently shed in the saliva of infected hosts. The virus infects B lymphocytes *via* the interaction of the viral glycoprotein gp350/220 with the cell surface receptor, CD21 (1). Latently infected cells, express a range of viral genes referred to as latency programs 0-III. Cells in latency III express 6 nuclear antigens (EBNAs), 3 latent membrane proteins (LMPs), a set of viral encoded miRNAs, and 2 non-coding RNAs (EBERs) (2). EBERs are ubiquitously expressed in all forms of latency and are often used as targets for the detection of EBV in tissues. Collectively, latent viral proteins expressed during latency III appear to be fundamental for EBV transforming capacity (3). In the face of a competent immune response, EBV shuts down the expression of all viral genes, with the exception of EBERs (4). Despite the predominance of the latent cycle, EBV infected cells occasionally undergo acute lytic replication, which aids the dissemination of the virus. Moreover, the lytic cycle contributes to transient viremia, and increased peripheral viral load during the acute phase of infection (5).

Acquisition of EBV during late adolescence or early adulthood can lead to symptomatic infectious mononucleosis (IM) (6), which is an important risk factor for the development of multiple sclerosis (MS) (7). MS is a disease that results in the destruction of myelin sheaths in the brain and spinal cord, a process known as demyelination. The influx of inflammatory immune cells and reactive gliosis are other important hallmarks of MS (8). In addition to demyelination and inflammation, EBV infection in the brain has been reported in MS cases (9–12). However, it is unclear how peripheral EBV travels to the CNS and what its consequences are on the CNS.

The CNS is no longer considered an immune privileged site, as once thought. Rather, there is a bi-directional intricate communication between the periphery and the CNS. Inflammation in the periphery can interfere with the blood-brain barrier (BBB) integrity and induce changes in the brain (13, 14). Similarly, when murine γ-herpesvirus 68 (MHV-68), a virus that naturally infects rodents and is biologically similar to EBV, is introduced directly into the brain of BALB/c mice, the virus can spread from the site of inoculation (i.e. brain) to the peripheral organs, including the spleen. Moreover, on reactivation of latent virus, MHV-68 can be readily detected in both the CNS, and the spleen (15).

Our laboratory has previously shown that intravenous inoculation of rabbits with EBV results in the virus establishing latency that mimics asymptomatic infection in humans (16). Upon primary infection, rabbits elicited a strong humoral response, correlating with undetectable levels of the virus in peripheral blood. However, immunosuppression of latently infected animals using cyclosporin A (CsA), resulted in reactivation and marked increase in peripheral viral load. EBV reactivation was associated with the expression of the immediate early lytic marker, BZLF1, and a handful of latent viral genes. These animals also showed pronounced infiltration of infected cells into the liver and the spleen (16).

In this study, we aimed to understand the impact of peripheral EBV infection on the CNS in the rabbit model. We proposed that latent EBV infection in rabbits could promote pathological alterations in the CNS that may predispose the infected animals to features seen in MS, such as inflammation and demyelination.

2 MATERIALS AND METHODS

2.1 Ethical Statement

All animal procedures in this study were reviewed and approved by the Institutional Review Board. Experiments were conducted on animals in adherence to the protocols approved by the Animal Research Ethics Committee of UAE University (Approval numbers: A-15-15; ERA-2018-5718).

2.2 Preparation of Virus Inoculum

B95-8, a B cell line of marmoset origin, was used to produce EBV for inoculation. The cells were grown in RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin solution (GIBCO, USA), 50 μg/ml gentamycin (Hyclone, USA), and 1× glutamine (GIBCO, USA) at 37°C, and 5% CO_2. Cells were then stressed by incubating at 30°C for 24hr to stimulate lytic cycle and virus shedding into the supernatant. The supernatant was centrifuged, and subsequently passed through 0.2-μm nylon filter (Thermo Fisher, USA). The filtered supernatant was used for intravenous (IV) injections following quantification of EBV copy number using qPCR.

2.3 Animals and Experimental Design

This study was divided into 2 parts:

a) investigating viral spread from the periphery to the CNS
b) investigating the dynamics of EBV infection over time

2.3.1 Investigating Viral Spread From the Periphery to the CNS

Four- to 8-week old New Zealand White (NZW) rabbits were obtained from a local supplier, and housed in our animal facility in the College of Medicine and Health Sciences (UAE University). Following a 2-week acclimatization, a total of 24 rabbits were randomly allocated to four groups (**Supplementary Figure 1A**):

Group 1 (EBV): eight animals were injected with 1×10^7 EBV copies, as determined by qPCR, *via* the marginal ear vein.

Group 2 (PBS control): four animals were IV injected with phosphate-buffered saline (PBS) (volume equivalent to that of EBV inoculum).

Group 3 (EBV+CsA): nine animals were injected with the same viral inoculum as for group 1 and treated with daily subcutaneous injections of cyclosporin A (CsA), (20mg/kg body weight, Sandimmune- Novartis).

Group 4 (CsA control): three animals were IV injected with PBS and immunosuppressed using daily CsA injections as in group 3.

Rabbits were monitored on a daily basis and sacrificed at day 14 post inoculation under Ketamine-Xylazine (40mg/kg and 5mg/kg, respectively) anesthesia. Whole blood was collected and separated into peripheral blood mononuclear cells (PBMCs) and plasma. Major organs including the spleen, brain and spinal cord were harvested.

2.3.2 Investigating the Dynamics of EBV Infection Over Time

In this set of experiments, NZW rabbits were divided into EBV group and PBS control group. At day 0, 15 animals in EBV group received the virus, and five animals in the control group received PBS *via* IV injection as described above. Three randomly selected rabbits from the EBV group and one rabbit from the control group were sacrificed at each of the following five time points: 3, 7, 14, 21 and 28 days post inoculation (**Supplementary Figure 1B**). Whole blood, spleen, brain, and spinal cord were collected.

2.4 DNA Extraction and qPCR for EBV Genome

PBMCs and plasma were isolated from whole blood samples using density gradient centrifugation on Histopaque-1077 (Sigma, Poole, UK). Genomic DNA (gDNA) was extracted from PBMCs, plasma, and biopsied tissues from spleen, brain, and spinal cord using QIAamp DNA Mini and Blood Mini Kit (QIAGEN), according to manufacturer's instructions.

Quantitative TaqMan PCR (Applied Biosystems) amplifying EBV BamHI fragment (17) was used to determine EBV copy number as previously described (18). The amplification reactions were run in duplicates on an Applied Biosystem 7500 real time thermocycler (Applied Biosystems). gDNA extracted from Namalwa cells was used to create a standard curve. Samples with undetermined Ct values were interpreted to have zero copy number for the purpose of statistical analysis.

2.5 RNA Extraction and RT-PCR

Total RNA was extracted from the brain using TRizol (Invitrogen, Germany). After determining the quantity and quality of the extracted RNA using NanoDrop 2000c (Thermo), 1μg of DNase-treated RNA was reverse-transcribed to cDNA using the Reverse Transcription System (Promega). SYBR Green Real-time PCR was performed, using Applied Biosystems QuantStudio™ 7 Flex System, to determine the relative mRNA expression of tumor necrosis factor α (TNFα), interleukin-1β (IL1β), IL2, and IL6 (19). The relative expression of latent EBV transcripts, EBER1, EBER2, EBNA1, and EBNA2 were also determined (20, 21). Samples were run in duplicates, and experiments were repeated twice. Relative expression was determined using comparative CT (ΔΔCt) method. Rabbit-specific GAPDH (housekeeping gene), and non-infected PBS samples (experimental controls) were used as reference.

2.6 Histology, EBER *In Situ* Hybridization (EBER-ISH), Immunohistochemistry, and Immunofluorescence

Formalin-fixed, paraffin-embedded (FFPE) tissues were cut into 5-μm sections and stained with hematoxylin and eosin (H&E) for basic histological examination. To identify viral proteins and cell populations contributing to inflammation in the CNS, a number of primary antibodies for viral and cellular markers were used (**Supplementary Table 1**).

For EBER-ISH, tissues were hybridized with a combination of 2 digoxin end-labelled probes complementary to EBER1 and EBER2, as previously described (18). Following blocking of the endogenous peroxidase activity, tissues were briefly digested with 0.1mg/ml proteinase K (Sigma). Sections were hybridized overnight with the probes, and two stringency washes were performed in 0.1×SSC buffer at 55°C. Mouse anti-digoxin antibody and Ultra-Sensitive ABC-Peroxidase Staining kit (Thermo Scientific, USA) were used for signal detection.

For chromogenic immunohistochemistry, heat-induced antigen retrieval was performed by incubating sections in boiling sodium citrate buffer (pH 6.2) for 10min. Endogenous peroxidase activity was quenched, followed by blocking in 5% BSA and 0.1% Triton-X 100 in 1×PBS for 1hr. Tissues were then incubated with primary antibodies at room temperature, overnight. Tissues were washed and incubated with appropriate secondary antibodies for 1hr. Diaminobenzidine (DAB) was used for signal detection and sections were counterstained with hematoxylin.

For immunofluorescence, sections were incubated with primary antibodies overnight. After washing, sections were incubated with fluorochrome-conjugated secondary antibodies for 1hr. Sections were washed, counterstained with DAPI and mounted using Fluoromount (Sigma). Fluorescence images were captured using fluorescence microscope (Zeiss).

For EBER-FISH and immunofluorescence, sections were hybridized overnight with EBER probes. After stringency wash,

sections were incubated with goat anti-IgG for 1hr. Fluorochrome-conjugated anti-digoxin and anti-goat antibodies were used as secondary antibodies.

2.7 Protein Extraction and ELISA

Homogenates of brain cortex were prepared in T-PER tissue protein extraction reagent (Thermo) and proteinase-inhibitor cocktail (Roche), using BeadBlaster™24 Homogenizer (Benchmark). Purified proteins were stored at -80°C until analysis. DuoSet ELISA development system for rabbit IL2 and IL6 were used according to the manufacturer's instructions (R&D Systems). All samples were assayed in duplicates, and experiments were repeated 3 times. A standard curve was included in each experiment and used to determine the quantity of cytokines in the test samples.

2.8 Statistical Analysis

Statistical analyses were performed using the GraphPad Prism Version 9.1.2 (GraphPad Software, San Diego, CA). Comparison between multiple groups was performed using one-way ANOVA or non-parametric multiple comparison, alpha= 0.05. Comparison between two groups was done using two-tailed unpaired t-test or nonparametric Mann-Whitney test. Data was displayed as mean± SEM. Spearman or Pearson correlation was used to correlate between 2 variables. P value ≤ 0.05 was considered statistically significant.

3 RESULTS

3.1 Investigating Viral Spread From the Periphery to the CNS

3.1.1 EBV Inoculated Rabbits Exhibit Viremia and High Viral DNA Load in the Periphery

The presence of EBV in MS brain has been reported in several studies (9–11, 18). How EBV in the periphery travels to the CNS is poorly understood. In order to determine whether peripheral EBV infection on its own can lead to CNS infection, we injected EBV intravenously into eight healthy NZW rabbits. In healthy animals, antiviral T cell responses act as a barrier that limits systemic viral dissemination. Therefore, in another nine rabbits, we injected EBV and immunosuppressed them using CsA. This was implemented to overcome anti-EBV T cell responses and increase the likelihood of EBV spreading to the CNS in these animals.

In the eight animals inoculated with EBV, no disease manifestations were observed during the 14-day study period, or at autopsy. In the EBV+CsA group however, 1/9 rabbits showed changed temperament, decreased activity, and major loss in body weight. To minimize animal suffering, this rabbit was euthanized at day 7, as opposed to the scheduled day 14. At autopsy, the spleen was found to be significantly enlarged with macroscopic white nodules in 4/9 rabbits in the EBV+CsA group, but not in any of the eight animals in the EBV group.

EBV infection in the spleen was confirmed by EBER *in situ* hybridization (EBER-ISH) in all animals inoculated with the virus. No EBER signal was seen in the spleen of any of the control rabbits that were injected with either, PBS or CsA (**Figure 1A**). This was further supported by the detection of the virus using qPCR. EBV genome was detected in the spleen of all the rabbits in both EBV and EBV+CsA groups (**Figures 1B, C**). Thus, peripheral EBV infection was established in 100% of the animals inoculated with EBV, regardless of the immune status.

Since viremia is an important event in predisposing to CNS infection (22), we quantified EBV in the PBMCs and plasma. All the animals in the EBV group (8/8) (**Figure 1B**) and 8/9 animals in EBV+CsA group (**Figure 1C**) had quantifiable, but variable viral load in the PBMCs. None of the PBS/CsA controls had detectable virus (**Figures 1B, C**). In the EBV+CsA group, the 1/9 animals which did not have detectable virus in the PBMCs, was the animal which was euthanized 1 week prematurely. This suggests that EBV detectability in peripheral blood may be suboptimal at 7dpi as opposed to 14dpi. As for plasma, 75% (6/8) of the animals in the EBV group (**Figure 1B**) and 78% (7/9) of EBV+CsA group (**Figure 1C**) were viremic (i.e. EBV DNA in plasma). We also found significant correlation between the levels of viremia and splenic viral load in the EBV group, but not in the EBV+CsA group (**Supplementary Table 2**). This implies the interfering effects of immunosuppression on viremia levels. Among the 3 peripheral compartments (plasma, PBMCs and spleen), plasma had the lowest level of EBV DNA (**Figures 1B, C**). This indicates that lytic shedding of the virus is relatively less frequent, and most of the virus is cell-associated.

3.1.2 Inflammatory Cell Aggregates Develop in the CNS Following Peripheral EBV Infection

To evaluate the impact of peripheral EBV infection on the CNS, we examined the brain and spinal cord for histopathological changes. Interestingly, we observed widespread presence of distinct cellular aggregates consisting of inflammatory cells and microglia nodules in the brain and spinal cord of (2/8) EBV and (7/9) EBV+CsA groups (**Figure 2**). These aggregates were not observed in any of the PBS controls. However, 2/3 CsA controls developed similar CNS aggregates. These observations suggest that peripheral EBV infection can promote neuroinflammation in some hosts, and is likely influenced by the host's genetics and immune system. Immunosuppression can also lead to neuroinflammation, possibly as a result of reactivation of latent infection(s) other than EBV. We also noted that the cellular aggregates in the spinal cord were less in number and smaller in size than cerebral aggregates (**Figure 2**). Moreover, the aggregates in the brain were widespread and present throughout the hemisphere, including the meninges and the cerebellum. Generally, these cellular aggregates were associated with CNS vasculature (**Supplementary Figure 2A**). The aggregates were observed more frequently in the cerebrum than in the cerebellum. The aggregates also formed in both hemispheres (**Supplementary Figure 2B**).

To determine the cellular makeup of the CNS aggregates, we immunostained sections of the brain and spinal cord for various cellular markers. CNS aggregates consisted of infiltrating macrophages ($RAM11^+$), microglia ($Iba1^+$), reactive astrocytes

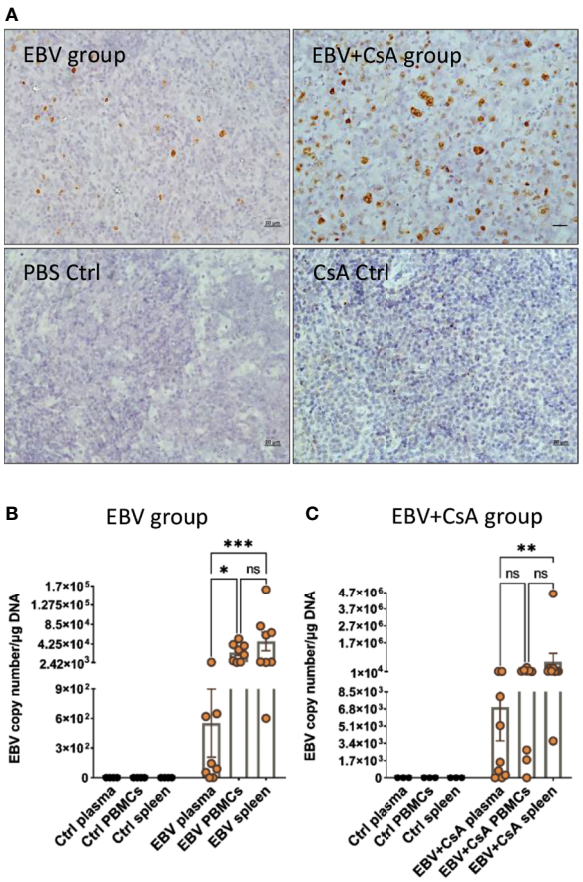

FIGURE 1 | EBV inoculated rabbits exhibit viremia and high viral DNA load in the periphery. **(A)** Representative images of EBER-ISH on rabbit spleen from EBV and EBV+CsA group and their corresponding controls. Scale bar=20µm. **(B)** EBV copy number measured using qPCR in plasma, PBMCs, and spleen of rabbits in EBV group (n=8) and PBS controls (n=4), and **(C)** EBV+CsA group (n=9) and CsA controls (n=3). Samples with undetermined EBV levels were plotted at y=0. EBV load is presented with mean ± SEM, and comparisons were made using the nonparametric Friedman test and Kruskal-Wallis. ns: $p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

(GFAP$^+$), and infiltrating B (CD21$^+$) and T lymphocytes (CD3$^+$), and neutrophils (**Figure 3A** and **Supplementary Figure 3**). Additionally, proliferating cells (PCNA$^+$) and infiltrating lymphocytes, including CD8$^+$ (but not CD4$^+$), IgG$^+$ (but not IgM$^+$) and EBI2$^+$ cells were dispersed within the aggregates (**Figure 3B**). Notably, the majority of these infiltrating immune cells were also diffusely scattered in the CNS parenchyma and formed several small clusters of loosely connected cells that lacked macrophage aggregation. While blood-derived macrophages/microglia appeared to make up the center of most, if not all, CNS aggregates, astrocytes and scattered proliferating B cells were mainly associated with the outer part of the aggregates (**Figures 4A, B**). Furthermore, to examine whether the presence of these aggregates is associated with demyelination, we stained for myelin basic protein (MBP). We observed disruption of myelin within the aggregates, but this did not extend beyond the aggregates (**Figure 5**). Collectively, these observations suggest that peripheral EBV infection can lead to immune cells trafficking into the CNS, and the formation of cellular aggregates. The aggregates consist of proliferating B cells, T-cells, and astrocytes, with blood derived macrophages occupying the center. Notably, the aggregates appear to be completely devoid of myelinated nerve fibers.

3.1.3 EBV Infected Cells Infiltrate the Brain of Both Immunocompetent and Immunosuppressed Animals

Since EBV infection of the brain has previously been shown in MS cases (9–11, 18, 23–27), we wanted to know whether primary peripheral infection can lead to infection of the brain. We stained brain sections, from EBV and EBV+CsA groups and PBS/CsA controls, for EBERs. EBV was detected in the brain of 6/8 animals in the EBV group, and 9/9 in the EBV+CsA group, independent of the presence of cellular aggregates. The 2/8 animals negative for EBV in the brain, contained no cellular aggregates. EBV infected cells were not seen in any of the PBS/CsA controls (**Figure 6A**). We also examined the distribution of transcriptionally active virus in the brain by staining a series of sections with anti-EBNA1. EBNA1$^+$ cells were seen dispersed throughout the brain (**Figure 6B**). Remarkably, massive infiltration of EBNA1$^+$ cells took place in the granular layer of the cerebellum (**Figure 6B**), suggesting that the cerebellum may be a vulnerable niche to EBV infection in the CNS (28). However, further work is required to evaluate the importance of the cerebellum in EBV infection.

In humans, EBV is primarily carried by circulating IgD$^-$ CD27$^+$ isotype-switched memory B cells (29, 30). To determine which cells are infected with EBV in the rabbit brain, we performed double staining for EBV (EBERs) and B-cells (IgM and IgG), in heavily infected brain sections. Both IgM$^+$ and IgG$^+$ cells were found to be EBV positive (**Figure 6C**). These findings indicate that primary peripheral EBV infection can be a sufficient event for EBV infected B cells to infiltrate the CNS.

3.2 Investigating the Dynamics of EBV Infection Over Time

3.2.1 EBV Load Peaks at Day 14 Post Infection in Peripheral and CNS Compartments

To evaluate infection dynamics and delineate changes in the incidence of CNS aggregates over time, we IV inoculated a new batch of rabbits with EBV and examined blood, spleen, brain, and spinal cord at five time points; 3, 7, 14, 21 and 28 days post infection (dpi).

In the peripheral compartment, EBV was detected in the PBMCs at all time points (**Figure 7**). In the spleen, however, virus reached detectable levels by day 7 and remained detectable throughout the next 3 time points. Interestingly, EBV in the plasma (indicative of viremia) could not be detected until 14 and 21dpi. In the CNS, the virus was detected in the brain at 7, 14, 21 and 28dpi, and in the spinal cord at 7, 14 and 21dpi. Notably, all 3 animals (100%) sacrificed at day 14 exhibited detectable high virus load in the plasma, PBMCs, spleen and brain. Thus, day 14 was the optimal time point for virus detection in the periphery

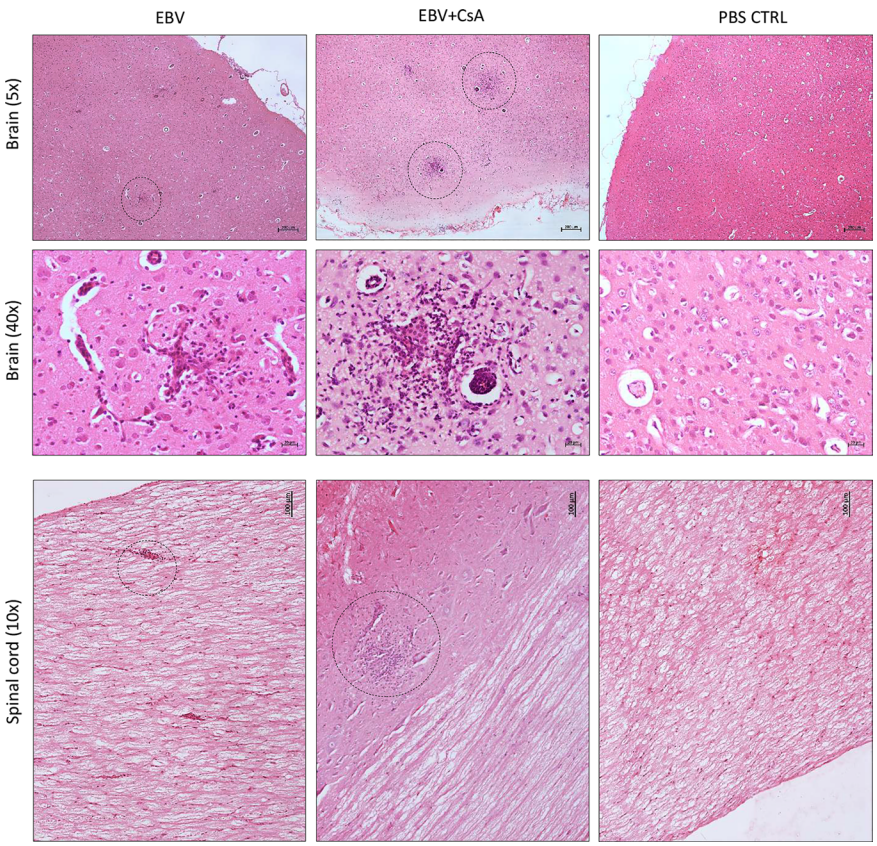

FIGURE 2 | Cell aggregation in the rabbit CNS. H&E staining of formalin-fixed, paraffin-embedded sections of a brain and a spinal cord harvested from EBV, EBV +CsA and PBS control groups. Dotted circles point to well-demarcated cellular aggregates. 40×, 10× and 5× images show scale bar of 20μm, 100μm, and 200μm, respectively.

and CNS. Additionally, EBV DNA load increased significantly at day 14 in plasma, PBMCs, spleen and brain (**Figure 7**). Furthermore, EBV DNA load in the brain correlated significantly with both splenic and PBMCs viral load (**Supplementary Table 3**). However, EBV load in the spinal cord correlated only moderately with EBV levels in the plasma (viremia) (**Supplementary Table 3**). Thus, increased viral load, in the spleen and PBMCs, may be a determinant for virus infection of the brain. This also highlights the importance of cell-associated virus, rather than free virus, in EBV trafficking to the brain.

3.2.2 The Occurrence of CNS Aggregates Peaks at Days 14 and 21 of Infection

We next examined coronal sections of the brain and cross sections of the spinal cord for the presence of inflammatory aggregates at 3, 7, 14, 21, and 28dpi. Although mild inflammation was frequently observed in the meninges and around blood vessels in the brain of infected animals, distinct cellular aggregates were only seen in the brain and spinal cord of animals sacrificed at days 14 and 21 (**Figure 8A**). Again, the aggregates in the spinal cord were smaller in size compared to the aggregates observed in the corresponding brain. Additionally, massive infiltration of the CNS by immune cells, including neutrophils, macrophages (**Supplementary Figure 4**), and B and T lymphocytes (**Supplementary Figure 5**) was observed at days 14 and 21 in sections with aggregates, but not in sections with limited mild inflammation. In agreement with part 1 of the study, the formation of aggregates was associated with disruption of myelin within aggregates, in both the brain (**Figure 9**) and spinal cord (**Supplementary Figure 6**).

To determine if there was a difference in the number of cerebral aggregates at different time points, we cut 1000, 5μm sections from all animals at each of the five time points. Aggregates were counted in sections at intervals of 50. Aggregates were observed to have great heterogeneity in morphology. Thus, for the purpose of counting, we defined an aggregate in the brain parenchyma as a clear continuous cluster of cells that is at least 60μm in diameter. The term aggregate also included any 2 cellular clusters that were connected by a thread of infiltrates. Thus, meningeal infiltrates, small vessels engorged with lymphocytes, clusters or perivascular cuffs that are less than 60μm in diameter were not counted. On average the number of aggregates reached the peak at 14 and 21dpi (**Figure 8B**). We next compared peripheral and CNS viral load in animals that developed CNS aggregates and those without aggregates. We found that

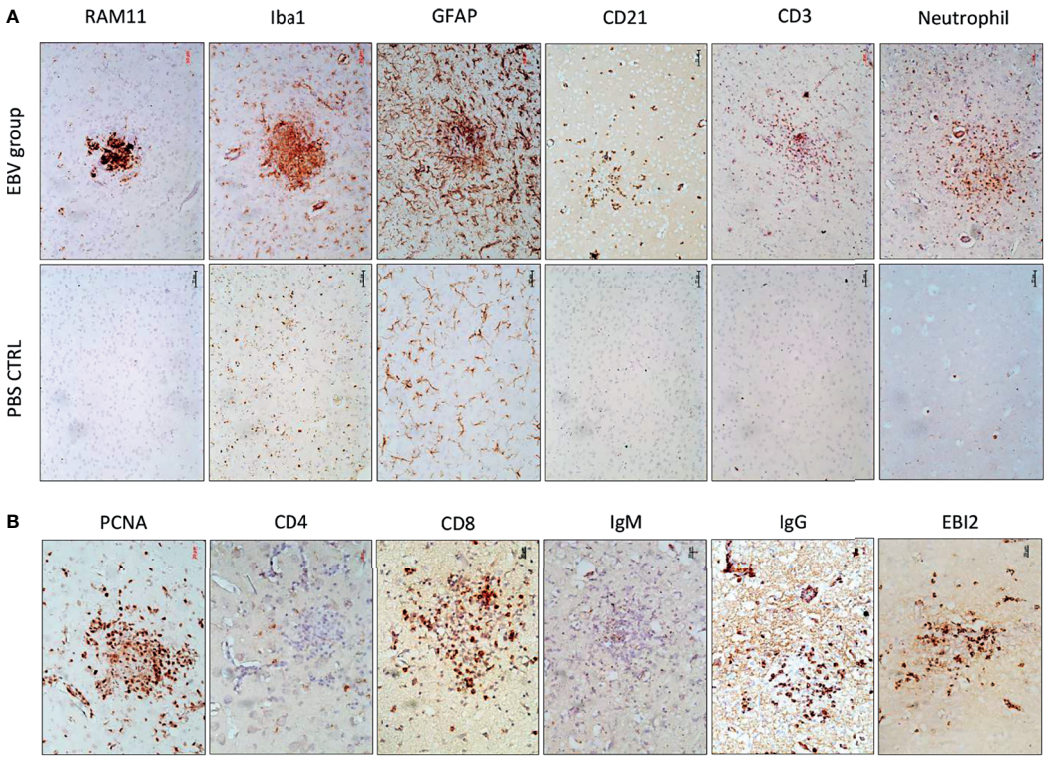

FIGURE 3 | The cellular makeup of cerebral aggregates in EBV infected rabbits. **(A)** Brain sections from EBV infected rabbits with cerebral aggregates and PBS controls were stained with rabbit-specific maker for macrophages (RAM11), microglia (Iba1), astrocytes (GFAP), B cells (CD21), T cells (CD3), and neutrophils. Scale bar=50μm. **(B)** Additional phenotypic characterization of lymphoid infiltrates by staining for the proliferation marker PCNA, T and B cell markers CD8, IgG, IgM, and Epstein-Barr virus-induced gene 2 (EBI2). Scale bar=20μm.

animals with aggregates had significantly elevated levels of EBV DNA in PBMCs compared to animals without aggregates (**Figure 8C**). This reflects a link between the level of cell-associated virus, but not cell-free virus, in the peripheral blood and the occurrence of CNS cellular aggregates. Thus, viremia may not be a determinant for the development of these structures in the CNS.

3.2.3 Peripheral EBV Infection Results in Altered Expression of Cytokines and Latent Viral Transcripts in the Brain

To understand the impact of peripheral EBV infection on the expression of proinflammatory cytokines in the brain, we performed qPCR for tumor necrosis factor α (TNFα), interleukin-1β (IL1β), interleukin-2 (IL2) and interleukin-6 (IL6). Analysis of relative expression over the 5 time points of infection revealed significant upregulation of TNFα, IL1β and IL2 at 28dpi, compared to the brain of non-infected PBS controls (**Figure 10A**). IL6, on the other hand, was significantly upregulated as early as 14dpi (**Figure 10A**). Furthermore, we determined whether the upregulation of these cytokines in the brain was coupled with altered expression of EBV latent transcripts. Similar to TNFα, IL1β and IL2, the expression of EBER1, EBER2 and EBNA1 was significantly elevated at day 28 (**Figure 10B**). EBER2, however, was significantly upregulated at day 14. This shows that the expression of proinflammatory cytokines correlate with that of viral transcripts in the brain.

Indeed, heat map of Spearman correlation of viral transcripts and cytokine expression in the brain, indicated a positive correlation between EBER1/2 and the cytokines IL6, IL2 and IL1β, (**Figure 10C**). Together, these results imply that increased mRNA expression of latent EBV transcripts is accompanied by increased expression of inflammatory cytokines in the brain.

We also examined the impact of EBV infection on the protein levels of IL2 and IL6 in the brain. Both 14 and 21dpi showed dramatic rise in the levels of IL6 protein, yet it only reached statistical significance at day 21, coinciding with the time points of aggregate occurrence (**Figure 10D**). Interestingly, there was a significant drop in the regulatory IL2 level at day 7 (**Figure 10D**), the time point that preceded aggregate formation. These observations suggest that the increased production of proinflammatory IL6 may be associated with the inflammation seen in the brain.

4 DISCUSSION

Some studies have found no indication of EBV infection in the MS brain (31–33). Whereas others have demonstrated the

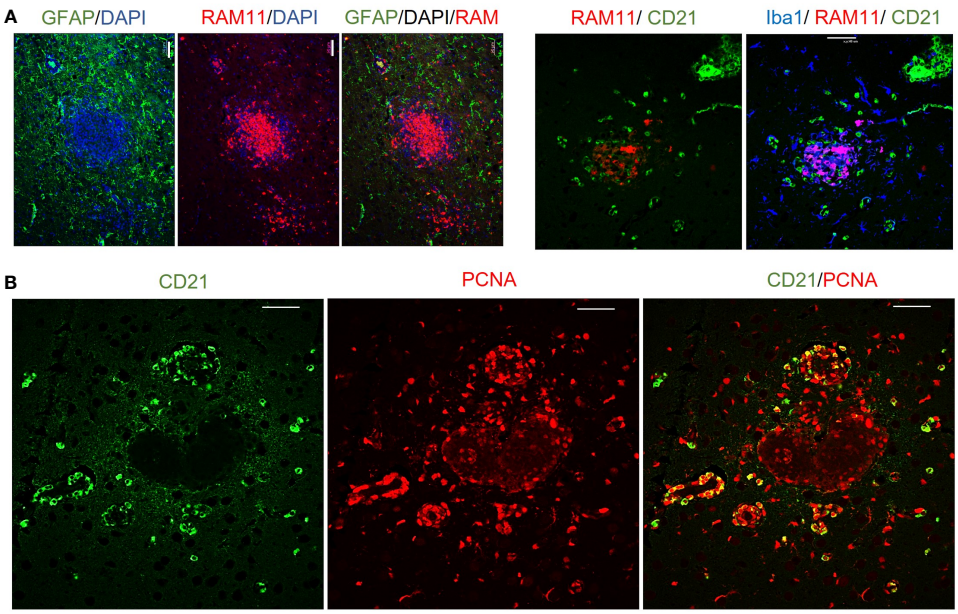

FIGURE 4 | Immunofluorescence double staining to determine the cellular makeup of cerebral aggregates in EBV infected rabbits. **(A)** Representative immunofluorescence staining of inflamed brain section with GFAP (green), RAM11 (red), and DAPI (blue). Scale bar=20μm. Location of B cells (green) in relation to macrophages (red) and microglia (blue) were identified by staining for CD21, RAM11 and Iba1, respectively. Scale bar=40μm. **(B)** Double positive CD21 (green) and PCNA (red) depict proliferating B lymphocytes in cerebral aggregates. Scale bar=40μm.

presence of the virus in the brain, implicating a role for the virus in the pathogenesis of this disease (9, 11, 18). However, the dynamics of virus trafficking to the brain, and its subsequent impact on disease development and/or progression, are poorly understood. Addressing these questions has been challenging due to the limited availability of an animal model that recapitulates the typically silent-mild infection seen in most humans. We and others have recently shown that rabbits are susceptible to EBV, and the infection mimics that observed in humans (16, 34–36). Previous studies have demonstrated the efficacy of intravenous (IV) inoculation in producing persistent infection in rabbits (35–37). This route of infection was shown to elicit antiviral humoral response and detectable virus and viral proteins in peripheral blood, spleen, and liver. Additionally, EBV levels in the periphery varied between different rabbits, and fluctuated overtime in a given animal (37). Similar to humans, the rabbit immune system does not completely eradicate EBV infection, as these animals remain latently infected with the virus (36). Attempts to infect rabbits *via* intranasal route were also successful, however, this was found to lead to lower expression of EBV proteins and milder infection compared to IV inoculation. Using the oral route, on the other hand, appeared less effective in establishing infection (35). Based on these observations, we used IV route in this study to ensure establishment of persistent EBV infection in rabbits.

Using the rabbit model, we explored the neuropathogenic potential of primary peripheral EBV infection. The findings uncovered several novel aspects of the dynamics of EBV infection in the periphery and CNS. 1) Intravenous inoculation of the virus resulted in widespread infection in all three peripheral compartments examined: spleen, PBMCs, and plasma. 2) Peripheral infection resulted in the virus traversing the brain. 3) Infection in the brain correlated with cell-associated virus, rather than circulating free virus in the plasma. 4) Peripheral EBV infection induced the formation of inflammatory cellular aggregates in the CNS, and these aggregates were composed of blood-derived macrophages surrounded by reactive astrocytes and infiltrating B and T lymphocytes.

Primary EBV infection during late adolescence can cause symptomatic infectious mononucleosis (IM). Both symptomatic and asymptomatic primary infection cause high viral load in the periphery. However, disrupted immunological profile is rather unique to IM (38–40). This emphasizes that EBV associated diseases emerge as a result of changes in the immune components triggered by the infection. Indeed, abnormalities in anti-EBV immune response exerted by $CD8^+$ T cells is believed to contribute to MS disease (41–43). In this study, we investigated primary EBV infection in healthy rabbits, and rabbits immunosuppressed with CsA. High viral load was detected in the peripheral compartments of all animals, particularly the immunosuppressed (EBV+CsA) group. This group also exhibited more than 10-fold higher levels of free virus in the plasma. However, the level of free virus did not correlate with brain infection. By contrast, there was a positive correlation between infected cells in the PBMCs/spleen, and brain infection. These findings support the idea that CNS infection is due to migrating infected lymphocytes, most probably B cells. van

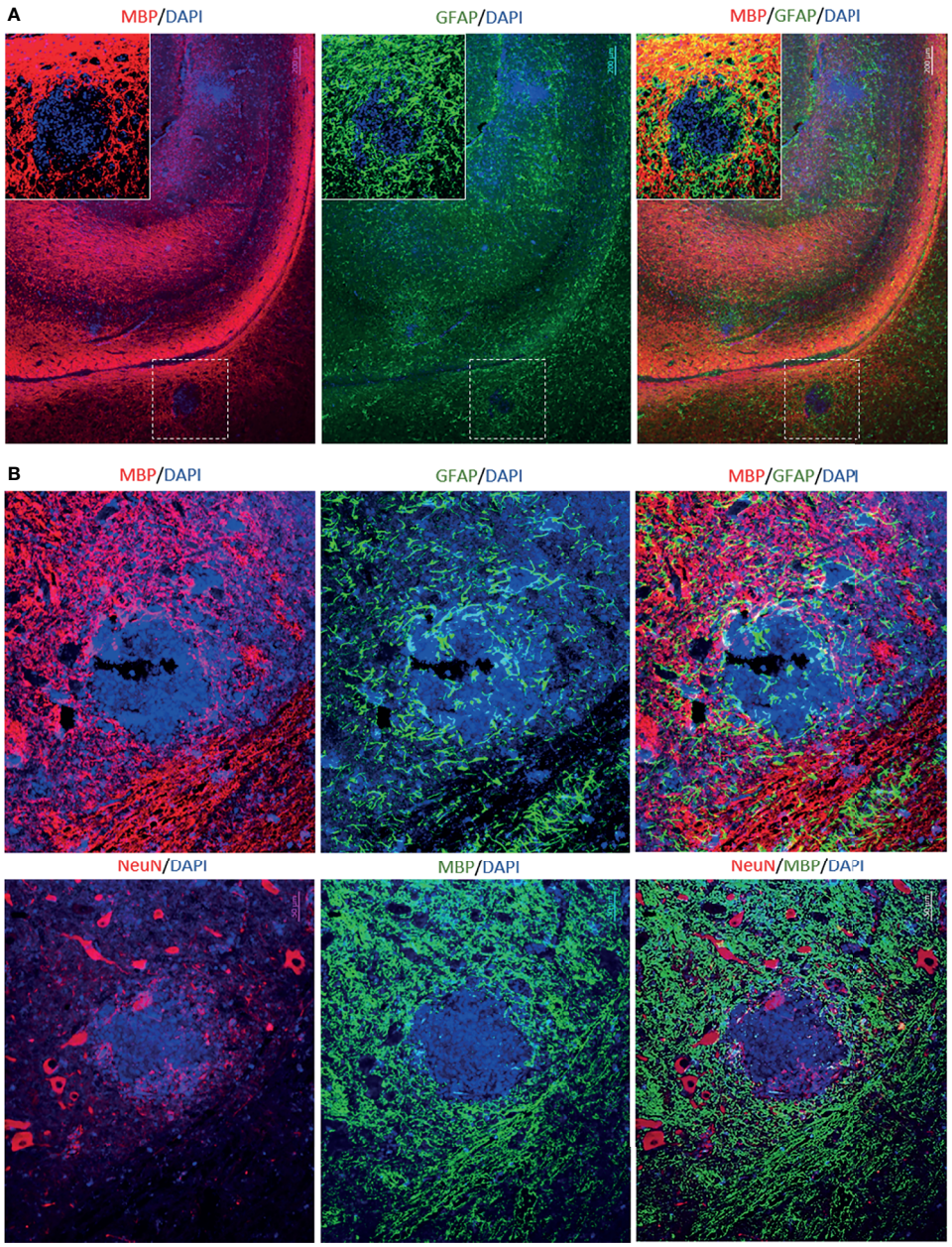

FIGURE 5 | Immunofluorescence double staining to assess demyelination in aggregates-positive case from EBV group. **(A)** Immunostaining for MPB and GFAP in the brain. Scale bar=200μm **(B)** Immunostaining for MBP, GFAP and NeuN in the corresponding spinal cord. The aggregates were completely devoid of myelinated nerve fibers. However, demyelination appeared to be restricted to the aggregates and it was not widespread. Scale bar=50μm.

Langelaar and coauthors have recently shown that there is a positive correlation between IgM^- IgD^- B cells expressing the chemokine CXCR3 and EBV load in the blood of MS patients who underwent bone marrow transplantation (44). This may mechanistically implicate this chemokine in the migration of virus infected cells to the CNS. Furthermore, EBV infected B cells with phosphoprotein 1/osteopontin gene upregulation have been found to have the potential of infiltrating the CNS (45). The gene upregulation in these cells is associated with epigenetic changes including histone modification. The migration of EBV infected cells from the periphery to the brain has also been reported recently in humanized mice (46). This was achieved by inoculating humanized mice with EBV and treating them with pembrolizumab, a monoclonal antibody, used clinically to block the immune checkpoint programmed death 1 (PD-1) receptor. Subsequently, virus propagation to the CNS led to the formation

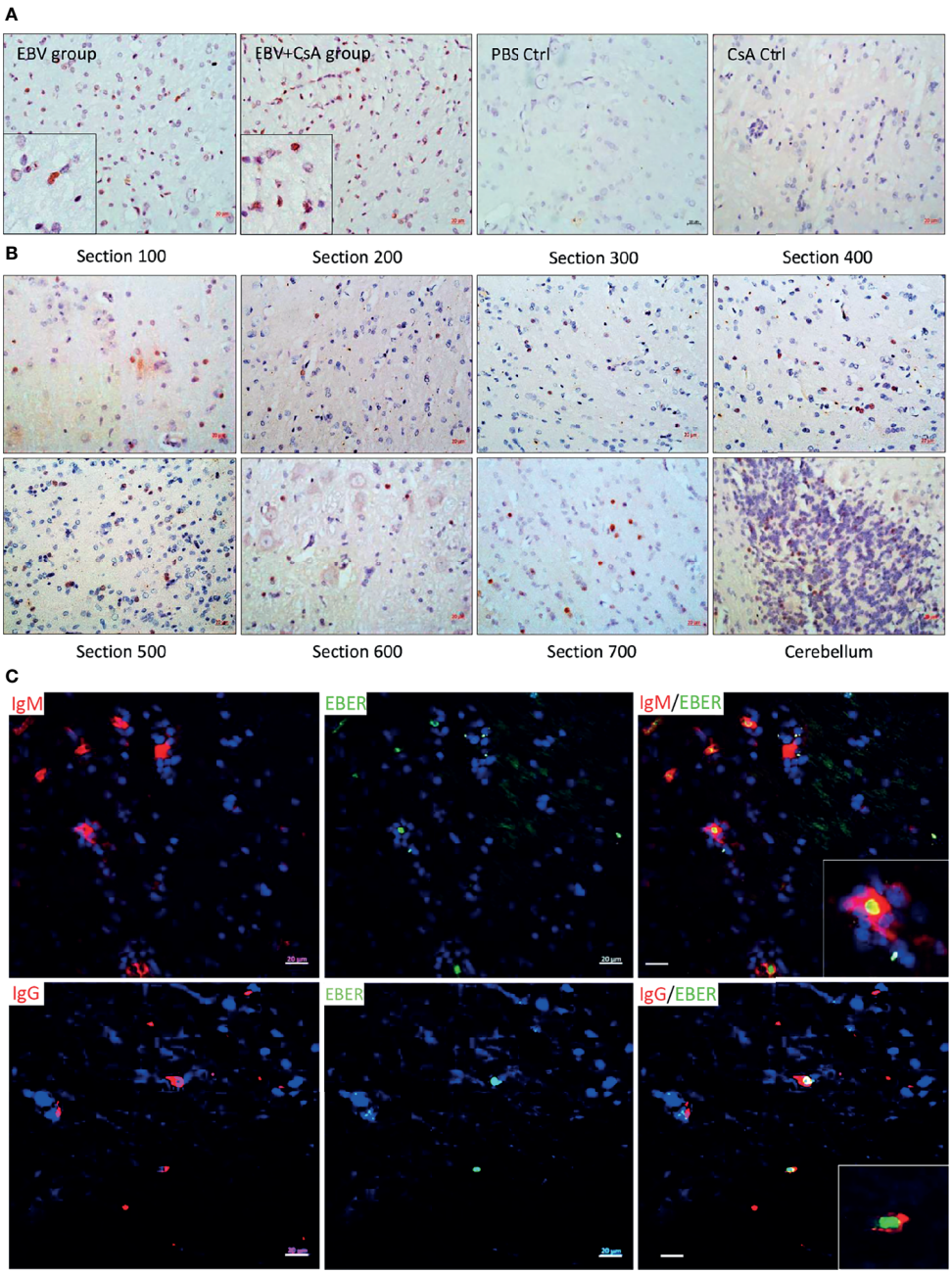

FIGURE 6 | EBV infected cells infiltrated the brain. **(A)** Representative images of EBER-ISH on brain sections from EBV and EBV+CsA groups and their corresponding PBS/CsA controls. Scale bar=20μm. **(B)** Immunohistochemistry for EBNA1 in a series of sections from a heavily infected cerebral hemisphere and cerebellum. Scale bar=20μm. **(C)** Double staining for IgG/IgM (red) and EBERs (green) to determine the phenotype of infected cells in the brain. Scale bar=20μm.

of EBER-rich lymphomas in the brain. These mice had low frequency of circulating T cells, many of which were exhausted (i.e. $TIM3^+$ and $LAG3^+$ T cells) (46).

Different animals have been used to study EBV infection in the brain. Some studies investigated intracerebral inoculation of MHV68 into mice (15, 47, 48). Animals exhibited signs of severe disease, which was more fatal in juvenile mice than in older ones (48). The direct introduction of the virus into the CNS was shown to result in mononuclear cell infiltrates and viral infection of the meninges, ependymal cells, oligodendrocytes, hippocampal pyramidal neurons, and the Bergmann glia cells in the cerebellum. The infection was also associated with damage to the white matter (47). Japanese macaques were also found to naturally develop an acute MS-like disease as a result of CNS

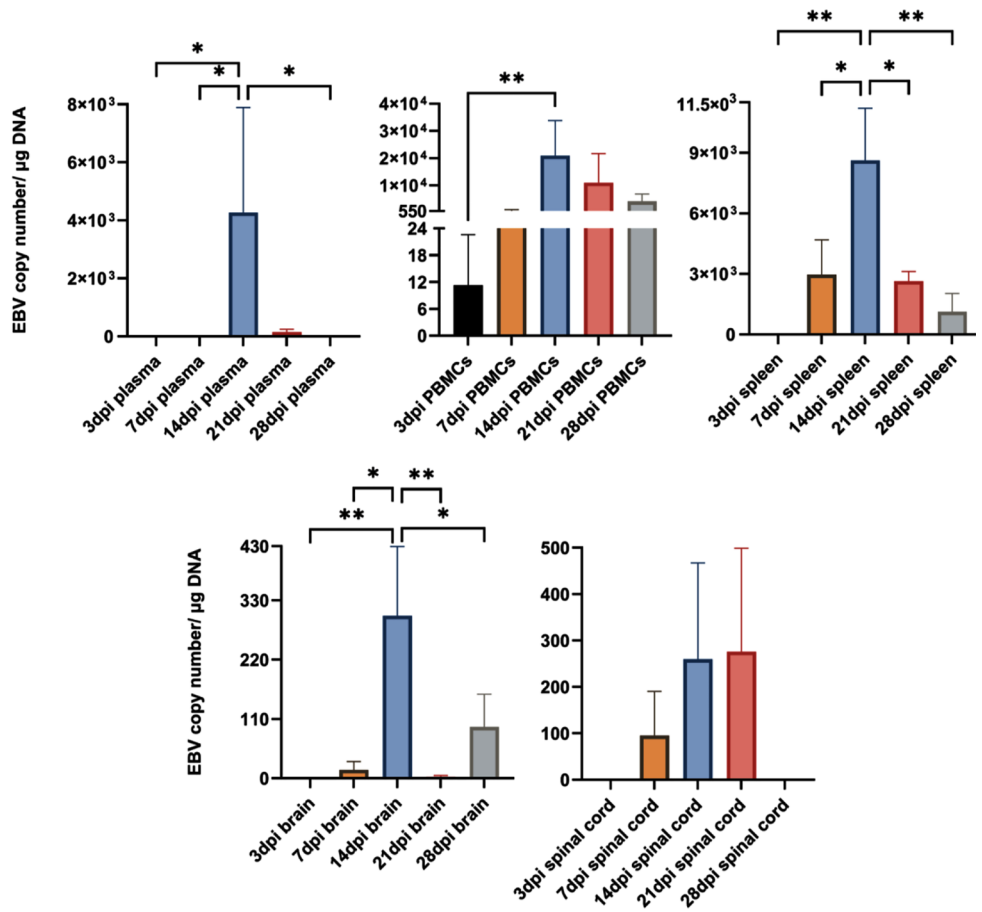

FIGURE 7 | EBV load peaks in the periphery and CNS at 14dpi. EBV copy number in plasma, PBMCs, spleen, brain and spinal cord determined by qPCR at 3, 7, 14, 21 and 28dpi (n=3 rabbits/time-point), and displayed as mean ± SEM. One-Way ANOVA and non-parametric Kruskal-Wallis test were used to compare between groups. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. Bars without asterisks are not significantly different.

infection with a newly identified γ-herpesvirus. The CNS contained several inflammatory demyelinating lesions (49). The CNS infiltrating $CD4^+$ T cells and $CD8^+$ T cells were later shown to elicit immune response against the myelin antigens MBP, myelin oligodendrocyte glycoprotein and proteolipid protein (50). Rhesus monkeys were also reported to develop inflammation in the brain (infiltration of T lymphocytes and macrophages in the parenchyma and meninges) when they were administered autologous B lymphoblastoid cell lines infected with a γ-herpesvirus pulsed *ex vivo* with MOG peptides (51).

In our study, immune cell aggregation developed in rabbit brains without overt signs of neurological deficits. Similarly, it has been reported that intranasal infection of 129/SvEv mice with rabies CVS-F3 does not result in neurological manifestations, despite the occurrence of neuroinflammation, BBB breakdown and the increased expression of the proinflammatory cytokines such as IL6 and TNFα (52). Importantly, cell aggregates formed only in some animals. Why only a fraction of infected animals developed CNS aggregates remain to be explored. However, our results suggest that EBV load in PBMCs may partly be linked to the formation of these structures. Additionally, host factors such as genetic background and the fitness of immune system to control viral infection are also likely to be important. HLA alleles are believed to interact with EBV to shape disease susceptibility in people with MS (53, 54), while peripheral EBV load is found to correlate positively with the MS risk allele HLA-DRB1*15 and negatively with the protective allele HLA-A*02 (55). Moreover, EBV latent protein EBNA2 is thought to interact with risk loci related to MS and other autoimmune diseases (56). Addressing the effect of HLA-DRB1*15, EBV infected humanized mice reconstituted with HLA-DR15+ immune system components were shown to exhibit poor control over the virus despite the increased activation and proliferation of T lymphocytes (57). Remarkably, some T lymphocytes from these animals were found to cross-react with the MBP (57).

Cell aggregates in the CNS of rabbits contained a heterogeneous cell population made up of brain resident cells, infiltrating macrophages, neutrophils and B and T lymphocytes. In general, aggregates were seen at dissimilar stages of evolution in a given section, and thus differed in composition. Most of the aggregates

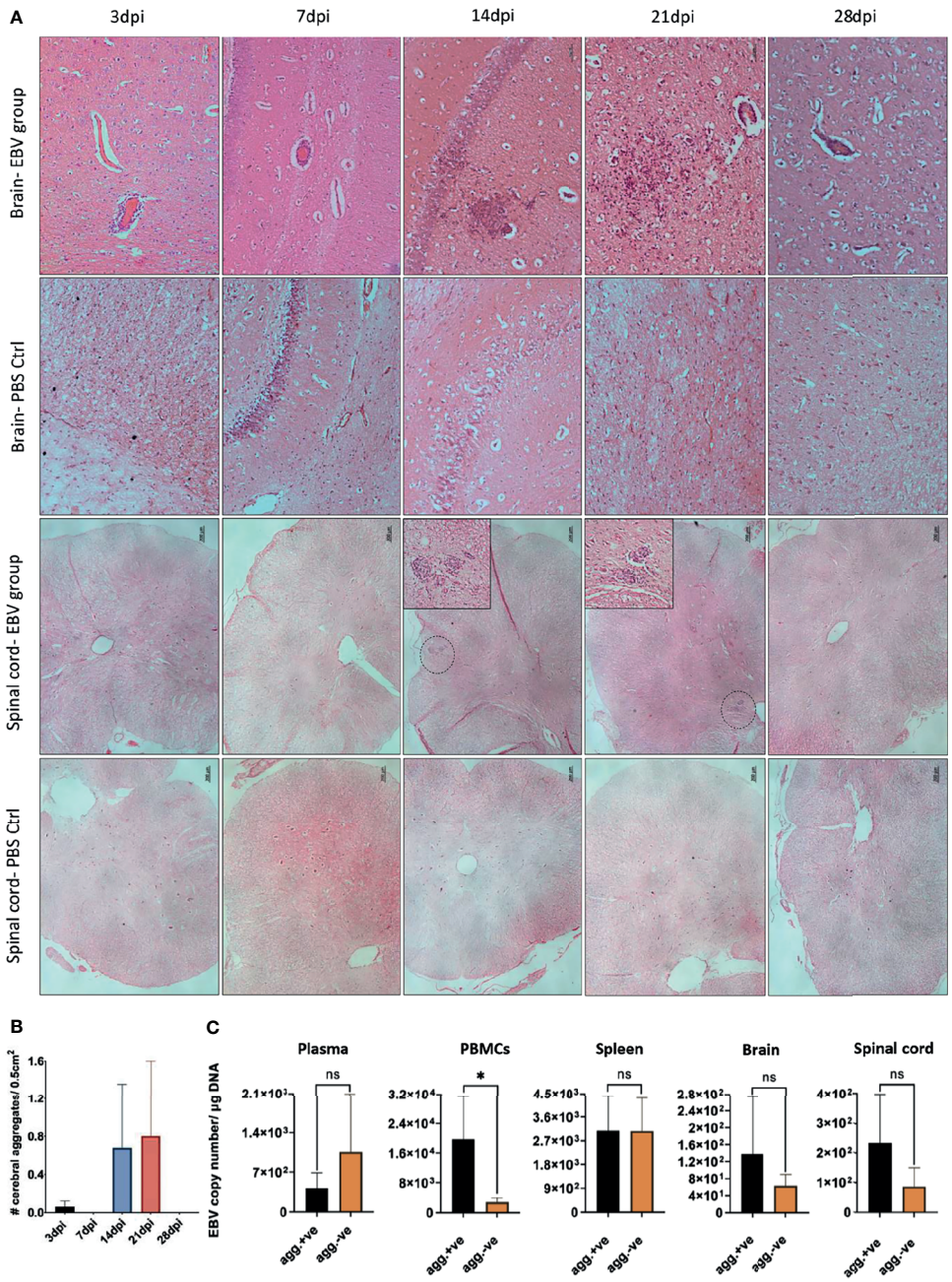

FIGURE 8 | CNS aggregate formation peaks at 14 and 21dpi. **(A)** Representative images of H&E staining of coronal brain sections (scale bar=50µm) and cross sections of spinal cord (scale bar=200µm) from EBV group and PBS control group (PBS ctrl) sacrificed at 3, 7, 14, 21 and 28dpi. **(B)** Quantification of cell aggregates in the brain. Aggregates were counted in one every 50 5µm-sections over a span of ~1000 brain sections from EBV infected animals sacrificed at 3, 7, 14, 21 and 28dpi (n=3 rabbits/time-point). Number of cell aggregates per 0.5cm^2 are displayed as mean ± SEM. Comparisons were made using the non-parametric Kruskal-Wallis test. **(C)** Comparison of EBV load in plasma, PBMCs, spleen, brain and spinal cord of animals with CNS aggregates and those without using two-tailed unpaired t test and Mann-Whitney test. ns: $p > 0.05$, $^*p \leq 0.05$.

had infiltrating macrophages as the prominent core surrounded by reactive astrocytes and dispersed lymphocytes. However, few aggregates lacked macrophage infiltration, but contained either a cluster of reactive glia or loosely connected lymphocytes. Brain-infiltrating T lymphocytes were mainly $CD8^+$ cells. The scarcity of $CD4^+$ cells within aggregates cannot be simply due to the effect of CsA, because the number of $CD4^+$ cells was also limited in aggregates formed in the EBV group. Only few $CD4^+$ cells were

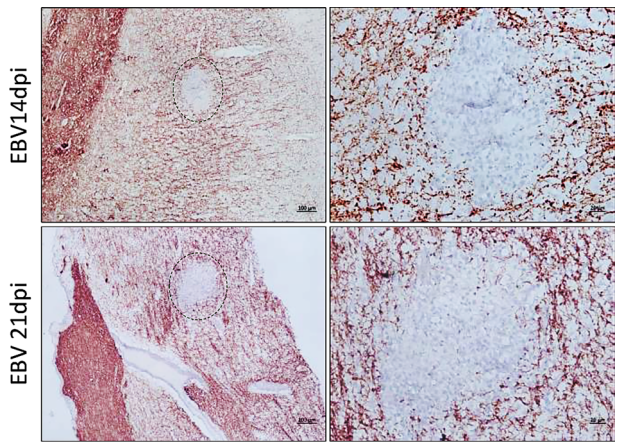

FIGURE 9 | Inflammatory aggregates in the EBV infected brain are free of myelinated nerve fibers. Brain sections were examined for demyelination in and around the aggregates by immunostaining for MBP. Demyelination was evident within aggregates developed at 14dpi and 21dpi. Scale bar at lower magnification=100μm. Scale bar at higher magnification=20μm.

scattered in the parenchyma. The data suggested that the contribution of $CD4^+$ cells to both CNS infiltration and aggregate formation was minimal.

Immune aggregates reminiscent of organized lymphoid structures were previously recognized in the meninges of MS brain, and have gained attention as a potential pathogenic feature of the disease (58, 59). In addition to MS, EBV infection has been associated with the formation and/or persistence of these immune aggregates (also known as ectopic lymphoid-like structures) in the inflamed tissue in certain organ-specific autoimmune diseases (9, 60–62). The ectopic lymphoid-like structures observed in meningeal inflammation in MS contained distinct clusters of $CD20^+$ B cells and $CD138^+$ plasma cells, intermingled with $CD35^+$ follicular dendritic cells and $CD3^+$ T cells (63). These structures expressed markers that determine the fate of B cells including CXCL13, CD27, and BAFF (25, 64). In rabbits, B lymphocytes contributed to the meningeal inflammation and aggregate formation in brain parenchyma. However, the cell organization and phenotypes observed in inflammation in rabbits did not mimic the typical organization of ectopic lymphoid-like structures reported in MS. B lymphocytes in the rabbit aggregates expressed proliferation marker PCNA, IgM, IgG and EBI2. Notably, EBI2 has been reported to be upregulated in activated T and B lymphocytes, and affects the movement of these cells (58–61). EBI2 expression by astrocytes was shown to promote the migration of macrophages (62). Moreover, the cellular aggregates observed in the rabbit CNS were entirely devoid of myelinated nerve fibers, suggesting that some form of demyelination was occurring within these aggregates. However, the underlying mechanisms for this demyelination remain to be further investigated.

We also observed that viral load peaked at day 14 post infection, both in the peripheral and CNS compartments. EBV load in peripheral blood, but not in CNS, correlated with aggregate formation. Small sample size could be one possible explanation for not seeing a significant difference in the CNS viral load between animals that developed aggregates and those that did not. Alternatively, it may be possible that the formation of aggregates is influenced by the expression of EBV transcripts and not the viral load. We noticed increased expression of IL6 mRNA and protein in aggregate positive brain at day 14 of infection. Moreover, IL6 expression strongly correlated with EBV-encoded EBERs. In agreement with our results, the viral load of Theiler's Murine Encephalomyelitis Virus (TMEV) in the CNS of mice has been shown not to correlate with the development of experimental autoimmune encephalitis (EAE) (65). Instead, disease outcome correlated well with immune response to viral components. Thus, virus trafficking into the CNS is not sufficient for the neuropathological changes to occur.

Another important finding from our rabbit model is the positive strong correlation between increased expression of viral latent transcripts, particularly the viral RNAs, EBERs, and the cytokines IL1β, IL6, and IL2 in the brain. By contrast, the lytic transcript BZLF1 could not be detected in the brain with or without aggregates at any of the time points, ruling out the role of the lytic cycle in inflammation. In some animals in EBV+CsA, however, BZLF1 was detected in few cells in the brain. On similar grounds, induction of EAE in mice infected with MHV-68 was shown to result in aggravated disease (66). The onset of disease course of EAE coincided with the virus establishing latency in mice, and not during the acute pre-latent infection. Mice that were infected with latency deficient MHV68 had significantly milder disease than those latently infected with the wild-type virus. The latent infection in mice was found to cause increased T lymphocyte infiltration into the CNS, and suppress the anti-inflammatory phenotype of T cells; regulatory T cells, both in the periphery and CNS (66).

We also observed a positive correlation between the expression of *EBNA1* and *EBNA2* and *TNFα* expression in the brain. A recent study reported that immunizing mice with EBNA1 amino acid region 411-426 led to neurological deficits reminiscent of EAE, and the development of MRI-confirmed cortical lesions (67). This region of EBNA1 was also found to trigger high antibody response in individuals with relapsing-remitting and secondary progressive MS, and these antibodies cross-reacted with MBP amino acid region 205-224 (67). Furthermore, EBV latent proteins were found to be upregulated in MS lesions (27). Virus reactivation in the MS brain was also associated with marked neuroinflammation and demyelination leading to fatal immune reconstitution inflammatory syndrome (68, 69). Our study and these reports support the hypothesis that transcriptionally active EBV in the brain promotes immunological alterations.

Additionally, we demonstrated elevated mRNA levels of IL1β and TNFα at the later stage of infection (28dpi). These Th1 cytokines (IL1β, and TNFα, IFNγ) were implicated in impaired BBB (52, 70–73). It has been suggested that virus infection of the CNS incites the generation of inflammatory cytokines, which in return compromises the integrity of BBB, for example by altering the expression of BBB tight junction proteins (74–77). Thus, BBB

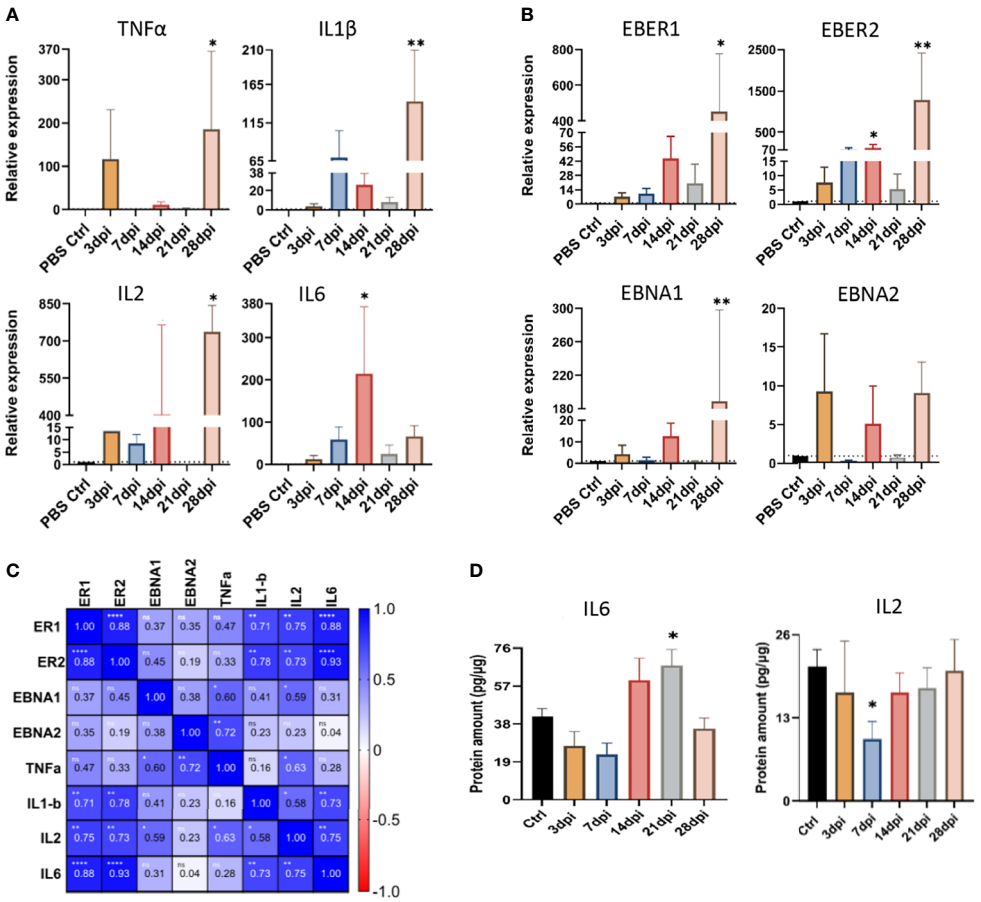

FIGURE 10 | Peripheral EBV infection results in altered expression of cytokines and viral transcripts in the brain. The expression of **(A)** cytokines (TNFα, IL1β, IL2, and IL-6), and **(B)** EBV latent transcripts (EBER1, EBER2, EBNA1 and EBNA2), in the brain tissue harvested at 3, 7, 14, 21, and 28dpi, examined using qPCR. Data displayed as mean ± SEM. Comparisons were made using one-way ANOVA with Fisher's LSD test or the nonparametric Kruskal-Wallis test. **(C)** Heat map of Spearman correlation of inflammatory and viral transcripts in the brain. Color mapping for positive and negative correlation are indicated in the legend on the right. ns: p >0.05, *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. **(D)** The levels of IL2 and IL6 proteins were measured by ELISA in brain tissues harvested at 3, 7, 14, 21 and 28dpi. Data displayed as mean ± SEM. Comparisons were made using one-way ANOVA with Fisher's LSD test or Welch and Brown-Forsythe test. *$p \leq 0.05$, **$p \leq 0.01$ compared to PBS controls. Bars without asterisks are not significantly different from the PBS controls.

breakdown could be a consequence of viral infection of the CNS (74, 76, 78). One could argue that increased mRNA levels of IL1β, and TNFα at 28dpi may be followed by increased BBB permeability and recurrent influx of immune cells into the CNS. Whether EBV infection disrupts BBB integrity warrants further investigation.

Another critical issue arising from this study is the need to determine antigen specificity of lymphoid infiltrates in the CNS. The functional characterization of virus-specific immune response could further explain the inflammatory response and identify the extent of the damage brought about by either virus infected cells or immune response directed against transcriptionally active virus (79). It has been shown that EBV-specific $CD8^+$ T cells make up ~0.5-2.5% of total brain-infiltrating $CD8^+$ T cells in MS (23). This frequency was found to be significantly higher than $CD8^+$ T cells reactive against MBP, CMV, or influenza virus. Further characterization of EBV-specific $CD8^+$ T cells showed the expression of degranulation marker CD107a, perforin, and granzyme B, indicating their cytotoxic nature (23).

5 CONCLUSIONS

In conclusion, our results support a neuropathogenic potential of EBV. The neuroinflammation and immunopathological aspects of EBV gleaned from the rabbit model will help us explore, otherwise poorly understood, viral-host interactions that can be essential for the pathogenesis of EBV-associated neuropathologies including MS. The flexibility of this model offers avenues to examine the CNS-periphery axis during viral infection, and to identify potential cofactors for EBV-associated neuropathology. Further studies are needed to determine the cellular behavior and events that are crucial in the formation of neuroinflammatory aggregates, the resulting

tissue damage, and the resolution of inflammation. Studying this cascade of events can provide us with an opportunity to critically evaluate potential and specific therapeutic targets that are essential to either halt the progression of EBV-associated neuropathologies or promote resolution of neuroinflammation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Ethics Committee of UAE University (Approval numbers: A-15-15; ERA-2018-5718).

AUTHOR CONTRIBUTIONS

Study conception and design was performed by GK. AH and NR performed animal experiments, sample collection, and data analysis and interpretation. SS provided technical guidance on histopathology examination, immunohistochemistry and immunofluorescence staining. AH and GK drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.764937/full#supplementary-material

REFERENCES

1. Vrzalikova K, Sunmonu T, Reynolds G, Murray P. Contribution of Epstein‐Barr Virus Latent Proteins to the Pathogenesis of Classical Hodgkin Lymphoma. *Pathogens* (2018) 7:59. doi: 10.3390/pathogens7030059
2. Long HM, Meckiff BJ, Taylor GS. The T-Cell Response to Epstein-Barr Virus-New Tricks From an Old Dog. *Front Immunol* (2019) 10:2193. doi: 10.3389/fimmu.2019.02193
3. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD. The Immunology of Epstein-Barr Virus-Induced Disease. *Annu Rev Immunol* (2015) 33:787–821. doi: 10.1146/annurev-immunol-032414-112326
4. Smatti MK, Al-Sadeq DW, Ali NH, Pintus G, Abou-Saleh H, Nasrallah GK. Epstein–Barr Virus Epidemiology, Serology, and Genetic Variability of LMP-1 Oncogene Among Healthy Population: An Update. *Front Oncol* (2018) 8:211. doi: 10.3389/fonc.2018.00211
5. Antsiferova O, Müller A, Rämer PC, Chijioke O, Chatterjee B, Raykova A, et al. Adoptive Transfer of EBV Specific CD8+ T Cell Clones can Transiently Control EBV Infection in Humanized Mice. *PLoS Pathog* (2014) 10:e1004333. doi: 10.1371/journal.ppat.1004333
6. Dunmire SK, Verghese PS, Balfour HH. Primary Epstein-Barr Virus Infection. *J Clin Virol* (2018) 102:84–92. doi: 10.1016/j.jcv.2018.03.001
7. Hedström AK, Huang J, Michel A, Butt J, Brenner N, Hillert J, et al. High Levels of Epstein-Barr Virus Nuclear Antigen-1-Specific Antibodies and Infectious Mononucleosis Act Both Independently and Synergistically to Increase Multiple Sclerosis Risk. *Front Neurol* (2019) 10:1368. doi: 10.3389/fneur.2019.01368
8. Bevan RJ, Evans R, Griffiths L, Watkins LM, Rees MI, Magliozzi R, et al. Meningeal Inflammation and Cortical Demyelination in Acute Multiple Sclerosis. *Ann Neurol* (2018) 84:829–42. doi: 10.1002/ana.25365
9. Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, et al. Dysregulated Epstein-Barr Virus Infection in the Multiple Sclerosis Brain. *J Exp Med* (2007) 204:2899–912. doi: 10.1084/jem.20071030
10. Tzartos JS, Khan G, Vossenkamper A, Cruz-Sadaba M, Lonardi S, Sefia E, et al. Association of Innate Immune Activation With Latent Epstein-Barr Virus in Active MS Lesions. *Neurology* (2012) 78:15–23. doi: 10.1212/WNL.0b013e31823ed057
11. Moreno MA, Or-Geva N, Aftab BT, Khanna R, Croze E, Steinman L, et al. Molecular Signature of Epstein-Barr Virus Infection in MS Brain Lesions. *Neurol Neuroimmunol Neuroinflamm* (2018) 5:e466. doi: 10.1212/NXI.0000000000000466
12. Lassmann H, Niedobitek G, Aloisi F, Middeldorp JM, Neuropromise EBV Working Group. Epstein-Barr Virus in the Multiple Sclerosis Brain: A Controversial Issue–Report on a Focused Workshop Held in the Centre for Brain Research of the Medical University of Vienna, Austria. *Brain* (2011) 134:2772–86. doi: 10.1093/brain/awr197
13. Liu Y, Zhang S, Li X, Liu E, Wang X, Zhou Q, et al. Peripheral Inflammation Promotes Brain Tau Transmission *via* Disrupting Blood-Brain Barrier. *Biosci Rep* (2020) 40:BSR20193629. doi: 10.1042/BSR20193629
14. Furutama D, Matsuda S, Yamawaki Y, Hatano S, Okanobu A, Memida T, et al. IL-6 Induced by Periodontal Inflammation Causes Neuroinflammation and Disrupts the Blood-Brain Barrier. *Brain Sci* (2020) 10:679. doi: 10.3390/brainsci10100679
15. Kang H-R, Cho H-J, Kim S, Song IH, Lee TS, Hwang S, et al. Persistent Infection of a Gammaherpesvirus in the Central Nervous System. *Virology* (2012) 423:23–9. doi: 10.1016/j.virol.2011.11.012
16. Khan G, Ahmed W, Philip PS, Ali MH, Adem A. Healthy Rabbits Are Susceptible to Epstein-Barr Virus Infection and Infected Cells Proliferate in Immunosuppressed Animals. *Virol J* (2015) 12:28. doi: 10.1186/s12985-015-0260-1
17. Ryan JL, Fan H, Glaser SL, Schichman SA, Raab-Traub N, Gulley ML. Epstein-Barr Virus Quantitation by Real-Time PCR Targeting Multiple Gene Segments: A Novel Approach to Screen for the Virus in Paraffin-Embedded Tissue and Plasma. *J Mol Diagnostics* (2004) 6:378–85. doi: 10.1016/S1525-1578(10)60535-1
18. Hassani A, Corboy JR, Al-Salam S, Khan G. Epstein-Barr Virus Is Present in the Brain of Most Cases of Multiple Sclerosis and may Engage More Than Just B Cells. *PLoS One* (2018) 13:e0192109. doi: 10.1371/journal.pone.0192109
19. Schnupf P, Sansonetti PJ. Quantitative RT-PCR Profiling of the Rabbit Immune Response: Assessment of Acute Shigella Flexneri Infection. *PLoS One* (2012) 7:e36446. doi: 10.1371/journal.pone.0036446
20. Wang C, Wang H, Zhang Y, Guo W, Long C, Wang J, et al. Berberine Inhibits the Proliferation of Human Nasopharyngeal Carcinoma Cells *via* an Epstein-

Barr Virus Nuclear Antigen 1-Dependent Mechanism. *Oncol Rep* (2017) 37:2109–20. doi: 10.3892/or.2017.5489
21. Tierney RJ, Shannon-Lowe CD, Fitzsimmons L, Bell AI, Rowe M. Unexpected Patterns of Epstein-Barr Virus Transcription Revealed by a High Throughput PCR Array for Absolute Quantification of Viral mRNA. *Virology* (2015) 474:117–30. doi: 10.1016/j.virol.2014.10.030
22. Archibald LK, Quisling RG. Central Nervous System Infections. In: *Textbook of Neurointensive Care*. London: Springer-Verlag (2013). p. 427–517. doi: 10.1007/978-1-4471-5226-2_22
23. Serafini B, Rosicarelli B, Veroni C, Mazzola GA, Aloisi F. Epstein-Barr Virus-Specific CD8 T Cells Selectively Infiltrate the Brain in Multiple Sclerosis and Interact Locally With Virus-Infected Cells: Clue for a Virus-Driven Immunopathological Mechanism. *J Virol* (2019) 93:e00980–19. doi: 10.1128/JVI.00980-19
24. Angelini DF, Serafini B, Piras E, Severa M, Coccia EM, Rosicarelli B, et al. Increased CD8+ T Cell Response to Epstein-Barr Virus Lytic Antigens in the Active Phase of Multiple Sclerosis. *PLoS Pathog* (2013) 9:e1003220. doi: 10.1371/journal.ppat.1003220
25. Serafini B, Severa M, Columba-Cabezas S, Rosicarelli B, Veroni C, Chiappetta G, et al. Epstein-Barr Virus Latent Infection and BAFF Expression in B Cells in the Multiple Sclerosis Brain: Implications for Viral Persistence and Intrathecal B-Cell Activation. *J Neuropathol Exp Neurol* (2010) 69:677–93. doi: 10.1097/NEN.0b013e3181e332ec
26. Magliozzi R, Serafini B, Rosicarelli B, Chiappetta G, Veroni C, Reynolds R, et al. B-Cell Enrichment and Epstein-Barr Virus Infection in Inflammatory Cortical Lesions in Secondary Progressive Multiple Sclerosis. *J Neuropathol Exp Neurol* (2013) 72:29–41. doi: 10.1097/NEN.0b013e31827bfc62
27. Veroni C, Serafini B, Rosicarelli B, Fagnani C, Aloisi F. Transcriptional Profile and Epstein-Barr Virus Infection Status of Laser-Cut Immune Infiltrates From the Brain of Patients With Progressive Multiple Sclerosis. *J Neuroinflamm* (2018) 15:18. doi: 10.1186/s12974-017-1049-5
28. Pruitt AA. Infections of the Cerebellum. *Neurologic Clinics* (2014) 32:1117–31. doi: 10.1016/j.ncl.2014.07.009
29. Joseph AM, Babcock GJ, Thorley-Lawson DA. EBV Persistence Involves Strict Selection of Latently Infected B Cells. *J Immunol* (2000) 165:2975–81. doi: 10.4049/jimmunol.165.6.2975
30. Hochberg D, Souza T, Catalina M, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Acute Infection With Epstein-Barr Virus Targets and Overwhelms the Peripheral Memory B-Cell Compartment With Resting, Latently Infected Cells. *J Virol* (2004) 78:5194–204. doi: 10.1128/JVI.78.10.5194-5204.2004
31. Torkildsen Ø, Stansberg C, Angelskår SM, Kooi E-J, Geurts JJG, van der Valk P, et al. Upregulation of Immunoglobulin-Related Genes in Cortical Sections From Multiple Sclerosis Patients. *Brain Pathol* (2010) 20:720–9. doi: 10.1111/j.1750-3639.2009.00343.x
32. Sargsyan SA, Shearer AJ, Ritchie AM, Burgoon MP, Anderson S, Hemmer B, et al. Absence of Epstein-Barr Virus in the Brain and CSF of Patients With Multiple Sclerosis. *Neurology* (2010) 74:1127–35. doi: 10.1212/WNL.0b013e3181d865a1
33. Willis SN, Stadelmann C, Rodig SJ, Caron T, Gattenloehner S, Mallozzi SS, et al. Epstein-Barr Virus Infection Is Not a Characteristic Feature of Multiple Sclerosis Brain. *Brain* (2009) 132:3318–28. doi: 10.1093/brain/awp200
34. Osborne AJ, Atkins HM, Balogh KK, Brendle SA, Shearer DA, Hu J, et al. Antibody-Mediated Immune Subset Depletion Modulates the Immune Response in a Rabbit (Oryctolagus Cuniculus) Model of Epstein-Barr Virus Infection. *Comp Med* (2020) 70:312–22. doi: 10.30802/AALAS-CM-20-000019
35. Okuno K, Takashima K, Kanai K, Ohashi M, Hyuga R, Sugihara H, et al. Epstein-Barr Virus can Infect Rabbits by the Intranasal or Peroral Route: An Animal Model for Natural Primary EBV Infection in Humans. *J Med Virol* (2010) 82:977–86. doi: 10.1002/jmv.21597
36. Kanai K, Takashima K, Okuno K, Kato K, Sano H, Kuwamoto S, et al. Lifelong Persistent EBV Infection of Rabbits With EBER1-Positive Lymphocyte Infiltration and Mild Sublethal Hemophagocytosis. *Virus Res* (2010) 153:172–8. doi: 10.1016/j.virusres.2010.07.026
37. Takashima K, Ohashi M, Kitamura Y, Ando K, Nagashima K, Sugihara H, et al. A New Animal Model for Primary and Persistent Epstein-Barr Virus Infection: Human EBV-Infected Rabbit Characteristics Determined Using Sequential Imaging and Pathological Analysis. *J Med Virol* (2008) 80:455–66. doi: 10.1002/jmv.21102
38. Abbott RJ, Pachnio A, Pedroza-Pacheco I, Leese AM, Begum J, Long HM, et al. Asymptomatic Primary Infection With Epstein-Barr Virus: Observations on Young Adult Cases. *J Virol* (2017) 91:e00382–17. doi: 10.1128/JVI.00382-17
39. Piriou E, Asito AS, Sumba PO, Fiore N, Middeldorp JM, Moormann AM, et al. Early Age at Time of Primary Epstein-Barr Virus Infection Results in Poorly Controlled Viral Infection in Infants From Western Kenya: Clues to the Etiology of Endemic Burkitt Lymphoma. *J Infect Dis* (2012) 205:906–13. doi: 10.1093/infdis/jir872
40. Dunmire SK, Grimm JM, Schmeling DO, Jr HHB, Hogquist KA. The Incubation Period of Primary Epstein-Barr Virus Infection: Viral Dynamics and Immunologic Events. *PLoS Pathog* (2015) 11:e1005286. doi: 10.1371/journal.ppat.1005286
41. Pender MP, Csurhes PA, Burrows JM, Burrows SR. Defective T-Cell Control of Epstein-Barr Virus Infection in Multiple Sclerosis. *Clin Transl Immunol* (2017) 6:e126. doi: 10.1038/cti.2016.87
42. Pender MP, Csurhes PA, Pfluger CM, Burrows SR. Deficiency of CD8+ Effector Memory T Cells is an Early and Persistent Feature of Multiple Sclerosis. *Mult Scler* (2014) 20:1825–32. doi: 10.1177/1352458514536252
43. Veroni C, Aloisi F. The CD8 T Cell-Epstein-Barr Virus-B Cell Trialogue: A Central Issue in Multiple Sclerosis Pathogenesis. *Front Immunol* (2021) 12:665718. doi: 10.3389/fimmu.2021.665718
44. van Langelaar J, Wierenga-Wolf AF, Samijn JPA, Luijks CJM, Siepman TA, van Doorn PA, et al. The Association of Epstein-Barr Virus Infection With CXCR3+ B-Cell Development in Multiple Sclerosis: Impact of Immunotherapies. *Eur J Immunol* (2021) 51:626–33. doi: 10.1002/eji.202048739
45. Soldan SS, Su C, Lamontagne RJ, Grams N, Lu F, Zhang Y, et al. Epigenetic Plasticity Enables CNS-Trafficking of EBV-Infected B Lymphocytes. *PLoS Pathog* (2021) 17:e1009618. doi: 10.1371/journal.ppat.1009618
46. Volk V, Theobald SJ, Danisch S, Khailaie S, Kalbarczyk M, Schneider A, et al. PD-1 Blockade Aggravates Epstein-Barr Virus+ Post-Transplant Lymphoproliferative Disorder in Humanized Mice Resulting in Central Nervous System Involvement and CD4+ T Cell Dysregulations. *Front Oncol* (2020) 10:614876. doi: 10.3389/fonc.2020.614876
47. Terry LA, Stewart JP, Nash AA, Fazakerley JK. Murine Gammaherpesvirus-68 Infection of and Persistence in the Central Nervous System. *J Gen Virol* (2000) 81:2635–43. doi: 10.1099/0022-1317-81-11-2635
48. Cho H-J, Kim S, Kwak S-E, Kang T-C, Kim H-S, Kwon H-J, et al. Age-Dependent Pathogenesis of Murine Gammaherpesvirus 68 Infection of the Central Nervous System. *Mol Cells* (2009) 27:105–11. doi: 10.1007/s10059-009-0011-5
49. Axthelm MK, Bourdette DN, Marracci GH, Su W, Mullaney ET, Manoharan M, et al. Japanese Macaque Encephalomyelitis: A Spontaneous Multiple Sclerosis-Like Disease in a Nonhuman Primate. *Ann Neurol* (2011) 70:362–73. doi: 10.1002/ana.22449
50. Govindan AN, Fitzpatrick KS, Manoharan M, Tagge I, Kohama SG, Ferguson B, et al. Myelin-Specific T Cells in Animals With Japanese Macaque Encephalomyelitis. *Ann Clin Transl Neurol* (2021) 8:456–70. doi: 10.1002/acn3.51303
51. Haanstra KG, Wubben JAM, Jonker M, 't Hart BA. Induction of Encephalitis in Rhesus Monkeys Infused With Lymphocryptovirus-Infected B-Cells Presenting MOG(34-56) Peptide. *PLoS One* (2013) 8:e71549. doi: 10.1371/journal.pone.0071549
52. Phares TW, Kean RB, Mikheeva T, Hooper DC. Regional Differences in Blood-Brain Barrier Permeability Changes and Inflammation in the Apathogenic Clearance of Virus From the Central Nervous System. *J Immunol* (2006) 176:7666–75. doi: 10.4049/jimmunol.176.12.7666
53. Teymoori-Rad M, Mozhgani S-H, Zarei-Ghobadi M, Sahraian MA, Nejati A, Amiri MM, et al. Integrational Analysis of miRNAs Data Sets as a Plausible Missing Linker Between Epstein-Barr Virus and Vitamin D in Relapsing Remitting MS Patients. *Gene* (2019) 689:1–10. doi: 10.1016/j.gene.2018.12.004
54. Nielsen TR, Rostgaard K, Askling J, Steffensen R, Oturai A, Jersild C, et al. Effects of Infectious Mononucleosis and HLA-DRB1*15 in Multiple Sclerosis. *Mult Scler* (2009) 15:431–6. doi: 10.1177/1352458508100037
55. Agostini S, Mancuso R, Guerini FR, D'Alfonso S, Agliardi C, Hernis A, et al. HLA Alleles Modulate EBV Viral Load in Multiple Sclerosis. *J Transl Med* (2018) 16:80. doi: 10.1186/s12967-018-1450-6
56. Harley JB, Chen X, Pujato M, Miller D, Maddox A, Forney C, et al. Transcription Factors Operate Across Disease Loci, With EBNA2

Implicated in Autoimmunity. *Nat Genet* (2018) 50:699–707. doi: 10.1038/s41588-018-0102-3
57. Zdimerova H, Murer A, Engelmann C, Raykova A, Deng Y, Gujer C, et al. Attenuated Immune Control of Epstein-Barr Virus in Humanized Mice Is Associated With the Multiple Sclerosis Risk Factor HLA-Dr15. *Eur J Immunol* (2021) 51:64–75. doi: 10.1002/eji.202048655
58. Mitsdoerffer M, Peters A. Tertiary Lymphoid Organs in Central Nervous System Autoimmunity. *Front Immunol* (2016) 7:451. doi: 10.3389/fimmu.2016.00451
59. Pikor NB, Prat A, Bar-Or A, Gommerman JL. Meningeal Tertiary Lymphoid Tissues and Multiple Sclerosis: A Gathering Place for Diverse Types of Immune Cells During CNS Autoimmunity. *Front Immunol* (2016) 6:657. doi: 10.3389/fimmu.2015.00657
60. Croia C, Serafini B, Bombardieri M, Kelly S, Humby F, Severa M, et al. Epstein-Barr Virus Persistence and Infection of Autoreactive Plasma Cells in Synovial Lymphoid Structures in Rheumatoid Arthritis. *Ann Rheum Dis* (2013) 72:1559–68. doi: 10.1136/annrheumdis-2012-202352
61. Cavalcante P, Serafini B, Rosicarelli B, Maggi L, Barberis M, Antozzi C, et al. Epstein-Barr Virus Persistence and Reactivation in Myasthenia Gravis Thymus. *Ann Neurol* (2010) 67:726–38. doi: 10.1002/ana.21902
62. Croia C, Astorri E, Murray-Brown W, Willis A, Brokstad KA, Sutcliffe N, et al. Implication of Epstein-Barr Virus Infection in Disease-Specific Autoreactive B Cell Activation in Ectopic Lymphoid Structures of Sjögren's Syndrome. *Arthritis Rheumatol* (2014) 66:2545–57. doi: 10.1002/art.38726
63. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of Ectopic B-Cell Follicles With Germinal Centers in the Meninges of Patients With Secondary Progressive Multiple Sclerosis. *Brain Pathol* (2004) 14:164–74. doi: 10.1111/j.1750-3639.2004.tb00049.x
64. Magliozzi R, Columba-Cabezas S, Serafini B, Aloisi F. Intracerebral Expression of CXCL13 and BAFF Is Accompanied by Formation of Lymphoid Follicle-Like Structures in the Meninges of Mice With Relapsing Experimental Autoimmune Encephalomyelitis. *J Neuroimmunol* (2004) 148:11–23. doi: 10.1016/j.jneuroim.2003.10.056
65. Myoung J, Bahk YY, Kang HS, Dal Canto MC, Kim BS. Anticapsid Immunity Level, Not Viral Persistence Level, Correlates With the Progression of Theiler's Virus-Induced Demyelinating Disease in Viral P1-Transgenic Mice. *J Virol* (2008) 82:5606–17. doi: 10.1128/JVI.02442-07
66. Casiraghi C, Citlali Márquez A, Shanina I, Steven Horwitz M. Latent Virus Infection Upregulates CD40 Expression Facilitating Enhanced Autoimmunity in a Model of Multiple Sclerosis. *Sci Rep* (2015) 5:13995. doi: 10.1038/srep13995
67. Jog NR, McClain MT, Heinlen LD, Gross T, Towner R, Guthridge JM, et al. Epstein Barr Virus Nuclear Antigen 1 (EBNA-1) Peptides Recognized by Adult Multiple Sclerosis Patient Sera Induce Neurologic Symptoms in a Murine Model. *J Autoimmun* (2020) 106:102332. doi: 10.1016/j.jaut.2019.102332
68. Serafini B, Scorsi E, Rosicarelli B, Rigau V, Thouvenot E, Aloisi F. Massive Intracerebral Epstein-Barr Virus Reactivation in Lethal Multiple Sclerosis Relapse After Natalizumab Withdrawal. *J Neuroimmunol* (2017) 307:14–7. doi: 10.1016/j.jneuroim.2017.03.013
69. Serafini B, Zandee S, Rosicarelli B, Scorsi E, Veroni C, Larochelle C, et al. Epstein-Barr Virus-Associated Immune Reconstitution Inflammatory Syndrome as Possible Cause of Fulminant Multiple Sclerosis Relapse After Natalizumab Interruption. *J Neuroimmunol* (2018) 319:9–12. doi: 10.1016/j.jneuroim.2018.03.011
70. Wong D, Dorovini-Zis K, Vincent SR. Cytokines, Nitric Oxide, and cGMP Modulate the Permeability of an *In Vitro* Model of the Human Blood-Brain Barrier. *Exp Neurol* (2004) 190:446–55. doi: 10.1016/j.expneurol.2004.08.008
71. Förster C, Burek M, Romero IA, Weksler B, Couraud P-O, Drenckhahn D. Differential Effects of Hydrocortisone and Tnfα on Tight Junction Proteins in an *In Vitro* Model of the Human Blood–Brain Barrier. *J Physiol* (2008) 586:1937–49. doi: 10.1113/jphysiol.2007.146852
72. Tsao N, Hsu HP, Wu CM, Liu CC, Lei HY. Tumour Necrosis Factor-α Causes an Increase in Blood-Brain Barrier Permeability During Sepsis. *J Med Microbiol* (2001) 50:812–21. doi: 10.1099/0022-1317-50-9-812
73. Daniels BP, Holman DW, Cruz-Orengo L, Jujjavarapu H, Durrant DM, Klein RS. Viral Pathogen-Associated Molecular Patterns Regulate Blood-Brain Barrier Integrity *via* Competing Innate Cytokine Signals. *mBio* (2014) 5:e01476–01414. doi: 10.1128/mBio.01476-14
74. Chai Q, He WQ, Zhou M, Lu H, Fu ZF. Enhancement of Blood-Brain Barrier Permeability and Reduction of Tight Junction Protein Expression Are Modulated by Chemokines/Cytokines Induced by Rabies Virus Infection. *J Virol* (2014) 88:4698–710. doi: 10.1128/JVI.03149-13
75. Bonney S, Seitz S, Ryan CA, Jones KL, Clarke P, Tyler KL, et al. Gamma Interferon Alters Junctional Integrity *via* Rho Kinase, Resulting in Blood-Brain Barrier Leakage in Experimental Viral Encephalitis. *mBio* (2019) 10:e01675–19. doi: 10.1128/mBio.01675-19
76. Li F, Wang Y, Yu L, Cao S, Wang K, Yuan J, et al. Viral Infection of the Central Nervous System and Neuroinflammation Precede Blood-Brain Barrier Disruption During Japanese Encephalitis Virus Infection. *J Virol* (2015) 89:5602–14. doi: 10.1128/JVI.00143-15
77. Minagar A, Long A, Ma T, Jackson TH, Kelley RE, Ostanin DV, et al. Interferon (IFN)-Beta 1a and IFN-Beta 1b Block IFN-Gamma-Induced Disintegration of Endothelial Junction Integrity and Barrier. *Endothelium* (2003) 10:299–307. doi: 10.1080/10623320390272299
78. Cain MD, Salimi H, Gong Y, Yang L, Hamilton SL, Heffernan JR, et al. Virus Entry and Replication in the Brain Precedes Blood-Brain Barrier Disruption During Intranasal Alphavirus Infection. *J Neuroimmunol* (2017) 308:118–30. doi: 10.1016/j.jneuroim.2017.04.008
79. Pender MP, Csurhes PA, Smith C, Douglas NL, Neller MA, Matthews KK, et al. Epstein-Barr Virus-Specific T Cell Therapy for Progressive Multiple Sclerosis. *JCI Insight* (2018) 3:e124714. doi: 10.1172/jci.insight.124714

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PERSPECTIVE
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The Multiple Sclerosis Prodrome: Evidence to Action

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A growing body of work points toward the existence of a clinically symptomatic prodromal phase in multiple sclerosis (MS) that might span 5–10 years or more. A prodrome is an early set of signs or symptoms predating the onset of classical disease, which in turn predates a definitive diagnosis. Evidence for a prodromal phase in MS could have major implications for prevention, earlier recognition and treatment, as well as an improved disease course or prognosis. This Perspective provides a succinct overview of the recent advances in our understanding of the MS prodrome and current key challenges. Many of the MS prodromal features characterized thus far are non-specific and are common in the general population; no single feature alone is sufficient to identify an individual with prodromal MS. Biomarkers may increase specificity and accuracy for detecting individuals in the MS prodromal phase, but are yet to be discovered or formally validated. Progress made in the elucidation of prodromal phases in other neurological and immune-mediated diseases suggests that these barriers can be overcome. Therefore, while knowledge of a prodromal phase in MS remains nascent, how best to move from the rapidly growing evidence to research-related action is critical. Immediate implications include refining the concept of the MS continuum to include a prodromal phase. This will help inform the true "at risk" period when considering exposures that might cause MS. Major long-term implications include the earlier recognition of MS, improved prognosis, through earlier disease management, and the future possibility of MS disease prevention.

Keywords: multiple sclerosis, prodrome, preclinical, prevention, risk

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INTRODUCTION

A prodrome is an early set of signs or symptoms predating the onset of classical disease (1), which in turn predates a definitive diagnosis. Until recently, it was thought that multiple sclerosis (MS) did not have a prodromal period (1, 2), even though prodromal phases are well-recognized in other neurological and immune-mediated chronic conditions (3–6). While the prodrome remains a nascent field in MS, understanding the nature of the prodrome is critical in defining the etiologically relevant period when searching for risk factors for MS. Future applications may also include identification of individuals at risk of MS and enhanced opportunity for early management of disease. This Perspective Article summarizes the current state of knowledge of the MS prodrome, with a focus on the actionable evidence. Together with reflections on lessons learned from other chronic disease fields will help pave the path forwards to effect meaningful change in MS.

BOX 1 | Search strategy and selection criteria

References for this article were identified by searching PubMed for journal articles published in English, with a focus on the last 5 years, using the following terms (and alternative spellings): *multiple sclerosis, prodrome, prodromal phase, pre-clinical, risk factor, RIS, CIS*. In addition, the reference lists of articles were reviewed along with the authors' own files and the most relevant articles were included within the article. The primary focus (selection criteria) were for peer-reviewed journal articles (original observational case-control, cohort or intervention studies, or other reviews of original work). Case reports and case series were excluded. Select older studies representing landmark advances were included, as necessary, in order to place current findings in context.

We focus here on the most recent literature, and studies not covered in detail in prior relevant articles **(Box 1)** (2, 7–10). We also include a brief overview of the most important or landmark findings to date, thus providing context to this rapidly emerging field. Each section concludes with a synopsis of the potential *actionable evidence*, thus providing an outline of how the field should harness knowledge of the MS prodrome to effect change, both now and in the future. Finally, we propose a refined timeline for MS, conceptualized as a continuum, which includes the prodromal phase (**Figure 1**).

BOX 2 | Identifying the MS prodromal phase

Definition of a prodrome: an early set of signs or symptoms related to a disease, but predating the onset of classical symptoms, which in turn predates a definitive diagnosis.

The challenge: identifying the onset of classical disease can be difficult and differs across studies. For the purposes of this article, we have summarized the most common used below, and indicate what the timing (date) of each likely represents:

• *MS symptom onset*: typically recorded by a MS neurologist in a patient's medical record and is based on a careful medical history.

Represents the closest to actual classical onset of MS, based on current knowledge.

• First *demyelinating diagnostic code*: typically captured in health administrative data (from hospital or physician billing records) or in electronic medical records.

Represents the first formal medical recognition of a demyelinating event.

• First *MS diagnostic code* (e.g., International Classification of Diseases (ICD)-9/10 340 or G35, or Read codes): typically captured as for a first demyelinating diagnostic code.

Represents the first formal medical recognition of MS.

For the purposes of this article, *'classical MS onset'* is used to refer to either MS symptom onset or a first demyelinating code, as needed (e.g., to describe studies that used both to determine the end of the possible prodromal phase). MS symptom onset is arguably the closest possible to classical MS onset, thus enabling studies to avoid capturing the period between classical MS onset and diagnosis. This period, while of interest, should not be considered part of the prodromal phase.

THE MS PRODROME: KEY FINDINGS

The MS Prodrome: Clinical Aspects and Potential Duration

The last 5 years have seen the emergence of population-based studies which objectively measured signs and symptoms occurring before classical MS onset (8, 11–18). Importantly, the designs of these studies minimize the potential for both selection and recall bias. Collectively these studies suggest that an MS prodromal phase is detectable at least 5 years before MS symptom onset (or 10 years before a first MS diagnostic code), and possibly up to 20 years in persons who develop primary progressive (PP) MS (11–18). Studies in persons with radiologically isolated syndrome (RIS) suggest that the prodromal phase is of variable duration and may begin as early as 10–15 years before MS symptom onset (19, 20).

A myriad of signs and symptoms have been identified as more common during the years leading up to MS (defined by various studies as MS symptom onset or a first demyelinating code or a MS diagnostic code, **Box 2**), as compared to persons without MS, and range from cognitive deficits, to psychiatric morbidity, fatigue, sleep disorders, pain, fibromyalgia, bowel/bladder and dermatological issues (8, 11–18). In young men, aged 18 or 19 years old, entering the Norwegian military, lower cognitive scores were found in the 2 years before MS symptom onset, relative to those who did not develop MS ($\Delta = 0.80$, 95% CI: 0.20–1.41, $p = 0.0095$, equivalent to a 6 IQ-point difference) (18). The mental health burden in the 5 years before a first demyelinating code or MS symptom onset, was measurable as ≈50% more visits to psychiatrics and ≈50% more mood disorder claims (based on physician-derived diagnostic ICD codes). Based on general practitioners records, depression may be more common up to 10 years before the first recorded MS or demyelinating diagnostic code (17). Intriguingly, the prodromal phase in children (first demyelinating diagnostic code <18 years of age) may have a negative impact on the mental health burden of their mothers; a possibility raised in one study (21). While the role of stress as a risk factor for MS onset remains unclear (22, 23), if a stressful event could trigger MS and also lead to mental-health related issues, this could provide an alternative explanation for findings. Finally, asymptomatic women at high ($n = 27$) vs. low ($n = 20$) risk of developing MS, based on a genes-environment score, exhibited poor vibration perception in their great toe [mean = 2.48 (SD: 0.60) vs. 1.83 (SD: 0.54), $p = 0.008$, age, height and test date adjusted] (24). Whether this represents a potential clinical sign of the MS prodrome is intriguing, but remains to be determined (24).

Patient Characteristics and the MS Prodrome

There is little research on whether the clinical presentation of the MS prodrome differs by age, sex, or the subsequent disease course (12, 16, 18). Current evidence suggests that pain is more evident in older adults while anemia is more pronounced in men, in the 5-years before a first demyelinating diagnostic code. The odds of pain increased from 1.76 (95% CI: 1.49–2.06) in those aged <30 years at their first event to 2.35 (95% CI: 2.13–2.60) in those ≥50 years (12), compared to matched controls without MS. The odds of anemia in men was higher [odds ratio

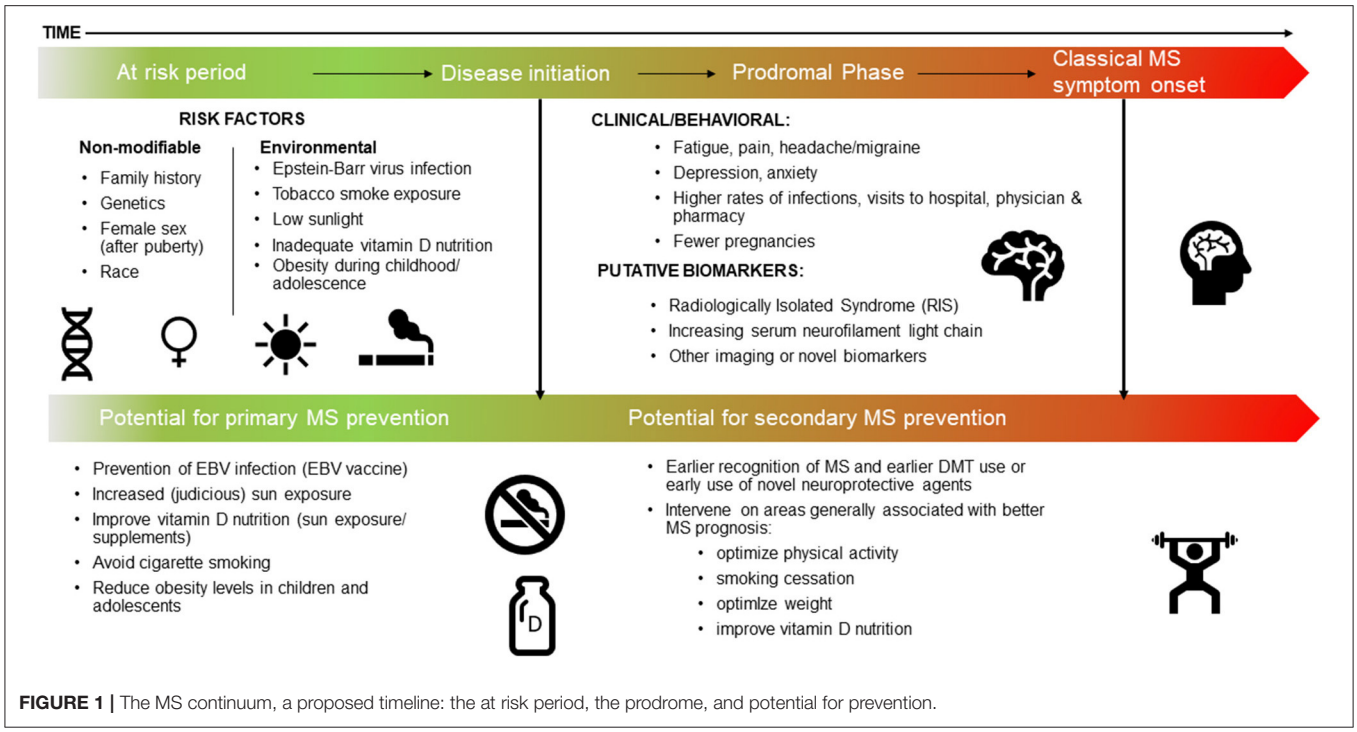

FIGURE 1 | The MS continuum, a proposed timeline: the at risk period, the prodrome, and potential for prevention.

(OR) 2.40; 95% CI: 1.68–4.29] than in women (OR: 1.23; 95% CI: 1.04–1.45), as compared to the general population. The sex-differences for anemia could simply reflect the higher prevalence of anemia among women, resulting in the lower relative estimate than men (12). Why anemia was more common for both men and women with MS during the prodrome is less clear. Findings could result from MS-related symptoms, such as fatigue, leading to an increase in detection of anemia among persons with MS (12). Intriguingly, recent work has suggested that red blood cells are active participants in the body's immune response (25), such that the inflammatory processes of MS could lead to a reduction in circulating red blood cells, leading to anemia.

Of the limited studies where disease course was examined (16, 18), those with either PP or relapsing-onset MS appeared to exhibit broadly similar prodromal features, with a notable exception for dermatological issues (16). In the 5 years before MS symptom onset, PP relative to relapsing-onset MS cases exhibited 47% lower rates of visits to dermatologists (rate ratio: 0.53; 95% CI: 0.30–0.96). Skin-related manifestations are recognized as relatively common in other immune-mediated diseases (26). Thus, whether these observations in MS indicate that early markers of inflammation differ by disease course, being lower in PP-onset MS cases is an intriguing possibility. Further, findings from the Norwegian military cohort suggest a much longer prodromal phase in PPMS; lower cognitive scores were measurable up to 20 years before PPMS symptom onset, relative to 2-years for the RRMS cases (21). For the PPMS cases, this was equivalent to 4.6-7 IQ-point difference compared to the control men who did not develop MS, $p = 0.045$ (21). While all these findings are interesting, confirmation in other, ideally larger populations is needed. Finally, no study to date has examined socio-demographic factors (e.g., race/ethnicity, socio-economic status, education or related health inequities), despite evidence that these are associated with MS outcomes after diagnosis (27, 28).

Misdiagnosis and Missed Opportunity

Evidence of potential misdiagnoses and missed opportunities for earlier recognition of MS is also apparent across studies. For example, for individuals who developed PPMS, a higher rate of nervous system-related physician claims (ICD codes) in the 5 years before MS symptom onset was observed compared to relapsing-onset MS (rate ratio = 3.00; 95% CI: 1.06–8.49) (16). This may, in part, represent a delay in medical recognition which is not uncommon, particularly in PPMS (29, 30). Others have explored the issue of missed opportunities for earlier recognition by examining ambulatory care records in the years before a first MS diagnostic code (ICD 340) in a subgroup of patients with no record of a CIS and found that many physician visits in these patients before MS diagnosis were, in hindsight, likely a demyelinating event (31). These studies provide further evidence that earlier recognition of MS may be possible (31, 32).

Actionable Evidence

Together, these studies demonstrate that clinical features suggestive of an MS prodrome can be objectively measured at the population-level. Clearly, many of the MS prodromal features identified are also non-specific and common in the general population; no single feature alone will be sufficient to identify an individual with prodromal MS. Findings also suggest

that an iterative approach is required; as earlier recognition and diagnosis of MS is achieved, then this could refine understanding of the MS prodrome. Thus, there is sufficient evidence to warrant further investment of resources and research funds in this area. The Table provides key examples. One low-cost, but valuable endeavor would be to re-evaluate previous studies for signs and symptoms suggestive of the MS prodrome.

PUTATIVE BIOMARKERS OF THE MS PRODROME

Given the wide range of common and non-specific clinical symptoms observed at the population-level before the onset of MS symptoms, biomarkers for prodromal MS would be tremendously helpful. Such biomarkers could increase specificity and accuracy for identifying individuals in the MS prodrome.

Neuroimaging and the Radiologically Isolated Syndrome

One potential biomarker is abnormal neuroimaging, such as in people with RIS. RIS is the clinical syndrome in which individuals underwent MRI scans of the brain for reasons other than suspected MS, resulting in an MRI finding suggestive of MS (i.e., this was an unexpected or incidental finding) (33). Formal criteria for RIS were proposed in 2009, which require that MRI findings meet the 2005 MRI criteria for dissemination in space (33). RIS differs from MS in that no classical MS symptoms are present. While some people with RIS are asymptomatic (e.g., they were participants in a research study), it can be inferred from the indications for obtaining MRIs that many have one or more non-specific symptoms, some of which potentially overlap with those of an MS prodrome. Such symptoms include mood disorders and, most commonly, headache (19, 20, 33). A substantial proportion of individuals with RIS (34% within 5 years and 51% within 10 years) subsequently developed a typical symptom of MS in sizeable multi-site studies (19, 20). While headache was not associated with an increased risk of subsequent clinical demyelination in one such study, the risk associated with other symptoms remains unknown (16). The precise relationship between RIS and an MS prodrome needs to be better understood, including whether they are distinct entities, overlapping entities, and/or part of a continuum (**Figure 1**). Given the possibility of overlap between potential symptoms of the MS prodrome (which commonly occur in the general population) and the non-specific symptoms reported in many people with RIS, RIS may emerge as being associated with prodromal MS. This possibility also provides rationale for exploring other neuroimaging biomarkers for the MS prodrome.

Advanced Neuroimaging Techniques

Advanced neuroimaging techniques, when studied in the context of RIS, may also be useful for identifying biomarkers of the MS prodrome. For instance, regional (cerebellum and thalamus), and whole brain volumes were generally lower in individuals with RIS compared to controls (34–37). One study found that cortical volumes were similar in individuals with RIS (n = 19) and MS (n = 26), but were lower in these 45 individuals together as compared to 21 controls (38). In those with RIS, lower cortical volumes correlated with reduced performance on cognitive testing, suggesting an important functional association with a potential prodromal symptom. Other case-control studies have shown microstructural changes in brain white matter using diffusion tensor imaging and altered metabolic pathways in individuals with RIS using brain proton magnetic resonance spectroscopy suggesting their potential utility (39, 40).

Brain white matter lesions on MRI commonly occur for reasons other than demyelinating pathology. Therefore, there is a need for biomarkers specifically for the white matter lesions due to MS. For example, central veins occurred more frequently in white matter lesions (detected on MRI using FLAIR* at 3T) in individuals with MS as compared to those with migraine in one study (41). Various definitions of the "central vein sign" also distinguished individuals with CIS and/or MS from those with other conditions (42, 43). It would be of value for future studies to determine whether central veins within MRI lesions are associated with increased risk for the subsequent development of clinical MS in people with RIS who also present with various symptoms, currently considered non-specific. Paramagnetic rims around lesions may also be a novel MRI biomarker of value during the MS prodrome (44).

Serum, CSF and Other Opportunities for Biomarker Discovery

Given that the pathobiology of MS has presumably started before the prodromal phase, exploring biomarkers associated with neuronal injury and loss, such as neurofilament light chain (NfL), while not specific to MS, may be useful for the prodrome. In a nested case-control study of US military personnel, serum NfL levels were elevated in 30 individuals who subsequently developed MS as compared to 30 matched controls (median 16.7 vs. 15.2 pg/mL, $p = 0.04$) (45). Serum samples were obtained a median of 6 years before MS symptom onset in cases.

While less easily acquired than serum, CSF is often obtained in the diagnostic workup of individuals with suspected MS and it is therefore worthwhile to consider potential CSF biomarkers for the MS prodrome. In a study of 75 individuals with RIS, both unique CSF oligoclonal bands and elevated CSF NfL level were associated with the earlier development of CIS (hazard ratios 14.7, 95% CI: 1.8–120.2; $p = 0.012$ and 1.02, 95% CI: 1.00–1.04; $p = 0.019$, respectively) (46). Preliminary studies suggest that novel CSF analyses including single cell RNA sequencing may also hold promise. In one study, single cell analyses discriminated between the CSF immune profiles of twins discordant for MS (47). Other emerging evidence indicates that the gut microbiome may be altered in MS, suggesting another potential biomarker of the prodrome (48). Other potential molecular biomarkers include serum/CSF glial fibrillary acidic protein, and serum-based micro-ribonucleic acids (miRNAs) (49–51). Finally, abnormal visual evoked responses (52, 53) and optical coherence tomography (54) may be biomarkers associated with abnormalities in the visual pathways.

Actionable Evidence

Together these findings suggest that there may be measurable biomarkers for the MS prodrome including those in serum, CSF, and on MRI that warrant further study (see **Table 1**). Exploration of biomarkers for the MS prodrome will likely result in an improved understanding of the pathology of MS itself as many of these biomarkers reflect underlying pathophysiological mechanisms.

RISK FACTOR (TRIGGER) OR PRODROMAL FEATURE?

The duration of the MS prodrome can be defined as the time between the initiation of MS pathology and the appearance of the classical clinical demyelinating events that eventually lead to an MS diagnosis (**Figure 1**). Knowledge of this period is critical to identify true causal risk factors for MS. Many environmental exposures assessed after the MS disease process begins may not be an accurate representation of the pre-pathological onset exposure. For example, during the prodromal phase, general feelings of unwellness may lead to changes in diet or physical activity, and any associations observed are more likely to be due to "reverse causation" and not a true causal risk factor.

There are currently four environmental risk factors for MS that evidence suggests may have a causal role in MS development: infection with Epstein-Barr virus (EBV), low sunlight exposure/low serum vitamin D levels, obesity in early life, and cigarette smoking (56). One necessary determinate of causality is temporality—i.e., the exposure must occur before the initiation of the disease process. While there is evidence supporting temporality for each of these factors based on childhood/adolescent exposure being associated with future MS risk, a closer look at the lower risk of MS with higher serum 25-hydroxyvitamin D (25(OH)D) levels is illustrative of the complexity of whether a risk factor is a trigger or prodromal feature. There have been four prospective studies of serum 25(OH)D measured in samples collected on average 5, (57) 8, (58) and 9 (59, 60) years before MS symptom onset with an overall range of less than one to up to 32 years and all found an inverse association between higher 25(OH)D levels and risk of MS onset. That the average time of sample collection before MS onset in these studies falls within 10 years before MS symptom onset, and the possibility that 25(OH)D levels decline during a prodromal phase (e.g., if an individual begins sun avoidance behaviors due to not feeling well), reverse causation cannot be ruled out as a possible explanation on the basis of these results alone. Results of two studies of 25(OH)D levels during pregnancy or at birth and future risk of MS in the offspring found that deficient serum vitamin D levels in mothers or in dried blood spots from neonates were associated with an increased risk of MS onset in the child (61, 62), and case-control studies of sun exposure have consistently found an inverse association between higher sun exposure in childhood/adolescence and lower MS risk (56, 63–65). Additionally, Mendelian randomization studies have found that genetically lower 25(OH)D is associated with an increased risk of MS in adults and children (66–68). Together, these studies suggest that exposure to low vitamin D levels may pre-date the onset of the prodromal phase and be a true risk factor for MS. EBV infection is also a risk factor for MS and the evidence for infection occurring prior to the onset of MS, and the prodromal phase, is strong (56). Individuals who are EBV seronegative have a near zero risk of having MS, and a prospective study among EBV seronegative young adults found the risk of MS increased only after infection with EBV (69). There was no increase in risk of MS with infection of cytomegalovirus (as measured serologically) over the same time period (69), suggesting the association is EBV specific rather than a general increased risk of infections.

Studies of other risk factors that have been measured within the presumed prodromal phase, i.e., within 5–10 years of MS symptom onset, include migraines, lower levels of physical activity, diet quality, pregnancy and oral contraceptive use (70–73). Pregnancy, for example, has been associated with a decreased MS risk, while oral contraceptive use associated with an increased risk in some studies (73), but studies of the MS prodromal phase suggest that women who develop MS may choose birth control or delay pregnancy simply because they are experiencing signs and symptoms of the prodromal phase (14). Similarly with diet quality before MS symptom onset, no association with MS risk was found, but if individuals make dietary improvements in response to prodromal sign and symptoms, reverse causation may be one explanation (70). Defining the true time of MS onset and studying exposures before that time is critical in teasing apart risk factors from prodromal features.

Actionable Evidence

Given the evidence that prodromal MS may precede MS symptom onset by 5 or more years, a review and re-evaluation of the MS environmental risk factor literature should be conducted to determine whether any associations (null or otherwise) may be explained by the exposure being measured in the presumed prodromal phase rather than before. Further, future study designs of environmental risk factors of MS need to factor in the time of a possible prodromal phase, assessing exposure at multiple time points prior—perhaps up to 10 years or more–to MS symptom onset, though this is not without challenges.

FUTURE PERSPECTIVES

Evidence for a prodromal phase of MS has major implications for prevention, earlier recognition and diagnosis of MS, as well as improved disease prognosis. Immediate implications include refining the conception of a timeline for MS that includes a prodromal phase as part of the MS continuum (**Figure 1**). This will help inform the true "at risk" period when considering risk factors that might trigger or cause disease initiation and onset of MS. As our understanding of the possible duration of the MS prodrome is refined, this will provide further clarity and advance capacity to potentially prevent MS though interventions implemented before disease initiation and the onset of clinical MS (that is during a "true" risk factor phase). Of note, it is feasible that there will be overlap between risk factors for MS

TABLE 1 | Actionable evidence and the MS prodrome: from general to specific examples.

Clinical aspects of the MS prodrome	
Evidence	Clinical features of the MS prodrome can be objectively measured 5–10 years before classical MS onset (8, 11–18).
Action	*Invest further resources to support research of the clinical features of the MS prodrome*
	Systematically re-evaluate previous studies for signs and symptoms suggestive of the MS prodrome
	Determine what the potential duration of the MS prodrome is, and whether, and how this differs across populations and within specific patient groups
	Refine the proposed timeline for MS, conceptualized as a continuum, to include the prodromal phase, see Figure (i.e., the at risk phase precedes disease initiation, which is then followed by the prodromal phase, classical MS symptom onset and diagnosis)
Evidence	At the population-level, specific clinical features (often derived from health administrative data or medical records) are more common for MS cases at least 5 years before MS symptom onset, and possibly up to 20 years in PP MS, relative to population controls (12–14, 16, 18).
Action	*Greater granularity is required to capture subtle signs and symptoms of the MS prodrome that do not necessarily prompt medical attention*
Evidence	Features of the MS prodrome may differ by sex, age and disease course (12, 16, 18)
Action	*Investigate how patient characteristics may influence presentation of the MS prodrome (e.g., age, sex, socio-economic status, race/ethnicity/culture, health inequity) and how features of the MS prodrome may differ by subsequent disease course*
Evidence	Some clinical features captured before MS symptom onset (16), or a first MS diagnostic code (31), are suggestive of misdiagnoses and missed opportunity for prompt recognition and earlier appropriate MS diagnosis
Action	*Need to better understand why and when these are occurring and if amenable to change, resulting in improved outcomes for people with MS*
	Iterative approach required; earlier recognition and diagnoses of MS will refine understanding of the MS prodrome
Biomarkers of the MS prodrome	
Evidence	Many individuals with radiologically isolated syndrome have symptoms that overlap with prodromal MS and subsequently develop classical MS symptom onset (19, 20, 33)
Action	*Determine the relationship between radiologically isolated syndrome and the MS prodrome*
	Other neuroimaging biomarkers for the MS prodrome warrant study
Evidence	Serum neurofilament light is elevated up to 6 years before MS symptom onset (45)
Action	*Establish whether other biomarkers in the CSF and serum as well as novel biomarkers, such as the composition of the gut microbiome, may be measurable prior to, and are associated with, subsequent classical MS symptom onset*
Risk factor (trigger) or prodromal feature?	
Evidence	Many studies focus on exposures during the few years before reported MS symptoms onset or MS diagnosis
Action	*Re-evaluate the MS environmental risk factor literature to determine whether associations may be due to exposure being measured in the presumed prodromal phase rather than the true "at risk" period*
	Future study designs of environmental risk factors need to factor in the timing of a possible prodromal phase
Prodromal phases in other diseases and implications for MS	
Evidence	Criteria exist to identify other prodromal diseases; e.g., validated research criteria to identify likely prodromal Parkinson's disease (55)
Action	*Examine the feasibility (including key gaps in knowledge), and the acceptability of developing research criteria for prodromal MS*
	Develop research criteria to identify the probability of a person having prodromal MS

and features of the MS prodrome. For example, it is reasonable to expect serum vitamin D to be low during the prodromal phase as people change behavior in response to increasing health concerns, and consequently spend less time outdoors. However, low serum vitamin D levels earlier in life may also increase the risk of developing MS, in certain populations.

While longer term implications of the MS prodrome include the potential for earlier recognition or diagnosis of MS, much more work is needed before this could be applied in clinical practice. Also, while studies to date have provided a "proof-of-principle" that an MS prodrome exists, many of the individual features identified are not specific to MS and are common in the general population. However, a tangible future goal, which could facilitate improving outcomes in MS, could be the development of *research* criteria for prodromal MS. A probability score, estimating the likelihood of an individual being in the prodromal phase of MS is envisaged. Building on current knowledge, including prior MS genetic risk scores (74–76), this prodromal probability score could be based on an optimal combination of prodromal clinical features (e.g., depression, anxiety, pain, dermatological issues or other combinations of features) with risk markers (e.g., age, sex, family history/genetics) and biomarkers (e.g., serum NfL, imaging markers [such as those observed in people with RIS], serum vitamin D). This approach is similar to the research criteria developed to identify prodromal Parkinson's disease (55, 77) and to those being tested/developed in other neurodegenerative and autoimmune diseases including dementia with Lewy bodies (78), Type 1 diabetes (79), and rheumatoid arthritis (4). Such research criteria could facilitate identification of high-risk individuals, defined using an acceptable threshold, e.g., 80 or 90% probability of having prodromal MS. This information is envisaged for research purposes only (not clinical practice). For example, these individuals could be offered enrollment in clinical trials of future neuroprotective drugs or other interventions (80). This would complement the ongoing clinical trials in

people with RIS in which disease-modifying drugs approved to treat MS are being tested for their ability to prevent or to delay classical MS symptom onset (e.g., NCT02739542, NCT03122652). Creation of validated research criteria for prodromal MS will require further research investment to provide greater granularity of the most relevant prodromal features (**Table 1**) and will ultimately require contributions from a broad range of international stakeholders, including multi-disciplinary researchers, clinician-scientists and the MS community.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

HT, KM, and NM drafted the original manuscript, reviewed and edited the text, Tables, Boxes, and Figure. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Matthews WB. *McAlpine's Multiple Sclerosis. 2nd ed*. London: Churchill Livingstone (1991).
2. Tremlett H, Marrie RA. The multiple sclerosis prodrome: Emerging evidence, challenges, and opportunities. *Mult Scler.* (2020) 2020:1352458520914844. doi: 10.1177/1352458520914844
3. Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* (2011) 7:280–92. doi: 10.1016/j.jalz.2011.03.003
4. Greenblatt HK, Kim HA, Bettner LF, Deane KD. Preclinical rheumatoid arthritis and rheumatoid arthritis prevention. *Curr Opin Rheumatol.* (2020) 32:289–96. doi: 10.1097/BOR.0000000000000708
5. Mokhtari M, Rajarethinam R. Early intervention and the treatment of prodrome in schizophrenia: a review of recent developments. *J Psychiatr Pract.* (2013) 19:375–85. doi: 10.1097/01.pra.0000435036.83426.94
6. Postuma RB, Berg D. Prodromal Parkinson's disease: The decade past, the decade to come. *Mov Disord.* (2019) 34:665–75. doi: 10.1002/mds.27670
7. Makhani N, Tremlett H. The multiple sclerosis prodrome. *Nat Rev Neurol.* (2021) 17:515–21. doi: 10.1038/s41582-021-00519-3
8. Yusuf FLA, Ng BC, Wijnands JMA, Kingwell E, Marrie RA, Tremlett H. A systematic review of morbidities suggestive of the multiple sclerosis prodrome. *Expert Rev Neurotherapeutics.* (2020) 2020:1–21. doi: 10.1080/14737175.2020.1746645
9. Makhani N. The multiple sclerosis prodrome is just unspecific symptoms in radiologically isolated syndrome patients - Commentary. *Mult Scler.* (2021) 2021:13524585211044951. doi: 10.1177/13524585211044951
10. Tremlett H, Okuda DT, Lebrun-Frenay C. The multiple sclerosis prodrome is just unspecific symptoms in radiologically isolated syndrome patients - No. *Mult Scler.* (2021) 2021:13524585211035951. doi: 10.1177/13524585211035951
11. Zhao Y, Wijnands JMA, Hogg T, Kingwell E, Zhu F, Evans C, et al. Interrogation of the multiple sclerosis prodrome using high-dimensional health data. *Neuroepidemiology.* (2020) 54:140–7. doi: 10.1159/000505331
12. Yusuf F, Wijnands JM, Kingwell E, Zhu F, Evans C, Fisk JD, et al. Fatigue, sleep disorders, anaemia and pain in the multiple sclerosis prodrome. *Mult Scler.* (2021) 27:290–302. doi: 10.1177/1352458520908163
13. Wijnands JMA, Kingwell E, Zhu F, Zhao Y, Hogg T, Stadnyk K, et al. Health-care use before a first demyelinating event suggestive of a multiple sclerosis prodrome: a matched cohort study. *Lancet Neurol.* (2017) 16:445–51. doi: 10.1016/S1474-4422(17)30076-5
14. Wijnands JM, Zhu F, Kingwell E, Zhao Y, Ekuma O, Lu X, et al. Five years before multiple sclerosis onset: Phenotyping the prodrome. *Mult Scler.* (2019) 25:1092–101. doi: 10.1177/1352458518783662
15. Hogg T, Wijnands JMA, Kingwell E, Zhu F, Lu X, Evans C, et al. Mining healthcare data for markers of the multiple sclerosis prodrome. *Mult Scler Relat Disord.* (2018) 25:232–40. doi: 10.1016/j.msard.2018.08.007
16. Wijnands JMA, Zhu F, Kingwell E, Zhao Y, Evans C, Fisk J, et al. Prodrome in relapsing-remitting and primary progressive multiple sclerosis. *Eur J Neurol.* (2019) 26:1032–6. doi: 10.1111/ene.13925
17. Disanto G, Zecca C, MacLachlan S, Sacco R, Handunnetthi L, Meier UC, et al. Prodromal symptoms of multiple sclerosis in primary care. *Ann Neurol.* (2018) 83:1162–73. doi: 10.1002/ana.25247
18. Cortese M, Riise T, Bjørnevik K, Bhan A, Farbu E, Grytten N, et al. Preclinical disease activity in multiple sclerosis: A prospective study of cognitive performance prior to first symptom. *Ann Neurol.* (2016) 80:616–24. doi: 10.1002/ana.24769
19. Lebrun-Frenay C, Kantarci O, Siva A, Sormani MP, Pelletier D, Okuda DT, et al. Radiologically isolated syndrome: 10-year risk estimate of a clinical event. *Ann Neurol.* (2020) 88:407–17. doi: 10.1002/ana.25799
20. Okuda DT, Siva A, Kantarci O, Inglese M, Katz I, Tutuncu M, et al. Radiologically isolated syndrome: 5-year risk for an initial clinical event. *PLoS ONE.* (2014) 9:e90509. doi: 10.1371/journal.pone.0090509
21. Marrie RA, O'Mahony J, Maxwell CJ, Ling V, Yeh EA, Arnold DL, et al. High rates of health care utilization in pediatric multiple sclerosis: A Canadian population-based study. *PLoS ONE.* (2019) 14:e0218215. doi: 10.1371/journal.pone.0218215
22. Artemiadis AK, Anagnostouli MC, Alexopoulos EC. Stress as a risk factor for multiple sclerosis onset or relapse: a systematic review. *Neuroepidemiology.* (2011) 36:109–20. doi: 10.1159/000323953
23. Riise T, Mohr DC, Munger KL, Rich-Edwards JW, Kawachi I, Ascherio A. Stress and the risk of multiple sclerosis. *Neurology.* (2011) 76:1866–71. doi: 10.1212/WNL.0b013e31821d74c5
24. Xia Z, Steele SU, Bakshi A, Clarkson SR, White CC, Schindler MK, et al. Assessment of early evidence of multiple sclerosis in a prospective study of asymptomatic high-risk family members. *JAMA Neurol.* (2017) 74:293–300. doi: 10.1001/jamaneurol.2016.5056
25. Lam LKM, Murphy S, Kokkinaki D, Venosa A, Sherrill-Mix S, Casu C, et al. DNA binding to TLR9 expressed by red blood cells promotes innate immune activation and anemia. *Sci Transl Med.* (2021) 13:eabj1008. doi: 10.1126/scitranslmed.abj1008
26. Rashtak S, Pittelkow MR. Skin involvement in systemic autoimmune diseases. *Curr Dir Autoimmun.* (2008) 10:344–58. doi: 10.1159/000131754
27. Amezcua L, McCauley JL. Race and ethnicity on MS presentation and disease course. *Multiple Sclerosis.* (2020) 26:561–7. doi: 10.1177/1352458519887328
28. Rivas-Rodríguez E, Amezcua L. Ethnic considerations and multiple sclerosis disease variability in the United States. *Neurol Clin.* (2018) 36:151–62. doi: 10.1016/j.ncl.2017.08.007

29. Kingwell E, Leung AL, Roger E, Duquette P, Rieckmann P, Tremlett H. Factors associated with delay to medical recognition in two Canadian multiple sclerosis cohorts. *J Neurol Sci.* (2010) 292:57–62. doi: 10.1016/j.jns.2010.02.007
30. Barin L, Kamm CP, Salmen A, Dressel H, Calabrese P, Pot C, et al. How do patients enter the healthcare system after the first onset of multiple sclerosis symptoms? The influence of setting and physician specialty on speed of diagnosis. *Mult Scler.* (2020) 26:489–500. doi: 10.1177/1352458518823955
31. Gasperi C, Hapfelmeier A, Daltrozzo T, Schneider A, Donnachie E, Hemmer B. Systematic assessment of medical diagnoses preceding the first diagnosis of multiple sclerosis. *Neurology.* (2021) 2021:12074. doi: 10.1212/WNL.0000000000012074
32. Solomon AJ, Ascherio A. Early diagnosis of multiple sclerosis: further evidence for missed opportunity. *Neurology.* (2021). doi: 10.1212/WNL.0000000000012087. [Epub ahead of print].
33. Okuda DT, Mowry EM, Beheshtian A, Waubant E, Baranzini SE, Goodin DS, et al. Incidental MRI anomalies suggestive of multiple sclerosis: The radiologically isolated syndrome. *Neurology.* (2009) 72:800–5. doi: 10.1212/01.wnl.0000335764.14513.1a
34. George IC, El Mendili MM, Inglese M, Azevedo CJ, Kantarci O, Lebrun C, et al. Cerebellar volume loss in radiologically isolated syndrome. *Mult Scler.* (2021) 27:130–3. doi: 10.1177/1352458519887346
35. Giorgio A, Stromillo ML, Rossi F, Battaglini M, Hakiki B, Portaccio E, et al. Cortical lesions in radiologically isolated syndrome. *Neurology.* (2011) 77:1896–9. doi: 10.1212/WNL.0b013e318238ee9b
36. Rojas JI, Patrucco L, Míguez J, Besada C, Cristiano E. Brain atrophy in radiologically isolated syndromes. *J Neuroimaging.* (2015) 25:68–71. doi: 10.1111/jon.12182
37. Azevedo CJ, Overton E, Khadka S, Buckley J, Liu S, Sampat M, et al. Early CNS neurodegeneration in radiologically isolated syndrome. *Neurol Neuroimmunol Neuroinflamm.* (2015) 2:e102. doi: 10.1212/NXI.0000000000000102
38. Amato MP, Hakiki B, Goretti B, Rossi F, Stromillo ML, Giorgio A, et al. Association of MRI metrics and cognitive impairment in radiologically isolated syndromes. *Neurology.* (2012) 78:309–14. doi: 10.1212/WNL.0b013e31824528c9
39. Stromillo ML, Giorgio A, Rossi F, Battaglini M, Hakiki B, Malentacchi G, et al. Brain metabolic changes suggestive of axonal damage in radiologically isolated syndrome. *Neurology.* (2013) 80:2090–4. doi: 10.1212/WNL.0b013e318295d707
40. Giorgio A, Stromillo ML, De Leucio A, Rossi F, Brandes I, Hakiki B, et al. Appraisal of brain connectivity in radiologically isolated syndrome by modeling imaging measures. *J Neurosci.* (2015) 35:550–8. doi: 10.1523/JNEUROSCI.2557-14.2015
41. Solomon AJ, Schindler MK, Howard DB, Watts R, Sati P, Nickerson JP, et al. "Central vessel sign" on 3T FLAIR* MRI for the differentiation of multiple sclerosis from migraine. *Ann Clin Transl Neurol.* (2016) 3:82–7. doi: 10.1002/acn3.273
42. Sinnecker T, Clarke MA, Meier D, Enzinger C, Calabrese M, De Stefano N, et al. Evaluation of the central vein sign as a diagnostic imaging biomarker in multiple sclerosis. *JAMA Neurol.* (2019) 76:1446–56. doi: 10.1001/jamaneurol.2019.2478
43. Ciotti JR, Eby NS, Brier MR, Wu GF, Chahin S, Cross AH, et al. Central vein sign and other radiographic features distinguishing myelin oligodendrocyte glycoprotein antibody disease from multiple sclerosis and aquaporin-4 antibody-positive neuromyelitis optica. *Mult Scler.* (2021) 2021:13524585211007086. doi: 10.1177/13524585211007086
44. Maggi P, Sati P, Nair G, Cortese ICM, Jacobson S, Smith BR, et al. Paramagnetic rim lesions are specific to multiple sclerosis: an international multicenter 3T MRI Study. *Ann Neurol.* (2020) 88:1034–42. doi: 10.1002/ana.25877
45. Bjornevik K, Munger KL, Cortese M, Barro C, Healy BC, Niebuhr DW, et al. Serum neurofilament light chain levels in patients with presymptomatic multiple sclerosis. *JAMA Neurol.* (2019) 77:58–64. doi: 10.1001/jamaneurol.2019.3238
46. Matute-Blanch C, Villar LM, Álvarez-Cermeño JC, Rejdak K, Evdoshenko E, Makshakov G, Nazarov V, et al. Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. *Brain.* (2018) 141:1085–93. doi: 10.1093/brain/awy021
47. Beltrán E, Gerdes LA, Hansen J, Flierl-Hecht A, Krebs S, Blum H, et al. Early adaptive immune activation detected in monozygotic twins with prodromal multiple sclerosis. *J Clin Invest.* (2019) 129:4758–68. doi: 10.1172/JCI128475
48. Mirza A, Forbes JD, Zhu F, Bernstein CN, Van Domselaar G, Graham M, et al. The multiple sclerosis gut microbiota: A systematic review. *Mult Scler Relat Disord.* (2020) 37:101427. doi: 10.1016/j.msard.2019.101427
49. Zailaie SA, Siddiqui JJ, Al Saadi RM, Anbari DM, A SA, Cupler EJ. Serum based miRNA as a diagnostic biomarker for multiple sclerosis: a systematic review and meta-analysis. *Immunol Invest.* (2021) 2021:1–16. doi: 10.1080/08820139.2021.1887888
50. Sun M, Liu N, Xie Q, Li X, Sun J, Wang H, et al. A candidate biomarker of glial fibrillary acidic protein in CSF and blood in differentiating multiple sclerosis and its subtypes: A systematic review and meta-analysis. *Mult Scler Relat Disord.* (2021) 51:102870. doi: 10.1016/j.msard.2021.102870
51. Momtazmanesh S, Shobeiri P, Saghazadeh A, Teunissen CE, Burman J, Szalardy L, et al. Neuronal and glial CSF biomarkers in multiple sclerosis: a systematic review and meta-analysis. *Rev Neurosci.* (2021) 32:573–95. doi: 10.1515/revneuro-2020-0145
52. Lebrun C, Bensa C, Debouverie M, Wiertlevski S, Brassat D, de Seze J, et al. Association between clinical conversion to multiple sclerosis in radiologically isolated syndrome and magnetic resonance imaging, cerebrospinal fluid, and visual evoked potential: follow-up of 70 patients. *Arch Neurol.* (2009) 66:841–6. doi: 10.1001/archneurol.2009.119
53. Miguel JM, Roldán M, Pérez-Rico C, Ortiz M, Boquete L, Blanco R. Using advanced analysis of multifocal visual-evoked potentials to evaluate the risk of clinical progression in patients with radiologically isolated syndrome. *Sci Rep.* (2021) 11:2036. doi: 10.1038/s41598-021-81826-z
54. Knier B, Berthele A, Buck D, Schmidt P, Zimmer C, Mühlau M, et al. Optical coherence tomography indicates disease activity prior to clinical onset of central nervous system demyelination. *Mult Scler.* (2016) 22:893–900. doi: 10.1177/1352458515604496
55. Heinzel S, Berg D, Gasser T, Chen H, Yao C, Postuma RB. Update of the MDS research criteria for prodromal Parkinson's disease. *Mov Disord.* (2019) 34:1464–70. doi: 10.1002/mds.27802
56. Ascherio A, Munger KL. Epidemiology of multiple sclerosis: from risk factors to prevention-an update. *Semin Neurol.* (2016) 36:103–14. doi: 10.1055/s-0036-1579693
57. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA.* (2006) 296:2832–8. doi: 10.1001/jama.296.23.2832
58. Biström M, Alonso-Magdalena L, Andersen O, Jons D, Gunnarsson M, Vrethem M, et al. High serum concentration of vitamin D may protect against multiple sclerosis. *Mult Scler J Exp Transl Clin.* (2019) 5:2055217319892291. doi: 10.1177/2055217319892291
59. Munger KL, Hongell K, Åivo J, Soilu-Hänninen M, Surcel HM, Ascherio A. 25-Hydroxyvitamin D deficiency and risk of MS among women in the Finnish Maternity Cohort. *Neurology.* (2017) 89:1578–83. doi: 10.1212/WNL.0000000000004489
60. Salzer J, Hallmans G, Nyström M, Stenlund H, Wadell G, Sundström P. Vitamin D as a protective factor in multiple sclerosis. *Neurology.* (2012) 79:2140–5. doi: 10.1212/WNL.0b013e3182752ea8
61. Munger KL, Åivo J, Hongell K, Soilu-Hänninen M, Surcel HM, Ascherio A. Vitamin D status during pregnancy and risk of multiple sclerosis in offspring of women in the finish maternity cohort. *JAMA Neurol.* (2016) 73:515–9. doi: 10.1001/jamaneurol.2015.4800
62. Nielsen NM, Munger KL, Koch-Henriksen N, Hougaard DM, Magyari M, Jørgensen KT, et al. Neonatal vitamin D status and risk of multiple sclerosis: A population-based case-control study. *Neurology.* (2017) 88:44–51. doi: 10.1212/WNL.0000000000003454
63. Tremlett H, Zhu F, Ascherio A, Munger KL. Sun exposure over the life course and associations with multiple sclerosis. *Neurology.* (2018) 90:e1191–e9. doi: 10.1212/WNL.0000000000005257
64. van der Mei IA, Ponsonby AL, Dwyer T, Blizzard L, Simmons R, Taylor BV, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *BMJ.* (2003) 327:316. doi: 10.1136/bmj.327.7410.316
65. Magalhaes S, Pugliatti M, Riise T, Myhr KM, Ciampi A, Bjornevik K, et al. Shedding light on the link between early life sun exposure and risk of

multiple sclerosis: results from the EnvIMS Study. *Int J Epidemiol.* (2019) 48:1073–82. doi: 10.1093/ije/dyy269
66. Gianfrancesco MA, Stridh P, Rhead B, Shao X, Xu E, Graves JS, et al. Evidence for a causal relationship between low vitamin D, high BMI, and pediatric-onset MS. *Neurology.* (2017) 88:1623–9. doi: 10.1212/WNL.0000000000003849
67. Rhead B, Bäärnhielm M, Gianfrancesco M, Mok A, Shao X, Quach H, et al. Mendelian randomization shows a causal effect of low vitamin D on multiple sclerosis risk. *Neurol Genet.* (2016) 2:e97. doi: 10.1212/NXG.0000000000000097
68. Mokry LE, Ross S, Ahmad OS, Forgetta V, Smith GD, Goltzman D, et al. Vitamin D and risk of multiple sclerosis: a mendelian randomization study. *PLoS Med.* (2015) 12:e1001866. doi: 10.1371/journal.pmed.1001866
69. Levin LI, Munger KL, O'Reilly EJ, Falk KI, Ascherio A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol.* (2010) 67:824–30. doi: 10.1002/ana.21978
70. Rotstein DL, Cortese M, Fung TT, Chitnis T, Ascherio A, Munger KL. Diet quality and risk of multiple sclerosis in two cohorts of US women. *Mult Scler.* (2019) 25:1773–80. doi: 10.1177/1352458518807061
71. Dorans KS, Massa J, Chitnis T, Ascherio A, Munger KL. Physical activity and the incidence of multiple sclerosis. *Neurology.* (2016) 87:1770–6. doi: 10.1212/WNL.0000000000003260
72. Kister I, Munger KL, Herbert J, Ascherio A. Increased risk of multiple sclerosis among women with migraine in the Nurses' Health Study II. *Multiple Sclerosis J.* (2011) 18:90–7. doi: 10.1177/1352458511416487
73. Yong HY, McKay KA, Daley CGJ, Tremlett H. Drug exposure and the risk of multiple sclerosis: A systematic review. *Pharmacoepidemiol Drug Saf.* (2018) 27:133–9. doi: 10.1002/pds.4357
74. Dobson R, Ramagopalan S, Topping J, Smith P, Solanky B, Schmierer K, et al. A risk score for predicting multiple sclerosis. *PLoS ONE.* (2016) 11:e0164992. doi: 10.1371/journal.pone.0164992
75. De Jager PL, Chibnik LB, Cui J, Reischl J, Lehr S, Simon KC, et al. Integration of genetic risk factors into a clinical algorithm for multiple sclerosis susceptibility: a weighted genetic risk score. *Lancet Neurol.* (2009) 8:1111–9. doi: 10.1016/S1474-4422(09)70275-3
76. Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet Neurol.* (2010) 9:727–39. doi: 10.1016/S1474-4422(10)70094-6
77. Berg D, Postuma RB, Adler CH, Bloem BR, Chan P, Dubois B, et al. MDS research criteria for prodromal Parkinson's disease. *Mov Disord.* (2015) 30:1600–11. doi: 10.1002/mds.26431
78. McKeith IG, Ferman TJ, Thomas AJ, Blanc F, Boeve BF, Fujishiro H, et al. Research criteria for the diagnosis of prodromal dementia with Lewy bodies. *Neurology.* (2020) 94:743–55. doi: 10.1212/WNL.0000000000009323
79. Herold KC, Bundy BN, Long SA, Bluestone JA, DiMeglio LA, Dufort MJ, et al. An Anti-CD3 antibody, teplizumab, in relatives at risk for type 1 diabetes. *N Engl J Med.* (2019) 381:603–13. doi: 10.1056/NEJMoa1902226
80. Allanach JR, Farrell JW, Mésidor M, Karimi-Abdolrezaee S. Current status of neuroprotective and neuroregenerative strategies in multiple sclerosis: A systematic review. *Mult Scler.* (2021) 2021:13524585211008760. doi: 10.1177/13524585211008760

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Predicting Multiple Sclerosis: Challenges and Opportunities

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Determining effective means of preventing Multiple Sclerosis (MS) relies on testing preventive strategies in trial populations. However, because of the low incidence of MS, demonstrating that a preventive measure has benefit requires either very large trial populations or an enriched population with a higher disease incidence. Risk scores which incorporate genetic and environmental data could be used, in principle, to identify high-risk individuals for enrolment in preventive trials. Here we discuss the concepts of developing predictive scores for identifying individuals at high risk of MS. We discuss the empirical efforts to do so using real cohorts, and some of the challenges-both theoretical and practical-limiting this work. We argue that such scores could offer a means of risk stratification for preventive trial design, but are unlikely to ever constitute a clinically-helpful approach to predicting MS for an individual.

Keywords: prediction, polygenic risk score, Multiple Sclerosis, genetics, environmental risk score

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INTRODUCTION

Multiple Sclerosis (MS) is a prototypical complex autoimmune disease of the central nervous system. It is the leading cause of non-traumatic neurological disability in young adults, and affects over 2 million people worldwide (1). Although the pathogenesis of MS is not completely understood, converging lines of evidence support roles for both genetic and environmental factors in determining MS susceptibility. A variety of environmental influences are associated with increased susceptibility to MS; the most consistent and replicated risk factors are smoking, childhood obesity, infectious mononucleosis, and lower serum vitamin D (2). The largest genome-wide association study (GWAS) of MS orchestrated by the International Multiple Sclerosis Genetics Consortium (IMSGC) discovered 233 genetic signals associated with MS, collectively explaining around 50% of MS heritability (3).

It may be possible to quantify an individual's susceptibility to MS based on their genetic data and exposure to certain risk factors. In principle, if it were possible to predict an individual's risk of developing MS routinely in clinical practice, this could transform all aspects of MS care, including diagnosis, treatment choices, and prognosis. Accurate and early prediction could also pave the way for trials of preventive therapies. In reality, predicting whether a given individual will develop MS may be a pipedream, as attempts to do so are constrained by several theoretical and practical challenges.

In this review we discuss previous efforts to develop MS prediction algorithms and explore the challenges facing these approaches. We present an optimistic but realistic view of how personalized prediction may enhance MS research and care over the coming decades.

THE GENETIC CONTRIBUTION TO MS RISK

Key Points

- MS is a complex genetic disease, with small effects of >200 loci contributing to the genetic component of risk
- Common genetic factors alone could explain up to ∼20% of MS susceptibility

Heritability estimates derived from the IMSGC meta-analysis suggest that around 19.2% of MS susceptibility is attributable to the additive effects of common variants across the genome (3), of which roughly 50% could be explained in terms of genome-wide significant and suggestive effects, leaving ∼50% of heritability unexplained. The strongest signal from GWAS data is for the HLA-DRB1*15:01 risk allele (Odds Ratio of 2.9) (3). Evidence from the IMSGC case-control exome chip study analyzing the role of rare coding variants suggests that a further ∼10% of MS heritability may be explained by rare (Minor Allele Frequency < 0.05) variants (4). Neither these substantial gene discovery efforts nor smaller pedigree designs have discovered any reproducible single-gene causes of MS (5–19). Although rare monogenic forms of MS not captured in these studies cannot be excluded, these data argue for MS being a prototypical complex and polygenic disease. The genetic component to susceptibility consists in a large number of individually small effects scattered across at least two hundred genetic loci.

GENETIC RISK SCORES, ENVIRONMENTAL RISK SCORES, AND PREDICTION OF MS

Key Points

- Genetic risk scores for MS can be calculated by summing an individual's number of risk alleles at each known risk locus
- Various methods exist for deciding which risk variants to include in the genetic risk score, and how to "weight" the contribution of individual variants
- Environmental risk score's can be calculated in the same fashion if the effect of a given risk factor is known from case-control/cohort studies, and an individual's exposure to the risk factor can be quantified
- Efforts to predict MS using risk scores comprising genetic and environmental risk factors have all failed to show meaningful predictive performance on an individual level

As genotyping costs continue to fall and large biobank-scale GWAS become available for a number of common traits and diseases, it is conceivable that genotyping could become a routinely-available clinical test to help predict an individual's risk of developing a complex disease (20). If the effects of genetic variants on the risk of a disease are known through large GWAS, and an individual can be genotyped at these variants, it is straightforward to calculate the individual's genetic risk of the disease by adding together the sum of their risk alleles, each weighted by its effect: for j SNPs, with β_j the effect of each SNP on MS (i.e., the log odds ratio per effect allele), and g_j the individual's allele count at that SNP (which could be 0, 1, 2, or an imputed dosage probability), the individual's polygenic risk score over all SNPs is given by

$$PRS = \sum_{n=1}^{j} \beta_j g_j \quad (1)$$

Various methods have been developed to enhance polygenic risk score prediction of complex traits (21). Although the principle is universal-to combine the effects of risk alleles across the genome using external weights derived from GWAS-these methods differ in terms of how variants are selected for inclusion in the score, and how the weights are tuned (22–25).

Large cohort and case-control studies, driven primarily by Scandinavian and North American cohorts/registry data, have consistently demonstrated that several environmental factors play a role in determining MS susceptibility (2). Such risk factors include low serum vitamin D, various aspects of EBV infection (prior infectious mononucleosis, higher anti-EBV antibody titres, EBV seropositivity in general), childhood obesity, smoking and various other putative factors such as head injury, solvent inhalation, and shift work (2). Interestingly, the effect of some of these factors appears to be potentiated by the high risk HLA allele, DRB1*15:01 (26–28). It is plausible that environmental risk factors for MS are modified by an individual's prior genetic risk, and if this is correct, risk models which account for gene-environment interactions are likely to outperform models which do not.

The earliest effort to predict MS using environmental and genetic data was published in 2009 (29). Since then, there have been several efforts incorporating increasingly refined genetic maps of MS susceptibility and applying this approach to novel datasets (**Table 1**) (29–36, 38, 39). Broadly, these studies support the view that genetic risk scores (GRS) / PRS can discriminate between cases and controls. All show moderate performance (areas under the curve, AUC, ranging from 0.52 to 0.8), but all fall short of clinically-useful diagnostic test thresholds. Efforts to demonstrate a correlation between PRS and subclinical evidence of demyelination have yielded mixed results, with the largest such cohort (∼30,000 healthy controls in UK Biobank) failing to demonstrate an association (33, 40, 41) (unpublished data, https://github.com/benjacobs123456/PRS_UKB_MRI). In order to have clinical utility, scores should be able to make predictions which are useful on an individual level. The addition of disease-relevant environmental variables (such as prior smoking and prior infectious mononucleosis) has been shown to enhance the discriminative performance of these models (33).

Although these efforts highlight the discriminative capacity of risk models *en masse*, the performance metrics are well short of what would be required for a diagnostic or predictive test. In general the methods for deriving and applying risk scores, and the reporting of the results of such analyses have been inconsistent in the literature. Few studies report absolute risk estimates within deciles of the risk scores and calibration statistics (predicted disease prevalence in each risk decile vs. observed disease prevalence). In addition there are discrepancies between studies in the methods for selecting which genetic and/or environmental factors to include

TABLE 1 | Comparison of PRS and ERS efforts in MS in literature.

References	Score type	MS GWAS used	Population validated	Results (AUC)
De Jager et al. (29)	PRS	16 (MHC + non-MHC)	3 populations: 2,215 cases, 1,340 cases, 143 cases	0.64–0.70
	PRS	15 (non-MHC)		0.57–0.61
	PRS + ERS	16 (MHC + non-MHC)		0.68–0.74
Jafari et al. (30)	PRS	6	Simulated 100,000 genotypes	0.64
	PRS	24		0.66
	PRS	53		0.69
Gourraud et al. (31)	PRS + Female sex	17 (MHC + non-MHC)	1,213 MS families (810 sporadic, 403 multi-case)	0.57
	PRS	17 (MHC + non-MHC)		0.55
	PRS	16 (non-MHC)		0.52
	PRS + Female sex	1 (MHC)		0.58
Disanto et al. (32)	PRS	60 (non-MHC)	70 patients, 79 HC	0.66
	PRS	110 (non-MHC)		0.69
	PRS	1 (MHC)		0.71
	PRS	61 (MHC + non-MHC)		0.77
	PRS	111 (MHC + non-MHC)		0.8
Dobson et al. (33)	PRS + ERS	1 (MHC)	78 patients, 121 unaffected siblings, 103 HC	0.77
	PRS + ERS	58 (MHC + non-MHC)		0.8
	PRS + ERS – vitamin D	1 (MHC)		0.8
	PRS + ERS – vitamin D	58 (MHC + non-MHC)		0.82
Ayati and Koyuturk (34)	PRS	8,267	975 cases	0.64–0.65
	NetPocos	243 Pocos: 3 SNPs per Pocos		0.62–0.63
Xia et al. (35)*	ERS	0	113 cases, 1,683 asymptomatic first degree relative	p val-0.10
	PRS	64 (MHC + non-MHC)		p val 1.5E-5
	PRS + ERS	64 (MHC + non-MHC)		p val 4.8E-6
Kulm et al. (36)	Covariates + PCA only	0	1,445 cases in UKB	0.62
	PRS + Covariates + PCA	23,309		0.73
Jacobs et al. (37)	Covariates + PCA only	0	2,276 MS cases, 486,000 controls	0.63
	PRS + Covariates + PCA	200 (non-MHC)		0.67
	PRS + Covariates + PCA	232 (MHC + non-MHC)		0.71
Barnes et al. (38)	Covariates	0	3 populations: 15 cases, 30 cases, 97 cases	0.61–0.70
	PRS + Covariates	127 (MHC + non-MHC)		0.70–0.77

**Xia et al. (35), no AUC available, results shown as p-values for discrimination between MS cases and controls using the risk score. PRS, Polygenic Risk Score; ERS, Environmental Risk Score; PCA, Principal Components Analysis; HC, Healthy Controls.*

in the score, the methods for generating polygenic risk scores, the statistical evaluation of the model performance, and the choice of / omission of confounding covariates such as age, sex, and genetic principal components in prediction models. Furthermore, these studies differ substantially in terms of how the data were generated, i.e., cohort characteristics, genotyping methods, and ascertainment of environmental variables. The recent development of consensus guidelines should help streamline further efforts to predict MS using risk scores (42). Given this heterogeneity in methods and reporting, it is difficult to make comparisons across published studies.

CHALLENGES AND OPPORTUNITIES FOR RISK PREDICTION ALGORITHMS

Key Points

- MS heritability places an upper bound on PRS performance
- Uncertainty about which variants are causal at a locus leads to inclusion of non-causal variants in PRS, which degrades performance
- Most PRS are restricted to common variants, and therefore may miss some of the susceptibility conferred by high-impact, low-frequency variants
- Modeling interactions between genetic and environmental factors may improve PRS performance over models assuming independence
- Cross-ancestry differences in LD structure and allele frequencies limit the performance of PRS in non-European ancestries
- Environmental risk factors may not be truly causal, are difficult to measure consistently, and may have varying effects over time, limiting their usefulness in risk scores
- The low prevalence of MS limits the clinical utility of all individual-level risk scores, and this is disguised by focussing on metrics like AUC, accuracy, and sensitivity/specificity rather than the positive predictive value

- Case-control definitions in biobank-scale datasets used for risk score evaluation may be imperfect
- If there are truly random processes which contribute to MS pathogenesis, these are difficult to capture with risk scores

MS Heritability Places an Upper Bound on PRS Performance

The broad-sense heritability of MS-the proportion of phenotypic variation explained by genetic variation-places a theoretical upper limit on the performance of polygenic risk score prediction alone (43). Whilst generous estimates from twin studies estimate a broad-sense heritability of 50% (44), SNP heritability-the proportion of phenotypic variation attributable to additive effects of all typed/imputed SNPs across the genome-was estimated at 19.2% in the most recent GWAS (3). Genome-wide significant and suggestive loci only explain ∼50% of this SNP heritability. These considerations emphasize the limitations of PRS generated using common, genome-wide significant markers. Even PRS which incorporate weaker effects across the genome are bounded by the h^2_{SNP} of 19.2%. There are several explanations for missing heritability, which we discuss below, some of which could be overcome to improve MS prediction scores.

Selecting Causal Variants for Inclusion in PRS

The classical "clumping-and-thresholding" approach to variant selection for PRS selects variants for inclusion at each independent locus (defined by an arbitrary 'clumping' linkage disequilibrium and physical distance window), selecting the variant with the strongest statistical association with the trait (i.e., lowest *P*-value). Unfortunately, the variant with the lowest *P*-value is unlikely to be the true causal variant / one of the causal variants at the locus (45). Unless the included variant is in perfect LD ($R^2 = 1$) with the true causal variant, the performance of the PRS will be vulnerable to the LD structure in the region, and may perform poorly even in the presence of subtly different LD (where the true causal effect will be less well-captured by the included variant). Methods incorporating local LD structure to estimate SNP effects, such as LDpred, overcome this concern to a degree and lead to appreciable improvements in prediction accuracy (23).

Rare Variation

Rare variation may account for some of the missing heritability and thus improve PRS performance. Realistically, however, rare variants may have large effects for individuals, but they are unlikely to explain substantial phenotypic variation on a population scale. A variant with an odds ratio of 8 but a minor allele frequency (MAF) of 0.001 will only be observed, on average, once in a population of 500 people. Although this may have a substantial impact on that individual's risk of MS, it has only a limited impact on the overall performance of the score in the population.

Although heritability estimates suggest that rare (MAF $<$ 0.05) coding variation may account for a sizeable proportion of MS heritability, the largest effort to date using the exome chip platform revealed only five associated variants within four genes outside of known MS risk loci (4). As the landscape of rare variant contributions to MS becomes clearer through large exome sequencing efforts, further performance gains may be derived from including rarer variation in PRS.

Interactions

A simple additive PRS does not account for gene-gene or gene-environment interactions. External weights taken from GWAS assume that the effects of SNPs are constant regardless of the individual's genetic background or exposure to environmental risk factors. Various methods have been developed to account for gene-gene and gene-environment interaction in determining PRS weights. Such methods include use of conditional summary statistics, e.g., those derived from the Conditional Joint Analysis (COJO) method, which calculates effect sizes for SNPs iteratively, conditioning on each SNP in turn, starting with the strongest association (46).

Non-linear machine learning methods, such as gradient-boosted trees and random forests, can also account for high order interactions between SNPs without needing to specify these interactions *a priori*, and have been shown to afford prediction gains for complex traits in large datasets (47). It remains unclear to what extent this approach will lead to improvements in MS prediction, as widespread gene-gene interactions have not been observed outside of the MHC region in the largest sample size GWAS (3, 48). The preliminary evidence for interactions between PRS and environmental risk factors for MS suggests that incorporating GxE interaction terms into risk models may lead to further power gains (37).

Cross-Ancestry Portability

Accurate risk estimation with PRS relies on the "true" SNP effects in the target population (i.e., the individual/s being tested) being similar to the estimated SNP effects from GWAS. Measured SNP effects in one population may differ substantially from the effect of the variant in a different ancestral population due to the different LD structures, different allele frequencies, or other factors (such as ancestry-specific gene-gene and gene-environment effects). This is a major problem for PRS derived from GWAS of individuals of European ancestry, and has been empirically demonstrated to result in poorer quality predictions for individuals of other ancestral backgrounds (49). Novel statistical methods can improve prediction in non-European populations, for instance by incorporating information from multiple ancestries (50) or prioritizing variants based on functional annotations (51). Preliminary evidence from small non-European MS cohorts suggests that the genetic architecture of MS is not identical for people with Hispanic or African ancestry (52–54). Larger GWAS of MS in non-European populations are likely to improve predictive scores for these populations.

Environmental Risk Factors

Intuitively, including established environmental risk factors for MS should lead to improvements in prediction accuracy over genetic risk models alone. Generally, efforts to combine PRS and environmental risk factors have shown modest but appreciable

improvements in discriminative performance (**Table 1**). Several problems limit the value of adding environmental variables to risk scores.

First, included variables may not represent truly causal risk/protective factors. Although a large number of putative environmental risk factors have been linked to MS, it remains unclear whether some of these associations are spurious, reflecting confounding and/or bias rather than causality. Mendelian randomization (MR)-an instrumental variable approach-can be used to provide further support for causality, and has added weight to the concepts that childhood obesity and low serum vitamin D are causal risk factors, whereas the evidence for smoking has been less conclusive (55–60). Clearly, inclusion of environmental risk factors which represent confounding or bias rather than causal associations may increase the noise in prediction scores and limit the utility of such scores.

Second, environmental risk factors are notoriously difficult to capture and record accurately in large cohort settings. Precise phenotype definitions, methods of testing, timing of the study (prospective vs. retrospective), and various cultural influences may lead to subtle heterogeneity in phenotype definition across cohorts, and thus the effect estimates for the effect of a risk factor in the original case-control/cohort setting may not be accurate when applied to the testing or validation cohort.

Third, unlike genetic variants which are (largely) static throughout life, environmental risk factors for MS are dynamic and time-dependent. Thus, the timing of the exposure may be critical in determining the effect on MS susceptibility. For instance, converging evidence from observational and MR designs suggests that obesity during adolescence is a risk factor for MS (59, 61, 62). Crude risk scores which consider environmental risk factors as static and binary, e.g., whether or not an individual has ever smoked or had IM prior to MS diagnosis, are a gross oversimplification and miss the time-varying effects of such exposures on the risk of MS.

Some further general concerns apply to the use of environmental risk scores, some of which also apply for genetic risk scores. These concerns include the stability and accuracy of effect estimates derived from finite sample sizes, the somewhat arbitrary choice of which variables to include, the difficulty in including relevant confounding covariates without introducing multicollinearity (e.g., controlling for socio-economic status to assess the effect of smoking status), and whether to include interaction terms in the model or consider effects as independent.

Interpreting Performance Statistics

Most studies report the discriminative performance of PRS/hybrid risk scores, often quantified using the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. The AUC can be thought of as the probability that a randomly selected case will have a higher score than a randomly selected control. Thus, the AUC is a relative measure of the risk distribution in cases vs. controls, but gives no sense of the absolute disease risk for any given individual at any point in the risk score distribution. Similarly, other metrics of overall PRS performance in a population disguise the fact that on an individual basis, prediction accuracy at an individual level often falls far short of that what would be required for a clinically-useful test. Such metrics include model fit metrics such as Nagelkerke's pseudo-R^2 (which quantifies the proportion of variation in disease liability explained by the risk model) and the odds ratios for disease at each given PRS quantile.

For relatively rare diseases such as MS (with a population prevalence ∼0.2% in the UK https://www.gov.uk/government/publications/multiple-sclerosis-prevalence-incidence-and-smoking-status/multiple-sclerosis-prevalence-incidence-and-smoking-status-data-briefing), the differences in absolute risk between deciles of the risk score are generally very small. For example, in our analysis of the >2,000 MS cases and >480,000 controls in UK Biobank, we report an impressive-sounding AUC of 0.71 for the best-performing PRS (including the MHC region). However this metric hides the fact that the difference in disease prevalence between the highest decile and lowest deciles of the PRS was only 1% (1.2% in the highest decile vs. 0.2% in the lowest decile) (37).

To illustrate this point, consider a sample population of 10,000 people with an MS prevalence of 0.5% (i.e., 50 people have MS, 9,950 people do not have MS). If the PRS distributions in cases and controls follow a standard normal, with mean = 0 in controls and mean = 3 in cases (NB this is an unrealistically large effect), a model based on PRS alone could discriminate cases from controls with an AUC of 0.98. For the purposes of a diagnostic or predictive test, a threshold needs to be established such that individuals over that threshold are considered high-risk, and those below considered low-risk.

Selecting a PRS threshold that yields sensitivity and specificity >90% identifies as high-risk all 50 people with MS (i.e., sensitivity is 100%), but also identifies 975 healthy controls as high-risk. Therefore, the positive predictive value (PPV) is only 5%, i.e., among individuals labeled as "high-risk" by the PRS cutoff, only 5% (50/975 + 50) would truly have MS.

The PPV, unlike sensitivity and specificity, depends on population prevalence (for these same parameters, the PPV would be 33% at a prevalence of 5, and 51% at a prevalence of 10%), and thus provides a more realistic means for appraising the potential clinical utility of a risk score. This illustration emphasizes why risk score prediction is more likely to be clinically useful for common traits and diseases. We have published a Shiny app to illustrate this problem (https://benjacobs.shinyapps.io/PRS_individual_predictions/).

Case Definition for Validation of Risk Models

The evaluation of predictive models requires a large sample of cases and controls. Other than specialized disease biobanks in which MS diagnoses are rigorously checked against the McDonald criteria, case definitions for prediction studies are often derived from electronic health record (EHR) data; this is the case for most large biobanks, such as UK Biobank. Although these biobanks offer large sample sizes, especially for controls, there is a concern that EHR diagnoses may not be as accurate as McDonald-defined MS, and that some individuals may be

misclassified as having MS. The high rate of MS misdiagnosis in clinical settings makes this a very real concern which could derail efforts to validate predictive scores in this setting (63).

Reassuringly, there is substantial similarity between individuals with self-reported MS and those with ICD-coded MS in UK Biobank, and the results of our analyses are unaffected by using more stringent criteria for classifying cases, e.g., restricting to individuals who have more than one source of diagnostic report (from self-report, GP records, Hospital Episode Statistics, and other sources). Although this will never achieve the accuracy of McDonald diagnosis, it is a necessary and passable simplification in our view that allows researchers to understand MS using biobank-scale data.

Modeling Stochastic Processes

Given a generous estimate of 50% for the broad-sense heritability of MS and the individually small effects of environmental risk factors (ORs <= 3.6) (2), it is likely that a sizable proportion of MS susceptibility will remain unexplained. As discussed, there are various explanations for this explanatory gap. A particularly plausible argument is that the pathogenesis of complex diseases like MS is akin to cancer in that it involves stochastic hits which may vary from individual to individual, and are therefore difficult to measure in large cohorts. The biological underpinnings of such a process are open to speculation, but could plausibly involve events such as somatic mutations in disease-relevant tissues, aberrant breaking of immune tolerance by lymphocytes, or encountering a particular pathogen (64). A recent controversial modeling study supported this view (65). If correct, some elements of MS pathogenesis may be near impossible to quantify in a predictive model and would limit the maximum possible performance of such a model.

PERSPECTIVES

Despite major advances in our understanding of environmental and genetic risk factors for MS, efforts to combine this information into predictive scoring systems has been disappointing. There are several theoretical reasons for this-the low population prevalence of MS, missing heritability, imprecisely-measured environmental effects, and possibly a stochastic contribution to pathogenesis which is challenging to quantify. However, there are several challenges which could be overcome. Novel approaches to polygenic risk scoring, modeling interactions between genetic and environmental factors, GWAS of non-European cohorts, and use of large biobank-scale datasets to tune and validate scores offer exciting avenues for MS prediction research. For reasons we have discussed, we are unlikely to be able to predict MS on an individual basis with an acceptable accuracy in the near future. Risk scores may, however, be useful to identify high-risk individuals to enrich populations for trials of preventive therapies, such as an EBV vaccine. In our worked example, we illustrate how a PRS could be used to identify a subset of individuals with >10x the prevalence of MS compared to the unselected population. Further work is required to ensure broad applicability of risk scores across different ancestral populations, to demonstrate the validity of such scores in prospective work, and to work with people with MS and other stakeholders to communicate the value of, and the considerable caveats surrounding, the use of predictive scoring systems in clinical settings.

AUTHOR CONTRIBUTIONS

BJ, LH, RD, and GG all helped conceive, write, and edit the manuscript. BJ wrote the code for the illustrations. LH wrote the first draft. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. GBD 2016 Multiple Sclerosis Collaborators. Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol.* (2019) 18:269–85. doi: 10.1016/s1474-4422(18)30443-5
2. Olsson T, Barcellos LF, Alfredsson L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol.* (2017) 13:25–36. doi: 10.1038/nrneurol.2016.187
3. International Multiple Sclerosis Genetics Consortium. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science.* (2019) 365:aav7188. doi: 10.1126/science.aav7188
4. International Multiple Sclerosis Genetics Consortium. Electronic address: chris.cotsapas@yale.edu, International Multiple Sclerosis Genetics Consortium. Low-frequency and rare-coding variation contributes to multiple sclerosis risk. *Cell.* (2018) 175:1679–87. doi: 10.1016/j.cell.2018.09.049
5. Sawcer S, Ban M, Maranian M, Yeo TW, Compston A, Kirby A, et al. A high-density screen for linkage in multiple sclerosis. *Am J Hum Genet.* (2005) 77:454–67. doi: 10.1086/444547
6. Vidmar L, Maver A, Drulović J, Sepčić J, Novaković I, Ristič S, et al. Multiple Sclerosis patients carry an increased burden of exceedingly rare genetic variants in the inflammasome regulatory genes. *Sci Rep.* (2019) 9:9171. doi: 10.1038/s41598-019-45598-x
7. Maver A, Lavtar P, Ristić S, Stopinšek S, Simčič S, Hočevar K, et al. Identification of rare genetic variation of NLRP1 gene in familial multiple sclerosis. *Sci Rep.* (2017) 7:3715. doi: 10.1038/s41598-017-03536-9
8. Bernales CQ, Encarnacion M, Criscuoli MG, Yee IM, Traboulsee AL, Sadovnick AD, et al. Analysis of NOD-like receptor NLRP1 in multiple sclerosis families. *Immunogenetics.* (2018) 70:205–7. doi: 10.1007/s00251-017-1034-2
9. Vilariño-Güell C, Zimprich A, Martinelli-Boneschi F, Herculano B, Wang Z, Matesanz F, et al. Exome sequencing in multiple sclerosis families identifies 12 candidate genes and nominates biological pathways for the genesis of disease. *PLoS Genet.* (2019) 15:e1008180. doi: 10.1371/journal.pgen.1008180
10. Wang Z, Sadovnick AD, Traboulsee AL, Ross JP, Bernales CQ, Encarnacion M, et al. Nuclear receptor NR1H3 in familial multiple sclerosis. *Neuron.* (2016) 90:948–54. doi: 10.1016/j.neuron.2016.04.039
11. International Multiple Sclerosis Genetics Consortium. Electronic address: cotsapas@broadinstitute.org, International Multiple Sclerosis

Genetics Consortium. NR1H3 p.Arg415Gln is not associated to multiple sclerosis risk. *Neuron.* (2016) 92:333–5. doi: 10.1016/j.neuron.2016.09.052
12. Sadovnick AD, Gu BJ, Traboulsee AL, Bernales CQ, Encarnacion M, Yee IM, et al. Purinergic receptors P2RX4 and P2RX7 in familial multiple sclerosis. *Hum Mutat.* (2017) 38:736–44. doi: 10.1002/humu.23218
13. Zrzavy T, Kovacs-Nagy R, Reinthaler E, Deutschländer A, Schmied C, Kornek B, et al. A rare P2RX7 variant in a hungarian family with multiple sclerosis. *Mult Scler Relat Disord.* (2019) 27:340–1. doi: 10.1016/j.msard.2018.10.110
14. Garcia-Rosa S, Trivella DB, Marques VD, Serafim RB, Pereira JG, Lorenzi JC, et al. A non-functional galanin receptor-2 in a multiple sclerosis patient. *Pharmacogenomics J.* (2019) 19:72–82. doi: 10.1038/s41397-018-0032-6
15. Dyment DA, Cader MZ, Chao MJ, Lincoln MR, Morrison KM, Disanto G, et al. Exome sequencing identifies a novel multiple sclerosis susceptibility variant in the TYK2 gene. *Neurology.* (2012) 79:406–11. doi: 10.1212/WNL.0b013e3182616fc4
16. Zrzavy T, Leutmezer F, Kristoferitsch W, Kornek B, Schneider C, Rommer P, et al. Exome-sequence analyses of four multi-incident multiple sclerosis families. *Genes.* (2020) 11:988. doi: 10.3390/genes11090988
17. Ramagopalan SV, Dyment DA, Cader MZ, Morrison KM, Disanto G, Morahan JM, et al. Rare variants in the CYP27B1 gene are associated with multiple sclerosis. *Ann Neurol.* (2011) 70:881–6. doi: 10.1002/ana.22678
18. Pytel V, Matías-Guiu JA, Torre-Fuentes L, Montero-Escribano P, Maietta P, Botet J, et al. Exonic variants of genes related to the vitamin D signaling pathway in the families of familial multiple sclerosis using whole-exome next generation sequencing. *Brain Behav.* (2019) 9:e01272. doi: 10.1002/brb3.1272
19. Mescheriakova JY, Verkerk AJ, Amin N, Uitterlinden AG, van Duijn CM, Hintzen RQ. Linkage analysis and whole exome sequencing identify a novel candidate gene in a Dutch multiple sclerosis family. *Mult Scler.* (2019) 25:909–17. doi: 10.1177/1352458518777202
20. Wray NR, Lin T, Austin J, McGrath JJ, Hickie IB, Murray GK, et al. From basic science to clinical application of polygenic risk scores: a primer. *JAMA Psychiatry.* (2021) 78:101–9. doi: 10.1001/jamapsychiatry.2020.3049
21. Choi SW, Mak TS-H, O'Reilly PF. Tutorial: a guide to performing polygenic risk score analyses. *Nat Protoc.* (2020) 15:2759–72. doi: 10.1038/s41596-020-0353-1
22. Paré G, Mao S, Deng WQ. A machine-learning heuristic to improve gene score prediction of polygenic traits. *Sci Rep.* (2017) 7:12665. doi: 10.1038/s41598-017-13056-1
23. Vilhjálmsson BJ, Yang J, Finucane HK, Gusev A, Lindström S, Ripke S, et al. Modeling linkage disequilibrium increases accuracy of polygenic risk scores. *Am J Hum Genet.* (2015) 97:576–92. doi: 10.1016/j.ajhg.2015.09.001
24. Mak TSH, Porsch RM, Choi SW, Zhou X, Sham PC. Polygenic scores via penalized regression on summary statistics. *Genet Epidemiol.* (2017) 41:469–80. doi: 10.1002/gepi.22050
25. Privé F, Vilhjálmsson BJ, Aschard H, Blum MGB. Making the most of clumping and thresholding for polygenic scores. *Am J Hum Genet.* (2019) 105:1213–21. doi: 10.1016/j.ajhg.2019.11.001
26. Hedström AK, Lima Bomfim I, Barcellos L, Gianfrancesco M, Schaefer C, Kockum I, et al. Interaction between adolescent obesity and HLA risk genes in the etiology of multiple sclerosis. *Neurology.* (2014) 82:865–72. doi: 10.1212/WNL.0000000000000203
27. Hedström AK, Bomfim IL, Barcellos LF, Briggs F, Schaefer C, Kockum I, et al. Interaction between passive smoking and two HLA genes with regard to multiple sclerosis risk. *Int J Epidemiol.* (2014) 43:1791–8. doi: 10.1093/ije/dyu195
28. Nielsen TR, Rostgaard K, Askling J, Steffensen R, Oturai A, Jersild C, et al. Effects of infectious mononucleosis and HLA-DRB1*15 in multiple sclerosis. *Mult Scler.* (2009) 15:431–6. doi: 10.1177/1352458508100037
29. De Jager PL, Chibnik LB, Cui J, Reischl J, Lehr S, Simon KC, et al. Integration of genetic risk factors into a clinical algorithm for multiple sclerosis susceptibility: a weighted genetic risk score. *Lancet Neurol.* (2009) 8:1111–9. doi: 10.1016/S1474-4422(09)70275-3
30. Jafari N, Broer L, van Duijn CM, Janssens ACJW, Hintzen RQ. Perspectives on the use of multiple sclerosis risk genes for prediction. *PLoS ONE.* (2011) 6:e26493. doi: 10.1371/journal.pone.0026493
31. Gourraud P-A, McElroy JP, Caillier SJ, Johnson BA, Santaniello A, Hauser SL, et al. Aggregation of multiple sclerosis genetic risk variants in multiple and single case families. *Ann Neurol.* (2011) 69:65–74. doi: 10.1002/ana.22323
32. Disanto G, Dobson R, Pakpoor J, Elangovan RI, Adiutori R, Kuhle J, et al. The refinement of genetic predictors of multiple sclerosis. *PLoS ONE.* (2014) 9:e96578. doi: 10.1371/journal.pone.0096578
33. Dobson R, Ramagopalan S, Topping J, Smith P, Solanky B, Schmierer K, et al. A risk score for predicting multiple sclerosis. *PLoS ONE.* (2016) 11:e0164992. doi: 10.1371/journal.pone.0164992
34. Ayati M, Koyutürk M. PoCos: population covering locus sets for risk assessment in complex diseases. *PLoS Comput Biol.* (2016) 12:e1005195. doi: 10.1371/journal.pcbi.1005195
35. Xia Z, White CC, Owen EK, Von Korff A, Clarkson SR, McCabe CA, et al. Genes and environment in multiple sclerosis project: a platform to investigate multiple sclerosis risk. *Ann Neurol.* (2016) 79:178–89. doi: 10.1002/ana.24560
36. Kulm S, Marderstein A, Mezey J, Elemento O. A systematic framework for assessing the clinical impact of polygenic risk scores. *bioRxiv medRxiv.* (2020). doi: 10.1101/2020.04.06.20055574
37. Jacobs BM, Noyce AJ, Bestwick J, Belete D, Giovannoni G, Dobson R. Gene-environment interactions in multiple sclerosis: a UK biobank study. *Neurol Neuroimmunol Neuroinflamm.* (2021) 8:e1007. doi: 10.1212/NXI.0000000000001007
38. Barnes CLK, Hayward C, Porteous DJ, Campbell H, Joshi PK, Wilson JF. Contribution of common risk variants to multiple sclerosis in Orkney and Shetland. *Eur J Hum Genet.* (2021) 29:1701-9. doi: 10.1038/s41431-021-00914-w
39. International Multiple Sclerosis Genetics Consortium (IMSGC), Bush WS, Sawcer SJ, de Jager PL, Oksenberg JR, McCauley JL, et al. Evidence for polygenic susceptibility to multiple sclerosis–the shape of things to come. *Am J Hum Genet.* (2010) 86:621–5. doi: 10.1016/j.ajhg.2010.02.027
40. de Mol CL, Jansen PR, Muetzel RL, Knol MJ, Adams HH, Jaddoe VW, et al. Polygenic multiple sclerosis risk and population-based childhood brain imaging. *Ann Neurol.* (2020) 87:774–87. doi: 10.1002/ana.25717
41. de Mol CL, Neuteboom RF, Jansen PR, White T. White matter microstructural differences in children and genetic risk for multiple sclerosis: a population-based study. *Mult Scler.* (2021) 13524585211034826. doi: 10.1177/13524585211034826
42. Wand H, Lambert SA, Tamburro C, Iacocca MA, O'Sullivan JW, Sillari C, et al. Improving reporting standards for polygenic scores in risk prediction studies. *Nature.* (2021) 591:211–9. doi: 10.1038/s41586-021-03243-6
43. Wray NR, Yang J, Hayes BJ, Price AL, Goddard ME, Visscher PM. Pitfalls of predicting complex traits from SNPs. *Nat Rev Genet.* (2013) 14:507–15. doi: 10.1038/nrg3457
44. Fagnani C, Neale MC, Nisticò L, Stazi MA, Ricigliano VA, Buscarinu MC, et al. Twin studies in multiple sclerosis: a meta-estimation of heritability and environmentality. *Mult Scler.* (2015) 21:1404–13. doi: 10.1177/1352458514564492
45. Schaid DJ, Chen W, Larson NB. From genome-wide associations to candidate causal variants by statistical fine-mapping. *Nat Rev Genet.* (2018) 19:491–504. doi: 10.1038/s41576-018-0016-z
46. Yengo L, Sidorenko J, Kemper KE, Zheng Z, Wood AR, Weedon MN, et al. Meta-analysis of genome-wide association studies for height and body mass index in 700000 individuals of European ancestry. *Hum Mol Genet.* (2018) 27:3641–9. doi: 10.1093/hmg/ddy271
47. Elgart M, Lyons G, Romero-Brufau S, Kurniansyah N, Brody JA, Guo X, et al. Polygenic risk prediction using gradient boosted trees captures non-linear genetic effects and allele interactions in complex phenotypes. *bioRxiv medRxiv.* (2021). doi: 10.1101/2021.07.09.21260288
48. Moutsianas L, Jostins L, Beecham AH, Dilthey AT, Xifara DK, Ban M, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. *Nat Genet.* (2015) 47:1107–13. doi: 10.1038/ng.3395
49. Duncan L, Shen H, Gelaye B, Meijsen J, Ressler K, Feldman M, et al. Analysis of polygenic risk score usage and performance in diverse human populations. *Nat Commun.* (2019) 10:3328. doi: 10.1038/s41467-019-11112-0
50. Márquez-Luna C, Loh P-R, South asian type 2 diabetes (SAT2D) Consortium, SIGMA Type 2 Diabetes Consortium, Price AL. Multiethnic polygenic risk scores improve risk prediction in diverse populations. *Genet Epidemiol.* (2017) 41:811–23. doi: 10.1002/gepi.22083
51. Amariuta T, Ishigaki K, Sugishita H, Ohta T, Koido M, Dey KK, et al. Improving the trans-ancestry portability of polygenic risk scores by prioritizing variants in predicted cell-type-specific regulatory

elements. *Nat Genet.* (2020) 52:1346–54. doi: 10.1038/s41588-020-00740-8
52. Isobe N, Madireddy L, Khankhanian P, Matsushita T, Caillier SJ, Moré JM, et al. An immunochip study of multiple sclerosis risk in African Americans. *Brain.* (2015) 138:1518–30. doi: 10.1093/brain/awv078
53. Beecham AH, Amezcua L, Chinea A, Manrique CP, Rubi C, Isobe N, et al. The genetic diversity of multiple sclerosis risk among hispanic and African American populations living in the United States. *Mult Scler.* (2019) 26:1329–39. doi: 10.1177/1352458519863764
54. Chi C, Shao X, Rhead B, Gonzales E, Smith JB, Xiang AH, et al. Admixture mapping reveals evidence of differential multiple sclerosis risk by genetic ancestry. *PLoS Genet.* (2019) 15:e1007808. doi: 10.1371/journal.pgen.1007808
55. Belbasis L, Bellou V, Evangelou E, Tzoulaki I. Environmental factors and risk of multiple sclerosis: Findings from meta-analyses and mendelian randomization studies. *Mult Scler.* (2020) 26:397–404. doi: 10.1177/1352458519872664
56. Harroud A, Morris JA, Forgetta V, Mitchell R, Smith GD, Sawcer SJ, et al. Effect of age at puberty on risk of multiple sclerosis: a mendelian randomization study. *Neurology.* (2019) 92:e1803–10. doi: 10.1212/WNL.0000000000007325
57. Harroud A, Richards JB. Mendelian randomization in multiple sclerosis: a causal role for vitamin D and obesity? *Mult Scler.* (2018) 24:80–5. doi: 10.1177/1352458517737373
58. Vandebergh M, Goris A. Smoking and multiple sclerosis risk: a Mendelian randomization study. *J Neurol.* (2020) 267:3083–91. doi: 10.1007/s00415-020-09980-4
59. Jacobs BM, Noyce AJ, Giovannoni G, Dobson R. BMI and low vitamin D are causal factors for multiple sclerosis: a mendelian randomization study. *Neurol Neuroimmunol Neuroinflamm.* (2020) 7:e662. doi: 10.1212/NXI.0000000000000662
60. Mitchell RE, Bates K, Wootton RE, Harroud A, Richards JB, Smith GD, et al. Little evidence for an effect of smoking on multiple sclerosis risk: a mendelian randomization study. *PLoS Biol.* (2020) 18:e3000973. doi: 10.1371/journal.pbio.3000973
61. Hedström AK, Olsson T, Alfredsson L. High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. *Mult Scler.* (2012) 18:1334–6. doi: 10.1177/1352458512436596
62. Munger KL, Bentzen J, Laursen B, Stenager E, Koch-Henriksen N, Sørensen TIA, et al. Childhood body mass index and multiple sclerosis risk: a long-term cohort study. *Mult Scler.* (2013) 19:1323–9. doi: 10.1177/1352458513483889
63. Kaisey M, Solomon AJ, Luu M, Giesser BS, Sicotte NL. Incidence of multiple sclerosis misdiagnosis in referrals to two academic centers. *Mult Scler Relat Disord.* (2019) 30:51–6. doi: 10.1016/j.msard.2019.01.048
64. Fatehi F, Kyrychko SN, Ross A, Kyrychko YN, Blyuss KB. Stochastic effects in autoimmune dynamics. *Front Physiol.* (2018) 9:45. doi: 10.3389/fphys.2018.00045
65. Goodin DS, Khankhanian P, Gourraud P-A, Vince N. The nature of genetic and environmental susceptibility to multiple sclerosis. *PLoS ONE.* (2021) 16:e0246157. doi: 10.1371/journal.pone.0246157

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Prevention of MS Requires Intervention on the Causes of the Disease: Reconciling Genes, Epigenetics, and Epstein Barr Virus

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Prevention of multiple sclerosis requires intervention on modifiable causes of the condition making it necessary to establish what those causes are. MS is often stated to be a polygenic disease, with causal contributions from environmental factors and gene-environment interactions, implying an additive and independent relationship of these factors. Mechanistically there are no independent contributions of genes or environmental factors to traits. This model is unrealistic but still useful and underlies the concept of heritability, a foundational parameter in population genetics. However, it perpetuates a debate on an irreconcilable dichotomy about whether MS is primarily genetic or environmental. In particular, epidemiological evidence now exists for a causal, possibly even necessary, role for Epstein Barr Virus in MS. The additive model makes it unintuitive to reconcile MS as a genetic disease but also independently a viral illness. In this perspective it is argued that starting from a realistic interaction only model, based on broadly accepted biological premises, and working forward to explain why the classical additive model gives useful results, there is actually no paradox. An integrated approach using population genetic studies, immunology and molecular virology offers a particularly promising route to establish the elusive role of EBV in MS pathology, as EBV is a large and complex virus and its latency, dysregulated in most EBV-related pathologies, is hard to study *in vivo*. This approach may offer a route to prevention of MS altogether.

Keywords: multiple sclerosis, genetics, environment, Epstein Barr Virus, virus, prevention, autoimmunity, neuroinflammation

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1. INTRODUCTION

Prevention of multiple sclerosis (MS) occurring altogether, rather than prevention of MS disability by early diagnosis and effective treatment, would require intervening on the modifiable causes of MS, making it critical to establish what those are. However, fortunately much is now known about specific factors contributing to variation in MS risk (1).

Evidence for MS susceptibility being genetic is incontrovertible, and converges from many sources: aggregation of risk in families (2–5), robust genotype-phenotype associations for particular HLA alleles and over 200 non-HLA loci (6), from adoption studies (7), etc. Likewise, changing

disease incidence over a small number of generations (8, 9), marked geographical variation in disease risk between and within countries coupled with findings of migration studies (10, 11), and serological [Epstein Barr Virus (EBV)] and robust lifestyle (smoking, adolescent obesity and vitamin D deficiency) associations indicate the importance of environmental factors even decades prior to diagnosis (12). Particularly, in the case of EBV infection, which is the only consistent, strong, and temporal association (genetic or environmental) that has thus far been suggested to be necessary for MS (13–25), the evidence for a causal relationship in at least most cases of MS is unusually strong as specific viruses are rarely *necessary* for clinical syndromes (and are perhaps never sufficient), so the apparent necessity of EBV in MS is strikingly unusual (26). The epidemiological association between EBV and MS has been reviewed previously (27–29), but it may be noted that genes and EBV do not appear to explain all of the epidemiological observations, and whilst the second half of this perspective will focus on opportunities to use genetic and immunological studies to understand the role of EBV in MS, this suggests that other important factors contribute to causing MS (12). Other "hits" may even be necessary.

Clearly both genes and environment are important, but currently it is usually not possible to intervene to modify an individual's genes to prevent disease and antenatal genetic screening as a method of prevention brings a myriad of serious ethical concerns. However, a powerful feature of understanding the genetic architecture of a disease is that it can lead to the identification of environmental factors which may be modifiable or can reveal the biological pathways that these factors are acting on. Drugs can be targeted to particular biology, and protective or harmful environmental factors can be epidemiologically identified by their genetic interactions and exposures modified. For a complex environmental factor like EBV infection, the pathobiology can still be an enigma even after the evidence for causation is strong. In this case, combining insights from genetics, virology, and immunology and reconciling the genetic and environmental factors into an integrated etiological model may be a very fruitful path to prevention of disease altogether.

2. THE ROOT OF THE NATURE VS. NURTURE FALSE DICHOTOMY

Acknowledging the epidemiological data, MS is often described as a complex genetic disease, with important causal contributions also coming from environmental factors and gene-environment interactions (30–37). This statement and paraphrases of it sound etiological but actually describe the classic additive model of population genetics which is primarily concerned with a related but different concept: partitioning the variation of a trait observed in a given population into the sources of that variation (Equation 1) (38, 39). Where total *variation* in phenotype (P) in the population, is statistically "explained" by the linear (independent or additive) combination of variation in phenotype due to genetic (G), and environmental factors (E), and their interactions ($G \times E$). In the case of MS, the phenotype is risk or liability to develop MS [a continuous unmeasured (or latent) variable], where exceeding some threshold liability leads to disease penetrance (**Figure 1**). This partitioning also underlies the concept of heritability, which, for the purposes of statistical genetics, is defined as the proportion of phenotype attributable to the genotype term (G/P). Heritability, is a key parameter in population genetics, but "heritable" had a common language meaning dating to the fourteenth century in English and an established legal meaning relating to the inheritance of property before it had a technical one limited to partitioning statistical variation. Consequently, it is often confusingly used with imprecise or interchanging meanings (40).

$$P = G + E + (G \times E) \tag{1}$$

To illustrate the problem with confusing this with an etiological model, we can consider the issue of gun crime. Gun crime is entirely an interaction between guns and criminals and there are no additional independent mechanistic contributions to gun crime from guns or criminals. All gun crime is 100% due to the interaction. However, if guns and criminals were modeled as independent factors to explain variation in gun crime, it might be possible to explain some % of the variation in gun crime across cities based on gun availability, even though it makes no mechanistic sense to say that (e.g.,) 60% of gun crime can be explained independently by the availability of guns and so gun crime is 60% caused by guns and 40% by criminals.

The fundamental problem with considering the model in Equation (1) as an etiological model, therefore, is that it implies that genes and environment contribute independently [in the first two terms (G) and (E) of Equation 1] when it is widely accepted that this is unrealistic. The addition of a more mechanistically plausible gene-environment (GxE) interaction term does not resolve the issue of the first two terms being mechanistically implausible. Population genetic studies typically partition both variation in phenotype due to genes and environment further: for example, into contributions from individual and shared environment; and into additive, epistatic, and dominant genetic influences. However, the initial partitioning into independent genetic and environmental terms is the root of the nature vs. nurture false dichotomy (41). This gives rise to an apparent paradox when a disease, such as MS, appears to have a strong genetic basis but also to be the result of a necessary environmental factor.

> *"Without environmental inputs, your genome would have created nothing more than a damp spot on the carpet."*
>
> ***Lykken, 1995***

> *"if there is no environment, no organism can develop to display any phenotype whatsoever. Likewise, without a genetic constitution, there will be no organism."*
>
> ***McLearn, 1964***

> *"It is needless to insist that neither [nature nor nurture] is self-sufficient."*
>
> ***Galton, 1874***

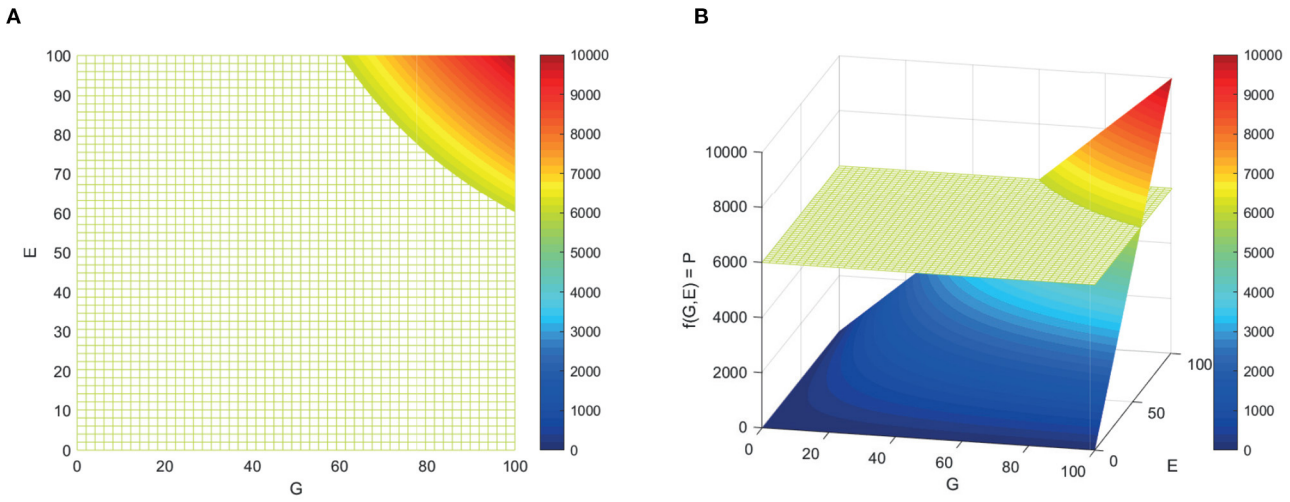

FIGURE 1 | Surface representing the f(G,E) where phenotype is an interaction of genotype and environment. **(A)** From above. **(B)** Angled.

The above quotations and compilation of views from many authors on the merits of heritability and the nature vs. nurture debate is available in Sesardic, Making sense of Heritability, 2005 (42), illustrating more than a century of consensus on the unrealistic nature of this model.

In the next section of this perspective, a simple derivation of the additive model (Equation 1) is derived from a more biologically realistic model and the historical context and implications for understanding MS epidemiology are discussed. Thereafter, it is argued that by focussing the tools of population genetics, immunology, and molecular epidemiology on the difficult problem of latent EBV, an elusive epigenetic master manipulator of memory B cells, and by investigating MS as both a complex genetic disease *and* probably a viral pathology simultaneously, exciting opportunities for attempting MS prevention may arise.

3. RECONCILING THE ADDITIVE MODEL OF POPULATION GENETICS WITH MOLECULAR GENETICS

3.1. Historical Perspective—The Additive Model Is Useful but Incomplete

The field of population genetics, and by extension MS genetics, is arguably founded on this model (Equation 1) and the heritability parameter, as they implicitly underpin the early work of pioneers Ronald Fisher and Sewall Wright. Particularly, Fisher's famous 1918 paper and subsequent work that set a mathematical foundation for reconciling Mendelian discrete units of inherited traits with the observations of continuous variation in traits like height measured by the early quantitative biometricians (43). Fisher's solution was to develop the statistical tools to partition the variance of traits in a population—variance could be quantified—into contributions from heritable (genetic) factors measured by the correlation between relatives and everything else. The concept and mathematics of correlation had been developed essentially for this purpose by Galton and Pearson, respectively. Fisher built on this, including by inventing the statistical technique of Analysis of Variance (ANOVA) which is based on the linear or additive segregation of contributions to variance. Wright's application of his method of path analysis, the ancestor of many modern techniques of causal inference, is analogous to Fisher's ANOVA in this respect as both chose to partition genes and environment as though they were independent (44) and all work based on the concept of heritability has carried the underlying partitioning since.

Initially, this approach was probably adopted because very little was known about the biochemical nature of genes in the era prior to the discoveries of Avery et al. (45) and Crick and Watson (46), and so it was perhaps as reasonable a choice as any other model. Although the prejudices of the scientific establishment were almost surely also relevant and fueled the eugenics movement of the time. If Fisher and contemporaries thought about the biological nature of the units of heredity whilst establishing the mathematics in the first four decades of the twentieth century, they probably thought they were likely to be proteins, discrete material units, capable of extraordinary complexity, that might actually have exerted independent effects to external environmental factors. However, biologists criticized this early mathematical work for being overly theoretical, and subsequently, when years later the nature of genes as code for biochemically interpreting the environment was discovered, as mechanistically unrealistic. However, by that time, decades of theory based on the additive model had proved to work successfully enough that attempts to point out it was unrealistic did not dissuade its use.

> *"I do not feel that this kind of work affects us biologists much at present. It is too much of the order of problem that deals with weightless elephants upon frictionless surfaces, where at the same*

time we are largely ignorant of the other properties of the said elephants and surfaces."

Biologist R. Punnett's lukewarm review of Fisher's now famous 1918 paper at the Royal Society of London.

3.2. All Models Are Wrong, but If This One Is Unrealistic Why Is It Useful

$$P = G \times E \tag{2}$$

To address the realism problem, that biology supports interaction only (Equation 2) and not independent contributions (Equation 1), population genetics texts offer the disclaimer that additive model and heritability tells us not about individuals or mechanistic causes but rather about the causes of variance in the specific populations $n > 1$ under investigation and that these findings are not necessarily valid if generalized to other populations or to individuals (as can be understood in the gun crime analogy) (40, 47).

But the idea that the sign can change from interaction (multiplicative) to addition just because the population $n > 1$ is surprising. The simplest population is two individuals, and Equation (1) is unrealistic here too, as the phenotypes of the population are the sum of the two individual phenotypes, generally $P = \sum_{i=1}^{n}(G_i \times E_i)$. So when is a population big enough for the additive approach to work and why? Perhaps more importantly, this risks underselling the mechanistic insights that are gained from studying the heritability of traits in populations. Genes that are associated with variance in the risk of MS in large GWAS, do inform as to the biological pathways mechanistically important for MS pathology in individuals because genes and environment act on individuals and do not have effects on the phenotypes in a population except via the sum of their effects on individuals. If a variant in a gene causes variation at the population level, it can only do so by being a mechanistic cause of the trait in at least some individuals.

Causes of variation in a population are, therefore, a subset of the mechanistic causes of that trait. However, other important mechanistic causes may not be responsible for any variation, for example, because they are ubiquitously experienced, or strongly associated with other causal factors that negate their associations: just as association does not imply causation, causation does not imply association.

The analogy of gun crime is useful because it highlights where the model stops being useful. If we did not know the mechanistic nature of gun crime, we might infer the importance of guns from discovering that their availability explained some of the variation across cities, whereas variation in access to spoons, kitchen chairs, or other household objects does not. But, if everyone had abundant and equal access to guns we would need a different approach despite guns still being a necessary cause of gun crime. Similar inferences can be drawn from the study of genetic variation and association with traits of interest at the population level for diseases like MS where the causes are less obvious. However, caution is particularly necessary not to discount the contribution of genes that are tightly conserved and environmental exposures that are ubiquitous or nearly so.

3.3. Deriving the Useful Additive Model From the Realistic Interaction Model

Starting from the premise that MS is exclusively the product of gene-environment interactions, gives the interaction model in Equation (2) which captures the reality of mechanism but otherwise is not useful. Working forward to derive the useful additive one (Equation 1) gives an insight into why the additive model gives useful results and makes the meaning of parameters derived from it (like heritability) more intuitive. A geometric representation of this is presented in **Figure 2** for the simplest possible population ($n = 2$). First, if we consider that all the causal factors for any phenotype can be partitioned into those that are also causing variation in the population and those that are causal but not causing variation (for example, because they are ubiquitous), then we can model the total (varying and non-varying) effects (P) and causes (G,E):

$$P_t = G_t \times E_t \tag{3}$$

where the subscript (t) denotes the total effects (P) or causes (G,E) for each term. Each term can then be partitioned into mutually exclusive and jointly exhaustive sets based on whether they are also observed to be varying (P) or causing variation (G,E) in the given population (v, varying) or not (c, constant). So the total phenotype P_t is the sum of the phenotype that is varying P_v and that which is not varying (common to everyone in the population) P_c, and similarly G_t is the sum of causal genes that are also causing variation G_v and those that are not causing variation G_c, and so on for environment. As below (Equation 4),

$$P_t = P_c + P_v;\ G_t = G_c + G_v;\ E_t = E_c + E_v \tag{4}$$

If we then say that we are only (for practical reasons) interested in the part of the phenotype that is varying, because that can be quantified using the units of population variance, then we need to subtract P_c, which will be only the component with no contributions to variation (v terms) (Equation 5).

$$P_c = G_c \times E_c \tag{5}$$

and so by rearranging the first Equation in (4), and substituting the right hand side of Equation (5), we can get,

$$P_v = P_t - (G_c \times E_c) \tag{6}$$

and by replacing P_t with the right hand side of (Equation 3) we get,

$$P_v = (G_t \times E_t) - (G_c \times E_c) \tag{7}$$

which expands using definitions for G_t and E_t in (Equation 4) to,

$$P_v = (G_v + G_c) \times (E_v + E_c) - (G_c \times E_c) \tag{8}$$

and after expansion of the first two bracketed terms, the constant terms ($Gc \times Ec$) cancel out. Giving,

$$P_v = (G_v \times E_c) + (E_v \times G_c) + (G_v \times E_v) \tag{9}$$

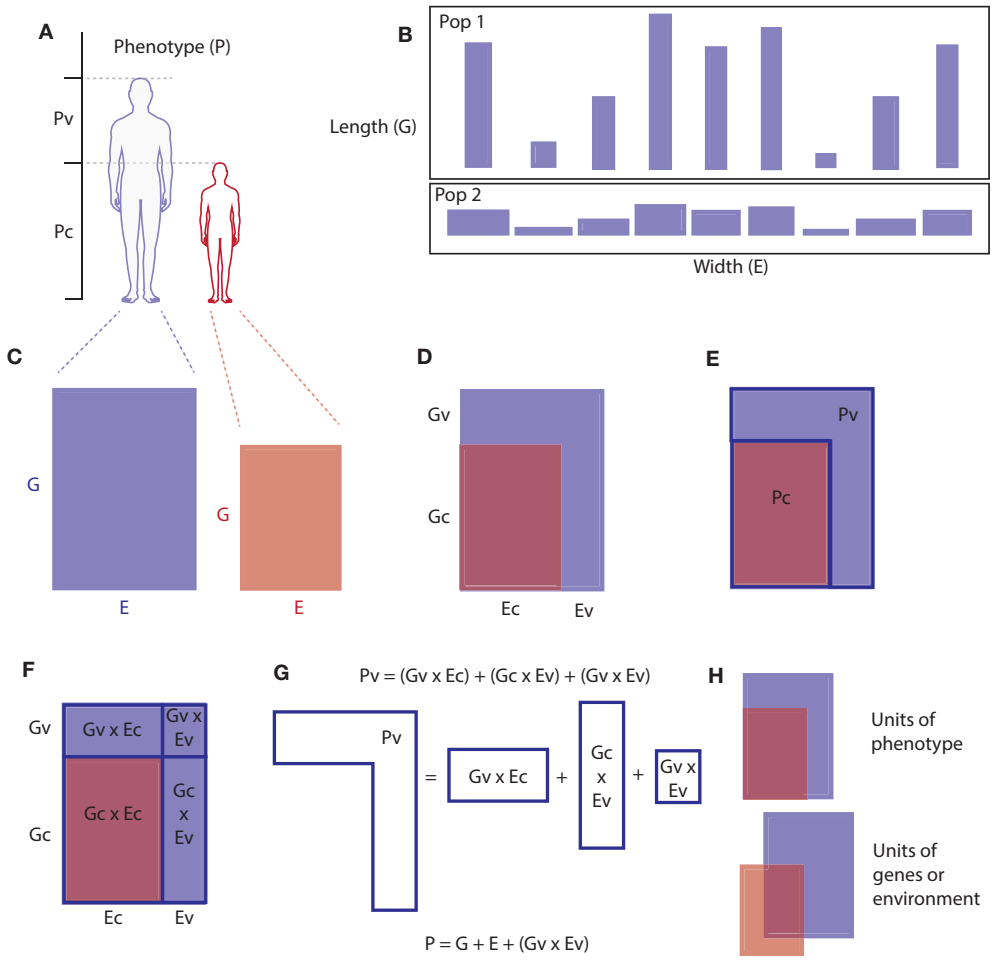

FIGURE 2 | Geometric representation of the partitioning of causes of a phenotype into genetic and environmental causes that are causing variation in the population and non-varying causes. Just as genes and environment are inseparable in causing phenotypes, the area of a rectangle cannot be considered to be mainly the product of either its length or width. However, if an individual's genes are represented on one axis (length) and their environment on an orthogonal axis (width) and phenotype represented by the area, then in a population of such individuals, most of the variation in area(phenotype) across the population can come from variation on one of the axes [e.g., the length (genes) axis]. For the simplest possible population, two individuals (two rectangles), we can see visually how Equations (1) and (3) are related. **(A)** Partitioning phenotype in a population (two individuals), phenotype of interest is represented as height, but any phenotype could be partitioned as such, **(B)** two populations of rectangles where the variation in area across the population is mostly the result of variation in length rather than width. **(C)** Phenotype represented as the product (area) of two necessary interacting factors. **(D–F)** Partitioning causal genes and environmental factors into common and varying contributions. **(G)** Decomposing the genetic and environmental contributions to phenotypic variation (Pv) into the additive model. **(H)** This model only works when the G and E terms are measured in terms of phenotype, when measured in units of genes or environment themselves the model would require a different (multivariate) model.

If we take any given population as having fixed conditions (for example, because they've already happened), then the constant terms can be treated as constants and ignored (as long as we remember that they do exist and may not necessarily be the same level or constant in other populations, see **Figure 2B**), then we get back to the classical additive model (Equation 1). All terms can then be measured or calculated in units of variation/deviation from the mean phenotype to allow them to be quantitatively interrogated. This is equivalent to mean centering (subtracting the mean value of phenotype (μ), scaling by standard deviation, and adding some random measurement error (ϵ), giving the familiar linear model:

$$P_v - \mu = (G_v - \mu) + (E_v - \mu) + ((G_v \times E_v) - \mu) + \epsilon \quad (10)$$

This demonstrates that the additive model (Equations 1 and 10) is an incomplete simplification of a realistic interaction model (Equation 9). It will be generalizable to populations other than the one it is fitted on only in the special circumstance where the environmental causes and genetic causes are the same and are partitioned in the same way. That is, when the same genetic and environmental causes are present and are also contributing to variation or not in both cohorts. Whether this is the case or not depends on what these factors are and how similar exposure profiles are in the populations in question. This implies that heritability is actually best understood as the proportion of variation explained by the interaction of genes that are causing variation in the population with ubiquitous causal environmental factors, not as

the proportion of variation explained by genetic variants acting independently.

3.4. Implications of This Model to MS Epidemiology

The first implication is that if a trait such as MS were to be 100% heritable it would only mean that the environmental factors causing it are not causing variation, not that it is a purely genetic disease and environmental causal factors are unimportant. Rather they will surely exist, but probably will have been ubiquitous or near ubiquitous, but without knowing what they are it is impossible to say whether they are modifiable, necessary for some other physiologically essential reason, or unavoidable environmental factors. Therefore, the necessity of a near ubiquitous virus like EBV is no barrier to observing a heritability considerably higher even than that which is actually observed in the case of MS. In fact, the more ubiquitous a necessary environmental factor is the higher the heritability will tend to be and the more aggregation in families that should be expected if causal genetic variants are also contributing to variation in the population. Therefore, there should be no theoretical conflict in accepting MS as a complex genetic disease and also the consequence of a viral infection simply on the basis of its heritability or aggregation in families.

The fact that the heritability of MS is much <100% probably speaks to the importance of other less ubiquitous environmental factors (beyond EBV) causing variation mostly by interacting with conserved genes $E_v \times G_c$ (the equivalent term to E in Equation 1) although it could in theory be possible that varying exposure to different strains of EBV (some pathogenic and some not) or timing of infection could account for some reduction of heritability. Whilst hypothetical, both have been suggested (48–50), and were it to be the case would add another layer of complexity, as when EBV is acquired in childhood, it is typically acquired from within the family unit. Therefore, as with human genes, EBV is also inherited identically by descent to an extent. This would mean that some aggregation due to shared ancestry could be mis-attributed to shared human genes, where the correlation between relatives in phenotype is also affected by the correlation between relatives in the pathogenicity of the strain of virus or timing of infection.

A second related implication, is that changes in exposure to environmental factors over time or space will affect estimates of heritability even where the frequency of gene variants are unchanged. Consider two otherwise identical hypothetical populations, where in one 30% of individuals smoke, and the other everyone does. Because smoking is an established risk factor for MS, the effect of increased smoking in the all-smoking cohort would be to inflate the ($Gv \times Ec$) term (Equation 9). In the classic additive model (Equations 1 and 10) this term is thought of as representing the genetic influences G. Therefore, an increase in environmental exposure, would counter-intuitively be reflected in higher estimates of genetic influence and heritability in the all-smoking cohort, and lower estimates of the influence of environmental factors and gene-environment interaction. At least, that is, if taking Equations (1) and (10) at face value (forgetting that each term does in fact represent a mechanistic G and E interaction). Higher estimates of heritability in locations with higher MS incidence has been demonstrated, and the more realistic model (Equation 9) explains the counter-intuitive but probably correct conclusion that the higher heritability in higher incidence populations is likely to reflect a higher burden of environmental exposures (51, 52). Although strictly speaking the heritability will increase when the environmental exposure is more constant and this could be higher or lower mean exposure or the same so long as less variation occurs (consider populations where 25% of individuals smoke one packet of 20 cigarettes per day vs. populations where 100% smoke either 1, 5, or 40 cigarettes per day). The latter three populations would all be expected to have higher heritability than the first cohort all else being equal.

A further implication is that despite replication in large genetic studies being important in eliminating associations caused by biases arising from observational nature of the study design, genotype-phenotype associations that do not replicate across cohorts may include some of the most interesting real causal associations. Because where genotype-phenotype associations do not replicate due to differences in environmental exposures captured under Ec between cohorts, it suggests that these environmental exposures are probably modifiable (despite not causing variation in either cohort independently). This could occur if, for example, a causal environmental factor is ubiquitously present in one cohort but not another. This may be of particular interest in the case of MS where there is wide variation in incidence between nations/regions/cultures (where ubiquitous exposures could plausibly differ), and where environmental exposures have been suggested to vary "at the population level" (31). If the two hypothetical near-identical-except-for-smoking cohorts in the previous paragraph, had smoking prevalences of 0% (not 30%) and 100%, then smoking would differ at the population level (i.e., between the populations) and not contribute to variability within either cohort. However, some of the gene-phenotype associations present in the all-smoking cohort [captured in the ($G_v \times E_C$) term], but not replicating in the non-smoking cohort would reveal genes that mediate the causal effect of smoking.

Critically, all terms (P,G,E) on both sides of these equations are in units of variation in phenotype, which may be important for considering how specific causal factors are divided between the terms. This means that a given environmental or genetic factor may fall into more than one category (c) or (v) in differing proportions, because the model would have to be specified differently (from the bottom up) if each gene or environmental factor were to be partitioned into one or other term (**Figure 2H**). For example, in a matched case-control GWAS study for MS 90% of controls will have EBV, and 100% of the cases will (if EBV is necessary for MS), so 90% of matched pairs will be concordant for the apparently necessary factor, the virus. EBV would be reflected in the E_C terms of Equation (8) for 90% of pairs, and for the 10% will contribute to the E_V containing terms. This would also mean that 10% of controls in these studies are therefore not at risk of MS due to being EBV naive, meaning that all estimates for genetic variants associated with MS will have effect sizes diluted or biased toward a null effect because some of the controls are not at risk

regardless of their genetic risk. Thus, if EBV is a necessary cause of MS, then the effect sizes genome-wide for SNP associations with MS are likely to be systematically underestimated by 10%. As the underlying models differ, this may explain some of the missing heritability phenomenon that occurs when top down and bottom up approaches to calculating heritability do not agree.

4. EPSTEIN BARR VIRUS: A MASTER EPIGENETIC MANIPULATOR OF B CELLS

4.1. Epidemiological Framework for Identifying Causal Associations

The epidemiological literature has been reviewed multiple times in the context of converging evidence, and is at least consistent with MS being a complication of EBV infection (27–29, 53, 54), but no definite counterfactual or experimental evidence exists to prove or disprove whether EBV causes MS, as no antiviral drug is known to clear latent EBV infection and as yet no vaccine protects from infection. However, classical causal theoretical frameworks can be considered strongly supportive. For example, the first four criteria set out by Bradford-Hill as the most important for judging an epidemiological association to be causal, overlap the criteria of a founder of causal philosophy, David Hume (55, 56). Hume identified strength, consistency, and temporality (cause before effect) as hallmarks of causal associations, and this insight underpins Bradford-Hill's attempt to establish a framework for epidemiological causal inference. Bradford-Hill made it clear that his nine criteria were not a checklist, but an ordered list with Hume's criteria three of the most important four determining when an association is likely to be due to cause and effect (56):

1. Strength of association (measured as ratio, not as an absolute difference): *"First upon my list I would put the strength of the association... in this situation I would reject the argument sometimes advanced that what matters is the absolute difference between the death rates of our various groups and not the ratio of one to the other. That depends upon what we want to know. If we want to know how many extra deaths from cancer of the lung will take place through smoking (i.e., presuming causation), then obviously we must use the absolute differences between the death rates... But it does not follow here... that this best measure of the effect upon mortality is also the best measure in relation to etiology. In this respect the ratios... are far more informative"*

The EBV-MS association is very strong, such that cases of EBV-naive MS, if they exist, are extremely rare, whereas 5–10% of the adult population will be EBV-naive (21). In fact, if one accepts that EBV *is* found in 100% of individuals with MS in large cohorts, if sufficiently sensitive methods are used (21), but only in 90% of healthy controls, then the point estimate on the odds ratio (odds of disease given exposed/odds of disease given unexposed) would be infinite and the lower bound on confidence very large. Given small biases cannot cause large effect sizes, Bradford-Hill argues that on this criteria alone, in the face of such a strong association, similar to that seen in the association between smoking and lung cancer, a non-causal explanation for the association, if it exists, should be obvious:

"Though there is good evidence to support causation it is surely much easier in this case to think of some feature of life that may go hand-in-hand with smoking—features that might conceivably be the real underlying cause or, at the least, an important contributor, whether it be lack of exercise, nature of diet, or other factors. But to explain the pronounced excess of cancer of the lung in any other environmental terms requires some feature of life so intimately linked with cigarette smoking and with the amount of smoking that such a feature should be easily detectable. If we cannot detect it or reasonably infer a specific one, then in such circumstances I think we are reasonably entitled to reject the vague contention of the armchair critic "you can't prove it," there may be such a feature."

2. Consistency: *"Next on my list of features to be specially considered I would place the consistency of the observed association"*

The EBV-MS association has been observed consistently across studies in various patient groups, geographies, ethnicities, ages, sexes, and sub-types of MS (14, 25, 57). This is important because the consistency across multiple studies improves the statistical (frequentist) confidence in the association, making it less likely to have occurred by chance, but also limits the alternative explanations to biases that would also be present across these multiple diverse settings.

3. Specificity: *"the specificity of the association, [is] the third characteristic which invariably we must consider"*

The lack of a consistent association with other saliva-transmitted viruses (e.g., CMV) reduces the probability that some large bias accounts for the observation and restricts the kind of bias that could be responsible. For example, this persuasively excludes many other "features of life" that could potentially result in higher exposure to EBV as an explanation because these features would also be non-specifically associated with exposures to other infectious agents and the necessary association observed is EBV specific (17, 25).

4. Temporal relationship: *"My fourth characteristic is the temporal relationship of the association"*

Evidence of EBV associations with MS in serosurveys has critically also been demonstrated to be temporal (EBV always before MS) in longitudinal studies (14, 18, 20, 58). This is further persuasive of a causal effect, and excludes many person-specific potential biases and reverse causality, e.g., shared susceptibility to EBV and MS, as it demonstrates that even for those who develop the disease (and so are susceptible to it) the risk of MS is extraordinarily low or nothing in these individuals prior to them contracting the virus.

Therefore, whatever the cause of MS is, it will ultimately have to explain why such a strong, consistent and temporal association with EBV is observed. If some bias accounts for the association making EBV simply a bystander, rather than a cause, then it begs the question, why is it not obvious what the explanation is?

4.2. Biological Plausibility for a Causal Association

The epidemiological case is further bolstered by the biological plausibility of EBV (plausibility being one of Bradford-Hill's lesser criteria) as both a known cause of serious pathology and

specifically as a cause of autoimmunity. Transient autoimmunity is recognized to occur at the time of primary EBV infection with infectious mononucleosis when the virus replicates to extremely high levels before the host adaptive immune system recognizes its presence (59–63).

In addition, several aspects of the natural history of MS fit the biology of EBV. As a persistent herpes virus infection which periodically reactivates from a quiet latency in a fluid compartment, the dissemination in time and space of neuroinflammatory attacks occurring over decades in persons living with relapse-remitting MS fits strikingly well. In addition, the site of life-long viral latency is strictly memory B cells, which are now known to be important for pathogenesis and a therapeutic target (64–67). Thus, a reasonable index of suspicion based on biology of the virus (prior probability) combined with a suitable likelihood from epidemiological evidence makes EBV a credible cause of MS.

EBV is a remarkably-successful, large, double-stranded DNA gammaherpes virus that for most of human history has been practically ubiquitous, now being only nearly ubiquitous by adulthood in high-income nations (67). In recent generations, EBV has transitioned from millions of years of equilibrium to a non-equilibrium virus with a reproductive number less than one ($R < 1$), reflected in the rising average age of infection and new evolutionary pressures. In low-income settings, almost everyone is still infected in early childhood, but in high income settings some 10–50% of individuals escape infection in childhood with most of these individuals acquiring the infection later in life (sometimes experiencing infectious mononucleosis) such that 90–95% of people are infected eventually. The virus has co-evolved with its human hosts in this ecological niche for millions of years (67, 68).

Consequently, EBV has specialized to make use of several unusual aspects of B cell physiology (**Figure 3**). Rather than rapidly replicating itself thousands of times (in the lytic cycle) on first infecting a B cell, as occurs with most viral infections, it expands the latently infected pool using a specialist repertoire of genes which drive the cell cycle, manipulating the pathways that B cells use to clonally expand and select for immunity in response to external stimuli (such as recognizing their cognate antigens) (67, 69). In order to do this EBV has acquired mimics of critical B cell specific signals, which essentially renders the infected B cells autonomous of T cell help and antigen (69, 70). Whilst manipulating the cell, the virus hides in the nucleus as a circular pseudochromosome (or episome), chromatinized and epigenetically marked, but not integrated into the human chromosomes. It is copied once and only once per cell cycle and faithfully segregated to daughter cells using only the host cell's replication machinery and a single viral protein. In effect, EBV immortalizes these cells whilst masquerading as a human chromosome and, transcribing only a very tightly controlled subset of its genes. This tight manipulation of both viral and cellular gene expression is a masterclass in epigenetic regulation, involving DNA methylation, histone modifications, and a complete 3-dimensional re-organization of chromatin environment within the nucleus of infected cells (71–73). This unusual strategy explains why so many EBV-related pathologies are lymphoproliferative, and why problems of dysregulated latency are common features of most EBV-related pathologies. Because as successful as this strategy is, driving cells to clonally expand and rendering them autonomous is risky, with a clear line of site to cancer and to autoimmunity. Thus, the absence of pharmacological tools that target latency, rather than lytic infection, is unfortunate (**Figure 3**).

Controlling latently-infected EBV is energy-intensive and a precarious immunological task, as evidenced by the Ĩ,000-fold increase in EBV-related malignancies in persons with CD4+ immunosuppression as a result of HIV infection, increase in latent cell number before and after some forms of (T cell affecting) immunosuppression, and the fact that a mutation in a single gene on the X-chromosome that leads to a defect in a protein important for T cell signaling, causes a lethal form of fulminant infectious mononucleosis called X-linked lymphoproliferative (XLP) syndrome in males after infection with EBV (67, 68, 74).

Unfortunately, studying the epigenetics of latency in MS patients and healthy controls, outwith the context of lymphoproliferative conditions, is challenging due to the virus being in equilibrium with T cell surveillance which maintains a low number of infected B cells (**Figure 3**). During established latency, the infected cell pool is a very small subset (in the region of 1–50 infected cells per million) of the total circulating B cells meaning that a large volume of blood needs to be collected to be sure of collecting even one virally infected cell. For example, an individual with a low normal B cell count and a low proportion of virally infected cells could have as few as five infected memory B cells in 50 mls of peripheral blood (**Figures 4**, **5**) (75). Further, when EBV amplifies itself by switching to lytic production, it linearizes and strips its DNA of its epigenetic marks for packaging into viral particles, essentially wiping it clean (68). Technologies are improving potentially allowing for single cell and rare cell approaches to address this, but it remains a technical hurdle.

For these reasons, using powerful but indirect methods (such as population genetics and immunology) to study the role of latent EBV in MS pathophysiology may yield valuable clues as to the nature of the pathology. Observations that many of the significant MS SNP-associations overlap with EBNA-2 transcription factor binding sites, for example, is interesting because this agrees with observations that anti-EBNA2 antibodies are part of the subset of EBV proteins that show a raised antibody profile in MS (23, 76–79). If these studies are interpreted as converging on a role for EBNA-2, then this gives an insight into the nature of the dysregulation of latency, as EBNA-2 is an essential for regulating viral latent gene expression and for EBV-driving lymphocytes into the cell cycle. Expression would be expected to increase EBV-infected cell number or turnover (67, 69).

A further example might be drawn from a study of cell-type specific transcriptomics (80), which identified genes involved in Neddylation as differentially expressed in the lymphocytes (T cells) of MS cases vs. controls. This is striking because Neddylation is evidently also a critical process for EBV and other herpes viruses, so much so that the EBV carries its

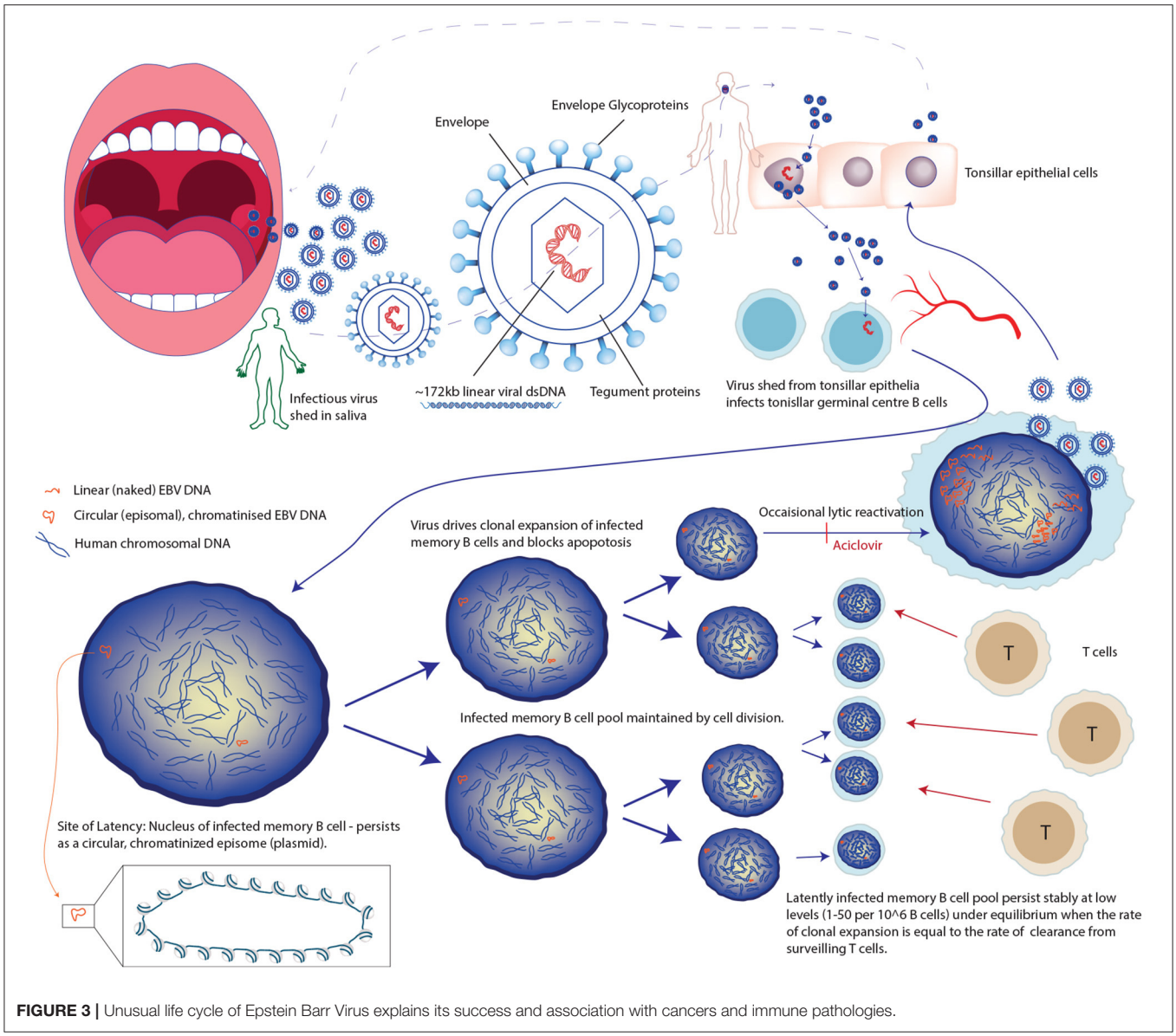

FIGURE 3 | Unusual life cycle of Epstein Barr Virus explains its success and association with cancers and immune pathologies.

own deneddylase enzyme amongst its tegument proteins, and antibodies against this viral protein (encoded by gene BPLF1) have recently been identified as a predictor of EBV viral load using an unbiased antibody screening method (80–82). Pharmacological targeting of viral enzymes is a potential therapeutic strategy, and so may be another fruitful avenue for further exploration. Intriguingly, the same drug targeting this pathway, has been proposed for trials in MS and in treating a human herpesvirus (81).

4.3. EBV-Focussed MS Prevention Possibilities

Even before the pathophysiological role of EBV in MS is established, it is possible to speculate generally on what a successful EBV-directed preventative strategy might look like.

4.3.1. Vaccination

Vaccination aimed at preventing infection with EBV would be clinically useful in a number of contexts beyond autoimmunity, for example, in preventing infectious mononucleosis or post-transplantation. These other indications may substantially de-risk the investment required to develop such a vaccine, given the EBV-MS association is not universally accepted as causal. In the context of prevention of MS, targeted vaccination for EBV-negative young adults who are at risk geographically and/or genetically (assessed either by polygenic risk or as a result of family history) of MS may be particularly worthwhile as using sophisticated models of known predictors of MS risk it has been demonstrated that individuals can be identified with much higher than population risk of MS (83). Targeting young adults pre-college, as is practized for meningococcal vaccination, may be particularly worthwhile given the high hazard rate of EBV exposure at this life stage.

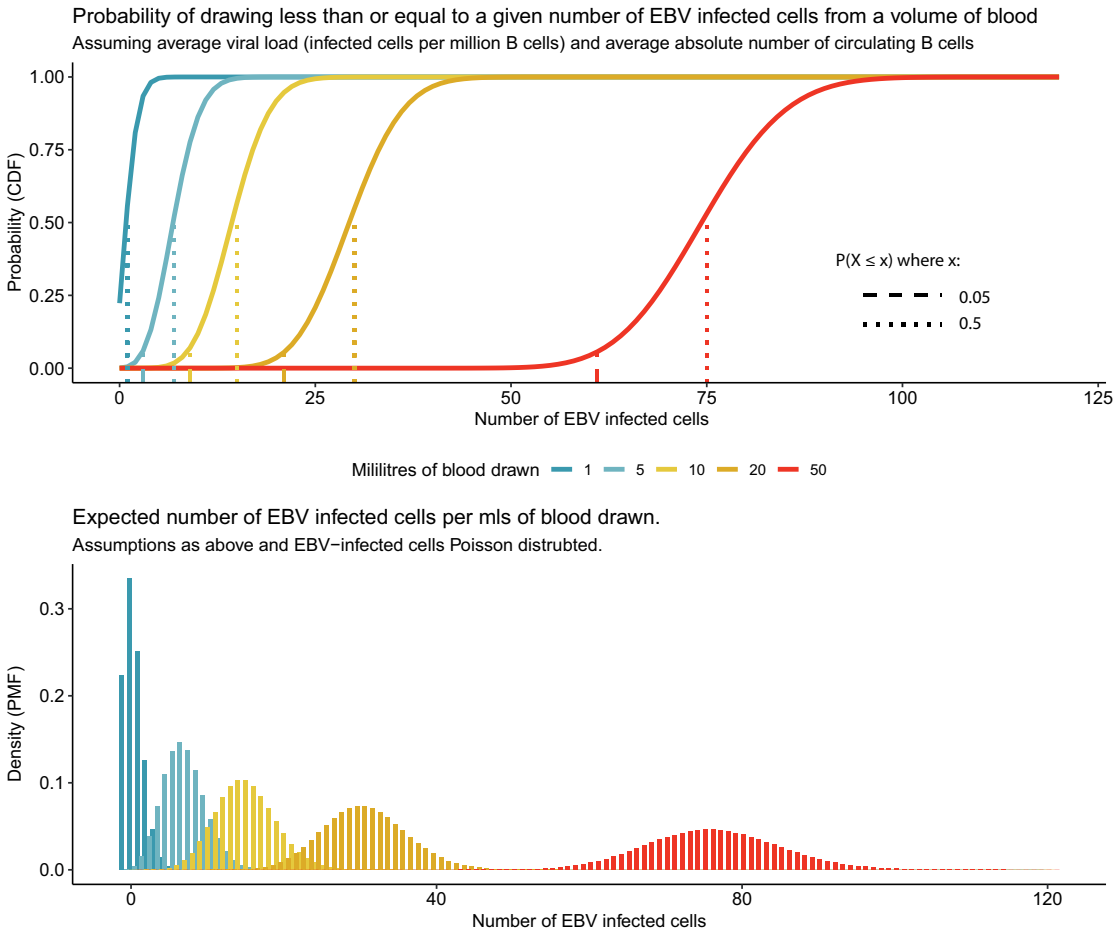

FIGURE 4 | Rarity of EBV infected cells in peripheral blood makes studying latent EBV challenging. Average person has 500,000 latently EBV-infected cells in circulation, resident in a small subset of circulating B cells. For different quantities of peripheral blood the 95 and 50% (expected mean number) probability of EBV-infected cells are represented for an individual with the average viral load (5 per million B cells infected) and average absolute number of circulating B cells (3×10^5 per ml).

Initial vaccination attempts for EBV used a recombinant peptide vaccine aimed at a single immunodominant glycoprtein (gp350) (84). Unfortunately, this was unable to prevent infection, however, a Phase I trial of a combination mRNA vaccine for six surface glycoproteins has recently entered clinical trials (clinicaltrials.gov/ct2/show/NCT05164094). As a large DNA virus, EBV mutates extremely slowly and has <1 transmission opportunity for selection per human lifetime, thus this progress is extremely exciting and whilst primary outcome of the trial is the prevention of infectious mononucleosis and EBV infection, if the vaccine is effective at preventing the latter then this immediately would open the door to prevention of MS in EBV-naive individuals.

Vaccination may also be fruitful in those already infected with EBV. One school of thought is that to maintain a latently infected pool of lymphocytes EBV has to maintain a low level of continuous new infection. In this circumstance vaccines aimed at reducing new infection could be of benefit. However, an alternative strategy would see vaccination aimed at restory cellular immunity and improving anti-viral T cell surveillance and latent-gene expressing cell clearance. This is roughly the principle on which the shingles vaccine targets another member of the human herpes family, to control latent infection. Thus, vaccination may not only be useful in naive individuals but could perhaps be a useful strategy to help rebalance the virus-immunity equilibrium in persistently-infected individuals prior to or even after the onset of MS.

4.3.2. Anti-virals and Anti-cancer Drugs

Latent EBV infection in growth transformed (rather than resting) cells profoundly alters the nuclear organization, cellular gene expression and the metabolism of infected cells in a manner similar to cancer. EBV-infected dividing cells, for example, show aerobic glycolysis a hallmark of cancer cells (85, 86). Where EBV-infected cells behave or can be triggered to behave differently from normal, healthy, uninfected cells, opportunities may arise for drugging pathways that infected cells are particularly sensitive to. In addition, many anti-viral drugs have broad activity beyond

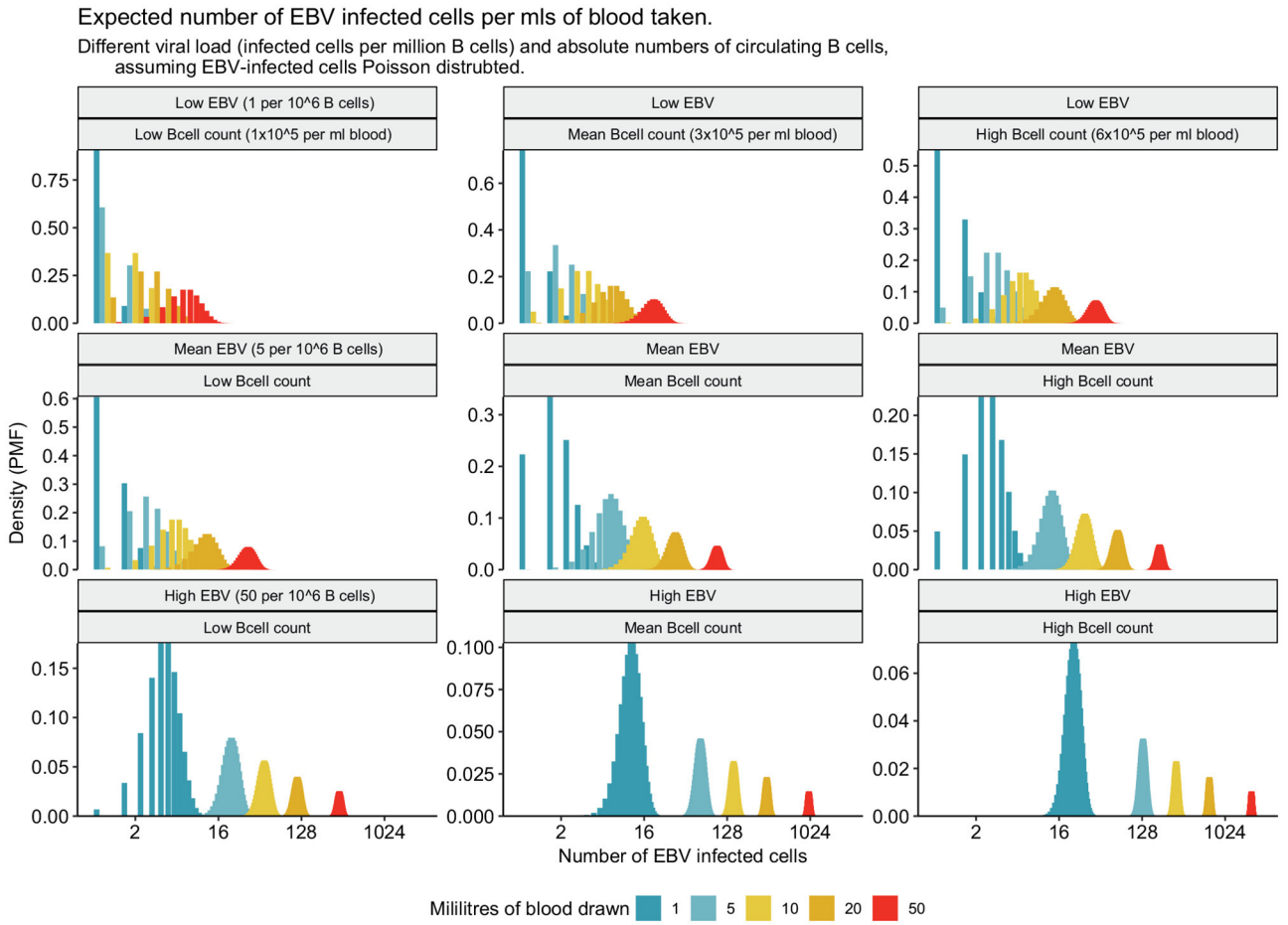

FIGURE 5 | Frequency of EBV infected cells for low, mean, and high B cell counts and EBV viral loads.

the classes of virus that they are licensed for use in. Therefore, there may be as-yet overlooked anti-EBV efficacy of other licensed or experimental drugs which may be repurposeable, or combinations of therapies that can force EBV-infected cells into a sensitive (e.g., dividing) state where they are druggable may be identifiable (87–89). The shock and kill anti-viral strategy.

4.3.3. Immunotherapies

Immunotherapies such as anti-CD20 monoclonal antibodies are effective treatments for B cell lymphoproliferative conditions and have been re-purposed and subsequently re-designed due to efficacy in treating autoimmune conditions. However, many of these, and other immunotherapies have direct or indirect effects against EBV-infected cells, and it is unknown whether some of their beneficial effects in autoimmunity are mediated by these effects. However, in addition to this, targeted immunotherapies specifically designed to target EBV-infected cells may be possible as both lytic and latent EBV infection appears to alter the infected cell proteomics considerably including for membrane proteins which could be targets of immunotherapies (86, 90).

4.3.4. Cell-Based Therapies

Cell-based therapies, finally, show great promise in treating latent EBV. This approach was successfully pioneered for the treatment of EBV post-transplant lymphoproliferative disease (a problem of poorly controlled EBV latency in the context of immunosuppression) (91–93). However, a recent clinical trial has shown exciting promise and satisfactory tolerability of *in vitro*-expanded autologous EBV-specific T cell therapies directed at a restricted subset of EBV latent proteins in persons living with secondary progressive MS (94), providing hope that this may also be a fruitful approach in treating even advanced MS.

Thus, whilst there is currently no licensed vaccine or therapy known to clear latent EBV infection, there is substantial promise on numerous fronts in this area. Understanding the pathophysiological role of EBV in MS may identify other potential routes to prevention altogether.

5. CONCLUSION

The limitations of the additive model of population genetics are well appreciated by geneticists, however, despite this

there have been frequent misunderstandings and unfortunate misapplications. One result is the continuation of irreconcilable debate as to whether multiple sclerosis is primarily a genetic disease or an environmental one, even in the face of intriguing evidence that implicates EBV as a necessary cause and the discovery of much of the genetic architecture explaining the heritable variation across populations. As MS is entirely the product of gene-environment interactions it is caused (100%) both by genes and environment. Partitioning the % into the sources of variation tells us nothing about whether the causes are modifiable or not. Here it is argued that an integrated model, accepting MS susceptibility as polygenic, and that the condition may be a complication of EBV infection, offers an opportunity to understand both the role of the virus and other environmental factors and may offer new preventative strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PK conceived of the presented work, wrote, and revised the manuscript.

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REFERENCES

1. Dobson R, Giovannoni G. Multiple sclerosis-a review. *Eur J Neurol*. (2019) 26:27–40. doi: 10.1111/ene.13819
2. Ebers G, Koopman W, Hader W, Sadovnick A, Kremenchutzky M, Mandalfino P, et al. The natural history of multiple sclerosis: a geographically based study: 8: familial multiple sclerosis. *Brain*. (2000) 123:641–9. doi: 10.1093/brain/123.3.641
3. Weinshenker B, Bulman D, Carriere W, Baskerville J, Ebers G. A comparison of sporadic and familial multiple sclerosis. *Neurology*. (1990) 40:1354. doi: 10.1212/WNL.40.9.1354
4. Hartung HP, Will R, Francis D, Grosse-Wilde H, Rudge P, Scaravilli F, et al. Familial multiple sclerosis. *J Neurol Sci*. (1988) 83:259–68. doi: 10.1016/0022-510X(88)90073-1
5. Mackay RP. The familial occurrence of multiple sclerosis and its implications. *Ann Intern Med*. (1950) 33:298–320. doi: 10.7326/0003-4819-33-2-298
6. International Multiple Sclerosis Genetics Consortium. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science*. (2019) 365:eaav7188. doi: 10.1126/science.aav7188
7. Ebers G, Sadovnick A, Risch N. A genetic basis for familial aggregation in multiple sclerosis. *Nature*. (1995) 377:150–1. doi: 10.1038/377150a0
8. Koch-Henriksen N, Sòrensen PS. The changing demographic pattern of multiple sclerosis epidemiology. *Lancet Neurol*. (2010) 9:520–32. doi: 10.1016/S1474-4422(10)70064-8
9. Marrie R, Yu N, Blanchard J, Leung S, Elliott L. The rising prevalence and changing age distribution of multiple sclerosis in Manitoba. *Neurology*. (2010) 74:465–71. doi: 10.1212/WNL.0b013e3181cf6ec0
10. Compston A, Confavreux C. The distribution of multiple sclerosis. *McAlpine's Multiple Sclerosis*. (2006) 2006:71–111. doi: 10.1016/B978-0-443-07271-0.50004-5
11. Munk Nielsen N, Corn G, Frisch M, Stenager E, Koch-Henriksen N, Wohlfahrt J, et al. Multiple sclerosis among first-and second-generation immigrants in Denmark: a population-based cohort study. *Brain*. (2019) 142:1587–97. doi: 10.1093/brain/awz088
12. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. *Ann Neurol*. (2007) 61:504–13. doi: 10.1002/ana.21141
13. Warner H, Carp R. Multiple sclerosis and Epstein-Barr virus. *Lancet*. (1981) 2:1290. doi: 10.1016/S0140-6736(81)91527-0
14. Ascherio A, Munch M. Epstein-Barr virus and multiple sclerosis. *Epidemiology*. (2000) 11:220–4. doi: 10.1097/00001648-200003000-00023
15. Alotaibi S, Kennedy J, Tellier R, Stephens D, Banwell B. Epstein-Barr virus in pediatric multiple sclerosis. *JAMA*. (2004) 291:1875–9. doi: 10.1001/jama.291.15.1875
16. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann Neurol*. (2006) 59:499–503. doi: 10.1002/ana.20820
17. DeLorenze GN, Munger KL, Lennette ET, Orentreich N, Vogelman JH, Ascherio A. Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. *Arch Neurol*. (2006) 63:839–44. doi: 10.1001/archneur.63.6.noc50328
18. Levin LI, Munger KL, O'Reilly EJ, Falk KI, Ascherio A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol*. (2010) 67:824–30. doi: 10.1002/ana.21978
19. Pender MP. The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis. *Neuroscientist*. (2011) 17:351–67. doi: 10.1177/1073858410381531
20. Munger K, Levin L, O'Reilly E, Falk K, Ascherio A. Anti-Epstein-Barr virus antibodies as serological markers of multiple sclerosis: a prospective study among United States military personnel. *Multiple Sclerosis J*. (2011) 17:1185–93. doi: 10.1177/1352458511408991
21. Pakpoor J, Giovannoni G, Ramagopalan SV. Epstein-Barr virus and multiple sclerosis: association or causation? *Expert Rev Neurotherap*. (2013) 13:287–97. doi: 10.1586/ern.13.6
22. Deuschle K, Hofmann J, Otto C, Bellmann-Strobl J, Scherner O, Klumbies K, et al. Are there Epstein-Barr virus seronegative patients with multiple sclerosis? *Multiple Sclerosis J*. (2013) 19:1242. doi: 10.1177/1352458512472751
23. Ruprecht K, Wunderlich B, GießR, Meyer P, Loebel M, Lenz K, et al. Multiple sclerosis: the elevated antibody response to Epstein-Barr virus primarily targets, but is not confined to, the glycine-alanine repeat of Epstein-Barr nuclear antigen-1. *J Neuroimmunol*. (2014) 272:56–61. doi: 10.1016/j.jneuroim.2014.04.005
24. Dobson R, Kuhle J, Middeldorp J, Giovannoni G. Epstein-Barr-negative MS: a true phenomenon? *Neurol Neuroimmunol Neuroinflamm*. (2017) 4:e318. doi: 10.1212/NXI.0000000000000318
25. Belbasis L, Bellou V, Evangelou E, Ioannidis JP, Tzoulaki I. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurol*. (2015) 14:263–73. doi: 10.1016/S1474-4422(14)70267-4
26. Rothman KJ. Causes. *Am J Epidemiol*. (1976) 104:587–92. doi: 10.1093/oxfordjournals.aje.a112335
27. Ascherio A, Munger KL. Epstein-Barr virus infection and multiple sclerosis: a review. *J Neuroimmune Pharmacol*. (2010) 5:271–7. doi: 10.1007/s11481-010-9201-3
28. Ruprecht K. The role of Epstein-Barr virus in the etiology of multiple sclerosis: a current review. *Expert Rev Clin Immunol*. (2020) 16:1143–57. doi: 10.1080/1744666X.2021.1847642
29. Bar-Or A, Pender MP, Khanna R, Steinman L, Hartung HP, Maniar T, et al. Epstein-Barr Virus in multiple sclerosis: theory

and emerging immunotherapies. *Trends Mol Med*. (2021) 27:410–1. doi: 10.1016/j.molmed.2019.11.003
30. Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. *Lancet Neurol*. (2004) 3:104–10. doi: 10.1016/S1474-4422(03)00663-X
31. Ebers G. Interactions of environment and genes in multiple sclerosis. *J Neurol Sci*. (2013) 334:161–3. doi: 10.1016/j.jns.2013.08.018
32. Sawcer S, Franklin RJ, Ban M. Multiple sclerosis genetics. *Lancet Neurol*. (2014) 13:700–9. doi: 10.1016/S1474-4422(14)70041-9
33. Van Der Mei I, Otahal P, Simpson Jr S, Taylor B, Winzenberg T. Meta-analyses to investigate gene-environment interactions in neuroepidemiology. *Neuroepidemiology*. (2014) 42:39–49. doi: 10.1159/000355439
34. Olsson T, Barcellos LF, Alfredsson L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol*. (2017) 13:25–36. doi: 10.1038/nrneurol.2016.187
35. Waubant E, Lucas R, Mowry E, Graves J, Olsson T, Alfredsson L, et al. Environmental and genetic risk factors for MS: an integrated review. *Ann Clin Transl Neurol*. (2019) 6:1905–22. doi: 10.1002/acn3.50862
36. Sadovnick D. The place of environmental factors in multiple sclerosis: genes, environment and the interactions thereof in the etiology of multiple sclerosis. *Rev Neurol*. (2019) 175:593–6. doi: 10.1016/j.neurol.2019.08.003
37. Jacobs BM, Noyce AJ, Bestwick J, Belete D, Giovannoni G, Dobson R. Gene-Environment Interactions in Multiple Sclerosis: a UK Biobank Study. *Neurol Neuroimmunol Neuroinflamm*. (2021) 8:e1007. doi: 10.1212/NXI.0000000000001007
38. Kempthorne O. *An Introduction to Genetic Statistics*. NY: John Wiley & Sons, Inc. (1957).
39. Kempthorne O. Heritability: uses and abuses. *Genetica*. (1997) 99:109–12. doi: 10.1007/BF02259514
40. Visscher PM, Hill WG, Wray NR. Heritability in the genomics ERA–concepts and misconceptions. *Nat Rev Genet*. (2008) 9:255–66. doi: 10.1038/nrg2322
41. Hutchinson M. The major cause of multiple sclerosis is environmental: genetics has a minor role-commentary. *Multiple Sclerosis J*. (2011) 17:1176. doi: 10.1177/1352458511421107
42. Sesardic N. *Making Sense of Heritability*. Cambridge, UK: Cambridge University Press (2005). doi: 10.1017/CBO9780511487378
43. Fisher RA. XV. The correlation between relatives on the supposition of Mendelian inheritance. *Earth Environ Sci Trans R Soc Edinburgh*. (1919) 52:399–433. doi: 10.1017/S0080456800012163
44. Wright S. The method of path coefficients. *Ann Math Stat*. (1934) 5:161–215. doi: 10.1214/aoms/1177732676
45. Avery OT, MacLeod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J Exp Med*. (1944) 79:137–58. doi: 10.1084/jem.79.2.137
46. Watson JD, Crick FH. Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature*. (1953) 171:737–8. doi: 10.1038/171737a0
47. Lewontin RC. The analysis of variance and the analysis of causes. *Int J Epidemiol*. (2006) 35:520–5. doi: 10.1093/ije/dyl062
48. Brennan R, Burrows J, Bell M, Bromham L, Csurhes P, Lenarczyk A, et al. Strains of Epstein-Barr virus infecting multiple sclerosis patients. *Multiple Sclerosis J*. (2010) 16:643–51. doi: 10.1177/1352458510364537
49. Mechelli R, Manzari C, Policano C, Annese A, Picardi E, Umeton R, et al. Epstein-Barr virus genetic variants are associated with multiple sclerosis. *Neurology*. (2015) 84:1362–8. doi: 10.1212/WNL.0000000000001420
50. Haahr S, Plesner A, Vestergaard B, Höllsberg P. A role of late Epstein-Barr virus infection in multiple sclerosis. *Acta Neurol Scand*. (2004) 109:270–5. doi: 10.1046/j.1600-0404.2003.00221.x
51. Ristori G, Cannoni S, Stazi MA, Vanacore N, Cotichini R, Alfó M, et al. Multiple sclerosis in twins from continental Italy and Sardinia: a nationwide study. *Ann Neurol*. (2006) 59:27–34. doi: 10.1002/ana.20683
52. Islam T, Gauderman WJ, Cozen W, Hamilton AS, Burnett ME, Mack TM. Differential twin concordance for multiple sclerosis by latitude of birthplace. *Ann Neurol*. (2006) 60:56–64. doi: 10.1002/ana.20871
53. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol*. (2007) 61:288–99. doi: 10.1002/ana.21117
54. Mechelli R, Umeton R, Policano C, Annibali V, Coarelli G, Ricigliano VA, et al. A "candidate–interactome" aggregate analysis of genome–wide association data in multiple sclerosis. *PLoS ONE*. (2013) 8:e63300. doi: 10.1371/journal.pone.0063300
55. Hume D. A treatise upon human nature. *London*. (1739) 1738:40. doi: 10.1093/oseo/instance.00046221
56. Hill AB. *The Environment and Disease: Association or Causation*? Vol. 58, Sage Publications (1965). doi: 10.1177/003591576505800503
57. Jacobs BM, Giovannoni G, Cuzick J, Dobson R. Systematic review and meta-analysis of the association between Epstein-Barr virus, multiple sclerosis and other risk factors. *Multiple Sclerosis J*. (2020) 26:1281–97. doi: 10.1177/1352458520907901
58. Pakpoor J, Disanto G, Gerber JE, Dobson R, Meier UC, Giovannoni G, et al. The risk of developing multiple sclerosis in individuals seronegative for Epstein-Barr virus: a meta-analysis. *Multiple Sclerosis J*. (2013) 19:162–6. doi: 10.1177/1352458512449682
59. Holborow E, Hemsted E, Mead S. Smooth muscle autoantibodies in infectious mononucleosis. *Br Med J*. (1973) 3:323–5. doi: 10.1136/bmj.3.5875.323
60. Sutton R, Emond R, Thomas D, Doniach D. The occurrence of autoantibodies in infectious mononucleosis. *Clin Exp Immunol*. (1974) 17:427.
61. Kataaha P, Holborow E, Edwards J. Incidence of anti-intermediate filament antibody in serum samples of students with suspected glandular fever. *J Clin Pathol*. (1985) 38:351–4. doi: 10.1136/jcp.38.3.351
62. Nagata K, Hayashi K. Epstein-Barr Virus reactivation-induced immunoglobulin production: significance on autoimmunity. *Microorganisms*. (2020) 8:1875. doi: 10.3390/microorganisms8121875
63. Kakalacheva K, Regenass S, Wiesmayr S, Azzi T, Berger C, Dale RC, et al. Infectious mononucleosis triggers generation of IgG auto-antibodies against native myelin oligodendrocyte glycoprotein. *Viruses*. (2016) 8:51. doi: 10.3390/v8020051
64. Miyashita EM, Yang B, Babcock GJ, Thorley-Lawson DA. Identification of the site of Epstein-Barr virus persistence *in vivo* as a resting B cell. *J Virol*. (1997) 71:4882–91. doi: 10.1128/jvi.71.7.4882-4891.1997
65. Greenfield AL, Hauser SL. B-cell therapy for multiple sclerosis: entering an era. *Ann Neurol*. (2018) 83:13–26. doi: 10.1002/ana.25119
66. Barun B, Bar-Or A. Treatment of multiple sclerosis with anti-CD20 antibodies. *Clin Immunol*. (2012) 142:31–7. doi: 10.1016/j.clim.2011.04.005
67. Young LS, Yap LF, Murray PG. Epstein-Barr virus: more than 50 years old and still providing surprises. *Nat Rev Cancer*. (2016) 16:789–802. doi: 10.1038/nrc.2016.92
68. Longnecker RM, Kieff E, Cohen JI. Epstein-Barr Virus. In: *Fields Virology*, 6th Edition. Philadelphia, PA: Lippincott Williams & Wilkins (2013).
69. Kang MS, Kieff E. Epstein-Barr virus latent genes. *Exp Mol Med*. (2015) 47:e131. doi: 10.1038/emm.2014.84
70. Faulkner GC, Krajewski AS, Crawford DH. The ins and outs of EBV infection. *Trends Microbiol*. (2000) 8:185–9. doi: 10.1016/S0966-842X(00)01742-X
71. Tempera I, Klichinsky M, Lieberman PM. EBV latency types adopt alternative chromatin conformations. *PLoS Pathog*. (2011) 7:e1002180. doi: 10.1371/journal.ppat.1002180
72. Tempera I, Lieberman PM. Epigenetic regulation of EBV persistence and oncogenesis. In: *Seminars in Cancer Biology*, Vol. 26. Elsevier (2014). p. 22–9. doi: 10.1016/j.semcancer.2014.01.003
73. Arvey A, Tempera I, Tsai K, Chen HS, Tikhmyanova N, Klichinsky M, et al. An atlas of the Epstein-Barr virus transcriptome and epigenome reveals host-virus regulatory interactions. *Cell Host Microbe*. (2012) 12:233–45. doi: 10.1016/j.chom.2012.06.008
74. Ko YH. EBV and human cancer. *Exp Mol Med*. (2015) 47:e130. doi: 10.1038/emm.2014.109
75. Decker LL, Klaman LD, Thorley-Lawson DA. Detection of the latent form of Epstein-Barr virus DNA in the peripheral blood of healthy individuals. *J Virol*. (1996) 70:3286–9. doi: 10.1128/jvi.70.5.3286-3289.1996
76. Harley JB, Chen X, Pujato M, Miller D, Maddox A, Forney C, et al. Transcription factors operate across disease loci, with EBNA2 implicated in autoimmunity. *Nat Genet*. (2018) 50:699–707. doi: 10.1038/s41588-018-0102-3
77. Afrasiabi A, Parnell GP, Fewings N, Schibeci SD, Basuki MA, Chandramohan R, et al. Evidence from genome wide association studies implicates reduced

control of Epstein-Barr virus infection in multiple sclerosis susceptibility. *Genome Med*. (2019) 11:1–13. doi: 10.1186/s13073-019-0640-z
78. Hong T, Parameswaran S, Donmez O, Miller D, Forney C, Lape M, et al. Epstein-Barr virus nuclear antigen 2 (EBNA2) extensively rewires the human chromatin landscape at autoimmune risk loci. *Genome Res*. (2021) 31:2185–98. doi: 10.1101/gr.264705.120
79. Mechelli R, Umeton R, Manfré G, Romano S, Buscarinu MC, Rinaldi V, et al. Reworking GWAS data to understand the role of nongenetic factors in MS etiopathogenesis. *Genes*. (2020) 11:97. doi: 10.3390/genes11010097
80. Kim K, Pröbstel AK, Baumann R, Dyckow J, Landefeld J, Kogl E, et al. Cell type-specific transcriptomics identifies neddylation as a novel therapeutic target in multiple sclerosis. *Brain*. (2021) 144:450–61. doi: 10.1093/brain/awaa421
81. Becker T, Le-Trilling VTK, Trilling M. Cellular cullin RING ubiquitin ligases: druggable host dependency factors of cytomegaloviruses. *Int J Mol Sci*. (2019) 20:1636. doi: 10.3390/ijms20071636
82. Venkataraman T, Valencia C, Mangino M, Morgenlander W, Clipman SJ, Liechti T, et al. Antiviral antibody epitope selection is a heritable trait. *bioRxiv [Preprint]*. (2021). doi: 10.1101/2021.03.25.436790
83. Dobson R, Ramagopalan S, Topping J, Smith P, Solanky B, Schmierer K, et al. A risk score for predicting multiple sclerosis. *PLoS ONE*. (2016) 11:e0164992. doi: 10.1371/journal.pone.0164992
84. Sokal EM, Hoppenbrouwers K, Vandermeulen C, Moutschen M, Léonard P, Moreels A, et al. Recombinant gp350 vaccine for infectious mononucleosis: a phase 2, randomized, double-blind, placebo-controlled trial to evaluate the safety, immunogenicity, and efficacy of an Epstein-Barr virus vaccine in healthy young adults. *J Infect Dis*. (2007) 196:1749–53. doi: 10.1086/523813
85. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
86. Wang LW, Shen H, Nobre L, Ersing I, Paulo JA, Trudeau S, et al. Epstein-Barr-virus-induced one-carbon metabolism drives B cell transformation. *Cell Metab*. (2019) 30:539–55. doi: 10.1016/j.cmet.2019.06.003
87. Drosu NC, Edelman ER, Housman DE. Tenofovir prodrugs potently inhibit Epstein-Barr virus lytic DNA replication by targeting the viral DNA polymerase. *Proc Natl Acad Sci USA*. (2020) 117:12368-12374. doi: 10.1073/pnas.2002392117
88. Chodosh J, Holder VP, Gan Yj, Belgaumi A, Sample J, Sixbey JW. Eradication of latent Epstein-Barr virus by hydroxyurea alters the growth-transformed cell phenotype. *J Infect Dis*. (1998) 177:1194–201. doi: 10.1086/515290
89. Jones K, Nourse J, Corbett G, Gandhi M. Sodium valproate in combination with ganciclovir induces lysis of EBV-infected lymphoma cells without impairing EBV-specific T-cell immunity. *Int J Lab Hematol*. (2010) 32(1 Pt 1):e169–74. doi: 10.1111/j.1751-553X.2008.01130.x
90. Ersing I, Nobre L, Wang LW, Soday L, Ma Y, Paulo JA, et al. A temporal proteomic map of Epstein-Barr virus lytic replication in B cells. *Cell Rep*. (2017) 19:1479–93. doi: 10.1016/j.celrep.2017.04.062
91. Haque T, Wilkie GM, Taylor C, Amlot PL, Murad P, Iley A, et al. Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells. *Lancet*. (2002) 360:436–42. doi: 10.1016/S0140-6736(02)09672-1
92. Burns DM, Crawford DH. Epstein-Barr virus-specific cytotoxic T-lymphocytes for adoptive immunotherapy of post-transplant lymphoproliferative disease. *Blood Rev*. (2004) 18:193–209. doi: 10.1016/j.blre.2003.12.002
93. Haque T, Taylor C, Wilkie GM, Murad P, Amlot PL, Beath S, et al. Complete regression of posttransplant lymphoproliferative disease using partially HLA-matched Epstein Barr Virus-specific cytotoxic T cells. *Transplantation*. (2001) 72:1399–402. doi: 10.1097/00007890-200110270-00012
94. Pender MP, Csurhes PA, Smith C, Douglas NL, Neller MA, Matthews KK, et al. Epstein-Barr virus-specific T cell therapy for progressive multiple sclerosis. *JCI insight*. (2018) 3:124714. doi: 10.1172/jci.insight.124714

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Preventing Multiple Sclerosis: The Pediatric Perspective

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Pediatric-onset multiple sclerosis (MS) is a predominantly relapsing-remitting neuroinflammatory disorder characterized by frequent relapses and high magnetic resonance imaging (MRI) lesion burden early in the disease course. Current treatment for pediatric MS relies on early initiation of disease-modifying therapies designed to prevent relapses and slow progression of disability. When considering the concept of MS prevention, one can conceptualize *primary prevention* (population- or at-risk population interventions that prevent the earliest facet of MS pathobiology and hence reduce disease incidence), or *secondary prevention* (prevention of disease consequence, such as reducing relapse frequency and lesion accrual, enhancing focal lesion repair, promoting CNS resilience against the more global facets of disease injury, and ultimately, preventing progression of neurological disability). Studying the pediatric MS population provides a unique opportunity to explore early-life exposures that contribute to the development of MS including perinatal and environmental risk determinants. Research is ongoing related to targeting these risk factors for potential MS *primary prevention*. Here we review these key risk factors, their proposed role in the pathogenesis of MS, and their potential implications for primary MS prevention.

Keywords: multiple sclerosis, pediatric multiple sclerosis (MS), preventative medicine, demyelinating disease, MS environmental risk factors

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INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating disease affecting the central nervous system that primarily affects adults; however, 3 to 10% of all patients diagnosed with MS experience their first demyelinating event prior to the age of 18 (1–3). In children, MS is characterized by a highly relapsing course though relatively complete or near-complete recovery from attacks is seen (4). Pediatric-onset MS patients face a lower risk for disability within the first 10 years of disease onset, and a longer time from onset to entry into secondary progressive disease compared to adult-onset patients. However, they develop disability overall at a younger age than when the disease starts in adulthood. Cognitive deficits, fatigue, and depression are also prominent features of pediatric-onset MS (1, 5) that impact quality of life. Current treatment strategies, particularly prompt initiation of highly effective disease modifying therapies, reduce relapses and may reduce the reduce relapses and reduce the likelihood of progressive disability.

The etiology of pediatric-onset MS is believed to be multifactorial involving a complex interplay between genetic and environmental risk factors (1, 6). The idea of studying early-life exposures and risk factors for MS to guide earlier targeted interventions against these risk determinants to prevent the disease is an important concept in the field. The most widely studied and most likely modifiable risk determinants associated with pediatric MS include environmental risk factors such as exposure to Epstein-Barr virus (EBV), low serum 25-(OH) hydroxyvitamin D /low sun exposure, diet and alterations in the gut microbiome, obesity, and exposure to cigarette smoking and other lung irritants (**Table 1**). Additionally, the role of perinatal risk factors in MS risk is still being evaluated. In this review we will discuss these key risk determinants and the potential therapeutic strategies to target these risk factors to prevent multiple sclerosis.

TABLE 1 | Pediatric onset MS risk factors.

Risk factor	Odds ratio or relative risk	Source(s)	Modifiable?
Breast feeding	4.43 (OR) No association (12)	Cross-sectional, case control (7) Case control study (12)	Yes
EBV exposure	4.5 (OR)	Meta-analyses (14, 15)	Potentially
High serum 25-(OH) hydroxyvitamin D	0.72 (OR)	Meta-analysis/Mendelian Randomzation (16)	Yes
Sun exposure	Adjusted RR 0.55	Case control study (17)	Potentially
Gut-microbiome	Not available	Pilot cohort study (18), case control study (19)	Yes
Body Mass Index (BMI)	1.60 (OR, females) 1.42 (OR, males); 1.17 (OR)	Case control study (20) Meta-analysis (16)	Yes
Passive smoking	2.12 (RR)	Population-based case control study (21)	Yes
Air pollutants	Carbon monoxide OR 5.45 Sulfur Dioxide OR 3.99 Fine particulate matter OR 7.53	Multicenter case control study (22)	Yes
Household exposures	Rodenticides OR 2.10 Weed Control agents OR 1.99 Plant/Tree agents OR 2.72	Cross sectional analysis of case control study (23)	Yes

PERINATAL RISK FACTORS: THE ROLE OF BREAST MILK

Breastfeeding is one of the earliest childhood exposures that has been investigated in relation to MS risk; however, its role in MS has yielded mixed results. In 2017, a small cross-sectional case control study performed at the University of Virginia analyzed the association of breastfeeding in infancy on future risk of pediatric onset MS by comparing 36 pediatric onset MS patients with 72 control patients (7). In this study, lack of breastfeeding was associated with a future diagnosis of pediatric onset MS (odds ratio-4.43; 95% confidence interval, $P = 0.003$) with 36% of the pediatric onset MS patients being breastfed compared to 71% of the controls. There have been other earlier studies to support the association between breastfeeding and decreased risk of MS (8, 9) as well as studies demonstrating a protective effect of breastfeeding against other autoimmune conditions such as Type 1 diabetes and inflammatory bowel disease (10). The hypothesized mechanism revolves around the idea that molecular mimicry between cow milk proteins and human self-antigens leads to the development of early MS. Thus, breast milk may provide protection from autoreactivity to cow milk (7). To support this hypothesis, there has been data suggesting increased abnormal T cell responses against certain cow milk proteins when compared to healthy controls in patients with type I diabetes, children with acquired demyelinating syndromes, and children with MS (11). Although this research provides compelling evidence for the benefits of breast milk, there was a large study by the US network of Pediatric MS centers that found no association of breast feeding with risk of pediatric MS (12). In this case control study, 265 pediatric MS cases were compared to 412 healthy controls, and after controlling for pregnancy-related factors, breast-feeding was not associated with MS (12). Nonetheless, for women that are able, breastfeeding may potentially reduce autoimmune dysregulation and has clear other benefits in the developing child including protection against infections, enhanced growth and development due to the balance of nutrients in breast milk, and improved brain growth and development (13). Early breastfeeding counseling to pregnant women, particularly to those who have a family history of MS, could be a potential preventative therapy against the development of MS while providing so many other benefits to both mother and child.

ENVIRONMENTAL RISK FACTORS: THE ROLE OF EPSTEIN-BARR VIRUS (EBV) EXPOSURE

There has been consistent evidence that past EBV infection is associated with increased risk of adult-onset multiple sclerosis including in meta-analyses (14, 15, 24–27). In a large national cohort study performed between 2004 and 2010 including 332 children, risk of MS was increased in children with past exposure to EBV (HR 2.04, 0.99–4.20) (27). Additionally, the US network of Pediatric MS centers published a large case control study that supported the association of remote EBV exposure and MS risk in children. This study found that EBV viral capsid antigen (EBV-VCA) seropositivity was associated

with increased odds of having MS by 7.4 times (28). In children, it has been reported that up to 15% of children with an MS diagnosis are EBV-seronegative; however, a recent study demonstrated that some of these EBV-seronegative children carrying an MS diagnosis may not have been appropriately diagnosed. In this study, 25 EBV-seronegative patients among 189 pediatric patients diagnosed with CIS/MS were re-evaluated clinically, serologically and radiographically. Upon re-evaluation, 11 of 25 (44%) of these patients were found to be myelin oligodendrocyte glycoprotein (MOG) antibody positive, 4 of the remaining 14 patients did not meet 2017 McDonald criteria for MS, and of the 10 remaining patients who did meet 2017 McDonald criteria for MS had clinical features that were unusual for an MS diagnosis (29). Ultimately this study concluded that a diagnosis of pediatric MS is exceedingly rare in patients who are EBV-seronegative. There are numerous hypotheses that have been proposed to explain how EBV may be involved in MS pathogenesis: (i) EBV leads to chronic latent (and intermittently reactive) infection of human B cells, which then may prime T cells to cross-react and recognize CNS antigens via molecular mimicry; (ii) EBV may drive pro-inflammatory responses in latently infected B cells leading to expression of pro-inflammatory cytokines and reduction in anti-inflammatory cytokines (notably interleukin-10); and (iii) EBV may elicit "bystander damage" *via* induction of an antiviral immune response against infected cells in the CNS (30). With regards to EBV infection being a "preventable" risk factor, EBV vaccine research is underway, however the development of an EBV vaccine faces challenges including safety concerns due to oncogenic potential, lack of a suitable animal model for EBV disease, incomplete understanding of the exact route and mechanism of EBV infection, and concern that an EBV vaccine would not be commercially viable (30–32).

ENVIRONMENTAL RISK FACTORS: THE ROLE OF SUN AND SERUM 25-(OH) HYDROXYVITAMIN D EXPOSURE

Decreased exposure to sunlight and low serum 25-(OH) hydroxyvitamin D levels have been implicated as important risk factors for the development of MS. Despite a large collection of data to support the association of both low sunlight and low serum 25-(OH) hydroxyvitamin D levels with MS risk, it has proven challenging to distinguish the independent effect from ultraviolet radiation (UVR) from that of 25-(OH) hydroxyvitamin D and vice versa as UVR is involved in the conversion of vitamin D into an active metabolite. There has been some work performed, however, that suggests independent effects on MS risk. In one early study performed in 2012, Baarnhielm et al. demonstrated that lower UVR exposure was associated with increase MS risk after correcting for serum 25-(OH) hydroxyvitamin D levels. In this population-based case control study, an increased MS risk was identified in those patients whom reported low UVR exposure (OR 2.2, 95% CI 1.5–3.3) and this association held true even after adjusting for 25-(OH) hydroxyvitamin D status (33). In a more recent study, sun exposure was examined over the life course of patients with and without MS. This study found that living in high ultraviolet-B (UV-B) areas before MS onset was associated with a 45% lower MS risk (adjusted RR 0.55, 95% CI 0.42–0.73) (17). Sunlight has been postulated to reduce MS risk through both 25-(OH) hydroxyvitamin D independent and dependent pathways. Independent from vitamin D, it has been proposed that sun exposure may lead to suppression of cell mediated immunity as well as modulating the release of cytokines and chemokines (34). The effects of UVR exposure has been studied in experimental autoimmune encephalomyelitis (EAE) models, our current model of MS in mice, showing a reduction in peripheral inflammation in these mice after UVR exposure (35). "Prescribing" sun exposure as a preventative measure for MS is challenging as the amount of sunlight, duration, and timing of exposure are unknown. Additionally, risk of skin cancer from this exposure presents safety concerns.

Serum 25-(OH) hydroxyvitamin D levels have been an extensively studied risk determinant for the development of MS. Several studies have discovered not only an association of serum 25-(OH) hydroxyvitamin D levels with risk of developing MS, but some studies have even proposed a causal role for low 25-(OH) hydroxyvitamin D in the pathogenesis of MS (16, 36, 37). In one such study, Mendelian randomization, an analysis that uses genetic associations to test the effects of biomarkers on the risk of a disease, was used to identify a potential causal relationship between serum 25-(OH) hydroxyvitamin D levels and risk of pediatric-onset MS. In this study, meta-analysis showed increasing levels of serum 25-(OH) hydroxyvitamin D (based on a vitamin D genetic risk score constructed using 3 single nucleotide polymorphisms associated with vitamin D levels) decreased the odds of pediatric-onset MS (for each additional risk SNP OR = 0.72, 95% CI: 0.55–0.94; $P = 0.02$) after controlling for sex, genetic ancestry, HLA-DRB1*15, and more than 100 single nucleotide polymorphisms identified as MS-risk variants (16). In a separate large prospective Canadian cohort study, it was also shown that baseline serum 25-(OH) hydroxyvitamin D status at the time of an incident demyelinating attack was associated with likelihood of further relapses confirming a diagnosis of pediatric-onset MS (27). More specifically, this study showed that a 10 mmol/L decrease in 25-(OH) hydroxyvitamin D was associated with a 20% relative increase in risk of pediatric MS compared to monophasic demyelination ($p = 0.006$) (27). Similar studies performed in adult patients with MS support a strong association of serum 25-(OH) hydroxyvitamin D and MS risk (36–38). One particular adult study, using data from the Finnish Maternity Cohort, showed that a 50 nmol/L increase in 25-(OH) hydroxyvitamin D was associated with a 39% reduced risk of MS (RR 0.61, 95% CI 0.44–0.85), $p = 0.003$. Additionally, it was shown that MS risk was 2-fold higher in women with 25-(OH) hydroxyvitamin D <30 nmol/L as compared to women with 25(OH)D ≥ 50 nmol/L (RR 2.02, 95% CI 1.18–3.45, $p = 0.01$) (38). The precise time in which

25-(OH) hydroxyvitamin D may exert its greatest influence on the risk of MS is unknown; however, there is evidence that lower serum 25-(OH) hydroxyvitamin D levels as early as the neonatal period are associated with a higher risk of MS. The influence of 25-(OH) hydroxyvitamin D in neonates was evaluated in two studies. In a Danish case-control study utilizing the Danish Newborn Screening Biobank (DNSB) and the Danish MS registry, MS risk was highest among patients with the lowest neonatal serum 25-(OH) hydroxyvitamin D levels (39). In a cohort study including 199 cases of MS, a 38% lower risk of MS was observed in women whose mothers drank 2–3 glasses of milk during pregnancy compared to mothers that drank little to no milk. This study also observed that daughters of mothers with higher 25-(OH) hydroxyvitamin D intake or predicted serum 25-(OH) hydroxyvitamin D levels during pregnancy had a lower risk of developing MS suggesting that 25-(OH) hydroxyvitamin D insufficiency can exert its effect as early as the in utero-stage of life (40). Similarly, in a prospective, nested case control study utilizing the Finnish Maternity Cohort (FMC), it was demonstrated that levels of 25(OH) hydroxyvitamin D that were greater than or equal to 75 (vs <75) nmol/L in women during pregnancy (collected between 10 and 14 weeks) were associated with a decreased risk of MS [odds ratio (OR) 0.39, 95% confidence interval (CI) 0.16–0.98] (41). There have been two studies that did not show an association between pregnancy/early neonatal vitamin D levels and future MS risk (42, 43). The first study, utilizing the Northern Sweden Maternity Cohort, showed there was no association between maternal 25-(OH) hydroxyvitamin D levels and risk of MS in offspring (42), however this study had a very small sample size and the association identified in this study had a large confidence interval making the results challenging to interpret. The second study, was a Swedish population case control study that evaluated neonatal blood samples used for phenylketonuria (PKU) to compare MS cases and controls. In this study it was shown that there was no association between neonatal 25-hydroxyvitamin D quintile and risk of multiple sclerosis (crude odds ratio = 1.0, 95% confidence interval = 0.68–1.44, for the highest quintile compared to the lowest) (43), however this study is limited in that some of the older samples showed evidence of 25-(OH) hydroxyvitamin D degradation which may have contributed to the null findings. There was also very low overall control participation in this particular study.

From a mechanistic standpoint, 25-(OH) hydroxyvitamin D is believed to attenuate the T-cell response to autoantigens, suppress the production of pro-inflammatory cytokines such as interferon-gamma and TH-17-interleukin 17, and increase T-regulatory cells (39, 44). Thus, this evidence suggests an important role of 25-(OH) hydroxyvitamin D in the development of MS, and one might propose implementation of 25-(OH) hydroxyvitamin D supplementation to individuals at higher risk of developing MS, such as those with a family history. Moreover, given evidence implicating a role for 25-(OH) hydroxyvitamin D as early as in utero, we might propose implementing 25-(OH) hydroxyvitamin D in at risk patients during pregnancy and/or at birth/infancy. 25-(OH) hydroxyvitamin D is a relatively safe and cost-effective therapy (45, 46) that has the potential to prevent MS, and like breast milk, has other potential health benefits.

ENVIRONMENTAL RISK FACTORS: THE ROLE OF DIET AND THE GUT-MICROBIOME

Diet plays an important role in the prevention of cardiovascular disease, stroke, and diabetes (47, 48), and emerging data suggests that diet and modulation of the gut microbiome may also influence the risk of pediatric MS. Specific dietary factors, other than vitamin D, have been investigated to identify potential roles in risk of MS. These studies may be confounded however due to reliance on patient/parents' recall of diet specifics and non-specific questionnaires. One case control study comparing 312 POMS cases with 456 controls, found that iron consumption below the recommended guidelines was associated with an increased risk of MS (odds ratio = 1.80, $p < 0.01$). This study also evaluated for associations between other dietary factors such as intake of fats, proteins, carbohydrates, sugars, fruits or vegetables; however, no significant difference in intake was identified between cases and controls (49). One case-control study (170 cases, 331 controls) has explored associations between sodium intake and pediatric MS. This study did not find an association between higher sodium intake and risk of POMS (50).

The role of the gut microbiome in CNS autoimmunity has become increasingly recognized (51). Studies have revealed that patients with MS may have a different gut microbiome composition compared to healthy controls suggesting modulation of the microbiome may help prevent MS (18, 52). In animal models, modulation of the gut microbiome appears to influence risk and severity of EAE (53–55) including two studies that showed germ-free mice (mice free of microbes) were less likely to develop EAE (53, 54). In a case-control study comparing children with new-onset MS and healthy controls, a significant increase in relative abundance for members of the *Desulfovibrionaceae* (*Bilophila*, *Desulfovibrio* and *Christensenellaceae*) and depletion in *Lachnospiraceae* and *Ruminococcaceae* in stool samples (all p and q < 0.000005) was identified in the children with MS irrespective of exposure to disease-modifying therapy. The changes identified in the microbiota of the pediatric MS patients support the idea that an increase in pro-inflammatory microbiota and a decrease in anti-inflammatory microbiota contribute to the early immune dysregulation seen in MS (19). Furthermore, differences in the relationships between immune markers and gut microbiota have been observed when comparing children with MS and control cases (18). In an additional case control study utilizing the same cohort of patients described above, children without MS were found to have an inverse correlation between gut microbiota evenness and Th17 and Th2 blood markers, with microbiota dominated by specific taxa being associated with decreased immune markers. This effect was lost in children with MS. Additionally, at the phylum level, Bacteroides was

inversely associated with Th17 in children with MS (r = 0.719, p = 0.008) but not controls (r = 0.320, p = 0.401). Alternatively, Fusobacteria was found to be positively correlated with T-regulatory cells in controls (r = 0.829, p = 0.006) but not in children with MS (r = −0.069, p = 0.808) (18). With regard to the potential function of gut microbiota in the pathogenesis of MS, evidence suggests, utilizing a validated algorithm (56), that enrichment of microbial genes involved in glutathione metabolism was observed in MS cases rather than control cases (19). Disruption of glutathione homeostasis has previously been reported as a possible mechanism of MS neurodegeneration (57).

Taken together, focusing on dietary modifications, adequate iron intake and diets that influence the microbiome in an "anti-inflammatory" manner may be plausible approaches to reduce the risk of MS in in children with a family history of MS. Further studies will be required to identify specific diets that alter the microbiome in a favorable manner by potentially increasing anti-inflammatory microbiota and decreasing pro-inflammatory microbiota. Additionally, it will be important to identify whether microbiome changes occur prior to onset or as a result of MS or initiation of MS therapies or some combination of these factors. This will be important to understand to determine the most optimal timing for implementation of potential preventative gut microbiome intervention.

ENVIRONMENTAL RISK FACTORS: THE ROLE OF OBESITY

Several studies have provided evidence that elevated BMI is associated with increased risk of childhood-onset MS (20, 58, 59). Elevated BMI has been associated with increased risk of pediatric MS in both post-pubertal girls (OR = 1.60, 95% confidence interval [CI]: 1.12, 2.27, P = 0.009) and boys (OR = 1.43, 95% CI: 1.08, 1.88, P = 0.011) in a case-control study of 254 pediatric-onset MS cases and 420 controls (20). An association of obesity and pediatric-onset MS was also suggested in a large meta-analysis of a US and Swedish cohort of POMS using BMI genetic risk scores (GRS) that incorporated the cumulative effect of 97 variants associated with BMI (16). Additionally, in a large longitudinal retrospective analysis of prospectively collected data, of 774 POMS cases, elevated BMI z-scores were associated with increased risk of MS in both girls ages 7-13 (HR 1.17 to 1.21) and boys ages 8–10 (HR 1.14 to 1.15) (58).

There have been several proposed mechanisms through which obesity may contribute to the pathogenesis of MS. The presence of increased adipose tissue hormone leptin is believed to play a role in the risk of MS given its pro-inflammatory properties (60). Animal models have also supported this mechanism demonstrating that leptin-deficient mice failed to develop EAE when stimulated with MOG 35-55 specific T-cells coinciding with decreased levels of pro-inflammatory cytokines (IL-2, IL-6, INF-γ, TNF-α) (61). Obesity has also been associated with IL-6 dependent TH17 production which has been shown to exacerbate EAE in mice (62). Another adipokine, adiponectin, has also been implicated in the pathogenesis of MS (63). One study found elevated levels of leptin and fatty acid binding protein-4 as well as reduced adiponectin in boys and girls with MS compared to age and sex-matched controls (64). A recent study comparing 33 children with MS to 54 children with MOGAD, and 29 healthy controls, observed significantly higher levels of adiponectin in the serum of MS patients compared to both the MOGAD and healthy control patients (p = 0.02) (59). This study also investigated the functional consequence of elevated adiponectin on immune cells and discovered that adiponectin from the serum of pediatric MS patients led to pro-inflammatory responses in CD14+ monocytes, T-cell activation, upregulation of CNS microglia pro-inflammatory markers, and downregulation of CNS microglial specific quiescent/anti-inflammatory markers (63) all evidence supporting a role of adiponectin to induce disease in children with MS. Finally, there has been evidence suggesting that obesity, through disruption of TH17/Treg balance, may alter the gut microbiome resulting in dysregulation of the intestinal immune response (65).

Promoting healthy weight in children is an intervention that should be enforced in all youth regardless of their potential MS risk. The benefits associated with healthy weight are plentiful, and with the added potential of possibly preventing a chronic neuroinflammatory disorder like MS, it is truly an essential therapeutic strategy. Additionally, the pathway to achieve healthy weight typically involves exercise and a healthy diet, two additional interventions that provide immense overall health benefits. Exercise alone, though beyond the scope of this review, has proven to have potential neurologic benefits including enhancing the production of neuroprotective trophic factors, promoting neuronal survival, promoting oligodendrocyte proliferation and repopulation, reducing neuronal injury, astrogliosis and modulation of cytokine production (66–68). In summary, counseling patients on the importance of a healthy lifestyle may have a significant impact on overall health and possibly contribute to the prevention of MS.

ENVIRONMENTAL RISK FACTORS: THE ROLE OF EXPOSURE TO CIGARETTE SMOKING AND OTHER AIR POLLUTION

Exposure to cigarette smoking, both active and passive, is another risk determinant that has consistently been identified to be associated with the risk of MS (21, 69, 70). As early as the 1960's, an association between active smoking and development of MS has been reported (71). More recently, in 2009, active smoking was identified as a risk factor for MS in a large European multi-national case-control study where the odds of MS in smokers was 1.5 (95% CI 1.3–1.8) in Sweden; 1.8 (95% CI 1.1–2.9) in Norway; and 1.3 (95% CI 1.0–1.9) in the UK (72). In children, where active smoking is generally less common (or at least, less commonly reported), the association of exposure to passive cigarette smoking and MS risk has been explored instead. In a population-based, case-control study conducted in France, the association of passive cigarette smoking exposure and risk of MS was assessed in 129 POMS cases and 1,038 age-matched

controls and found that passive exposure to parental smoking was associated with increased risk of MS (relative risk (RR) 2.12, 95% CI 1.43–3.15). An increased risk of MS was significantly associated with longer duration of passive smoke exposure (RR 2.49, CI 1.53–4.08) (21). In another study, an association between secondhand smoke exposure and MS was evaluated in a cohort of 216 children with monophasic demyelination and 81 children with MS and found that secondhand smoke exposure was not an independent risk factor for the development of MS, but when combined with the presence of *HLA-DRB1*15*, the odds of MS significantly increased [odds ratio (OR) = 3.7; 95% confidence interval (CI): 1.17–11.9] suggesting a gene-environment interaction (70).

The major proposed mechanisms of exposure to cigarette smoking and development of MS include direct neurotoxicity, demyelination and immune modulation (21, 65, 70, 71). Cyanide, a component of cigarette smoke, has been shown to cause demyelination in rat models (69). Cigarette smoke may also be involved in the pathogenesis of MS through modulation of cellular and humoral immune responses (73). Smoking promotes T-cell activation and proliferation in the lungs and thus may contribute to increased immune activation (74). Given this association of both active and passive smoking with the development of MS, one could postulate that simply counseling patients (and housemates and/or parents) to avoid cigarette smoking could prevent the development of MS in many young individuals. This benign intervention may also have a beneficial effect on the smoker's overall health and the health of those around them.

In addition to cigarette smoke, air pollution and lung irritants such as fine particulate matter, carbon monoxide, and sulfur dioxide have been evaluated for associations with MS risk. In a multicenter case-control study performed in 2018, it was shown that fine particulate matter, carbon monoxide, sulfur dioxide and lead air emissions were associated with increased odds for pediatric MS ($P < 0.01$) (22). Similar work evaluating household chemical exposures and MS risk also demonstrated evidence for exposure to rodenticides (OR 2.10), weed control agents (OR 1.99) and products for plant/tree disease control (OR 2.72) to be associated with increased MS risk in childhood (23). There has been some conflicting evidence however, with some studies including a study using the Nurses' Health Study showing that particulate matter was not statistically associated with MS risk (75). Nonetheless lung irritants are exposures that can be potentially prevented ad thus warrants further investigation. Proposed mechanisms for how these irritants can influence MS risk include release of pro-inflammatory cytokines, promotion of oxidative stress, and stimulation of the immune response to activate auto-aggressive T cells to enter the central nervous system (22, 76).

DISCUSSION

Although the precise etiology of pediatric-onset MS has yet to be identified, it has become increasingly more evident that multiple risk determinants may play a crucial role. Investigating these environmental determinants in the pediatric-onset MS population allows one to evaluate the earliest influences on MS development and pathogenesis in individuals that are temporally closer to the biologic inciting event(s) of the disease. Moreover, studying risk determinants in this population eliminates some of the challenges with recall bias, as children are temporally closer to the incident exposures being studied.

Some of the risk determinants associated with POMS may be modifiable at the population level to potentially prevent disease onset. Implementation of sun exposure and 25-(OH) hydroxyvitamin D supplementation as well as recommendations for healthy diet and avoidance of exposure to cigarette smoke are simple, yet potentially effective strategies to not only improve general health, but to also reduce risk of MS. The possibility of an EBV vaccine is intriguing; however, much research will be required to create a vaccine that is safe and effective. The question of whom and when to enforce these strategies remains unclear. We propose based on the current literature that patients with a genetic predisposition to MS (i.e., first-degree relative with MS), may be the population that would benefit the most from these recommendations. In regard to timing of these interventions, we propose initiating these recommendations when planning conception and throughout gestation, childhood and adolescence. Ultimately these interventions are safe with low potential for adverse effects, and notably, could also have other health benefits including, but not limited to, improved cardiovascular health, pulmonary health, mental health, and energy. Further studies, specifically in the form of randomized clinical trials, will be required to provide more definitive evidence of exactly whom, when, and how much sun exposure/ 25-(OH) hydroxyvitamin D supplementation should be given for primary prevention of MS. Another question is how might we measure the effect of implementing these strategies in neonates? Answering this question will require collaborative efforts to compare incidence of pediatric MS diagnoses in those individuals provided with these interventions vs. absence of these interventions.

AUTHOR CONTRIBUTIONS

DH, TC, EW, and BB all contributed to the conception, organization, and content material that was included in this manuscript. DH wrote the first draft of the manuscript. All authors contributed to manuscript revision and have read and approved the final version of this manuscript.

REFERENCES

1. Lee J, Chitnis T. Pediatric multiple sclerosis. *Semin Neurol.* (2016) 36:148–53. doi: 10.1055/s-0036-1579738
2. Otallah S, Banwell B. Pediatric multiple sclerosis: an update. *Curr Neurol Neurosci Rep.* (2018) 18:76. doi: 10.1007/s11910-018-0886-7
3. Yeshokumar AK, Narula S, Banwell B. Pediatric multiple sclerosis. *Curr Opin Neurol.* (2017) 30:216–21. doi: 10.1097/WCO.0000000000000452

4. Chitnis T, Aaen G, Belman A, Benson L, Gorman M, Goyal MS, et al. Improved relapse recovery in paediatric compared to adult multiple sclerosis. *Brain.* (2020) 143:2733–41. doi: 10.1093/brain/awaa199
5. Fadda G, Armangue T, Hacohen Y, Chitnis T, Banwell B. Paediatric multiple sclerosis and antibody-associated demyelination: clinical, imaging, and biological considerations for diagnosis and care. *Lancet Neurol.* (2021) 20:136–49. doi: 10.1016/S1474-4422(20)30432-4
6. Cappa R, Theroux L, Brenton JN. Pediatric multiple sclerosis: genes, environment, and a comprehensive therapeutic approach. *Pediatr Neurol.* (2017) 75:17–28. doi: 10.1016/j.pediatrneurol.2017.07.005
7. Brenton JN, Engel CE, Sohn MW, Goldman MD. Breastfeeding during infancy is associated with a lower future risk of pediatric multiple sclerosis. *Pediatr Neurol.* (2017) 77:67–72. doi: 10.1016/j.pediatrneurol.2017.09.007
8. Ragnedda G, Leoni S, Parpinel M, Casetta I, Riise T, Myhr KM, et al. Reduced duration of breastfeeding is associated with a higher risk of multiple sclerosis in both Italian and Norwegian adult males: the EnvIMS study. *J Neurol.* (2015) 262:1271–7. doi: 10.1007/s00415-015-7704-9
9. Conradi S, Malzahn U, Paul F, Quill S, Harms L, Then Bergh F, et al. Breastfeeding is associated with lower risk for multiple sclerosis. *Mult Scler.* (2013) 19:553–8. doi: 10.1177/1352458512459683
10. Davis MK. Breastfeeding and chronic disease in childhood and adolescence. *Pediatr Clin North Am.* (2001) 48:125–ix. doi: 10.1016/S0031-3955(05)70289-3
11. Banwell B, Bar-Or A, Cheung R, Kennedy J, Krupp LB, Becker DJ, et al. Abnormal T-cell reactivities in childhood inflammatory demyelinating disease and type 1 diabetes. *Ann Neurol.* (2008) 63:98–111. doi: 10.1002/ana.21244
12. Graves JS, Chitnis T, Weinstock-Guttman B, Rubin J, Zelikovitch AS, Nourbakhsh B, et al. Maternal and perinatal exposures are associated with risk for pediatric-onset multiple sclerosis. *Pediatrics.* (2017) 139:e20162838. doi: 10.1542/peds.2016-2838
13. Department of Health and Human Service Office on Women's Health. Benefits of breastfeeding. *Nutr Clin Care.* (2003) 6:125–31.
14. Almohmeed YH, Avenell A, Aucott L, Vickers MA. Systematic review and meta-analysis of the sero-epidemiological association between epstein barr virus and multiple sclerosis. *PLoS ONE.* (2013) 8:e61110. doi: 10.1371/journal.pone.0061110
15. Belbasis L, Bellou V, Evangelou E, Ioannidis JPA, Tzoulaki I. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurol.* (2015) 14:263–73. doi: 10.1016/S1474-4422(14)70267-4
16. Gianfrancesco MA, Stridh P, Rhead B, Shao X, Xu E, Graves JS, et al. Evidence for a causal relationship between low vitamin D, high BMI, pediatric-onset MS. *Neurology.* (2017) 88:1623–9. doi: 10.1212/WNL.0000000000003849
17. Tremlett H, Zhu F, Ascherio A, Munger KL. Sun exposure over the life course and associations with multiple sclerosis. *Neurology.* (2018) 90:e1191–9. doi: 10.1212/WNL.0000000000005257
18. Tremlett H, Fadrosh DW, Faruqi AA, Hart J, Roalstad S, Graves J, et al. Associations between the gut microbiota and host immune markers in pediatric multiple sclerosis and controls. *BMC Neurol.* (2016) 16:182. doi: 10.1186/s12883-016-0703-3
19. Tremlett H, Fadrosh DW, Faruqi AA, Zhu F, Hart J, Roalstad S, et al. Gut microbiota in early pediatric multiple sclerosis: a case–control study. *Eur J Neurol.* (2016) 23:1308–21. doi: 10.1111/ene.13026
20. Chitnis T, Graves J, Weinstock-Guttman B, Belman A, Olsen C, Misra M, et al. Distinct effects of obesity and puberty on risk and age at onset of pediatric MS. *Ann Clin Transl Neurol.* (2016) 3:897–907. doi: 10.1002/acn3.365
21. Mikaeloff Y, Caridade G, Tardieu M, Suissa S. Parental smoking at home and the risk of childhood-onset multiple sclerosis in children. *Brain.* (2007) 130:2589–95. doi: 10.1093/brain/awm198
22. Lavery AM, Waubant E, Casper TC, Roalstad S, Candee M, Rose J, et al. Urban air quality and associations with pediatric multiple sclerosis. *Ann Clin Transl Neurol.* (2018) 5:1146–53. doi: 10.1002/acn3.616
23. Mar S, Liang S, Waltz M, Casper TC, Goyal M, Greenberg B, et al. Several household chemical exposures are associated with pediatric-onset multiple sclerosis. *Ann Clin Transl Neurol.* (2018) 5:1513–21. doi: 10.1002/acn3.663
24. Makhani N, Banwell B, Tellier R, Yea C, McGovern S, O'Mahony J, et al. Viral exposures and MS outcome in a prospective cohort of children with acquired demyelination. *Mult Scler.* (2016) 22:385–8. doi: 10.1177/1352458515595876
25. Pakpoor J, Giovannoni G, Ramagopalan SV. Epstein-Barr virus and multiple sclerosis: association or causation? *Expert Rev Neurother.* (2013) 13:287–97. doi: 10.1586/ern.13.6
26. Pakpoor J, Disanto G, Gerber JE, Dobson R, Meier UC, Giovannoni G, et al. The risk of developing multiple sclerosis in individuals seronegative for Epstein-Barr virus: a meta-analysis. *Mult Scler.* (2013) 19:162–6. doi: 10.1177/1352458512449682
27. Banwell B, Bar-Or A, Arnold DL, Sadovnick D, Narayanan S, McGowan M, et al. Clinical, environmental, and genetic determinants of multiple sclerosis in children with acute demyelination: a prospective national cohort study. *Lancet Neurol.* (2011) 10:436–45. doi: 10.1016/S1474-4422(11)70045-X
28. Nourbakhsh B, Rutatangwa A, Waltz M, Rensel M, Moodley M, Graves J, et al. Heterogeneity in association of remote herpesvirus infections and pediatric MS. *Ann Clin Transl Neurol.* (2018) 5:1222–8. doi: 10.1002/acn3.636
29. Nourbakhsh B, Cordano C, Asteggiano C, Ruprecht K, Otto C, Rutatangwa A, et al. Multiple sclerosis is rare in epstein–barr virus–seronegative children with central nervous system inflammatory demyelination. *Ann Neurol.* (2021) 89:1234–9. doi: 10.1002/ana.26062
30. Bar-Or A, Pender MP, Khanna R, Steinman L, Hartung HP, Maniar T, et al. Epstein–Barr virus in multiple sclerosis: theory and emerging immunotherapies. *Trends Mol Med.* (2020) 26:296–310. doi: 10.1016/j.molmed.2019.11.003
31. Smith C, Khanna R. The development of prophylactic and therapeutic EBV vaccines. *Curr Top Microbiol Immunol.* (2015) 391:455–73. doi: 10.1007/978-3-319-22834-1_16
32. Balfour HH, Schmeling DO, Grimm-Geris JM. The promise of a prophylactic Epstein–Barr virus vaccine. *Pediatr Res.* (2020) 87:345–52. doi: 10.1038/s41390-019-0591-5
33. Bäärnhielm M, Hedström AK, Kockum I, Sundqvist E, Gustafsson SA, Hillert J, et al. Sunlight is associated with decreased multiple sclerosis risk: no interaction with human leukocyte antigen-DRB1*15. *Eur J Neurol.* (2012) 19:955–62. doi: 10.1111/j.1468-1331.2011.03650.x
34. Hedström AK, Olsson T, Kockum I, Hillert J, Alfredsson L. Low sun exposure increases multiple sclerosis risk both directly and indirectly. *J Neurol.* (2020) 267:1045–52. doi: 10.1007/s00415-019-09677-3
35. Becklund BR, Severson KS, Vang SV, DeLuca HF. UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production. *Proc Natl Acad Sci USA.* (2010) 107:6418–23. doi: 10.1073/pnas.1001119107
36. Mokry LE, Ross S, Ahmad OS, Forgetta V, Smith GD, Leong A, et al. Vitamin D and risk of multiple sclerosis: a mendelian randomization study. *PLOS Med.* (2015) 12:e1001866. doi: 10.1371/journal.pmed.1001866
37. Rhead B, Bäärnhielm M, Gianfrancesco M, Mok A, Shao X, Quach H, et al. Mendelian randomization shows a causal effect of low vitamin D on multiple sclerosis risk. *Neurol Genet.* (2016) 2:e97. doi: 10.1212/NXG.0000000000000097
38. Munger KL, Hongell K, Åivo J, Soilu-Hänninen M, Surcel HM, Ascherio A. 25-Hydroxyvitamin D deficiency and risk of MS among women in the finnish maternity cohort. *Neurology.* (2017) 89:1578–83. doi: 10.1212/WNL.0000000000004489
39. Nielsen NM, Munger KL, Koch-Henriksen N, Hougaard DM, Magyari M, Jørgensen KT, et al. Neonatal vitamin D status and risk of multiple sclerosis. *Neurology.* (2017) 88:44–51. doi: 10.1212/WNL.0000000000003454
40. Mirzaei F, Michels KB, Munger K, O'Reilly E, Chitnis T, Forman MR, et al. Gestational vitamin D and the risk of multiple sclerosis in offspring. *Ann Neurol.* (2011) 70:30–40. doi: 10.1002/ana.22456
41. Munger KL, Åivo J, Hongell K, Soilu-Hänninen M, Surcel HM, Ascherio A. Vitamin D status during pregnancy and risk of multiple sclerosis in offspring of women in the finnish maternity cohort. *JAMA Neurol.* (2016) 73:515–9. doi: 10.1001/jamaneurol.2015.4800
42. Salzer J, Hallmans G, Nyström M, Stenlund H, Wadell G, Sundström P. Vitamin D as a protective factor in multiple sclerosis. *Neurology.* (2012) 79:2140–5. doi: 10.1212/WNL.0b013e3182752ea8
43. Ueda P, Rafatnia F, Bäärnhielm M, Fröbom R, Korzunowicz G, Lönnerbro R, et al. Neonatal vitamin D status and risk of multiple sclerosis. *Ann Neurol.* (2014) 76:338–46. doi: 10.1002/ana.24210

44. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients*. (2013) 5:2502–21. doi: 10.3390/nu5072502
45. Berezowska M, Coe S, Dawes H. Effectiveness of vitamin D supplementation in the management of multiple sclerosis: a systematic review. *Int J Mol Sci*. (2019) 20:1301. doi: 10.3390/ijms20061301
46. Tinelli M, Pugliatti M, Antonovici A, Hausmann B, Hellwig K, Quoidbach V, et al. Averting multiple sclerosis long-term societal and healthcare costs: the value of treatment (VoT) project. *Mult Scler Relat Disord.* (2021) 54:103107. doi: 10.1016/j.msard.2021.103107
47. Mozaffarian D. Dietary and policy priorities for cardiovascular disease, diabetes, and obesity. *Circulation.* (2016) 133:187–225. doi: 10.1161/CIRCULATIONAHA.115.018585
48. Guzik A, Bushnell C. Stroke epidemiology and risk factor management. *Continuum.* (2017) 23:15–39. doi: 10.1212/CON.0000000000000416
49. Pakpoor J, Seminatore B, Graves JS, Schreiner T, Waldman AT, Lotze TE, et al. Dietary factors and pediatric multiple sclerosis: a case-control study. *Mult Scler.* (2018) 24:1067–76. doi: 10.1177/1352458517713343
50. McDonald J, Graves J, Waldman A, Lotze T, Schreiner T, Belman A, et al. A case-control study of dietary salt intake in pediatric-onset multiple sclerosis. *Mult Scler Relat Disord*. (2016) 6:87–92. doi: 10.1016/j.msard.2016.02.011
51. Colpitts SL, Kasper LH. Influence of the gut microbiome on autoimmunity in the central nervous system. *J Immunol.* (2017) 198:596–604. doi: 10.4049/jimmunol.1601438
52. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep.* (2016) 6:28484. doi: 10.1038/srep28484
53. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci*. (2011) 108(Suppl. 1):4615–22. doi: 10.1073/pnas.1000082107
54. Berer K, Mues M, Koutrolos M, Rasbi, AlZ, Boziki M, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature.* (2011) 479:538–41. doi: 10.1038/nature10554
55. Kennedy EA, King KY, Baldridge MT. Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Front Physiol.* (2018) 9:1534. doi: 10.3389/fphys.2018.01534
56. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol.* (2013) 31:814–21. doi: 10.1038/nbt.2676
57. Carvalho AN, Lim JL, Nijland PG, Witte ME, Van Horssen J. Glutathione in multiple sclerosis: more than just an antioxidant? *Mult Scler J.* (2014) 20:1425–31. doi: 10.1177/1352458514533400
58. Munger KL, Bentzen J, Laursen B, Stenager E, Koch-Henriksen N, Sørensen TI, et al. Childhood body mass index and multiple sclerosis risk: a long-term cohort study. *Mult Scler J.* (2013) 19:1323–9. doi: 10.1177/1352458513483889
59. Gianfrancesco MA, Acuna B, Shen L, Briggs FBS, Quach H, Bellesis KH, et al. Obesity during childhood and adolescence increases susceptibility to multiple sclerosis after accounting for established genetic and environmental risk factors. *Obes Res Clin Pract.* (2014) 8:e435–47. doi: 10.1016/j.orcp.2014.01.002
60. Huitema MJD, Schenk GJ. Insights into the mechanisms that may clarify obesity as a risk factor for multiple sclerosis. *Curr Neurol Neurosci Rep.* (2018) 18:18. doi: 10.1007/s11910-018-0827-5
61. Galgani M, Procaccini C, De Rosa V, Carbone F, Chieffi P, La Cava A, et al. Leptin modulates the survival of autoreactive CD4 $^{+}$ T cells through the nutrient/energy-sensing mammalian target of rapamycin signaling pathway. *J Immunol.* (2010) 185:7474–9. doi: 10.4049/jimmunol.1001674
62. Winer S, Paltser G, Chan Y, Tsui H, Engleman E, Winer D, et al. Obesity predisposes to Th17 bias. *Eur J Immunol*. (2009) 39:2629–35. doi: 10.1002/eji.200838893
63. Nyirenda MH, Fadda G, Healy LM, Mexhitaj I, Poliquin-Lasnier L, Hanwell H, et al. Pro-inflammatory adiponectin in pediatric-onset multiple sclerosis. *Mult Scler J*. (2021) 27:1948–59. doi: 10.1177/1352458521989090
64. Keyhanian K, Saxena S, Gombolay G, Healy BC, Misra M, Chitnis T. Adipokines are associated with pediatric multiple sclerosis risk and course. *Mult Scler Relat Disord*. (2019) 36:101384. doi: 10.1016/j.msard.2019.11384
65. Manzel A, Muller DN, Hafler DA, Erdman SE, Linker RA, Kleinewietfeld M. Role of "Western Diet" in inflammatory autoimmune diseases. *Curr Allergy Asthma Rep.* (2014) 14:404. doi: 10.1007/s11882-013-0404-6
66. Zafonte RD, Shih SL, Iaccarino MA, Tan CO. Neurologic benefits of sports and exercise. *Handb Clin Neurol.* (2018) 158:463–71. doi: 10.1016/B978-0-444-63954-7.00042-2
67. White LJ, Castellano V. Exercise and brain health – implications for multiple sclerosis. *Sports Med.* (2008) 38:179–86. doi: 10.2165/00007256-200838030-00001
68. Guo LY, Lozinski B, Yong VW. Exercise in multiple sclerosis and its models: focus on the central nervous system outcomes. *J Neurosci Res.* (2020) 98:509–23. doi: 10.1002/jnr.24524
69. Hernan MA. Cigarette smoking and incidence of multiple sclerosis. *Am J Epidemiol.* (2001) 154:69–74. doi: 10.1093/aje/154.1.69
70. Lavery AM, Collins BN, Waldman AT, Hart CN, Bar-Or A, Marrie RA, et al. The contribution of secondhand tobacco smoke exposure to pediatric multiple sclerosis risk. *Mult Scler.* (2018) 25:515–22. doi: 10.1177/1352458518757089
71. Antonovsky A. Epidemiologic study of multiple sclerosis in Israel. *Arch Neurol.* (1965) 13:183–93. doi: 10.1001/archneur.1965.00470020073010
72. Hedström, Baarnhielm M, Olsson T, Alfredsson L. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. *Neurology.* (2009) 73:696–701. doi: 10.1212/WNL.0b013e3181b59c40
73. Arnson Y, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. *J Autoimmun.* (2010) 34:J258–65. doi: 10.1016/j.jaut.2009.12.003
74. Öckinger J, Hagemann-Jensen M, Kullberg S, Engvall B, Eklund A, Grunewald J, et al. T-cell activation and HLA-regulated response to smoking in the deep airways of patients with multiple sclerosis. *Clin Immunol*. (2016) 169:114–20. doi: 10.1101/054502
75. Palacios N, Munger KL, Fitzgerald KC, Hart JE, Chitnis T, Ascherio A, et al. Exposure to particulate matter air pollution and risk of multiple sclerosis in two large cohorts of US nurses. *Environ Int.* (2017) 109:64–72. doi: 10.1016/j.envint.2017.07.013
76. Giovannoni G, Hawkes CH, Lechner-Scott J, Levy M, Pohl D. Air pollution and multiple sclerosis risk. *Mult Scler Relat Disord*. (2021) 48:102797. doi: 10.1016/j.msard.2021.102797

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SYSTEMATIC REVIEW
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A Systematic Review of Tissue and Single Cell Transcriptome/Proteome Studies of the Brain in Multiple Sclerosis

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Multiple sclerosis (MS) is an inflammatory demyelinating and degenerative disease of the central nervous system (CNS). Although inflammatory responses are efficiently treated, therapies for progression are scarce and suboptimal, and biomarkers to predict the disease course are insufficient. Cure or preventive measures for MS require knowledge of core pathological events at the site of the tissue damage. Novelties in systems biology have emerged and paved the way for a more fine-grained understanding of key pathological pathways within the CNS, but they have also raised questions still without answers. Here, we systemically review the power of tissue and single-cell/nucleus CNS omics and discuss major gaps of integration into the clinical practice. Systemic search identified 49 transcriptome and 11 proteome studies of the CNS from 1997 till October 2021. Pioneering molecular discoveries indicate that MS affects the whole brain and all resident cell types. Despite inconsistency of results, studies imply increase in transcripts/proteins of semaphorins, heat shock proteins, myelin proteins, apolipoproteins and HLAs. Different lesions are characterized by distinct astrocytic and microglial polarization, altered oligodendrogenesis, and changes in specific neuronal subtypes. In all white matter lesion types, *CXCL12, SCD, CD163* are highly expressed, and STAT6- and TGFβ-signaling are increased. In the grey matter lesions, TNF-signaling seems to drive cell death, and especially *CUX2*-expressing neurons may be susceptible to neurodegeneration. The vast heterogeneity at both cellular and lesional levels may underlie the clinical heterogeneity of MS, and it may be more complex than the current disease phenotyping in the clinical practice. Systems biology has not solved the mystery of MS, but it has discovered multiple molecules and networks potentially contributing to the pathogenesis. However, these results are mostly descriptive; focused functional studies of the molecular changes may open up for a better interpretation. Guidelines for acceptable quality or awareness of results from low quality data, and standardized computational and biological

pipelines may help to overcome limited tissue availability and the "snap shot" problem of omics. These may help in identifying core pathological events and point in directions for focus in clinical prevention.

Keywords: multiple sclerosis, systems biology, transcriptome, proteome, single cell, brain lesions, NAWM, NAGM

1 INTRODUCTION

Multiple sclerosis (MS) is a common cause of neurological disability among young adults that evolves in clinically different stages termed radiologically isolated syndrome (RIS), clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), secondary progressive phase (SPMS), and primary progressive MS (PPMS). However, this classification may not directly reflect the pathological mechanisms similarly to another classification that only considers clinical/radiological activity and disability progression (1).

MS has a heterogeneous, multifactorial origin that involves interactions between the immune and nervous system impacted by the genetic background (2) and by the environment (3, 4). The main pathological features are accumulation of lesions in the grey and white matter (GM, WM). These are characterized by different degrees of inflammation, demyelination, neuronal and axonal degeneration, oligodendrocyte loss, gliosis/glia activity, and remyelination. Additional features are diffuse inflammation in the normal-appearing (NA) tissues, meningeal infiltrates, and global CNS atrophy (5). Especially in early relapsing MS, influx of systemic immune cells into the CNS induces inflammatory demyelinating lesions (6, 7). As the disease progresses, the number of chronic active lesions increases, and they inversely correlate with the number of remyelinating/repairing lesions (8–10). Lesions in cortical and deep GM areas and neuronal loss become prominent in the progressive phase (11). At this stage, inflammation becomes more compartmentalized and is governed primarily by microglia, astrocytes, and tissue-resident lymphocytes (12, 13).

Approved MS treatments impact systemic adaptive immune responses and work effectively in the early phase (14). However, their passage through the blood-brain barrier is limited, and most of them do not affect innate immune responses in the CNS. Their effect on compartmentalized immune responses is largely unknown. Such limitations are also reflected by their poor impact in the progressive phase. Neuro- and oligodendrocyte-protective treatments that inhibit or reverse degenerative processes are basically missing. To develop efficient treatments for the progressive phase, understanding the molecular mechanisms of pathological events within the CNS is essential. This has shifted focus of MS research to CNS-specific events. Recent advances in omics will hopefully integrate several levels of spatiotemporal data, and may help to understand, how multiple factors can converge into phenotypically similar disease states. Such knowledge may also fuel novel treatments (15, 16). To accomplish such goals, several challenges have to be overcome, e.g. experimental and computational pipelines have to be standardized, and large amount of descriptive biological data should be functionally interpreted. Here, we systemically review the transcriptome and proteome studies in the MS brain and discuss gaps and obstacles.

2 METHODS

2.1 Search Strategies

A systematic electronic search was conducted in PubMed with the following search terms from as far back as possible (earliest identified study was from 1997) to October 2021: category one "multiple sclerosis"; category two "brain", "lesions", "white matter", "grey matter"; category three "omics", "profiling", "transcriptome", "array", "next generation sequencing" "proteome"; category four "human" and NOT "review". The search was also complemented by reference lists of articles identified by this search strategy.

2.2 Selection Criteria

Studies were included, if they fulfilled the following criteria: (i) the study was performed on human brain tissue from patients with MS; (ii) the study used next-generation sequencing, mass spectrometry or arrays on the human brain tissue; (iii) article written in English.

Studies were excluded if the study design was not clearly stated.

3 RESULTS

Omics studies on MS brain tissue are few. An overview of the different methods is illustrated in **Figure 1**. Advantages and disadvantages of different omics techniques are listed in **Table 1**. A flowchart summarizing the identification of relevant studies according to PRISMA is presented in **Figure 2**.

3.1 Transcriptional (mRNA, ncRNA, MicroRNA) Approaches to Examine Pathological Mechanisms in the MS Brain Tissue

In the late 1990s, the first large-scale gene expression profiles were performed on different WM lesions from both autopsies and biopsies using microarrays (**Table 2**). They revealed alterations in cell metabolism, shifts in cytokines and cell adhesion molecules (17, 18), new inflammatory (19, 20, 44) and oxidative damage markers (23). In the 2010s, the number of samples increased, and microdissected tissues were also analyzed in designed systems biology studies; these included vessels near lesions (27), chronic active rim areas (33), or specific cell types like astrocytes (32). Single-cell/nucleus technologies,

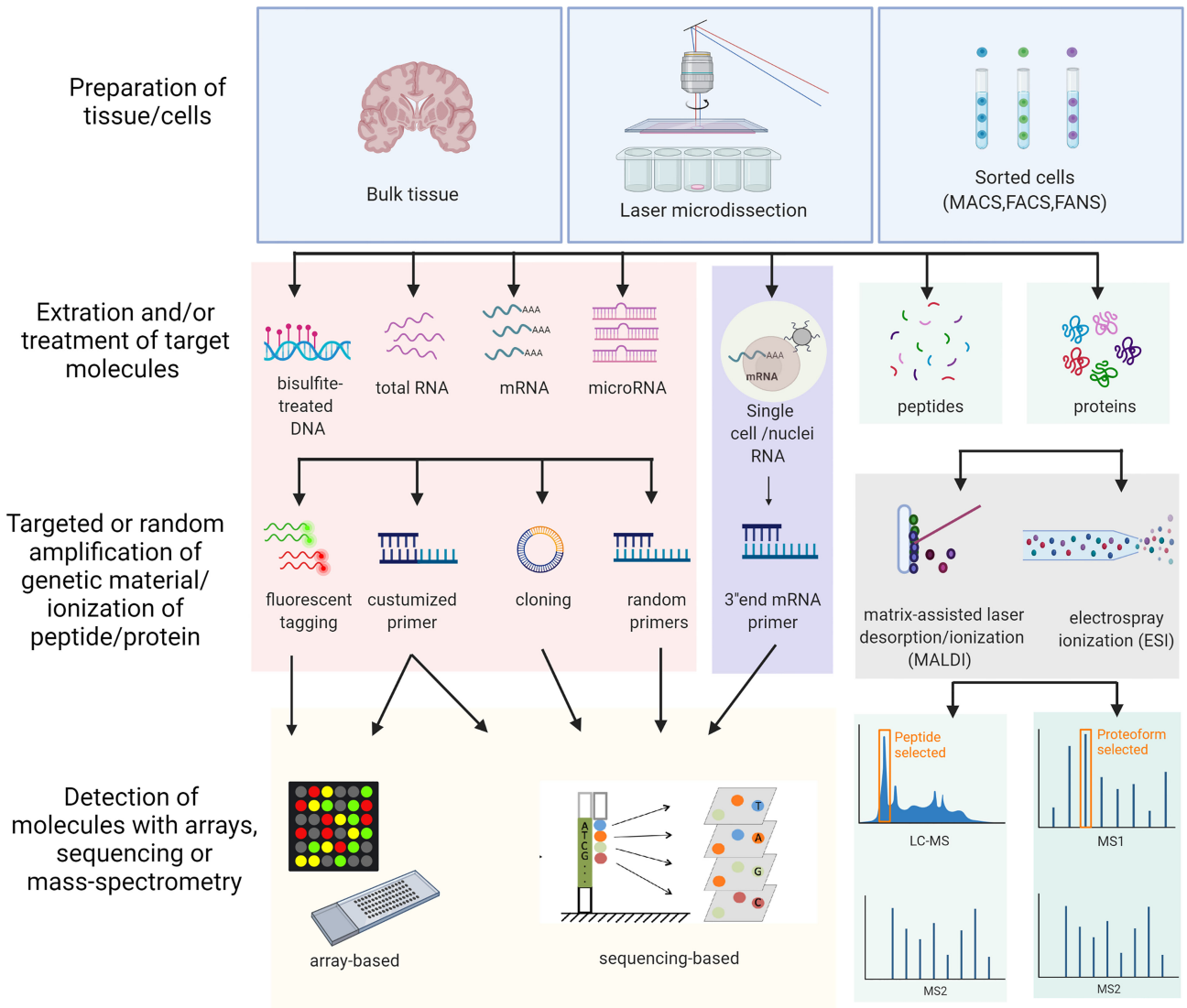

FIGURE 1 | Schematic overview of the different omics approaches used in the study. The overview includes all the different methods used in the studies included in this review. MACS, Magnetic-activated cell sorting; FACS, Fluorescence-activated cell sorting; FANS, Fluorescence-activated nucleus sorting; LC-MS, Liquid chromatography–mass spectrometry. Created with BioRender.com.

such as single-cell/nucleus RNA sequencing (sc/nRNA-seq, spatial-seq) that promote identification of novel cell types and cell state transitions have been published since 2019 (56–58).

We highlight the main findings in eight sections based on tissue types: (i) brain regional differences; (ii) NAWM; (iii) NAGM; (iv) WM lesions; (v) GM lesions; (vi) cell-specific changes; (vii) non-human transcripts, (viii) databases.

3.1.1 Brain Regional Differences

Corpus callosum and optic chiasm were the most significantly affected CNS regions in a study, and myelinating oligodendrocytes were most enriched with differentially expressed genes (51). Heat shock proteins were upregulated in all examined brain regions (*HSPA1A, HSPA1B, HSPA7, HSPA6, HSPH1, HSPA4L*) (52). Genes important in antigen-presentation, inflammation and hypoxia-induced responses were altered in the corpus callosum and optic chiasm (*TAPBP, IRF4, CTSB, CD79A*), while *STAT6* and *HLA-DRB5* were only increased in the optic chiasm. However, these regional differences may also reflect the presence of different cell types expressing different types of regional specific "housekeeping genes" with distinct physiological functions and purpose.

DNA methylation was altered, and RNA levels of DNA mehyltransferase were increased in MS hippocampus following demyelination (40). This study identified hypomethylation upstream of six genes including *ANKA*, a major regulator of CD40-CD40L, and hypermethylation upstream of ten genes e.g. *WDR81*, *NHLH2, PLCH1* involved in neuronal survival, synaptic density and memory.

TABLE 1 | An overview of the advantages and disadvantages of the omics techniques.

Omics	Target	Definition	Technology	Application	Temporal variance	Disadvantages	Advantages
Genomics	DNA	Assessment of variability in the DNA sequences of the genome	Whole genome sequencing	Genome-wide mutational analysis	None	Limited information about the MS state and prognosis	SNP variability is stable during life
			Exome sequencing (1.5% of the genome)	Exome-wide mutational analysis		Limited information about the MS state and prognosis, only information within the exons	
Epigenomics	Molecular changes on the DNA	Assessment of variability of factors that regulate the genome without changing the DNA sequence	WGBS (whole genome bisulfite-treated DNA sequencing)	Methylome-wide pattern and alterations	Moderate	Complex data analysis, lack of functional knowledge on methylation at other sites	Whole methylome state on single base pair level
			RRBS (bisulfite-treated CpG enriched region sequencing (3% of the genome))	Methylome pattern of CpG enriched regions based on restriction enzymes		Missing areas, difficulties in comparing between samples due to unpredictable cleavage and enrichment, no information at other bases (A, T, C)	Focused methylation status at CpG regions
			TBS (bisulfite-treated hybridized target DNA region sequencing)	Targeted methylation analysis of selected candidate genes		Need prior knowledge on candidate areas	Parallel investigation of many candidate genes
			Microarray (hybridization of ~850,000 probes at methylation sites)	Interrogation of pre-selected methylation sites across the genome		Limited to the probes available, no information at other bases (A,T, C), high background noise, not fully compatible across platforms	Cost efficient, methylome of 95% of CpG islands, high coverage of enhancer regions
			ATAC-seq (Tn5 transposase treated DNA sequencing)	Identification of accessible chromatin regions in genome-wide, including transcription factors, histone modifications.		Time-consuming, poor repeatability, signal-to-noise ratio is low	Unbiased identification of a real time profile of all active regulatory sequences in the genome using a small amount of cells
			ChIP-seq (chromatin immunoprecipitated DNA sequencing)	Analyze protein interactions with DNA by genome-wide mapping of epigenetic marks, transcription factors, or other DNA-binding proteins		Require good antibody for target protein, high amount and high quality of tissue	Map global binding sites precisely for any protein of interest, analyze the interaction pattern of any protein with DNA, or the pattern of any epigenetic chromatin modifications
			Sc/snATAC-seq (Tn5 transposase treated DNA sequencing within intact single nuclei)	Identification of accessible chromatin regions within single cells		Require high quality tissue, unclear if it is a limited subset of open chromatin sites in single cells	As ATAC-seq, but provides examination of cell-to-cell variability in chromatin organization,
Transcriptomics	Activated genes/ RNA	Assessment of variation on composition and abundance of the transcriptome	Microarray (cDNA hybridization of targets of interest to probes)	Differential gene expression analysis of protein-coding-genes (~18,700) or designed probes of interest	High	Limited dynamic range (probe-dependent), problems with competitive hybridization, high background, low sensitivity, not fully compatible across platforms	Well-defined protocols and analysis pipelines
			Next generation RNA-seq (cDNA sequencing of RNA with rRNA removal or mRNA enriched)	Genome-wide differential gene expression analysis of total RNA or mRNA		PCR amplified biases, lack of standardization between sequencing platforms (effect dynamic range and reproducibility), do not capture the whole transcriptome (small drop-outs)	Unbiased insight into all transcripts (novel and non-coding), accurately measuring expression level changes, ability to detect expression changes in non-coding genes
			EST (expressed sequencing tags of randomly selected clones sequenced	Differential gene expression analysis of the partial mRNA pool of the sample		Only partial profiles of the gene expression, a large numbers of housekeeping genes, neglect rare transcripts	Suitable for gene discovery, rapid and easy protocols

(Continued)

TABLE 1 | Continued

Omics	Target	Definition	Technology	Application	Temporal variance	Disadvantages	Advantages
			from cDNA libraries (total RNA or poly (A) RNA))				
			Amplicon (targeted sequencing based on probes designed for targets of interest)	Differential gene expression analysis of targets of interest		Prior knowledge of target RNAs	Multiplexing of hundreds to thousands of amplicons per reaction, less sequencing with high coverage
			Sc/snRNA-seq (poly(A) tagging, 5′-end, 3'-end or total RNA-sequencing within intact single nuclei or cell)	Gene expression profiles of individual cells		More time-consuming, require high quality tissue, identifies fewer transcripts than bulk RNA-seq (high drop-out), imperfect coverage can lead to a biased quantification, complex analyses	Transcriptomic profiling of heterogeneous tissue, or dynamic processes in single and within cell groups, sensitive, interrogate nuances of cell signaling pathways
			Spatial transcriptomics (sequencing of released tissue mRNA captured on spotted histology slides to combine gene activity with spatial resolution)	Spatially-resolved transcriptomics		Intact good quality tissue block, not single cell level (each spot represent 10-100 cells), complex analyses, time-consuming, good microscope	Map out gene expression in spatial context, capture how gene expression data might reflect the spatial relationships among multiple cells
Proteomics	Proteins	Assessment of variation on composition and abundance of the proteome	Mass spectrometry (identify (u)known peptides/proteins via separation of gaseous ions according to their differing in mass and charge)	Identification and quantification of proteins in a sample	High	Time-consuming complex data analysis, protein detection is affected by high abundance proteins and peptide ionization	Incredibly sensitive (parts per million), excellent for identifying unknown components or confirming their presence and abundance
			Array (binding of targets of interest to peptides (up to tens of thousands in several copies))	Identification and quantification of proteins of interest in a sample		Limited to prior knowledge (not discovery)	Profiling multiple proteins without disturbance of high abundance proteins, high number of arrays available for a wide range of applications.
			Sc mass cytometry (simultaneous measurement of more than 40 proteins at single-cell resolution)	Multiplexed and quantitative measurements of proteins and their modifications on single cells		Low dimension, prior knowledge of targets, limited target number (40), significant variation in signal intensity over time and across machines	Highly multiplexed and quantitative measurements of proteins and modifications, good pipelines for analysis

In the choroid plexus (CP), 17 genes were significantly upregulated in progressive MS patients (54). These genes were related to hypoxia, neuroprotection and secretion (e.g. *CXCL2, LYVE1, SNHG15, MT1X*, non-coding *HIFA1-AS3*), while strong inflammatory reactions were absent.

3.1.2 NAWM

Comparing NAWM to control WM, 465 genes were differentially expressed (48). Among the top ten upregulated genes were immune-related (*IGHG1, HLA-DRB5, GPNMB, CD163*) and mitochondria-related (*MTRNR2L12, MTRNR2L8*). NAWM was also different from control WM by a global defense against oxidative stress based on upregulation of *STAT6, HIFα* and its target genes (21, 26, 61). Genes in the STAT-6 signaling were upregulated in oligodendrocytes (61) (**Figure 3**). These alterations were accompanied by upregulation of *nNOS, HO-1* and *HLA-DR*, suggesting an inflammatory and oxidative-stress related reaction in oligodendrocytes outside of lesions. A combined methylome and transcriptome study found downregulation and hypermethylation of oligodendrocyte survival genes in NAWM (*BCL2L2, NBRG1*) (46). Besides oligodendrocytes, several dysregulated genes in MS suggested alterations in subcortical WM neurons (21).

NAWM microglia upregulated *STAT4* and *HLA-DRα* (26), and had a lipid metabolic gene expression profile (e.g. *EEPD1, PPARG, LPL*) with unchanged expression of the homeostatic signature (*P2RY12* and *TMEM119*) (50). Additionally, a subtype of microglia (48) had increased expression of *CD26/DPP4* in the

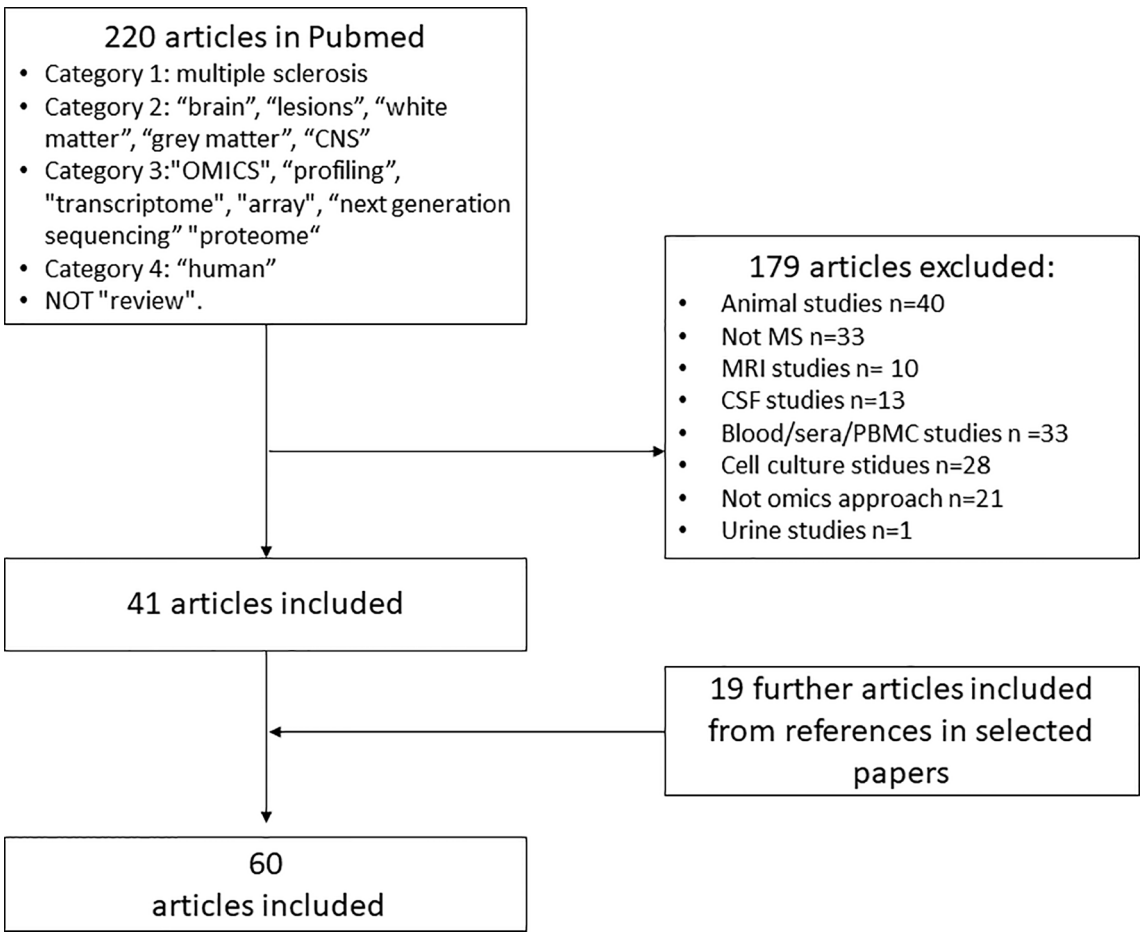

FIGURE 2 | Flowchart for identification and inclusion of relevant studies for systematic review. PMD, postmortem-delay; RIN, RNA integrity number.

NAWM (46). Astrocytic markers (*GFAP*, *AQP4*) were also altered in the NAWM (61) (**Figure 3**).

Genes of several chemokines and cytokines (21, 26) were upregulated in NAWM reflecting the low level inflammation even without lesion formation. A mild disease course was also associated with a different molecular profile with altered expression of genes related to immune-regulation, myelination, anti-oxidative mechanism and neuroprotection together with a high hypothalamus-pituitary-adrenal (HPA) axis activity (35).

3.1.3 NAGM

The difference in WM *vs.* GM microglia gene expression was significantly lower in MS compared to non-neurological disease brains. This suggests that microglia cells are losing region-specific profile in MS (50). However, while NAWM microglia have a lipid signature, NAGM microglia have increased expression of genes related to glycolysis and iron homeostasis (*SCL25A37, ABCB6*) and a neurodegenerative profile (*CXCR4, GPNMB, OPN/SPP1*) (**Figure 3**). Furthermore, in HLADRB1*15:01 positive patients, HLA-DRB1 and B5 were the highest expressed genes in NAGM (37).

3.1.4 WM Lesions

A continuum of dysfunctional homeostasis (e.g. *VIM, HBB, MAF*) and inflammatory changes (e.g. *CASP1, IRF5, MMP2*) between active lesions and NAWM supports the concept of MS involving the whole CNS (24). However, the lesions differed from NAWM by high expression of genes related to immunoglobulin synthesis (*IGKC, IGL, IGGL1, ILR6*) and neuroglial differentiation (*SNAP25, CAP2, NFL/M*) (24). Upregulated genes in active lesions compared to NAWM also included chemokine genes and receptors (*MIP-a, RANTES, CCR1, CCR4, CCR5, VLA-4, CCR8*) genes, interferon- and tumor-necrosis factor receptors (17), and cytokines (*TGFB, IL-3, OPN, IL-5, IL6*) (18, 44, 53) (**Figure 4**). Two highly expressed genes encoded the Th cell marker (*CD4*) and the antigen-presenting gene (*HLA-DRa*) (18). Additionally, CD8+ T cells containing cytotoxic granules were suggested to communicate with mononuclear phagocyte cell expressing CD163 and CD11b in the lesions (49). Genes encoding multiple autoantigens were also found in MS lesions indicating a secondary autoimmune stimulation that could exacerbate the ongoing inflammation (43).

TABLE 2 | An overview of the studies (n=49) that examined the transcriptome profile in human MS brain tissue.

Microarray of tissue (laser captured or macrodissected) and isolated cells: mRNA				
Authors	**Patients**	**Quality (PMD, RIN)**	**Methodology**	**Key findings**
Whitney et al. (17)	- 2 lesions from Becker et al. (43) - 1 NAWM from same patient	PMD: 8h	Tissue mRNA array	- 20 DEGs in lesion vs. NAWM related to cell metabolism, cytokines and cell adhesion molecule.
Baranzini et al. (18)	- 8 MS samples with active demyelination - 8 controls (non-MS)	–	Tissue mRNA array	- 31 DEGs in MS. - *CD4* was the most overexpressed gene. - Predominant expression pattern of Th1 cytokines mainly represented by *MIP-1a, RANTES, caspase-1, IL-1B, IL-18 IL-5, IL-6.*
Whitney et al. (19)	- 2 lesions from PPMS [from Becker et al. (43)] - 1 RRMS with chronic silent lesion - NAWM from the two patients	–	Tissue mRNA array	- *Arachidonate 5-LO* overexpressed in both microarray and EAE disease states but not NAWM or normal mouse brain.
Lock et al. (20)	- 1 active, 3 chronic active, 3 chronic inactive from 4 progressive MS patients - 2 control subjects	PMD: 1.5-8h	Tissue mRNA array	- *MAPK2* and *GM-CSF* were higher expressed in acute than chronic active lesion. - *FcRy* was higher expressed in chronic than acute lesion.
Graumann et al. (21)	- 12 NAWM in 10 MS - 8 WM in 7 control subjects	PMD: 5-22h	Tissue mRNA array	- DEGs in NAWM were involved in energy metabolism, neuroprotection, oxidative stress and ischemic preconditioning, axonal transport and synaptic transmission: *HIF1a, CREB, PI3K/Aktm VEGF, hexokinase 1, Cl-transporter, adenosine A1 receptor, GABA-A/B R, 14-3-3, STAT6+MCSF, IL-1, TNFa and GSH, ROS/RNS NF-L NF-M, synaptophysin, SCG10.*
Mycko et al. (22)	- 2 chronic active (marginal and centre) and 2 silent (marginal and centre) lesions from 4 SPMS	PMD: <8h	Tissue mRNA array	- Pathological events differ in the centre and at the edge of the chronic lesions. - 9 DEGs in in the marginal zone of chronic active lesions were highlighted: *CD4, IFNg, MAPKK1, Caspase 9, Cbl-b, EDDR1, HSP90, FLT3 ligand, adenosine A1 receptor.*
Tajouri et al. (23)	- 2 acute and 3 chronic active lesions from 5 SPMS - 4 control areas from non-MS	PMD: 4-24h	Tissue mRNA array	- Upregulation of immune-related DEGs: *MAL, VIL2, CXCL10, CXCR3* in MS. - Detection of genes related to oxidative damage protection: *TF, SOD1, GPX1, GSTP1.*
Lindberg et al. (24)	- 5 active lesions and 5 NAWM lesions from 6 SPMS - 12 WM from 12 control subjects	PMD: 3:45-9:20h	Tissue mRNA array	- Lesions and NAWM shared downregulated DEGs of anti-inflammatory property*: EGFR*, *TGFB3, cre-bp-1.* - Lesions differed from NAWM by higher *Ig* level and *IL-6R.* - Lesions had DEGs related to neuroglial development: *NF-L/M, STMN2, a/b-tubulin, dynamin, CAP2.*
Mycko et al. (25)	Same data as Mycko et al. (22)	PMD: <8h	Tissue mRNA array	- The centre of chronic active and inactive lesions had fewer genes differentially expressed and less infiltration. - *TNF* and *IL-6* were underrepresented in chronic inactive, but upregulation of *bcl-xm GFR2, hsp90A hsp60.*
Zeis et al. (61)	- 11 NAWM from 11 MS - 8 controls	PMD: 6-26h	Tissue mRNA array	Upregulation of both pro-inflammatory response: *STAT4, IL-1B, MCP-1, ICAM-1, RANTES, HLA-DR*; and anti-inflammatory response: *IL-10, TGFB2, STAT6, IL4R, IL13R.*
Zeis et al. (26)	- 4 biopsy from both lesion and non-demyelination in MS patient - 8 NAWM autopsy MS patients - 2 biopsy controls	–	Tissue mRNA array	- Active astrocytes (*GFAP, AQP4, HLA-DRA*) and active oligodendrocytes (*PLP, MAG,STAT6, nNOS, HO-1*) are strongly up-regulated in non-demyelinated WM during a very early acute phase of MS.
Cunnea et al. (27)	- Chronic active, chronic inactive and NAWM from 4 PPMS and 8 SPMS - WM from 5 controls	PMD: 8-33h	Microarray of microdissected vessels	- 113 genes involved in all aspects of endothelial cell biology, and 50% of those were DEGs from chronic active or inactive compared to NAWM or control. - Upregulated genes in chronic active and inactive were among others *VEGFA, MMP1*, *MMP14* and *ICAMs.*

(Continued)

TABLE 2 | Continued

Fischer et al. (28)	3 microdissected active lesions of patients with fulminant acute MS	–	Tissue mRNA array	Array detected genes of mitochondrial injury together with gene expression of various nicotinamide adenine dinucleotide phosphate oxidase subunits. The data suggest inflammation-associated oxidative burst in activated microglia and macrophages.
Mycko et al. (29)	5 CA lesions (marginal and centre) compared with NAWM from 5 SPMS	PMD: <8h RIN:6-7.5	Tissue mRNA array	- 45 heat-shock protein (HSP) genes of all 8 major families were present, and the pattern of HSP differed between centre and margin of the chronic active lesions.
Mohan et al. (30)	- 6 demyelinated inactive lesion from 4 MS - 4 remyelinated lesions from 3 MS - 4 demyelinated active lesions from 3 MS - 6 WM from 4 controls	–	Tissue mRNA array	- *FGF1* was the most abundant gene in remyelinating lesions compared to demyelinating and WM control tissue.
Licht-Mayer et al. (31)	WM study: - 4 acute MS cases each with NAWM, initial demyelinating lesions, late active lesions - 4 control cases GM study: - 3 SPMS each with cortical lesions - 3 control cases	–	Tissue mRNA array	- Nrf2 is upregulated in active MS lesions, especially in oligodendrocytes, while few number of Nrf2-postive neurons were detected. - A number of Nrf2-responsive genes involved in protection against oxidative stress were upregulated in initial demyelinating lesions. - Expression pattern of Nrf2-induced genes differed between WM and GM.
Waller et al. (32)	- 5 samples with astrocytes in NAWM from MS - 5 samples with astrocytes in WM from controls	PMD:5-33h RIN:>3	mRNA array of GFAP positive cells	Genes upregulated in NAWM astrocytes were related to scavenge transition metal ions and free radicals (*MT1,MT2*), transport and storage of iron (*FTL, TF*) and immune related ischaemic preconditioning (*TGF-B3, MAPKAPK2, MAPK4*), while gene encoding COX2 enzyme (*PTGS2*) was downregulated.
Hendrickx et al. (33)	- rim and perilesional-NAWM of 7 chronic active and 8 inactive lesions from 12 RRMS, 1 PPMS, and 2 with unknown MS disease course - 10 WM from 10 control subjects	PMD: 8:23±2.51-9:03±0.45h RIN: 5.79±0.62-7.42±0.67	Tissue mRNA array	- Upregulation of DEGs in rim of lesions involved in immune function, lipid binding, lipid uptake, and neuroprotective functions - Identified a set of genes that are related to lesion activity and expansion*: CHIT1, GPNMB, CCL18, OLR1, CD68, MSR1, CXCL16, CXCR4, NPY, KANK4, NCAN, TKTL1, ANO4*.
Zeis et al. (34)	- 9 active lesions, 9 NAWM, 7 remyelinating lesions and 5 inactive lesions from 7 PMS patients	PMD: 9-27h RIN:>7	Tissue mRNA array	- Increased expression of STAT6-singaling gens in active, remyelinating and inactive lesions - Expression of genes involved in oligodendrogliogenesis were qualitative and quantitative differently expressed in the different WM lesions
Melief et al (35)	- NAWM from 18 MS - WM from 9 controls	PMD: 4:15-13:20h RIN: 7.4-7.8	Tissue mRNA array	In MS patients with mild MS and high HPA-axis, the NAWM expression profile reflected genes involved in regulation of inflammation, myelination, anti-oxidant mechanisms and neuroprotection.
Magliozzi et al. (36)	- 20 MS motor cortex with and without substantial meningeal inflammation - 10 controls	PMD: 3-44h RIN: >7	Tissue mRNA array	A changing balance of TNF signalling in the cortex depending on the degree of inflammation.
Enz et al. (37)	64 NAGM samples of 25 MS patients and 42 control GM samples of 14 controls	PMD: 3-28h RIN: >6	Tissue mRNA array	*HLA-DRB1* is significantly higher expressed in MS NAGM and the protein expression is increased in HLADRB1*. 15:01-positive cases in grey matter on microglia based on immunofluorescence colocalization.
Jäckle et al. (38)	- 8 chronic active, 8 NAWM and 1 lesion rim af a chonic inactive lesion	PMD: 9-34h RIN: >3	Tissue mRNA array	- Accumulation of M1 microglia phenotype at lesion rim. - Upregulation at *ALOX15B, MME* and *TNFRSF25* in the lesion rim.
Microarray of tissue (laser captured or macrodissected): microRNA and methylome				
Authors	**Patients**	**Quality (PMD, RIN)**	**Methodology**	**Key findings**
Junker et al. (39)	- 16 active and 5 inactive white matter multiple sclerosis brain lesions - 9 control white matter specimens.		Tissue microRNA array	- miRNA signatures of active and inactive brain lesions of patients with MS. - microRNA-34a, microRNA-155 and microRNA-326 were upregulated in active MS lesions and related to the CD47 in microglia/macrophages.

(Continued)

TABLE 2 | Continued

Chomyk et al. (40)	9 myelinated and 7 demyelinated regions of hippocampus from 15 MS patients	PMD: 4-12h	Tissue methylation array	Genes involved in synaptic plasticity and neuronal survival were altered by methylation changes following demyelination in MS hippocampus. Here among hypomethylation of 6 genes (*AKNA, EBPL, FLJ42709, HERC6, OR52M1, SFRP1*) in demyelinated regions.
Tripathi et al. (41)	5 myelinated and 5 demyelinated WM lesions 6 SPMS patients	PMD: 9-37h	Tissue microRNA array	- Discovery of 11 pathogen-related and 12 protection-related miRNAs previously identified in sera and correlating with WM MRI abnormalities. - 7 of the 12 microRNAs related to protection were decreased in the MS lesions.
Kular et al. (42)	- Neuronal nuclei isolated from 14 MS patients (incl. NAWM, active, chronic active, chronic lesions) and 12 controls	PMD: 11± 11.4-23±3.7h	Tissue methylation array	- DNA methylation alterations in WM-neurons from MS patients compared to control. - Potential impaired CREB-mediated neuro-axonal integrity due to hypo-5mC and hyper-5hmC in MS neurons.
Fritsche et al. (64)	- 7 subpial lesions, 7 leucocortical lesions, 7 chronically inactive WM lesions and NAWM from 18 MS brains - Subpial and leucocortical areas of normal GM and normal WM from 12 age-matched controls		Tissue microRNA array	- 5 of 7 significantly upregulated miRNAs in grey matter lesions (miR-330-3p, miR-4286, miR-4488, let-7e-5p, miR-432-5p) shared the common target synaptotagmin7 (Syt7).
Tripathi et al. (41)	miRNA study: 5 NAGM and 5 MS demyelinating cortical lesions mRNA study: 8 NAGM from 6 MS brains and 8 cortical lesions from 8 MS brains	PMD: 3-9h	Tissue microRNA array	- 10 significant up- and 17 significant downregulated microRNAs in demyelinated GM vs. NAGM. - Predicted target mRNAs belonged to TGF-β signalling and FOXO signalling. - mir149, mir20a, mir29c and mir24 were key regulators based on PPI network analysis.

Next generation sequencing (NGS) of tissue (laser captured or macrodissected) and isolated cells: mRNA and total RNA

Authors	Patients	Quality (PMD, RIN)	Methodology	Key findings
Becker et al. (43)	- 3 lesions from 1 PPMS - 2 areas from healthy adult brain	PMD: 8h	Expressed sequencing tag (EST)	- 56 DEGs related to immune activation in PPMS. - Discovery of *MIP-1a* and *RANTES*.
Chabas et al. (44)	- 2 acute and 1 chronic lesion from 3 MS patients - 1 control subject		EST	- 50 DEGs in MS as *GFAP, MBP, HSP70, CRYAB* and *OPN* (osteopontin). - Degree of *OPN* expression correlated with severity of EAE disease.
Schmitt et al. (45)	- 7 WM lesions from 6 MS - 7 WM areas from 7 controls	PMD: 4:50-12h	Next generation amplicon sequencing	- No significantly different transcription patterns, when comparing HERV-W transcription in brain lesions from MS to healthy.
Huynh et al. (46)	- 28 NAWM from MS - 19 WM from controls	PMD: ≥31h RIN: ≥7	Tissue NGS (mRNA) and methylation array	- Downregulated and hypermethylated genes in NAWM were related to oligodendrocyte and neuronal function (*BCL2L2, HAGHL, NDRG1*). - Upregulated and hypomethylated genes in NAWM were encoding for cysteine proteases (*CTSZ, LGMN*).
Kriesel et al. (47)	Frozen brain tissue from: - 14 demyelinating brains: PPMS (n=11), SPMS (n=1), NMO (n=2) - 14 controls - 7 OND: herpes encephalitis (n=3), unknown encephalitis (n=2), subacute sclerosing pan encephalitis (n=2)	PMD: 4-24h	Tissue NGS (total RNA)	- Overexpression of HERV in demyelinating and OND brain samples compared to normal brain. Specific HERV and KRAB sequences were overexpressed in the demyelinating group.
Elkjaer et al. (48)	- 21 NAWM, 16 active, 17 chronic active, 14 inactive, 5 remyelinating lesion from 10 progressive MS patients - 25 WM of non-neurological disease subjects	PMD: 8-30h RIN: 6±1.7	Tissue NGS (total RNA)	- chronic active lesions were the most distinct from control WM based on the highest number of unique DEGs (n=2213), and differed the most from remyelinating lesions, indicating end of the spectrums in lesion evolution. - CD26/DPP4 was expressed by a subpopulation of microglia in the NAWM. - TGFβ-R2 was the central hub in the *de novo* network of common lesion DEGs, and it was expressed by astrocytes in remyelinating lesions.
Konjevic Sabolek et al. (49)	Laser-microdissected target areas of CD8 and perforin in active MS lesions of 4 patients		NGS (mRNA) of cells communicating with CD8+ cells	- Communication between CD8+ T cells and mononuclear phagocyte cells expressing *CD163* and *CD11b*.

(Continued)

TABLE 2 | Continued

Van der Poel et al. (50)	- 5 NAGM (occipital cortex), 10 NAWM (CC) of MS - 5 GM (occipital cortex), 11 WM (CC) of non-neurological disease	PMD: 6:06±0.018h (control) 9:17±0.18h (MS) RIN: 7.3±0.4, 7± 0.5 (control) 8.1±0.3, 6.3± 0.8 (MS)	NGS (mRNA) of isolated microglia	- Microglia show a clear region-specific profile between WM and GM. - Homeostatic profile of microglia was maintained in the normal appearing tissues (no changes in P2RY12, TMEM119). - Different regional transcriptional changes in MS microglia: microglia in NAWM had genes related to lipid metabolism; NAGM microglia had genes related to glycolysis and iron homeostasis.
Voskuhl et al. (51)	5 MS patients and 5 controls with regions including corpus callosum, optic chiasm, internal capsule, hippocampus, frontal cortex, and parietal cortex	RIN: 5.1-8.3 (control) 6.1-8.7 (MS)	Tissue NGS (mRNA)	- Corpus callosum and optic chiasm were the most significantly affected CNS regions in MS. - Myelinating oligodendrocytes were the cell type most enriched with DEGs in MS.
Chiricosta et al. (52)	Six different brain areas (corpus callosum, hippocampus, optic chiasm, internal capsule, frontal cortex and parietal cortex)from 5 MS and 5 controls (data from Voskuhl et al. 2019)	RIN: 5.1-8.3 (control) 6.1-8.7 (MS)	Tissue NGS (mRNA)	*HSPA1A, HSPA1B, HSPA7, HSPA6, HSPH1* and *HSPA4L*, encoding for HSP70s, are significantly upregulated in corpus callosum, hippocampus, internal capsule, optic chiasm, and frontal or parietal cortex, between healthy individuals and MS patients.
Frisch et al. (53)	The MS Atlas of Elkjaer et al. (48)	PMD: 8-30h RIN: 6±1.7	Tissue NGS (total RNA)	*VLA-4* is highly expressed in active lesions in non-treated PMS patients.
Rodríguez-Lorenzo et al. (54)	Choroid plexus samples from 6 PMS patients and 6 controls	PMD: 4.33-11h RIN: ≥ 6.5	NGS (mRNA)	- 17 genes increased in CP of PMS, here among the ncRNA, *HIF1A-AS2*. - Transcript alterations were related to hypoxic responses and secretion of neuroprotective peptides.
Elkjaer et al. (55)	71 MS brain samples and 25 control WM samples from Elkjaer et al. (48)	PMD: 8-30h RIN: 6±1.7	Tissue NGS (total RNA)	2.73% of the transcripts mapped to HERV transcripts. Here among HERV-W and HERV-H transcripts located close to the MS genetic risk locus at chromosome 7 regions were uniquely expressed in MS lesions.
Elkjaer et al. (55)	73 MS brain samples and 25 control WM samples from Elkjaer et al. (48)	PMD: 8-30h RIN: 6±1.7	Tissue NGS (total RNA)	*APOC1* was significantly increased in active MS lesions and *PTPRG* significantly increased in all WM MS brain tissue, while both encoding proteins were upregulated in the CSF of multiple MS subtypes.
Manuel et al. (94)	- Isolated microglia from 10 MS NAWM and 11 controls from van der Poel et al. (50) - 7 chronic active perilesional MS NAWM and 10 controls [from Hendrickx et al. (33)]		NGS data from both tissue and microglia in NAWM and WM	- Cross dataset evaluation suggested MAPK and JAK/STAT3 pathways as potential drug targets in MS. - *CDK4, IFITM3, MAPK1 MAPK3, METTL12B* were enriched colocalized genes in *de novo* network. - Rubidomycin hydrochloride and zafirlukast were suggested as potential medications for drug repositioning strategies.

Single nucleus RNA next-generation sequencing (snRNA-seq) of tissue and isolated cells

Authors	Patients	Quality (PMD, RIN)	Methodology	Key findings
Jakel et al. (56)	- 3 active, 3 chronic inactive, 4 chronic active, 3 NAWM, 2 remyelinating lesions from 4 progressive MS patients - 5WM from 5 controls	RIN: 4.04±.41	Tissue snRNA-seq	- Fewer nuclei from OPCs in all MS lesions and in NAWM compared to control. - The intermediate Oligo6 cells were highly reduced in MS. - Skewing in the subclusters of mature oligodendrocytes between MS and control tissue: the Oligo1 cluster was depleted in MS, whereas the Oligo2, Oligo3, Oligo5 and ImOLG clusters were enriched.
Masuda et al. (57)	- 5 patients with early active multiple sclerosis - 5 from healthy brain tissue removed during surgery for epilepsy	–	snRNA-seq of isolated microglia	- Microglia in MS had downregulation of homeostatic signature: *TMEM119, CX3CR1, P2RY12 and SLC2A5*. - Microglia could be separated into subsets with specialized functions as APC function, matrix-remodelling function, dampen cytotoxic functions.
Schirmer et al. (58)	- 12 MS tissue samples (entire tissue blocks including lesion and non-lesion GM and WM areas plus meningeal tissue) - 9 tissue samples from control individuals	PMD:6-27h RIN: 6.8-9.1	Tissue snRNA-seq	- CUX2+ excitatory neurons in cortical layers 2-3 were the cell type predominantly lost - WM astrocytes underwent broad transcriptional changes in the areas surrounding the lesion rim, such as upregulation of GFAP and CD44.

(Continued)

TABLE 2 | Continued

				-Microglia were dramatically increased in number in MS. - Myelinating oligodendrocytes at lesions had signatures of cell stress, iron accumulation and MHC class I presentation.
Wheeler et al. (59)	CNS samples from 4 MS and 5 controls (included datasets from other scRNA-seq studies: cortical and cerebellar astrocytes from 20 MS and 28 controls)	RIN: 6.3±.80	Tissue snRNA-seq	- An expanded astrocyte population in MS vs control characterized by decreased NRF2 activation and increased MAFG activation, DNA methylation, GM-CSF signalling and pro- inflammatory pathways activity.
Absinta et al. (60)	- 6 chronic active rim, 5 chronic inactive rim, 2 lesion core, 4 periplaque from 5 patients with progressive MS - 3 WM from 3 sex-matched controls	PMD: 6-12h	Tissue snRNA-seq	- High glial and immune cell diversity between lesion cores, active or inactive rim, and periplaque WM. Discovery of a lymphocyte-microglia-astrocyte axis with the key involvement of C1q in chronic active rim. - Two main microglia subsets identified: MIMS-foamy and MIMS-iron. Additionally, microglia signatures in MS overlap with neurodegenerative diseases suggesting similar mechanisms between primary and secondary degermation. - MIMS target genes were regulated by lymphocytes with the involvement of C1q, and C1q- blocking antibody gave a more homeostatic microglia phenotype.

Mitochondrial injury in initial WM lesions was indicated by increase of *ND1-6, CYTB, COX1, CYBA, MPO, PTGS1, PXDN, GPX4, PRDX1, SGK2, ALOX12, EPHX2* expression, which were related to degeneration of oligodendrocytes and neurons and contributed to reactive oxygen species production by activated microglia and macrophages (28) (**Figure 4**).

Active and chronic active lesions shared upregulation of a number of genes coding for e.g. iron-binding protein (*TF*), chemokine and its receptor important for T cell accumulation in CNS (*CXCR3, CXCL10*), the myelin-binding protein (*MBP*), the first subcomponent of the complement system (*C1QB*), oxidative protection (*GPX1, SOD1*) and cytokines (*IL-6, IL-17, INFg*) (20, 23). However, 70 uniquely differentially expressed genes were also found: e.g. coding for the receptor related to differentiation (*EPHB6*), the granulocyte-macrophage colony-stimulating factor (*GM-CSF*), and a MHC class I molecule (*HLA-A*) in active lesions or e.g. genes coding for the chaperone protein (*HSPA1A*), component of MHC class I (*B2M*) or complement factor 4B (*C4B*) in chronic active lesions.

Differences have also been found on an epigenetic level, as the microRNA profile was different between active and inactive lesions (39). In the active lesions, microRNA-34a, -155 and -326 were all upregulated and targeted the *CD47* in brain resident cells to release inhibitor control and promote phagocytosis (**Figure 4**). Moreover, upregulated miR-22, miR-320 in active lesion and upregulated miR-30d in inactive lesions (39) were related to pathogenic changes (41), while downregulation of miR-18a, miR23b in inactive lesions (39) were related to protective changes correlating with MRI abnormalities (41).

An in-depth investigation of different lesion types (active, early remyelinating, chronic active, inactive) in the WM showed extreme diverse events at transcriptome level. More differential expressed genes were unique than shared. Among the 282 altered genes common to all lesion types were genes related to inflammation (*STAT6, CXCL12, TNFs, DPP4/CD26, ITGA4, GPNMB, IL16, HLA-DRB5, MAFB, IGHG1, IGF2, MMP2*), phagocytosis (*SCD, CD163, MERTK*) complement pathway (*CFH, C7, CFI*), apoptosis/necroptosis (*FADS1, CASP1,-4, MLKL*) (48). Immunoglobulin genes were among the top 10 in all WM MS tissues, but the most heterogeneous expression pattern was detected in early remyelinating lesions. *TGFBR2* was the major molecular hub of the largest shared lesion network and was highly expressed in remyelinating lesions by astrocytes (48) (**Figure 4**). The most different signatures were found between remyelinating and chronic active lesions. Chronic active lesions had the highest number of unique genes reflecting intrinsic neuronal alterations, and *de novo* networks suggested an end-stage exhaustion (48). Most of the uniquely expressed genes in the early remyelinating lesions were non-coding RNAs, while others were related to lymphocytes and NKT cells (e.g. *CD8a, TIAM1, CTSW, CCL5/RANTES*), growth and development (e.g. *PEG10, BMP4, GDF10*), vascular changes and remodeling (e.g. *PLAU, VEGFA, CTGF*), mitochondria and protective stress responses (e.g. *NDUFA4, NOSTRIN*), lipid metabolism (e.g. *ACACA, ACOX2, ADH6, CA3*), and neurons (e.g. *NEUROD1, NLGN1, GRIA3*) (**Figure 5**). Another study found *CXCL12*, *SCD*, *STAT6* increased in all lesion types, and transcriptional differences between lesion types reflected a heterogeneous oligodendrogliogenesis (34). Quantitative changes of oligodendrocyte regulators were also found in remyelinating lesions (30). Compared to demyelinating lesions, remyelination was accompanied by significant changes in the expression of myelin proteins (*CNP, MAG, MBP, MOBP, MOG, OMG, PLP1)*, anti-inflammatory *IL10*, and semaphorins (*SEMA3C, SEMA4D, SEMA6A, SEMA6D, SEMA7A*) (**Figure 5**). The growth factor gene *FGF1* was significantly increased in remyelinating lesions compared to both control WM and demyelinating lesions. In functional experiments, FGF1 promoted both developmental myelination and remyelination by inducing LIF and CXCL8 in

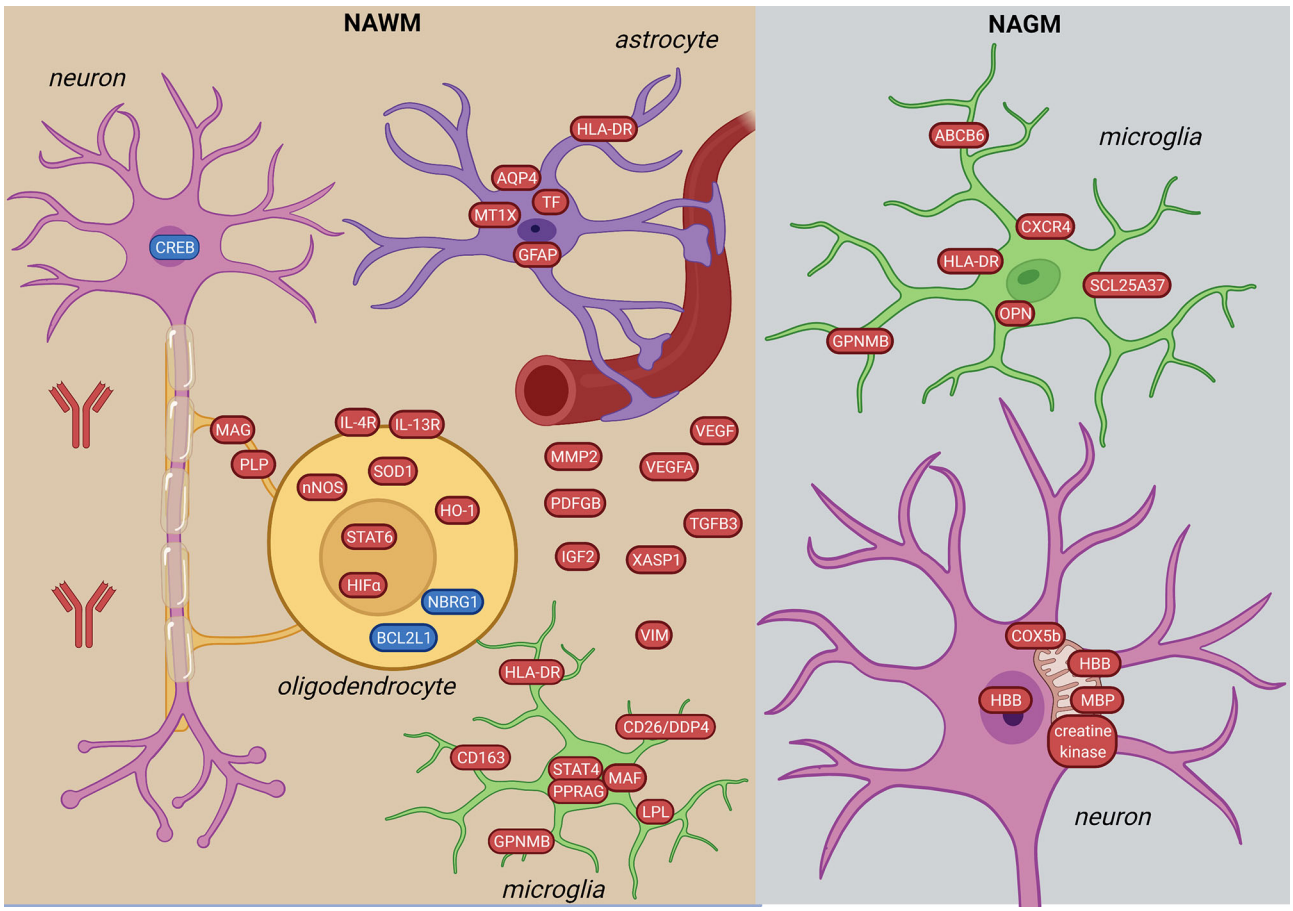

FIGURE 3 | Signature of NAWM and NAGM in the MS brain based on transcriptome and proteome studies. In the NAWM, alterations in all brain resident cells were observed. Oligodendrocytes are characterized by altered myelin transcripts and upregulate anti-inflammatory and hypoxia-induced pathways (STAT6-, HIFα-signaling). Microglia upregulate pro-inflammatory molecules (STAT4-signaling, HLA-DR, GPNMB, CD163). Inflammatory astrocytes have iron- and oxidative stress-related profiles. In the NAGM, microglia have a distinct inflammation-induced neurodegenerative profile from NAWM (CXCR4, ABCB6, SCL25A37). Neurons in the NAGM express hemoglobin β (HBB) and have alterations in mitochondrial proteins. The figure was created by compiling data from several articles, and therefore molecules may not be expressed at the same time. Created with BioRender.com.

astrocytes to recruit oligodendrocytes. *GFAP* was also significantly increased in active and remyelinating lesions (55) (**Figures 4**, **5**). The glia receptor protein tyrosine phosphatase gene *PTPRG* was increased in all MS WM tissues, and was also significantly increased in the CSF of MS patients compared to healthy and other neurological disease controls (55). *CHI3L1* was increased in astrocytes in the chronic active lesion rim (55), and by microglia in active lesions compared to NAWM (50).

In a single-nucleus study of WM lesions, the majority of cells were oligodendrocytes, and oligodendrocytes represented the most heterogenous cell population (56). One of the seven oligodendrocyte populations was termed immune oligodendroglia (imOLG) due to expression of *APOE* and *CD74*. OPCs were reduced in lesions and NAWM compared to control WM. One oligodendrocyte population was depleted, whereas three others and imOLG were enriched in MS. Several myelin protein genes were upregulated in mature oligodendrocytes in MS, however some of those (e.g. *CNP, MAG*) were downregulated in remyelinating lesions.

Excessive expression of the antioxidant transcription factor *NRF2* in oligodendrocytes indicated oxidative stress and degeneration at sites of initial demyelination in active lesions (31). *NRF2* in astrocytes and macrophages were mainly seen in the later stages of active lesions with profound loss of oligodendrocytes. *NRF2* in neurons was low or absent despite NRF2-positve oligodendrocytes in close proximity indicating cellular differences in reaction to oxidative stress and inflammation (**Figure 4**).

In chronic active lesions,14 genes were significantly upregulated in the rim *vs* the center (e.g. *IFNG, NGF2, CD4, CASP9, MAPKK1*) (22, 25, 29) (**Figure 6**). Inflammatory genes were upregulated in chronic active lesion center (*CCL4, IL6, CD27, TNFA*) (**Figure 6**), while upregulation of *NCAM, CSF1, HSP60, HSP90A, BCL2L1* in inactive lesion center and rim highlighted different inflammatory responses, beside apoptosis and stress (**Figure 5**). Heat shock protein genes in inactive lesions (48) and in the rim of chronic active lesions were upregulated, especially the heat shock factor 4 (HSF4) (29).

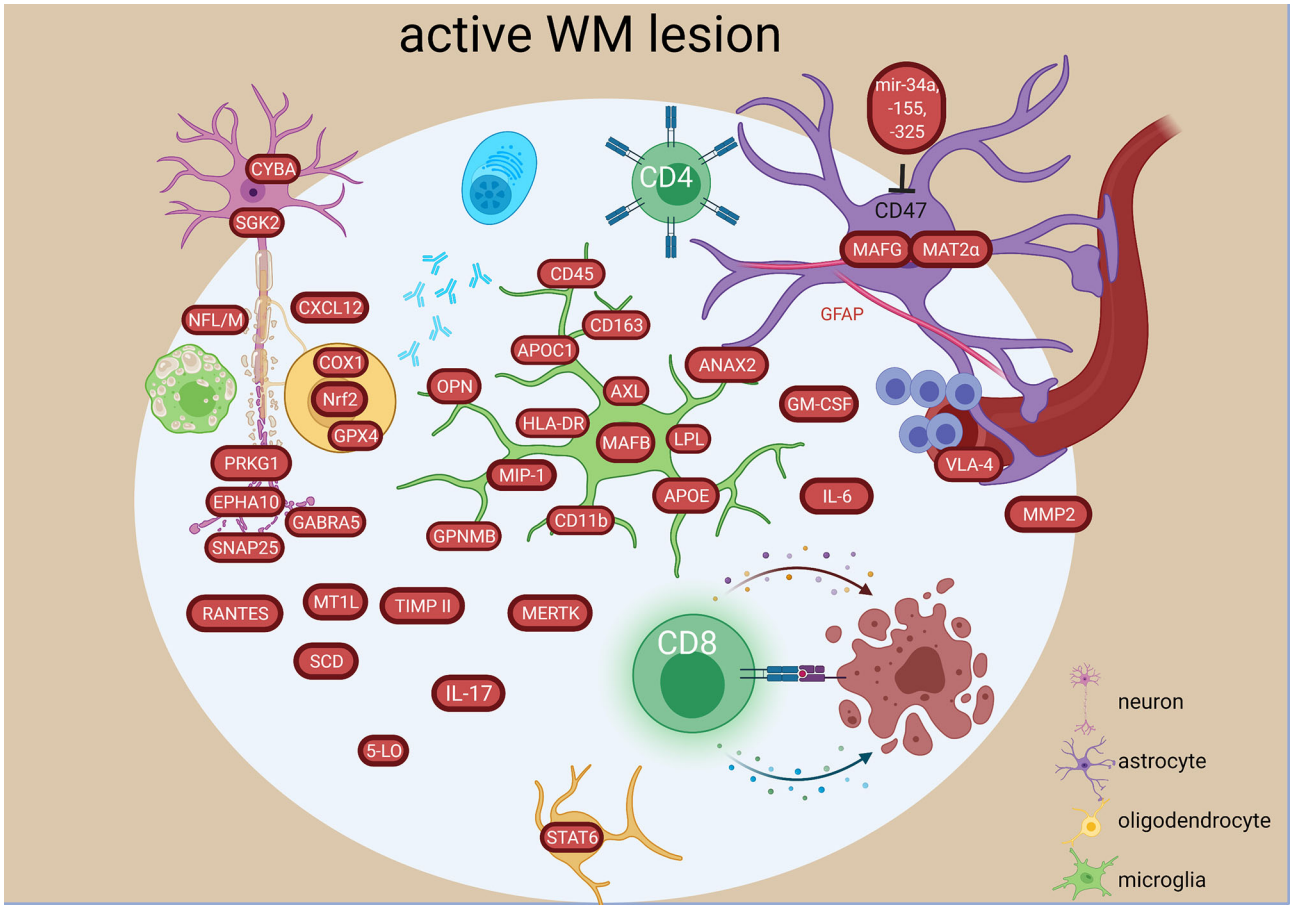

FIGURE 4 | Signature of active WM lesion in the MS brain based on transcriptome and proteome studies. In the active lesion, an increase in both innate and adaptive inflammatory responses are present characterized by different molecular components in resident and infiltrating cells. An oxidative stress and degenerative profile especially in the oligodendrocytes and neurons have also been detected. The figure was created by compiling data from several articles, and therefore molecules may not be expressed at the same time. Created with BioRender.com.

Upregulation of 165 genes and downregulation of 35 genes were identified in the chronic active lesion/slowly expanding lesions compared to inactive as well as NAWM (38). The upregulated genes suggested accumulation of microglia with proinflammatory differentiation at the lesion edge (e.g. *CD163, CD68, CSF3R, IGFBP5, ALOX15B, MME, TNFRSF25*) (**Figure 6**). A study that investigated the rim and peri-lesional regions of both chronic active and inactive lesions, found upregulation of previously not reported genes in the rim of chronic active lesions (*NPY, KANK4, NCAN, TKTL1, ANO4*) (33) (**Figure 6**). They also found that foamy macrophages in the rim upregulated genes involved in lipid binding and uptake indicating the expansion of demyelination (e.g. *MSR1, CD68, CXCL16, OLR1, CHIT1, GPNMB* all (**Figure 6**). Stressed oligodendrocytes with iron overload, reactive astrocytes and activated phagocytosing cells were also detected in the rim of chronic active lesions (58). These findings were confirmed and elaborated in a recent snRNA-study, where they found immunological-active OPCs, inflamed astrocytes (AIMS) and microglia (MIMS) in the chronic active rim (60). These were strongly connected to a high number of T cells and plasma cells suggesting an active role of the adaptive immune system in lesion expansion in collaboration with the glia cells in the smoldering inflammatory lesions (60). Microglia consisted of two distinct functional subtypes: the MIMS-foamy characterized by myelin phagocytosis and clearance properties, and the MIMS-iron, characterized by expression of complement C1q-complex, antigen-presentation and direct propagation of inflammatory damage at the lesion edge. The inflamed astrocytes were enriched for response to lipid, corticosteroids, wounding and expression of C3 similar to the A1 phenotype identified in the GM (62).

3.1.5 GM Lesions

A combined microRNA and mRNA profiling in GM lesions *vs* NAGM found significantly regulated microRNAs in GM lesions, which target genes of axonal guidance, TGFβ-signaling and FOXO signaling (63). Out of 27 significantly altered microRNAs, four microRNAs (mir149, mir20a, mir29c, mir25) and their targets (e.g. *HIF1A, VEGFA, TGFBR1, TGFBR2, NFKBIB, FGFR1, TNFSF10,*

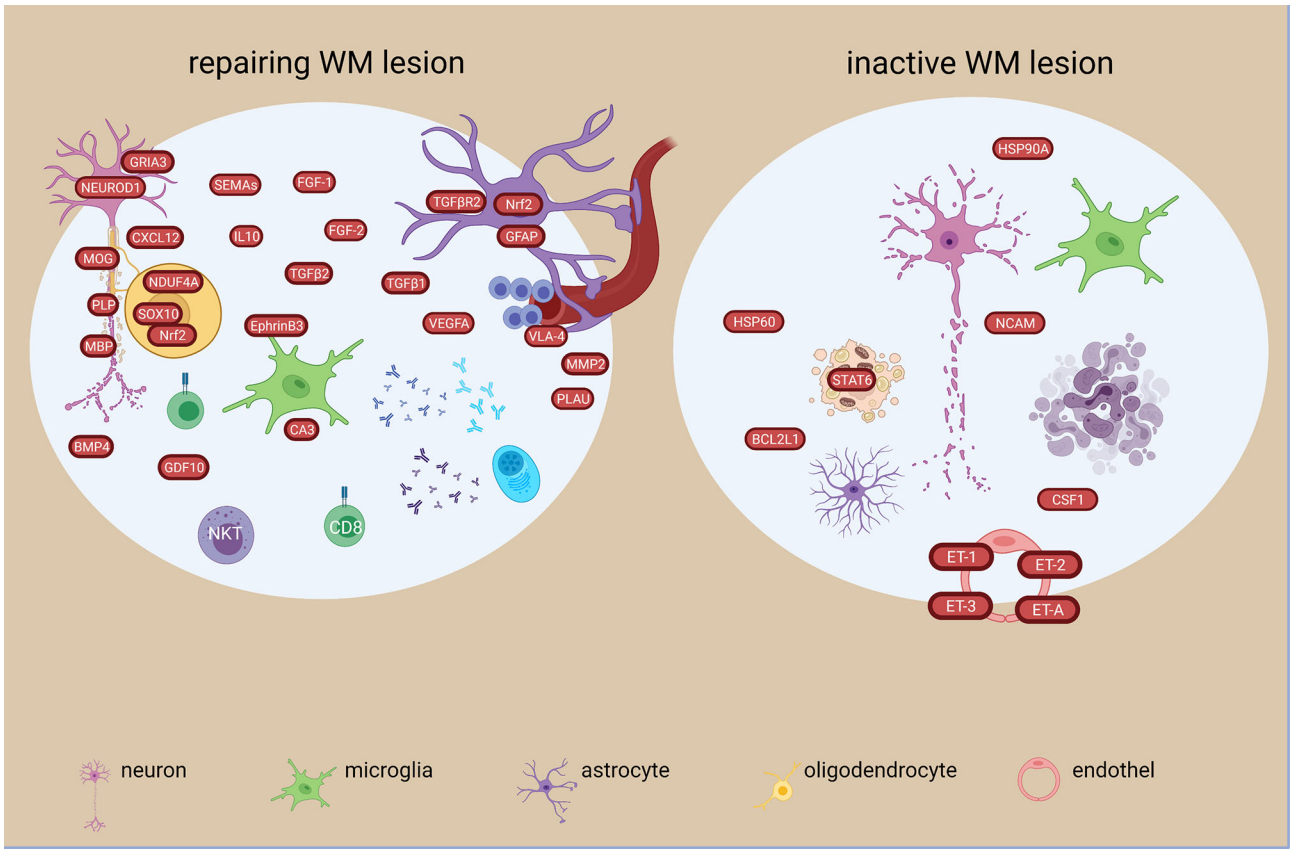

FIGURE 5 | Signatures of repairing/remyelinating and inactive WM lesion types in the MS brain based on transcriptome and proteome studies. Remyelinating signatures are characterized among others by soluble growth factors and reparatory molecules such as FGF-1, -2, TGFB1,-2, BMP4 and GDF10. Oxidative and anti-oxidative responses are present, as well as a heterogenous immune response. In the inactive lesion, different heat shock proteins are present together with changes in endothelin transcripts. The figure was created by compiling data from several articles, and therefore molecules may not be expressed at the same time. Created with BioRender.com.

BCL2, MAP2K4, STAT3, MMP2, PTEN, CD44) were associated with GM atrophy (**Figure 7**) (63). Three of the 27 significantly altered microRNAs were also detected in another GM lesional microarray study (mir181b, mir129-5p, mir1180) (64). Additionally, miR-330-3p, miR-4286, miR-4488, let-7e-5p and miR-432-5p shared the same mRNA target, the Syt7 gene coding for the neuroaxonal protein normally transported to synapses. These 5 microRNAs may be protective against Syt7 accumulation in the soma resulting in disturbed axonal transport.

TNF signaling was also significantly increased in GM lesions. Increased meningeal inflammation was associated with a shift from TNFR1/TNFR2 and NFkB-mediated anti-apoptotic pathways towards TNFR1- and RIPK3-mediated pro-apoptotic/pro-necroptotic signaling (36) (**Figure 7**). TNFR1 was expressed by neurons and oligodendrocytes, while TNFR2 was predominantly expressed by astrocytes and microglia. The authors suggest that immune cells in meninges generate a milieu of increased demyelination and neurodegeneration by changing the balance of TNF signaling.

Another study found a selective loss of neurons expressing the transcription factor *CUX2* in upper cortical layer lesions associated with pronounced meningeal B cell infiltration (58). These neurons expressed markers of cellular stress (*PPIA, NORAD, PUMILIO, RBMX*), and their loss may be a key event in MS progression and cortical atrophy (**Figure 7**).

3.1.6 Cell-Specific Changes

A study focused on endothelial cells in vessels found 52 genes significantly altered in chronic active or inactive lesions compared to control WM or NAWM (27). The majority of these genes belonged to endothelial cell activation, while *VEGFA* was the only one belonging to angiogenesis. Most of the genes were highly expressed in chronic active lesions compared to control WM (*ANXA5, CSF3, FGF1,-2, FLT1,-4, ICAM1, MMP1, -2*) (**Figure 6**) and compared to NAWM (*FGF2, FLT1,-3, MMP14, PLAU, RIPK1*). Several endothelin genes (*1,2,3,A*) involved in constriction of blood vessels and supply were increased in inactive lesions compared to NAWM (**Figure 5**).

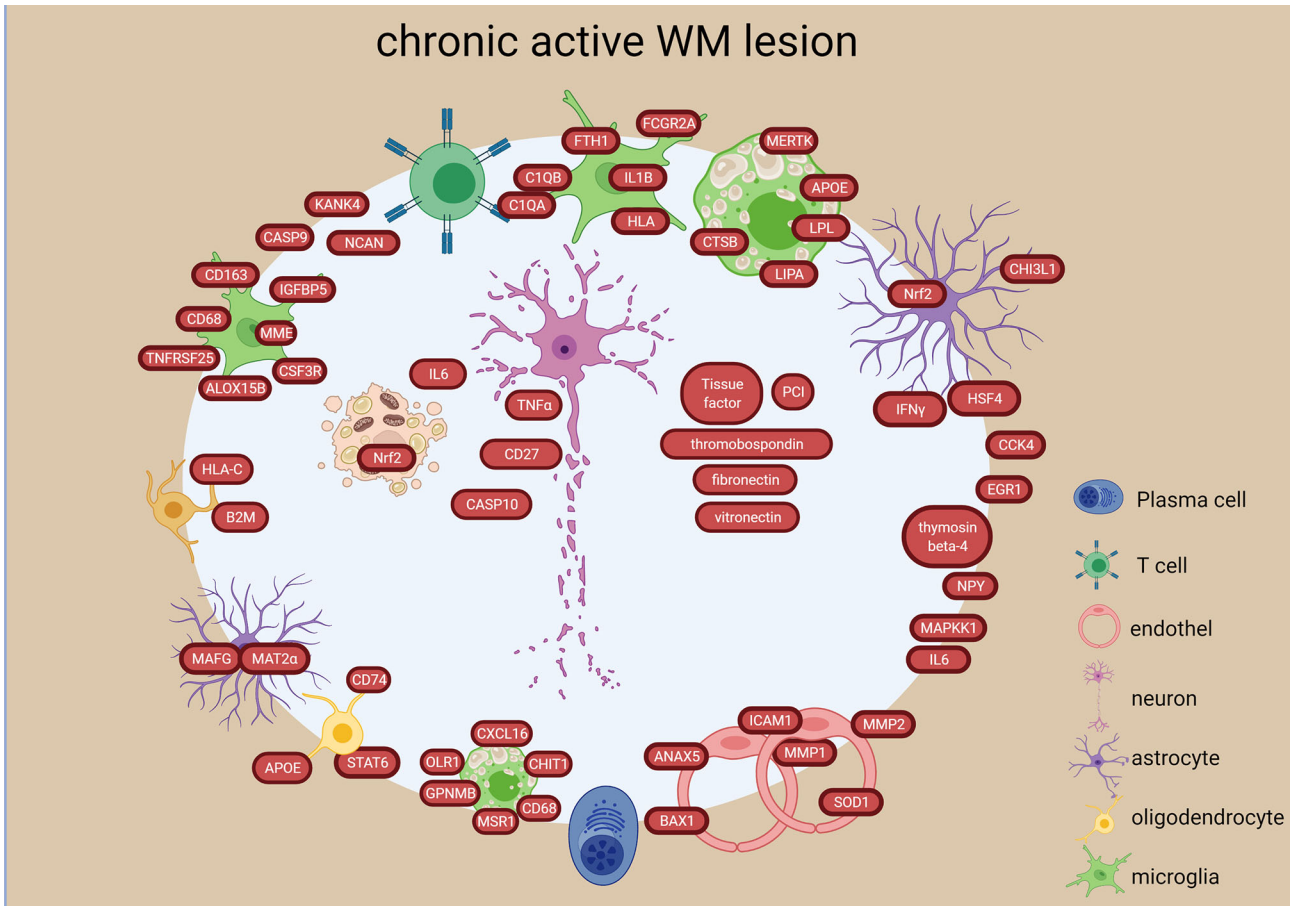

FIGURE 6 | Signatures of chronic active lesion in the WM lesion types in the MS brain based on transcriptome and proteome studies. Chronic active lesion has a different molecular profile in rim *vs* center. Most activity is present in the rim with stressed astrocytes and oligodendroglia, proinflammatory microglial polarization and foamy macrophages. Additionally, presence of coagulation factors and endothelial alterations are detected. The chronic active lesion displayed the highest number of neuronal/axonal intracellular components. The figure was created by compiling data from several articles, and therefore molecules may not be expressed at the same time. Created with BioRender.com.

Transcriptional profiling of isolated astrocytes in NAWM also revealed increased gene expression related to iron metabolism, oxidative stress, and inflammatory response (32) (**Figure 3**). An astrocyte single-cell study identified an expanded astrocyte population in active lesions characterized by decreased *NRF2* and increased *MAFG*, GM-CSF signaling, pro-inflammatory pathway activity and DNA methylation (*DNMT1*) (59) (**Figure 4**). This astrocyte population is characterized by a MAFG/MAT2α-driven pro-inflammatory genomic program contributing to the pathology and may be induced by GM-CSF produced by infiltrating T cells (**Figure 4**). This corresponds to the high *GM-CSF* in active lesions (23), and low *NRF2* in astrocytes in initial demyelinating lesions (31).

Seven microglia cell populations expressing the core microglial genes (*TMEM119, P2RY12*) in the WM were discovered in a single-cell study (57). Two of these clusters were enriched in brains of MS patients and one was associated with MS. These three populations had increased levels of *APOE* and *MAFB* (**Figures 4**, **8**). The MS-associated microglia subset highly expressed *CTSD, APOC1, GPNMB, ANAX2, LGALS1*, while the two MS-enriched clusters showed high expression of either *CD74, HLA-DRA, HLA-DRB1* or *OPN/SPP1, PADI2, LPL* (**Figure 8**). These findings suggest distinct disease-related subtypes of microglia in the MS brain, which were similar to microglia subtypes in a demyelination model. However, subsets of microglia varied substantially between individual patients indicating high inter-individual heterogeneity. Additionally, the different microglia populations appeared as a transcriptional continuum of the local populations, which could reflect the ability of microglia to easily adapt to changes in the surroundings.

Methylome changes within neuronal nuclei in WM suggested alterations in axonal guidance, synaptic plasticity and CREB signaling in MS (42). The CREB activity was reduced in NAWM compared to WM neurons suggesting alteration of CREB signaling prior focal tissue damage (**Figure 3**). Neurons from MS patients displayed epigenetic alterations affecting several

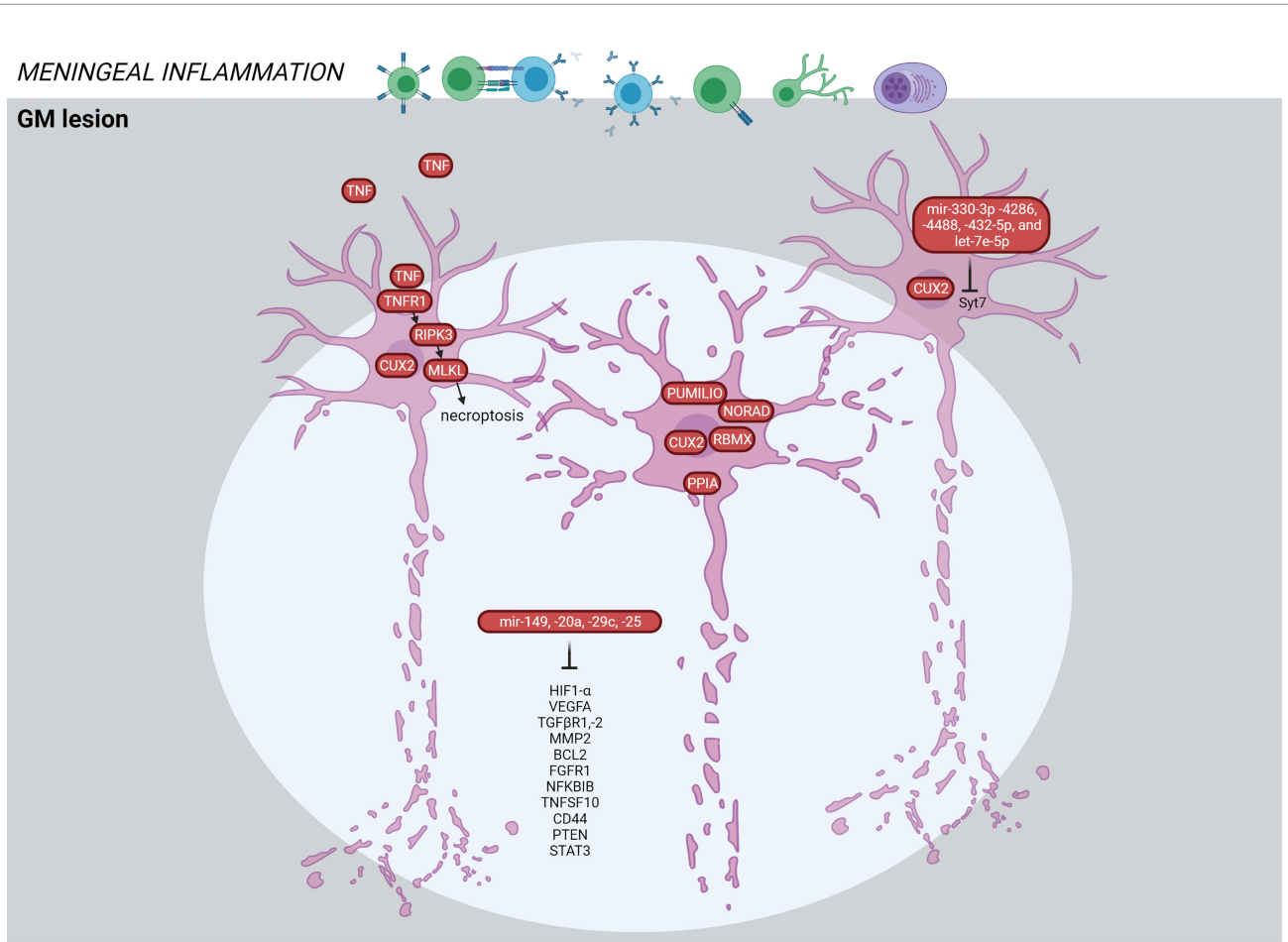

FIGURE 7 | Signature of the GM lesion in the MS brain based on transcriptome and proteome studies. GM lesions are characterized by neuronal death mediated through TNF signaling. The CUX2-expressing cells are particularly vulnerable for degeneration. Alterations in microRNAs have been detected in the GM lesions associated with cortical atrophy. The figure was created by compiling data from several articles, and therefore molecules may not be expressed at the same time. Created with BioRender.com.

genes of the glutamate/GABA signaling along with interconnected cellular networks (semaphoring/plexin, Slit/ROBO, Shh/Wnt signaling). Lesion-associated changes in genes implicated neuronal projections and synaptic processes (e.g. *GABRA5, PRKG1, DLGAP3/SAPAP3*) (42) (**Figure 4**).

3.1.7 Non-Human Transcripts

Amplicon next-generation sequencing of the human endogenous retrovirus (HERV)-W group found very similar transcript level between of WM lesions and control WM but evidence for interindividual differences in HERV-W transcript levels (45). In another study, genome-wide HERVs expression level between MS WM and control WM was not different (65). However, transcripts of HERV-W were reduced in chronic active and repairing lesions. Additionally, four different transcripts of HERV-W on chromosome 7 were only present in the MS brain (65). Another study found HERVs significantly overexpressed in demyelinating brain tissue including several retroviral domains (core, envelope, integrase, reverse transcriptase) (47). However, the overexpression was small. Due to multiple similar HERV transcripts incorporated and spread out throughout the human genome, examination of them is difficult.

Presence of microbial RNA sequences and bacterial antigens were associated with demyelinating brain lesions (66). In the study, they found 29 MS microbial candidate genera from 11 different phyla, most of which were anaerobic.

3.1.8 Databases

Based on these transcriptomics data, novel interactive online databases were generated. The MS Atlas (www.msatlas.dk), comprises comprehensive high-quality transcriptomic profiles of 98 different WM lesion types (53). The user-friendly MS Atlas was designed to provide information about significant expression of candidate genes and their participation in *de novo* protein-protein interaction networks in different MS lesions (53, 67). The OligoInternode database (https://ki.se/en/mbb/oligointernode), and the single cell expression atlas (https://www.ebi.ac.uk/gxa/sc/experiments/E-HCAD-35/results/tsne) give information about gene expression from single cells in MS lesions.

3.2 Systems Proteomics to Examine Pathological Mechanisms in the MS Brain Tissue

Proteomics has also been developed as a large-scale unbiased tool for identifying final products of cells and post-translational modifications such as phosphorylation, glycosylation and acetylation associated with MS (68, 69). Despite various proteome studies in brains of animal models of MS, only a few proteome studies of MS CNS tissue have been performed (**Table 3**).

3.2.1 WM Immune Activity

A proteome study found that 109 proteins could separate WM lesions from adjacent NAWM and control WM (70). Overlap was only observed between NAWM and WM lesions, but not between NAWM and control WM.

To characterize the MHC-bound peptide repertoire in MS brains, proteomics was performed on captured HLA-A, B, C, and DRs. 118 amino acid sequences from MHC I and 191 from MHC II were eluted corresponding to 174 identified proteins including both known and novel autoantigens (72). Some were involved in apoptosis (annexin A1, BCL2-associated TF1), enzymes (GDH, GS, G3PD, NADH dehydrogenase), cytoskeleton (actin, α-ubulin), immune responses (CXCR1, IL12R), CNS structure (NFL, GFAP, MBP, α-synuclein), and serum proteins/iron-related/coagulation (APOD, APOE, ferritin, transferrin, von Willebrand factor). These proteins within the MHC ligandome mirror the proteins involved in different features of the MS pathology.

Combined proteomics and genomics on two acute MS autopsied brain samples detected seven unique mutations of PLP1 (68). This was confirmed with in-depth genomic analysis on mRNA, but not in the genomic DNA, highlighting how results from integrative approaches can strengthen the discovery of specific and precise pathogenic mechanisms in MS.

Myeloid cells from active lesions, NAWM and WM in progressive MS (PMS) were analyzed using single-cell mass cytometry and found lower abundance of microglial homeostatic proteins in active lesions (P2Y12, TMEM119, CXC3R1, GPR56) (79). The myeloid cells in the active lesions were highly phagocytotic and activated indicated by upregulation of CD45, HLADR, CD44, CD114, CD11c, CD68, MS4A4A, CCR2, CD64, CD32, AXL, NFAT1, CD95, Clec7a, CD47, MIP-1β (CCL4) OPN (SPP1) (**Figure 4**). However, infiltrating myeloid cells were scarce in active lesions in PMS. Additionally, the TNF^{hi} microglia population was reduced in active lesions compared to NAWM.

3.2.2 Two Proteins Important in Remyelination?

Unsupervised clustering of proteomics data led to discovery of cortical lesions, which were not detectable by routine histology (77). They identified tymosin beta-4 mainly expressed in macrophages and activated microglia at the rim of chronic active WM lesions and in the GM (**Figure 6**). Tymosin beta-4 is involved in neurite extension and plays a role in restoring and remodeling neurons and in remyelination.

Another study found upregulation of the receptor tyrosine kinase Ephrin3 in the MS lesions. Tissue extracts from MS lesions inhibited OPC, while antibody-mediated masking of EphrinB3 epitopes promoted it (76) (**Figure 5**). These proteomics studies suggest that EphrinB3 and tymosin beta-4 may be potential targets to promote remyelination.

3.2.3 Coagulation and Hemoglobin β

Proteomics of microdissected active, chronic active and inactive lesions showed that chronic active lesions displayed the highest number of uniquely dysregulated proteins, and proteins of unknown function made up more than half of the unique proteins (71). This was supported by an independent study in 2011 (73). Five proteins involved in coagulation were unique to chronic active lesions (tissue factor, PCI, thromobospondin, fibronectin, vitronectin) (71) (**Figure 6**). Coagulation factors in the CNS interfere with synaptic homeostasis and neuronal networks, and act pleiotropic on different receptors of both resident and circulating cells as well as the extracellular matrix (80).

Another study found dysregulated proteins associated with extracellular matrix, oxidative stress and myelin sheath (73). There was decreased abundance of MAG (oligodendrocytes) and contactin-1 (neurons), while increase in GFAP (astrocytes) in the chronic active lesions in a milieu with abundant anti-oxidant PRX6 and metabolic processes (alfa-enolase).

Proteome studies with co-immunoprecipitation have discovered that hemoglobin β may play a role in neuronal energetics by interacting with histones in the nucleus and by binding to proteins in mitochondria (74, 75) (**Figure 7**).

3.2.4 Post-Translational Protein Modifications – A Missing Link

Studies on post-translational modifications will be the next layer of valuable information. Recently, a comprehensive analysis of citrullinated peptides in WM and GM of MS patients identified novel citrullinated sites of MBP, GFAP and vimentin, but their functional role remains unknown (78).

4 DISCUSSION

Omics studies of MS brain tissue in the last four decades support MS as a global brain disease with inflammation, iron-disturbances, cellular-stress and hypoxia. However, some regions are more affected than others and the biggest transcriptional changes were detected in the corpus callosum and the optic chiasm (51). While microglia seem to lose the regional specificity in MS, there are similarities between MS microglia phenotypes and the microglia phenotypes during de- and remyelination in the cuprizone model, which also affects mainly the corpus callosum (57, 81). The most affected cell type seems to be oligodendrocyte (30, 34, 51, 56). This may not be surprising as the disease is characterized by demyelination. However, there is a bias towards a higher number of studies investigating the WM than GM. Considering the altered genes, the cell type may be more important than the tissue location, although the local environment, architecture and milieu may continuously drive the cell types into different phenotypic and functional subsets to adapt to the local surroundings.

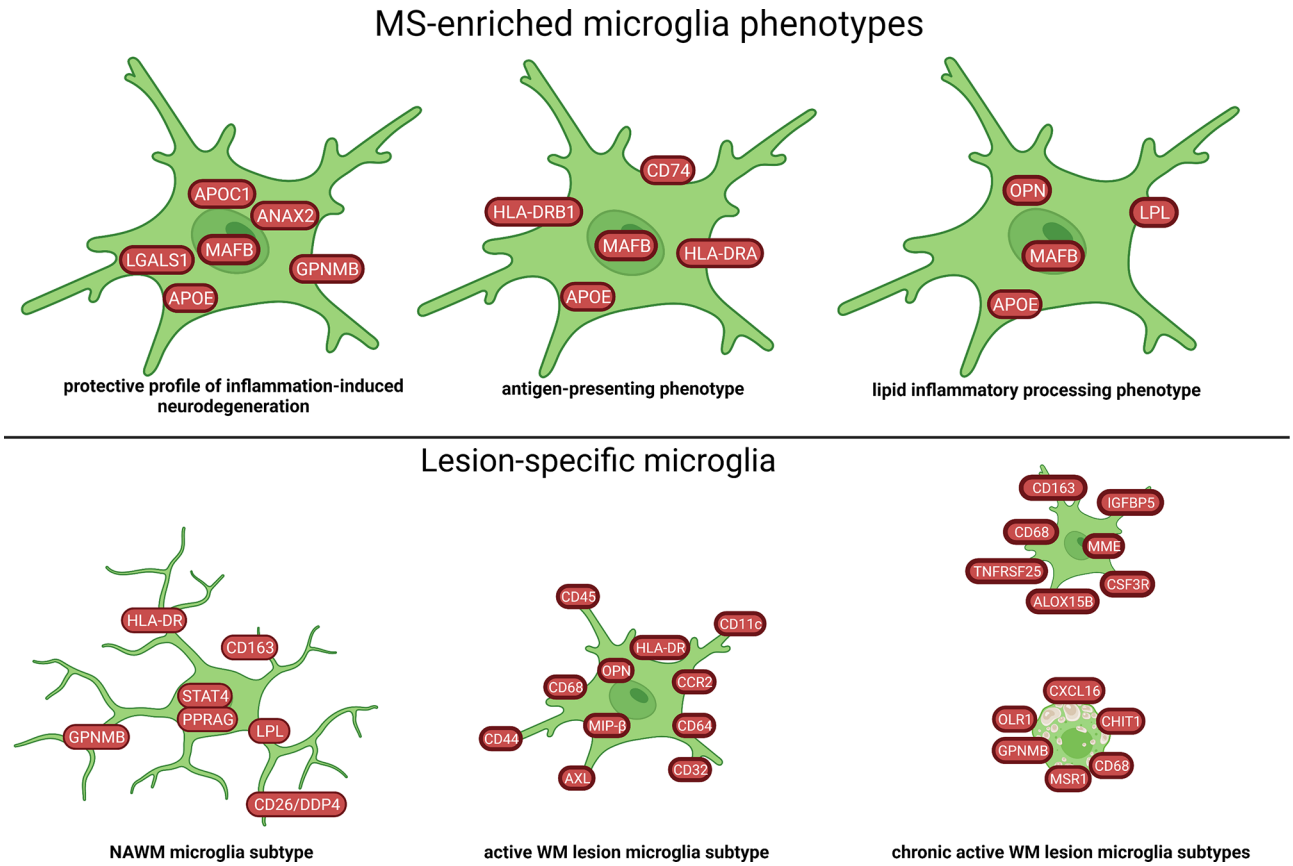

FIGURE 8 | Signature of microglia subtypes in the WM of MS brain based on transcriptome and proteome studies. Different profiles of microglia in WM tissue of MS have been identified. The NAWM microglia subtype in the figure was created by compiling data from several articles, and therefore molecules may not be expressed at the same time. Created with BioRender.com.

Molecular components of TGFβ signaling and CREB signaling are altered in addition to multiple changes in semaphorin-, heat shock-, myelin-, APO- and especially multiple types of HLA-transcripts/proteins. Key differentially expressed molecules found multiple times independent of lesion stage are related to inflammatory responses (CD163, OPN, GPNMB, MIP-α/β), lipid metabolism (SCD, LPL, SOD1) cell trafficking (MMP2, CXCL12, VEGFA), but there has been bias in the selection of the examined tissue/cell types.

4.1 Oligodendrocytes

Even in the NAWM, oligodendrocytes have a different molecular profile similar to a survival mode against virtual hypoxia. They upregulate the hypoxia induced HIFα-signaling pathway and the STAT6-signaling pathway, which is associated with anti-inflammatory IL-4 and IL-13 receptor expression (21, 26, 46). The STAT6-signaling seems to be even more increased in oligodendrocytes in all lesion types (34, 48). However, there is a heterogeneity of oligodendrocyte subtypes between different lesion types, where even an immunological phenotype appears (56, 58). This immunophenotypic OPC was also seen at the rim of chronic active lesions (60).

Myelin proteins are altered in all studies including even the GM mitochondrial proteome (74). Nevertheless, different studies showed contradicting results: myelin transcripts and proteins can be reduced in remyelinating lesions (34, 48), while others found them upregulated (30, 76). This could be due to the different stages of remyelinating and remodeling processes captured by omics studies as static snapshots. Understanding the molecular mechanisms in remyelinating lesion using omics may be complicated, as non-coding RNAs dominate and no known predefined pathways have been found (34, 48), but for OPC differentiation FGF1-signaling through astrocytes, EphrinB3 and thymosin beta-4 may be important (30, 76, 82). Mapping the genetic programs of OPC and oligodendrocyte development/polarization in MS may help to unlock and even direct the remyelination process.

4.2 Microglia

Microglia play a role during all stages of lesion evolution in both the GM and WM. Even far from lesions, there are highly activated distinct microglia subtypes (26, 48, 50). This suggests an early activation of their local function, most probably cleanup, which may have been catalyzed by low level of chemokines and

TABLE 3 | An overview of the studies (n=11) that examined the proteome profile in human MS brain tissue.

Authors	Patients	Quality (PMD)	Methodology	Key findings
Newcombe et al. (70)	- 3 WM lesions and adjacent NAWM from 3 blocks of 1 MS patient - 3 blocks of control CM from 2 controls	PMD: 8-15h	LC-MS/MS (MALDI-ToF) with reduction of abundant cytoskeletal proteins	- Cluster analysis based on 109 proteins showed three clusters: WM, NAWM and lesion. - WM samples or lesion samples could cluster with NAWM, but MS lesion and WM samples did not cluster together
Han et al. (71)	- 2 Active, 2 chronic active and 2 chronic lesions of fresh-frozen from 6 MS patients - Normal WM from 2 controls	PMD: 4-24h	LCM, LC-MS/MS (ESI)	- Number of unique proteins in the major lesion types: 158 for active, 416 for chronic active and 236 for chronic lesions. - Revealed 5 proteins involved in coagulation unique for chronic active lesions: tissue factor, PCI, thrombospondin, fibronectin and vitronectin.
Fissolo et al. (72)	- 8 samples from 8 MS patients	PMD: 8-38h	LC-MS/MS (ESI) with antibodies against HLAs	- Identified processed peptides presented on MHC I and II molecules from MS brains as self-antigens of diverse MBP peptides as well as GFAP, NFL, APOD, APOE, ferritin, transferrin - By characterizing the MHC ligandome of MS brain tissue, they identified 118 amino acid sequences from self-proteins from MHC I and 191 from MHC II molecules.
Ly et al. (73)	- 12 chronic active lesions, 8 chronic periplaque WM (PPWM), 12 late reyelinating lesions (LRM), 11 LRM PPWM from 3 MS patients (areas within same category were pooled within patient samples) - 6 WM areas from 4 controls	PMD: 8-58h	LCM, LC-MS/MS (ESI) with iTRAQ labelling	- Myelin-associated glycoprotein was significantly downregulated in chronic demyelinated lesions compared to late remyelinated lesions, NAWM and WM. - The number of protein identifications obtained from chronic lesions was significantly higher than in all other lesional/NAWM areas. - Contactin was downregulated in the NAWM surrounding chronic lesions compared to WM. - GFAP was upregulated in chronic lesions compared to NAWM and DWM. - HAPLN2 was downregulated in late remyelinated lesions and NAWM vs WM. - Upregulation of PRX-6 in chronic lesions vs chronic NAWM.
Broadwater et al. (74)	- parietal, Brodmann areas 1-3, frontal cortex and Brodmann area from 8 MS brains and 8 control brains	PMD: 3-30h	LC-MS/MS (SELDI-ToF)	- 4 proteins differentially expressed: COX5b, brain specific creatine kinase, hemoglobin-b-chain and MBP.
Brown et al. (75)	- 5 postmortem cortical MS tissue - 5 cortical areas from control brains	PMD: 3-23h	LC-MS/MS (ESI)	- 15 proteins including hemoglobin β subunit (Hbb) were identified. - Hbb was enriched in pyramidal neurons in internal layers of the cortex, and interacted with subunits of ATP synthase, histones, and a histone lysine demethylase.
Syed et al. (76)	- 3 chronic active, 3 active lesions, 2 peri-lesional WM and 1 NAWM from MS	PMD: 7-22h	LCM, LC-MS/MS (ESI)	- Ephrin3, an oligodendrocyte differentiation inhibitor, was expressed in demyelinated WM lesions.
Maccarrone et al. (77)	Discovery cohort: - NAWM, NAGM, and lesions with different extent of remyelination from 1 SPMS Validation cohort: - 12 PMS blocks	PMD: 8-24h	MALDI-IMS LC-MS/MS (ESI)	- Lesions with low remyelination had compounds of molecular weights smaller than 5300 Da, whereas completely remyelination had molecular weights of more than 15200 Da. - Tymosin beta-4 was highly expressed in demyelinated lesion rim.
Qendro et al. (68)	- brain lesions of 2 acute MS patients	PMD: 4-24h	LC-MS/MS (ESI) Peptide microarray Exom sequencing	- Mutated forms of proteolipid protein 1 (PLP1).
Faigle et al. (78)	- GM samples from 6 controls and 6 MS cases, WM samples from 3 controls and 9 MS cases.	PMD: 5-22h	LC-MS/MS (ESI)	- Identification of novel citrullinated peptides and already described citrullinated proteins: MBP, GFAP, and vimentin. - Modified proteins in MS WM was higher than control tissue and increased citrullination in WM compared to GM.
Böttcher et al. (79)	10 WM lesions and 10 NAWM from PMS 8 WM from controls	PMD: 4:21-10:20h	Single-cell mass cytometry with CyTOF of isolated microglia	- decreased abundance of homeostatic microglial markers, while increased expression of APC-, phagocytosis-, inflammatory- and apoptosis-related markers in active lesions. - TNFhi microglial cluster was higher in NAWM compared to active lesion - monocyte-derived macrophages were scarce in active lesions

cytokines detected throughout the brain. In active lesions, the microglia profile is highly activated, and seems to be the dominated by signal transduction (*CD45)*, immunomodulation (*OPN, CD11*), antigen-presentation (*HLADR*) and phagocytotic properties *(AXL, CD68, CD163*) (79). The MS microglia expressing *APOE* and *MAFB* were divided into three subgroups: a protective profile of inflammation-induced neurodegeneration, an antigen-presenting phenotype and an inflammatory lipid-processing phenotype (57). However, there was a decrease in the TNF^{high} microglia subgroup in active lesion compared to NAWM (79). In the rim of the chronic active lesions, microglia may have a damaging *vs* repairing functional phenotype, and by mapping the interactome, microglia strongly interacted with immune cells with involvement of the C1q providing evidence for a lymphocyte-glia axis of lesion progression (60).

4.3 Astrocytes

Being the most abundant cells in the CNS, astrocytes also have altered phenotypes in MS with spatial molecular differences (58). Astrocytes have multiple key functions depending on the surrounding cells and tissue architecture (83). In the NAWM, astrocytes express transcripts associated with iron homeostasis, oxidative stress and immune-related genes (32). *GFAP* is also increased in remyelinating WM lesions (30, 55). In the GM, astrocytes upregulate the *NRF2* and its anti-oxidant target molecules, implying a reparatory and neuroprotective effect (31). However, a pathogenic pro-inflammatory subtype of astrocytes has also been detected and is characterized by reduced expression of *NRF2* and increased expression of *MAFG/MAT2α*. In the chronic active rim, reactive and inflamed astrocytes (AIMS) were detected expressing C3 and an A1-proinflammatory profile and in close interaction with the inflammatory microglia (60). This suggests that astrocytes can polarize to very distinct activation states, which are either damaging or beneficial in the MS pathogenesis. A detailed description of processes towards astrocytic polarization and functional changes are needed, as they can promote brain repair.

4.4 Neurons

Neuronal pathology and axonal injury are hallmarks of MS and major contributors to progression and permanent disability. Neurons in the NAWM have altered expression of genes involved in axonal and synaptic guidance as well as the CREB-mediated neuroprotective signaling pathway (42). NFL and α-synuclein as autoantigens also suggest direct immune attack against neurons (72).

In the GM tissue, TNF signaling seem to play a crucial role, where released TNF binds to TNFR1 on neurons and oligodendrocytes and activates pro-apoptotic/pro-necroptotic pathways leading to brain atrophy (36, 84). *CUX2*-expressing neurons in the upper cortical layers are most vulnerable for cell stress and death (58). Hemoglobin β in the MS neurons works as an epigenetic regulator and interacts with mitochondrial proteins, both ultimately controlling the energy metabolism (75).

4.5 The Mystery of the Chronic Active Rim

The number of chronic active lesions is increased in the progressive phase and is associated with aggressive disease course and poor clinical prognosis (85). However, it is unclear if the active rim purely expands the lesion, or it represents a cellular/molecular wall to halt progression, or even a battle in between. Moreover, data suggest that even though chronic active lesions are histologically similar, there may be differences on a genomic programming level. As snapshots, omics studies cannot answer if such differences represent distinct molecular mechanisms leading to lesion evolution or rather halting those. Based on multiple transcriptome and proteome studies, chronic active lesion is the most unique WM lesion type: it has the highest number of differentially regulated genes and proteins that may represent end-stage exhaustion, and it differs the most from control WM on molecular levels (48, 71, 73). Some of the unique proteins in chronic active lesions are involved in anti-oxidation and coagulation (71, 73), while many of the transcripts are neuronal/axonal (48). The uniqueness of chronic active lesions has also been identified by distinct and diverse cell populations connected through a lymphocyte-microglia-astrocyte axis that may be responsible for the smoldering inflammation (60).

4.6 Unbalanced Rate of Discovery Research *vs* Functional Research

Omics studies of tissue alone are very unlikely to lead to new treatments. However, the rate for finding differentially expressed transcripts/proteins and molecular networks is much faster than establishing their functional roles in a specific cell and in a given context. Thus, interpretation can end up with crude functional annotations, and therefore may even confuse results. Interpretation of omics in MS is often annotated to immune cells or immunological properties, even though molecules may have different functions in the brain depending on cell type. Therefore, functional experiments can enhance the interpretation of omics findings in the context of CNS.

4.7 Limitations, Considerations, and Recommendations of Multi-Omics

At least four main problems need to be solved: (i) sample size and quality, (ii) the "snapshot" characteristics of omics (iii) analytic obstacles, integration and gaps of data, (iv) relationship between clinical/pathological classification and tissue systems biology (endophenotypes).

4.7.1 Quality

Sample size is often low due to high experimental cost, the need of specific laboratory equipment, and limited access to human MS brain tissue. Most studies conducted on brain tissue include a restricted number of patients, and overlapping these studies is also complicated due to inter-individual and inter-study variations. Additionally, availability of tissue from the early timeframe of the disease course or from the transition to progression is largely missing. Autopsy brain tissues often represent advanced stages of disease from older patients, while biopsy brain tissue is very limited, taken from specific sites and most often from patients with atypical MS. The postmortem delay of tissue varies considerably even within the same study (**Tables 2**, **3**). In transcriptomic studies, the RNA integrity number (RIN) value is often not mentioned, but the threshold

for integrity also depends on which downstream approach is used (**Tables 2**, **3**). Qualities and quantities also differ, where most identified proteins are the highly abundant (86, 87), and low abundant proteins, likely to be involved in the distinct specific processes, remain to be discovered. Consequently, to find the true pathological signatures, reproducible and robust results are needed generated by well-designed studies including sample size power calculation, standardization of experimental as well as computational pipelines and independent validation. Furthermore, the high experimental costs and limited material demand consortiums and larger studies in collaborations across disciplines and nations using experimental and computational consensus pipelines. This kind of international network of MS experts have already begun as with the "Mystery Solved Project" (88).

4.7.2 "Snapshot"

Omics provides only static snapshots of cells at different states in a limited area: only a moment is captured of the highly dynamic variations derived from the cell state kinetics, daily biological rhythms and even stratification of patient populations over time. Longitudinal studies or individual cell trajectory tools might be helpful, but the same cell can only be measured once. To overcome this, increasing the data size by learning a latent factor model would be necessary, which encodes some unknown cell state coupled with the cell type for deconvolution. This leads to another problem, where the rapidly produced comprehensive omics data challenge the current computational methods and tools for integrative analyses.

4.7.3 Analytic Obstacles, Integration and Gaps of Data

There is a danger that too much trust is given to the output data without comprehending, how those data were obtained. Especially, there is no criteria for the sample size, the quality and standardized computational pipelines. Difficulties in combining different datasets have also been emphasized by a comparison of proteome, mRNA and protein abundance profiles of oligodendrocytes and myelin (89). The challenges to develop true robust integrative methods include different modalities, batch effects between experiments, low sequencing depth and high-modality interactions.

Furthermore, directly translate changes in the transcriptome to the dysregulated proteome is improper due to posttranscriptional regulations and spatial and temporal differences in the production of RNA and proteins. On top of that, protein function and turnover are intensely regulated by posttranslational modifications. Phosphorylation and cysteine modifications regulate protein activity; glycosylation affects protein-protein interactions; and ubiquitination affects protein localization and turnover. Activity of a protein, and its abundance in a cell cannot be deduced with certainty from the level of the corresponding mRNA.

Another challenge is to clarify, how single features are associated through multiple interactions across distinct systems and networks, and how to validate them in simplified "artificial" functional assays and models. Functional follow-up studies of the discovered networks and molecules in the right context are required to obtain specific functional annotations as discussed in *4.6*. A potential approach to gain full mechanistic insight will require coordinated sets of molecular and cellular multilayer omics data obtained at multiple time points and collected from disease-relevant tissues representing different stages of damage or repair. Additionally, combination of different omics in different human compartments, and combination of omics in the human disease with animal models may help to assess the biological significance (55, 81). However, such combination of omics techniques needs high-level integration. Combination of data-driven and knowledge-driven models into integrative models may define, whether the altered pathways are related to cause or effect. Here, *in situ* RNAseq will also help in elucidating these aspects of cell-cell interaction without the need of artificial *in silico* and *in vitro* modeling.

With the rapid acquirement of data, the concept to understand the heterogeneity of MS may change, starting from the causative molecular signature rather than the clinical phenotype (90). The classic approach (*analytical forward approach*) (**Figure 9**) applies omics of a patient group with a particular phenotype and determines, which variants these people have in common. In contrast, analyses may also start from large omics datasets by examination what human variants have in common in a clinical setting and connect it to endophenotypes (*biological reverse approach*) (**Figure 9**). Applying this latter strategy for understanding the mechanism behind MS phenotypes, the interaction of functional subsets of single cells and their unique intracellular systems should be analyzed, where macromolecules and key hubs interact with each other in networks. The observed heterogeneity of cell subtypes (endophenotypes) in individual MS lesions may be responsible for the evolution of different lesion types, and the heterogenous composition of these lesion types may contribute to changes in specific brain networks that are ultimately responsible for the clinical heterogeneity (**Figure 9**). However, here the snapshot problem will also still be an obstacle.

4.7.4 Clinical/Pathological Classification *vs* Tissue Systems Biology

Finally, MS disease classification is only based on clinical phenotypes and not endophenotypes. Differential signatures in the CSF may reflect the presence of particular lesion types in the brain but also highlight the heterogeneity of lesion/pathogenesis subtypes (endophenotypes) in phenotypically similar patient groups. However, such heterogeneity may also arise from the timepoint of sampling. Avoiding this, repeated analyses of samples with large sample size are needed. While solving the "snap-shot problem", and also adding the endophenotypic signatures for the patients may specify the pathological events, and thereby use more targeted therapies. A recent study found strong association between severe cortical pathology and a distinctive CSF inflammatory profile (91). Additionally, using positron-emission tomography (PET), potential future targets for biomarkers could be identified in different MS lesion types *in vivo*. By combining different sources of information, such as

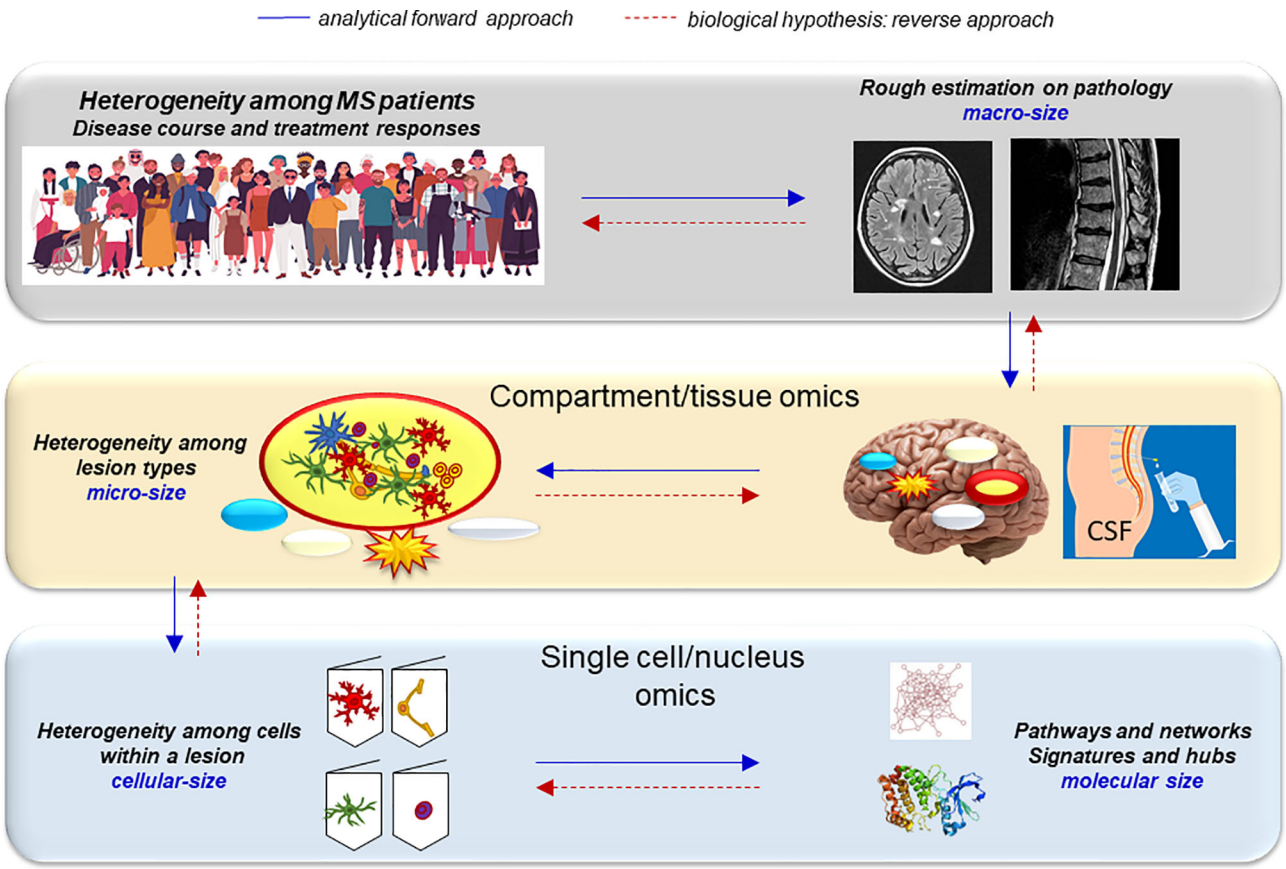

FIGURE 9 | Decoding the heterogeneity of MS with a reverse genetics approach. Analytical forward approach (blue arrows): The heterogeneity of the MS population is reflected by the heterogeneous course of MS and treatment responses. The hallmark of MS, WM brain lesions look similar on conventional MRI scans, but their histopathology is very different: characterized as active, inactive, chronic active and remyelinating/repairing lesions. This heterogeneity is most likely caused by the different cell types present in the lesions that is controlled by the heterogeneity of different networks and pathways activated within the cells and determined by some major hubs and molecular signatures. Biological hypothesis, reverse approach (red arrows): To decode this complexity, a reversed biological approach can be an alternative strategy. It can start from genetic regulation and molecular changes within individual cells that contribute to their fate. This will determine the evolution of lesions, and such complexity of lesion types will determine the individual MS brain and clinical outcomes. MS fate thus ultimately may depend on the interaction of singular cells.

omics and structural/functional neuroimaging, it may be possible to obtain a new integrated picture of the pathophysiological process in MS that could span from molecular alterations to cognitive manifestations.

5 CONCLUSION

Systems biology approach on MS brain tissue may not yet have reached as far as hoped due to tissue availability including different tissue sampling, divergent methodologies, analytic obstacles, gaps of data, and integration of datasets from various sources. Therefore, despite omics studies in MS have been present for decades, it can still be difficult to present an economical summary. However, it clearly revealed that MS is a global brain disease, where all resident brain cells are altered in different degrees. It showed that MS is a more complex and heterogenous disease on molecular level compared to the clinical classification. Paradoxically, this is also reflected in the difficulties of finding validated biomarkers based on omics approaches. Defining endophenotypes may help to disentangle the observed heterogeneity and find common patterns and dysregulated pathways: overcoming the snapshot problem is necessary for such functional interpretations.

Some of the consistent and/or key findings achieved by the systems biology investigations are inflammation within the brain of progressive MS, high levels and multiple types of HLA expression, high neuronal changes in both WM and GM, where TNF signaling is important and that CUX-2-expressing neurons are the most vulnerable; marked oligodendrocyte heterogeneity in the different WM lesion types; pathological/molecular changes in microglia within the NAWM before lesion evolution and distinct functional subgroups during lesion evolution; different astrocyte and microglia polarizations even in slowly expanding lesion rim; and high expression of CXCL12, SCD, STAT6, CD163 and TGFβR2 in all types of WM lesions.

The main power of systems biology is the comprehensive and unbiased approach at a time when out-of-the-box hypotheses for the disease course and progression are needed. Omics-driven data in MS are exponentially growing and if solutions to the major limitations (e.g. sample size, snap-shot problem) are solved, novel hypothesis-driven data can emerge. Applying innovative integrative methods to tissue and single-cell multi-omics combined with extensive interdisciplinary and international collaboration is a logical step forward. This will help give direction for functional experiments and in-depth molecular biological studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ME made the systematic article search and wrote the manuscript and made the figures. ZI provided critical feedback and helped shape the manuscript. JB and RR came with input and comments to the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sørensen PS, Thompson AJ, et al. Defining the Clinical Course of Multiple Sclerosis: The 2013 Revisions. *Neurology* (2014) 83:278–86. doi: 10.1212/WNL.0000000000000560
2. Patsopoulos N, Baranzini S, Santaniello A, Shoostari P, Cotsapas C, Wong G, et al. The Multiple Sclerosis Genomic Map: Role of Peripheral Immune Cells and Resident Microglia in Susceptibility. *bioRxiv* (2017) 143933. doi: 10.1101/143933
3. Wallin MT, Culpepper WJ, Coffman P, Pulaski S, Maloni H, Mahan CM, et al. The Gulf War Era Multiple Sclerosis Cohort: Age and Incidence Rates by Race, Sex and Service. *Brain* (2012) 135:1778–85. doi: 10.1093/brain/aws099
4. Langer-Gould A, Brara SM, Beaber BE, Zhang JL. Incidence of Multiple Sclerosis in Multiple Racial and Ethnic Groups. *Neurology* (2013) 80:1734–9. doi: 10.1212/WNL.0b013e3182918cc2
5. Lassmann H. Targets of Therapy in Progressive MS. *Mult Scler J* (2017) 23:1593–9. doi: 10.1177/1352458517729455
6. Lassmann H. Pathology and Disease Mechanisms in Different Stages of Multiple Sclerosis. *J Neurol Sci* (2013) 333:1–4. doi: 10.1016/j.jns.2013.05.010
7. Reynolds R, Roncaroli F, Nicholas R, Radotra B, Gveric D, Howell O. The Neuropathological Basis of Clinical Progression in Multiple Sclerosis. *Acta Neuropathol* (2011) 122:155–70. doi: 10.1007/s00401-011-0840-0
8. Frischer JM, Weigand SD, Guo Y, Kale N, Parisi JE, Pirko I, et al. Clinical and Pathological Insights Into the Dynamic Nature of the White Matter Multiple Sclerosis Plaque. *Ann Neurol* (2015) 78:710–21. doi: 10.1002/ana.24497
9. Kuhlmann T, Lassmann H, Brück W. Diagnosis of Inflammatory Demyelination in Biopsy Specimens: A Practical Approach. *Acta Neuropathol* (2008) 115:275–87. doi: 10.1007/s00401-007-0320-8
10. Luchetti S, Fransen NL, van Eden CG, Ramaglia V, Mason M, Huitinga I. Progressive Multiple Sclerosis Patients Show Substantial Lesion Activity That Correlates With Clinical Disease Severity and Sex: A Retrospective Autopsy Cohort Analysis. *Acta Neuropathol* (2018) 135:511–28. doi: 10.1007/s00401-018-1818-y
11. Kutzelnigg A, Lucchinetti CF, Stadelmann C, Brück W, Rauschka H, Bergmann M, et al. Cortical Demyelination and Diffuse White Matter Injury in Multiple Sclerosis. *Brain* (2005) 128:2705–12. doi: 10.1093/brain/awh641
12. Milstein JL, Barbour CR, Jackson K, Kosa P, Bielekova B. Intrathecal, Not Systemic Inflammation Is Correlated With Multiple Sclerosis Severity, Especially in Progressive Multiple Sclerosis. *Front Neurol* (2019) 10:1232. doi: 10.3389/fneur.2019.01232
13. Machado-Santos J, Saji E, Tröscher AR, Paunovic M, Liblau R, Gabriely G, et al. The Compartmentalized Inflammatory Response in the Multiple Sclerosis Brain Is Composed of Tissue-Resident CD8+ T Lymphocytes and B Cells. *Brain* (2018) 141:2066–82. doi: 10.1093/brain/awy151
14. Rommer PS, Milo R, Han MH, Satyanarayan S, Sellner J, Hauer L, et al. Immunological Aspects of Approved MS Therapeutics. *Front Immunol* (2019) 10. doi: 10.3389/fimmu.2019.01564
15. Civelek M, Lusis AJ. Systems Genetics Approaches to Understand Complex Traits. *Nat Rev Genet* (2014) 15:34–48. doi: 10.1038/nrg3575
16. Gibson G, Marigorta UM, Ojagbeghru ER, Park S. PART of the WHOLE: A Case Study in Wellness-Oriented Personalized Medicine. *Yale J Biol Med* (2015) 88:397–406.
17. Whitney LW, Becker KG, Tresser NJ, Caballero-Ramos CI, Munson PJ, Prabhu VV, et al. Analysis of Gene Expression in Mutiple Sclerosis Lesions Using cDNA Microarrays. *Ann Neurol* (1999) 46:425–8. doi: 10.1002/1531-8249(199909)46:3<425::AID-ANA22>3.0.CO;2-O
18. Baranzini SE, Elfstrom C, Chang SY, Butunoi C, Murray R, Higuchi R, et al. Transcriptional Analysis of Multiple Sclerosis Brain Lesions Reveals a Complex Pattern of Cytokine Expression. *J Immunol (Baltimore Md 1950)* (2000) 165:6576–82. doi: 10.4049/jimmunol.165.11.6576
19. Whitney LW, Ludwin SK, McFarland HF, Biddison WE. Microarray Analysis of Gene Expression in Multiple Sclerosis and EAE Identifies 5-Lipoxygenase as a Component of Inflammatory Lesions. *J Neuroimmunol* (2001) 121:40–8. doi: 10.1016/S0165-5728(01)00438-6
20. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-Microarray Analysis of Multiple Sclerosis Lesions Yields New Targets Validated in Autoimmune Encephalomyelitis. *Nat Med* (2002) 8:500–8. doi: 10.1038/nm0502-500
21. Graumann U, Reynolds R, Steck AJ, Schaeren-Wiemers N. Molecular Changes in Normal Appearing White Matter in Multiple Sclerosis Are Characteristic of Neuroprotective Mechanisms Against Hypoxic Insult. *Brain Pathol* (2003) 13:554–73. doi: 10.1111/j.1750-3639.2003.tb00485.x
22. Mycko MP, Papoian R, Boschert U, Raine CS, Selmaj KW. cDNA Microarray Analysis in Multiple Sclerosis Lesions: Detection of Genes Associated With Disease Activity. *Brain* (2003) 126:1048–57. doi: 10.1093/brain/awg107
23. Tajouri L, Mellick AS, Ashton KJ, Tannenberg AEG, Nagra RM, Tourtellotte WW, et al. Quantitative and Qualitative Changes in Gene Expression Patterns Characterize the Activity of Plaques in Multiple Sclerosis. *Mol Brain Res* (2003) 119:170–83. doi: 10.1016/j.molbrainres.2003.09.008
24. Lindberg RLP, De Groot CJA, Certa U, Ravid R, Hoffmann F, Kappos L, et al. Multiple Sclerosis as a Generalized CNS Disease–Comparative Microarray Analysis of Normal Appearing White Matter and Lesions in Secondary Progressive MS. *J Neuroimmunol* (2004) 152:154–67. doi: 10.1016/j.jneuroim.2004.03.011
25. Mycko MP, Papoian R, Boschert U, Raine CS, Selmaj KW. Microarray Gene Expression Profiling of Chronic Active and Inactive Lesions in Multiple Sclerosis. *Clin Neurol Neurosurg* (2004) 106:223–9. doi: 10.1016/j.clineuro.2004.02.019

26. Zeis T, Graumann U, Reynolds R, Schaeren-Wiemers N. Normal-Appearing White Matter in Multiple Sclerosis Is in a Subtle Balance Between Inflammation and Neuroprotection. *Brain* (2008) 131:288–303. doi: 10.1093/brain/awm291
27. Cunnea P, McMahon J, O'Connell E, Mashayekhi K, Fitzgerald U, McQuaid S. Gene Expression Analysis of the Microvascular Compartment in Multiple Sclerosis Using Laser Microdissected Blood Vessels. *Acta Neuropathol* (2010) 119:601–15. doi: 10.1007/s00401-009-0618-9
28. Fischer MT, Sharma R, Lim JL, Haider L, Frischer JM, Drexhage J, et al. NADPH Oxidase Expression in Active Multiple Sclerosis Lesions in Relation to Oxidative Tissue Damage and Mitochondrial Injury. *Brain* (2012) 135:886–99. doi: 10.1093/brain/aws012
29. Mycko MP, Brosnan CF, Raine CS, Fendler W, Selmaj KW. Transcriptional Profiling of Microdissected Areas of Active Multiple Sclerosis Lesions Reveals Activation of Heat Shock Protein Genes. *J Neurosci Res* (2012) 90:1941–8. doi: 10.1002/jnr.23079
30. Mohan H, Friese A, Albrecht S, Krumbholz M, Elliott CL, Arthur A, et al. Transcript Profiling of Different Types of Multiple Sclerosis Lesions Yields FGF1 as a Promoter of Remyelination. *Acta Neuropathol Commun* (2014) 2:168. doi: 10.1186/s40478-014-0168-9
31. Licht-Mayer S, Wimmer I, Traffehn S, Metz I, Brück W, Bauer J, et al. Cell Type-Specific Nrf2 Expression in Multiple Sclerosis Lesions. *Acta Neuropathol* (2015) 130:263–77. doi: 10.1007/s00401-015-1452-x
32. Waller R, Woodroofe MN, Wharton SB, Ince PG, Francese S, Heath PR, et al. Gene Expression Profiling of the Astrocyte Transcriptome in Multiple Sclerosis Normal Appearing White Matter Reveals a Neuroprotective Role. *J Neuroimmunol* (2016) 299:139–46. doi: 10.1016/j.jneuroim.2016.09.010
33. Hendrickx DAEE, van Scheppingen J, van der Poel M, Bossers K, Schuurman KG, van Eden CG, et al. Gene Expression Profiling of Multiple Sclerosis Pathology Identifies Early Patterns of Demyelination Surrounding Chronic Active Lesions. *Front Immunol* (2017) 8:1810. doi: 10.3389/fimmu.2017.01810
34. Zeis T, Howell OW, Reynolds R, Schaeren-Wiemers N. Molecular Pathology of Multiple Sclerosis Lesions Reveals a Heterogeneous Expression Pattern of Genes Involved in Oligodendrogliogenesis. *Exp Neurol* (2018) 305:76–88. doi: 10.1016/j.expneurol.2018.03.012
35. Melief J, Orre M, Bossers K, van Eden CG, Schuurman KG, Mason MRJ, et al. Transcriptome Analysis of Normal-Appearing White Matter Reveals Cortisol- and Disease-Associated Gene Expression Profiles in Multiple Sclerosis. *Acta Neuropathol. Commun* (2019) 7:1–19. doi: 10.1186/S40478-019-0705-7
36. Magliozzi R, Howell OW, Durrenberger P, Aricò E, James R, Cruciani C, et al. Meningeal Inflammation Changes the Balance of TNF Signalling in Cortical Grey Matter in Multiple Sclerosis. *J Neuroinflamm* (2019) 16:1–16. doi: 10.1186/S12974-019-1650-X
37. Enz LS, Zeis T, Schmid D, Geier F, van der Meer F, Steiner G, et al. Increased HLA-DR Expression and Cortical Demyelination in MS Links With HLA-Dr15. *Neurol Neuroimmunol Neuroinflamm* (2020) 7. doi: 10.1212/NXI.0000000000000656
38. Jäckle K, Zeis T, Schaeren-Wiemers N, Junker A, van der Meer F, Kramann N, et al. Molecular Signature of Slowly Expanding Lesions in Progressive Multiple Sclerosis. *Brain* (2020) 143:2073–88. doi: 10.1093/brain/awaa158
39. Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, Bittner R, et al. MicroRNA Profiling of Multiple Sclerosis Lesions Identifies Modulators of the Regulatory Protein CD47. *Brain* (2009) 132:3342–52. doi: 10.1093/brain/awp300
40. Chomyk AM, Volsko C, Tripathi A, Deckard SA, Trapp BD, Fox RJ, et al. DNA Methylation in Demyelinated Multiple Sclerosis Hippocampus. *Sci Rep* (2017) 7:8696. doi: 10.1038/s41598-017-08623-5
41. Tripathi A, Volsko C, Datta U, Regev K, Dutta R. Expression of Disease-Related miRNAs in White-Matter Lesions of Progressive Multiple Sclerosis Brains. *Ann Clin Transl Neurol* (2019) 6:854–62. doi: 10.1002/acn3.750
42. Kular L, Needhamsen M, Adzemovic MZ, Kramarova T, Gomez-Cabrero D, Ewing E, et al. Neuronal Methylome Reveals CREB-Associated Neuro-Axonal Impairment in Multiple Sclerosis. *Clin Epigenet* (2019) 11. doi: 10.1186/s13148-019-0678-1
43. Becker KG, Mattson DH, Powers JM, Gado AM, Biddison WE. Analysis of a Sequenced cDNA Library From Multiple Sclerosis Lesions. *J Neuroimmunol* (1997) 77:27–38. doi: 10.1016/S0165-5728(97)00045-3
44. Chabas D. The Influence of the Proinflammatory Cytokine, Osteopontin, on Autoimmune Demyelinating Disease. *Science (80-)* (2001) 294:1731–5. doi: 10.1126/science.1062960
45. Schmitt K, Richter C, Backes C, Meese E, Ruprecht K, Mayer J. Comprehensive Analysis of Human Endogenous Retrovirus Group HERV-W Locus Transcription in Multiple Sclerosis Brain Lesions by High-Throughput Amplicon Sequencing. *J Virol* (2013) 87:13837–52. doi: 10.1128/JVI.02388-13
46. Huynh JL, Garg P, Thin TH, Yoo S, Dutta R, Trapp BD, et al. Epigenome-Wide Differences in Pathology-Free Regions of Multiple Sclerosis-Affected Brains. *Nat Neurosci* (2014) 17:121–30. doi: 10.1038/nn.3588
47. Kriesel J, Bhetariya P, Chan B, Wilson T, Fischer K. Enrichment of Retroviral Sequences in Brain Tissue From Patients With Severe Demyelinating Diseases. *J Emerg Dis Virol* (2017) 3. doi: 10.16966/2473-1846.132
48. Elkjaer ML, Frisch T, Reynolds R, Kacprowski T, Burton M, Kruse TA, et al. Molecular Signature of Different Lesion Types in the Brain White Matter of Patients With Progressive Multiple Sclerosis. *Acta Neuropathol Commun* (2019) 7:205. doi: 10.1186/s40478-019-0855-7
49. Konjevic Sabolek M, Held K, Beltrán E, Niedl AG, Meinl E, Hohlfeld R, et al. Communication of CD 8 $^{+}$ T Cells With Mononuclear Phagocytes in Multiple Sclerosis. *Ann Clin Transl Neurol* (2019) acn3.783. doi: 10.1002/acn3.783
50. van der Poel M, Ulas T, Mizee MR, Hsiao CC, Miedema SSM, Adelia, et al. Transcriptional Profiling of Human Microglia Reveals Grey–White Matter Heterogeneity and Multiple Sclerosis-Associated Changes. *Nat Commun* (2019) 10:1139. doi: 10.1038/s41467-019-08976-7
51. Voskuhl RR, Itoh N, Tassoni A, Matsukawa MA, Ren E, Tse V, et al. Gene Expression in Oligodendrocytes During Remyelination Reveals Cholesterol Homeostasis as a Therapeutic Target in Multiple Sclerosis. *Proc Natl Acad Sci USA* (2019) 116:10130–9. doi: 10.1073/pnas.1821306116
52. Chiricosta L, Gugliandolo A, Bramanti P, Mazzon E. Could the Heat Shock Proteins 70 Family Members Exacerbate the Immune Response in Multiple Sclerosis? An in Silico Study. *Genes (Basel)* (2020) 11:1–12. doi: 10.3390/genes11060615
53. Frisch T, Elkjaer ML, Reynolds R, Michel TM, Kacprowski T, Burton M, et al. Multiple Sclerosis Atlas: A Molecular Map of Brain Lesion Stages in Progressive Multiple Sclerosis. *Netw Syst Med* (2020) 3:122–9. doi: 10.1089/nsm.2020.0006
54. Rodríguez-Lorenzo S, Ferreira Francisco DM, Vos R, Van Het Hof B, Rijnsburger M, Schroten H, et al. Altered Secretory and Neuroprotective Function of the Choroid Plexus in Progressive Multiple Sclerosis. *Acta Neuropathol. Commun* (2020) 8. doi: 10.1186/s40478-020-00903-y
55. Elkjaer ML, Nawrocki A, Kacprowski T, Lassen P, Simonsen AH, Marignier R, et al. CSF Proteome in Multiple Sclerosis Subtypes Related to Brain Lesion Transcriptomes. *Sci Rep* (2021) 11. doi: 10.1038/s41598-021-83591-5
56. Jäkel S, Agirre E, Mendanha Falcão A, van Bruggen D, Lee KW, Knuesel I, et al. Altered Huaman Oligodendrocyte Heterogeneity in Multiple Sclerosis. *Nature* (2019) 566:543–7. doi: 10.1038/s41586-019-0903-2
57. Masuda T, Sankowski R, Staszewski O, Böttcher C, Amann L, Sagar, et al. Spatial and Temporal Heterogeneity of Mouse and Human Microglia at Single-Cell Resolution. *Nature* (2019) 566:388–92. doi: 10.1038/s41586-019-0924-x
58. Schirmer L, Velmeshev D, Holmqvist S, Kaufmann M, Werneburg S, Jung D, et al. Neuronal Vulnerability and Multilineage Diversity in Multiple Sclerosis. *Nature* (2019) 573:75–82. doi: 10.1038/s41586-019-1404-z
59. Wheeler MA, Clark IC, Tjon EC, Li Z, Zandee SEJ, Couturier CP, et al. MAFG-Driven Astrocytes Promote CNS Inflammation. *Nature* (2020) 578:593–9. doi: 10.1038/s41586-020-1999-0
60. Absinta M, Maric D, Gharagozloo M, Garton T, Smith MD, Jin J, et al. A Lymphocyte–Microglia–Astrocyte Axis in Chronic Active Multiple Sclerosis. *Nature* (2021) 597:709–14. doi: 10.1038/s41586-021-03892-7
61. Zeis T, Probst A, Steck AJ, Stadelmann C, Brück W, Schaeren-Wiemers N. Molecular Changes in White Matter Adjacent to an Active Demyelinating Lesion in Early Multiple Sclerosis: Molecular Changes in MS Periplaque White Matter. *Brain Pathol* (2009) 19:459–66. doi: 10.1111/j.1750-3639.2008.00231.x
62. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic Reactive Astrocytes Are Induced by Activated Microglia. *Nature* (2017) 541:481–7. doi: 10.1038/nature21029

63. Tripathi A, Pandit I, Perles A, Zhou Y, Cheng F, Dutta R. Identifying miRNAs in Multiple Sclerosis Gray Matter Lesions That Correlate With Atrophy Measures. *Ann Clin Transl Neurol* (2021) 8:1279–91. doi: 10.1002/acn3.51365
64. Fritsche L, Teuber-Hanselmann S, Soub D, Harnisch K, Mairinger F, Junker A. MicroRNA Profiles of MS Gray Matter Lesions Identify Modulators of the Synaptic Protein Synaptotagmin-7. *Brain Pathol* (2020) 30:524–40. doi: 10.1111/bpa.12800
65. Elkjaer ML, Frisch T, Tonazzolli A, Röttger R, Reynolds R, Baumbach J, et al. Unbiased Examination of Genome-Wide Human Endogenous Retrovirus Transcripts in MS Brain Lesions. *Mult Scler J* (2021) 27:1829–37. doi: 10.1177/1352458520987269
66. Kriesel JD, Bhetariya P, Wang ZM, Renner D, Palmer C, Fischer KF. Spectrum of Microbial Sequences and a Bacterial Cell Wall Antigen in Primary Demyelination Brain Specimens Obtained From Living Patients. *Sci Rep* (2019) 9. doi: 10.1038/s41598-018-38198-8
67. Boscia F, Elkjaer ML, Illes Z, Kukley M. Altered Expression of Ion Channels in White Matter Lesions of Progressive Multiple Sclerosis: What Do We Know About Their Function? *Front Cell Neurosci* (2021) 15. doi: 10.3389/fncel.2021.685703
68. Qendro V, Bugos GA, Lundgren DH, Glynn J, Han MH, Han DK. Integrative Proteomics, Genomics, and Translational Immunology Approaches Reveal Mutated Forms of Proteolipid Protein 1 (PLP1) and Mutant-Specific Immune Response in Multiple Sclerosis. *Proteomics* (2017) 17:1600322. doi: 10.1002/pmic.201600322
69. Singh V, Stoop MP, Stingl C, Luitwieler RL, Dekker LJ, Van Duijn MM, et al. Cerebrospinal-Fluid-Derived Immunoglobulin G of Different Multiple Sclerosis Patients Shares Mutated Sequences in Complementarity Determining Regions. *Mol Cell Proteomics* (2013) 12:3924–34. doi: 10.1074/mcp.M113.030346
70. Newcombe J, Eriksson B, Ottervald J, Yang Y, Franzén B. Extraction and Proteomic Analysis of Proteins From Normal and Multiple Sclerosis Postmortem Brain. *J Chromatogr B Anal Technol Biomed Life Sci* (2005) 815:191–202. doi: 10.1016/j.jchromb.2004.10.073
71. Han MH, Hwang S-I, Roy DB, Lundgren DH, Price JV, Ousman SS, et al. Proteomic Analysis of Active Multiple Sclerosis Lesions Reveals Therapeutic Targets. *Nature* (2008) 451:1076–81. doi: 10.1038/nature06559
72. Fissolo N, Haag S, De Graaf KL, Drews O, Stevanovic S, Rammensee HG, et al. Naturally Presented Peptides on Major Histocompatibility Complex I and II Molecules Eluted From Central Nervous System of Multiple Sclerosis Patients. *Mol Cell Proteomics* (2009) 8:2090–101. doi: 10.1074/mcp.M900001-MCP200
73. Ly L, Barnett MH, Zheng YZ, Gulati T, Prineas JW, Crossett B. Comprehensive Tissue Processing Strategy for Quantitative Proteomics of Formalin-Fixed Multiple Sclerosis Lesions. *J Proteome Res* (2011) 10:4855–68. doi: 10.1021/pr200672n
74. Broadwater L, Pandit A, Clements R, Azzam S, Vadnal J, Sulak M, et al. Analysis of the Mitochondrial Proteome in Multiple Sclerosis Cortex. *Biochim Biophys Acta* (2011) 1812:630–41. doi: 10.1016/j.bbadis.2011.01.012
75. Brown N, Alkhayer K, Clements R, Singhal N, Gregory R, Azzam S, et al. Neuronal Hemoglobin Expression and Its Relevance to Multiple Sclerosis Neuropathology. *J Mol Neurosci* (2016) 59:1–17. doi: 10.1007/s12031-015-0711-6
76. Syed YA, Zhao C, Mahad D, Möbius W, Altmann F, Foss F, et al. Antibody-Mediated Neutralization of Myelin-Associated EphrinB3 Accelerates CNS Remyelination. *Acta Neuropathol* (2016) 131:281–98. doi: 10.1007/s00401-015-1521-1
77. Maccarrone G, Nischwitz S, Deininger SO, Hornung J, König FB, Stadelmann C, et al. MALDI Imaging Mass Spectrometry Analysis—A New Approach for Protein Mapping in Multiple Sclerosis Brain Lesions. *J Chromatogr B Anal Technol Biomed Life Sci* (2017) 1047:131–40. doi: 10.1016/j.jchromb.2016.07.001
78. Faigle W, Cruciani C, Wolski W, Roschitzki B, Puthenparampil M, Tomas-Ojer P, et al. Brain Citrullination Patterns and T Cell Reactivity of Cerebrospinal Fluid-Derived CD4+ T Cells in Multiple Sclerosis. *Front Immunol* (2019) 10. doi: 10.3389/fimmu.2019.00540
79. Böttcher C, van der Poel M, Fernández-Zapata C, Schlickeiser S, Leman JKH, Hsiao CC, et al. Single-Cell Mass Cytometry Reveals Complex Myeloid Cell Composition in Active Lesions of Progressive Multiple Sclerosis. *Acta Neuropathol Commun* (2020) 8. doi: 10.1186/s40478-020-01010-8
80. De Luca C, Virtuoso A, Maggio N, Papa M. Neuro-Coagulopathy: Blood Coagulation Factors in Central Nervous System Diseases. *Int J Mol Sci* (2017) 18. doi: 10.3390/ijms18102128
81. Martin NANA, Nawrocki A, Molnar V, Elkjaer MLML, Thygesen EKEK, Palkovits M, et al. Orthologous Proteins of Experimental De- and Remyelination Are Differentially Regulated in the CSF Proteome of Multiple Sclerosis Subtypes. *PloS One* (2018) 13:e0202530. doi: 10.1371/journal.pone.0202530
82. Maccarrone G, Nischwitz S, Deininger S-O, Hornung J, König FB, Stadelmann C, et al. MALDI Imaging Mass Spectrometry Analysis-A New Approach for Protein Mapping in Multiple Sclerosis Brain Lesions. *J Chromatogr B Analyt Technol Biomed Life Sci* (2016) 1047:1–10. doi: 10.1016/j.jchromb.2016.07.001
83. Kim Y, Park J, Choi YK. The Role of Astrocytes in the Central Nervous System Focused on BK Channel and Heme Oxygenase Metabolites: A Review. *Antioxidants* (2019) 8. doi: 10.3390/antiox8050121
84. Picon C, Jayaraman A, James R, Beck C, Gallego P, Witte ME, et al. Neuron-Specific Activation of Necroptosis Signaling in Multiple Sclerosis Cortical Grey Matter. *Acta Neuropathol* (2021) 141:585–604. doi: 10.1007/s00401-021-02274-7
85. Absinta M, Sati P, Masuzzo F, Nair G, Sethi V, Kolb H, et al. Association of Chronic Active Multiple Sclerosis Lesions With Disability in Vivo. *JAMA Neurol* (2019) 76:1474–83. doi: 10.1001/jamaneurol.2019.2399
86. Stoop MP, Singh V, Dekker LJ, Titulaer MK, Stingl C, Burgers PC, et al. Proteomics Comparison of Cerebrospinal Fluid of Relapsing Remitting and Primary Progressive Multiple Sclerosis. *PloS One* (2010) 5:e12442. doi: 10.1371/journal.pone.0012442
87. Kroksveen AC, Guldbrandsen A, Vedeler C, Myhr KM, Opsahl JA, Berven FS. Cerebrospinal Fluid Proteome Comparison Between Multiple Sclerosis Patients and Controls. *Acta Neurol Scand* (2012) 126:90–6. doi: 10.1111/ane.12029
88. *Mystery Solved Project | European Charcot Foundation*. Available at: https://www.charcot-ms.org/initiatives/mystery-solved-project (Accessed: 19th October 2021).
89. Jahn O, Siems SB, Kusch K, Hesse D, Jung RB, Liepold T, et al. The CNS Myelin Proteome: Deep Profile and Persistence After Post-Mortem Delay. *Front Cell Neurosci* (2020) 14:239. doi: 10.3389/fncel.2020.00239
90. Lyu J. Reverse Genetics: Wheat Solution. *Nat Plants* (2017) 3:17005. doi: 10.1038/nplants.2017.5
91. Magliozzi R, Hametner S, Facchiano F, Marastoni D, Rossi S, Castellaro M, et al. Iron Homeostasis, Complement, and Coagulation Cascade as CSF Signature of Cortical Lesions in Early Multiple Sclerosis. *Ann Clin Transl Neurol* (2019) 6:2150–63. doi: 10.1002/acn3.50893

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BRIEF RESEARCH REPORT
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Smoking Attributable Risk in Multiple Sclerosis

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Tobacco smoke is an important modifiable environmental risk factor for multiple sclerosis (MS) risk. The population attributable fraction (AF) of MS due to smoking can be used to assess the contribution of smoking to the risk of MS development. We conducted a matched case-control study, including individuals with MS and population-based controls. Overall, sex- and genetic risk score-stratified AF due to smoking were calculated by fitting logistic regression models. We included 9,419 individuals with MS and 9,419 population-based matched controls. At the time of MS onset 44.1% of persons with MS and 35.9% of controls ever regularly smoked of which 38.1% and 29.2% were still smoking. The overall AF was 13.1% (95%CI: 10.7 to 15.4). The AF was 10.6% (95% CI: 7.4 to 13.7) in females and 19.1% (95%CI: 13.1 to 25.1) in males. The AF was 0.6% (95%CI: 0.0 to 2) in ex-smokers. In those having human leucocyte antigen (HLA) and non-HLA risk scores above the median levels of controls, the AF was 11.4% (95%CI: 6.8 to 15.9) and 12% (95%CI: 7.7 to 16.3), respectively. The AF was 17.6% (95%CI: 10.2 to 24.9) and 18.6% (95%CI: 5.5 to 31.6) in those with HLA and non-HLA risk scores below the median levels in controls, respectively. We noticed a decline in AF in recent birth cohorts. This study indicates that at least 13% of cases of MS could be prevented through the avoidance of tobacco smoking. Considering the prevalence of MS, this represents a very large group of people in absolute number.

Keywords: multiple sclerosis, attributable fraction, risk factor, tobacco, smoking

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INTRODUCTION

Multiple sclerosis (MS) is the result of a complex interplay between genetic and environmental risk factors. Prevention of MS is becoming a reasonable aspiration, but it depends on the extent risk factors can be modified or mitigated. Cigarette smoking remains the most important single cause of preventable mortality and morbidity in the world. Exposure to cigarette smoke is also an important modifiable environmental risk factor for MS development and its clinical course (1–4), with epidemiological studies reporting a 50% higher risk of MS development in ever-smokers compared to never-smokers. (5)

The population attributable fraction (AF) of disease due to exposure, or the proportion of the disease in a population attributable to smoking, can be used to assess the contribution of smoking to

the risk of disease development. Estimating the AF could be a strong incentive to prevent disease by measuring the population burden associated with a given exposure. For example, the AF for smoking in people with lung cancer is 85% (6), indicating that a substantial number of lung cancers would not have occurred and relatively less lives would be lost if people did not smoke.

For smoking-associated diseases such as MS, the smoking AF increases with a higher prevalence of smoking in the population. Estimating the AF of MS due to smoking would determine the proportion of MS that could be avoided if people did not smoke. In this study, we estimated the AF due to smoking of MS in the Swedish population.

METHOD

Data Source

Our study population included individuals with MS and population-based controls participating in two large Swedish cohorts, the Epidemiological Investigation of Multiple Sclerosis (EIMS) and the Genes and Environment in Multiple Sclerosis (GEMS). (7) In the EIMS study, individuals with newly diagnosed MS (fulfilling the McDonald criteria), aged between 16 and 70 years, were identified at neurology clinics throughout Sweden and invited to participate in the study by completing a questionnaire and donating a blood sample. (8) The GEMS study includes prevalent cases of MS fulfilling the McDonald criteria who were identified from the Swedish National MS registry. (9) There were no overlap of cases between EIMS and GEMS. Controls were randomly selected from the national population register matched for age (equivalent of age at the diagnosis in cases), gender, and residential area at the time of the disease diagnosis. In both studies, information on exposure to tobacco smoking was obtained by asking about current and previous smoking habits. We defined "smokers" as those who have ever smoked cigarettes regularly before MS onset or the equivalent age in controls.

Adjustment for Genetic Risk Score

For a subset with available genetic data, the weighted human leucocyte antigen (HLA) specific and non-HLA genetic risk scores were calculated. Individuals were genotyped using an Illumina custom array (>90,000 SNPs), which focuses on MS genetic risk loci, particularly the HLA region on chromosome 6. (10) Standard marker and individual quality controls were performed using PLINK, and population outliers were identified using SmartPCA. After quality control and exclusion of outliers, classical HLA allele variants were imputed using HLAIMP*03. (10) Each individual's genetic susceptibility to MS was determined using a polygenic risk score (GRS) defined by the sum of all risk alleles carried (0, 1, and 2). Total risk and separated scores for HLA allele variants and non-HLA SNPs were calculated using established risk factors from previous interaction and genome-wide association studies. (10, 11) Scores were also weighted by their effect size controlled for population stratification and possible confounding genetic factors.

Statistical Analysis

For the calculation of AF, we used the method suggested by Dahlqwist et al. (12) In short, the method calculates confounder adjusted AF estimates for case-control study design. For each case of MS we identified one exact calendar year of birth and sex matched control while adjusting all the models for calendar year of birth in five groups. This was mainly done to account for changes in the prevalence of smoking in the Swedish general population over the years. Overall and sex stratified AF were calculated in the first instant. We also calculated AF stratified by HLA and non-HLA genetic burden. The genetic risk scores were dichotomized by the median score of the population-based controls. In a subset of individuals with information on HLA DRB1*15:01, we calculated smoking AF due to interaction (carriage of HLA DRB1*15:01 and being a smoker).

RESULTS

We included 9,419 individuals with MS and 9,419 population-based exact calendar year of birth and sex matched controls. 28% of the population were male with mean (standard deviation, SD) calendar year of birth of 1960 (± 14). 44.1% of persons with MS and 35.9% of controls had ever-smoked prior to onset or index age respectively. At the time of MS onset (and equivalent time in controls) 38.1% of cases and 29.2% of controls were still smoking (current smokers). The mean number of pack-years cigarette smoked was significantly higher in cases compared to controls [4.2 (± 7.2) vs. 3.2 (± 6.7), $P < 0.001$]. Cases smoked on average 5.7 (± 7.5) cigarettes per day for the duration of 6.2 (± 9.1) years. The average number of cigarettes smoked was 4.4 (± 6.9) for the duration of 4.9 (± 8.6) years in controls. The HLA and non-HLA genetic risk scores were available in 5,916 controls and 6,885 persons with MS (**Figure 2**). The risk of MS in ever-smokers was increased by 41% (95% confidence intervals (CI): 1.33 to 1.50) compared to never smokers.

The overall AF was 13.1% (95%CI: 10.7 to 15.4). The AF was 10.6% (95%CI: 7.4 to 13.7) in females and 19.1% (95%CI: 13.1 to 25.1) in males. The AF was less than 1% (AF: 0.6%, 95%CI: 0 to 2) in ex-smokers indicating beneficial effects of smoking cessation. **Figure 1** illustrates the AF over five-year birth-cohort strata along with percentage of smokers amongst cases in each birth-cohort. When investigating the impact of smoking intensity, we observed that the AF was 9.3% (95%CI: 7.1 to 11.5) in those smokers with pack-years smoked above the median and 7.1% (95%CI: 4.9 to 9.3) in those who smoked below the median smoking intensity.

In those having HLA and non-HLA risk scores above the median levels of controls, the AF was 11.4% (95%CI: 6.8 to 15.9) and 12% (95%CI: 7.7 to 16.3), respectively. The AF was 17.6% (95%CI: 10.2 to 24.9) and 18.6% (95%CI: 5.5 to 31.6) in those with HLA and non-HLA risk scores below the median levels in controls, respectively. The stratified AF estimates are summarized in **Figure 2**. Results of interaction analysis between HLA DRB1*15:01 and smoking revealed that approximately half of the AF due to smoking is independent of

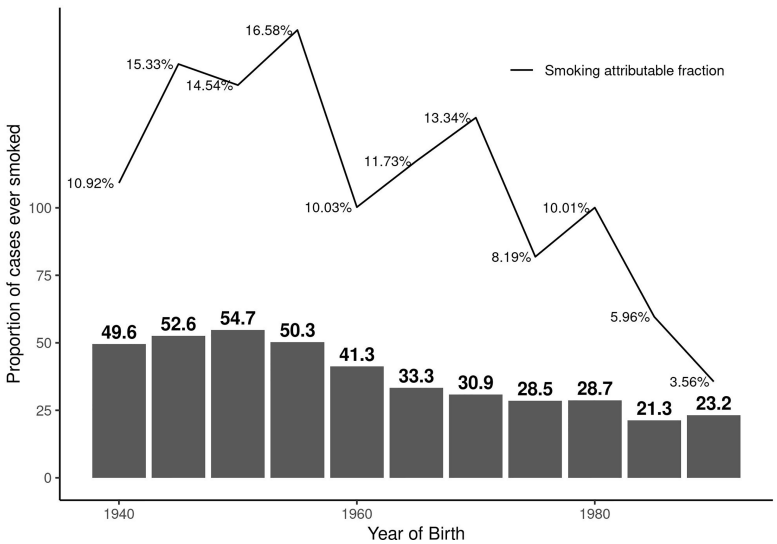

FIGURE 1 | Proportion of persons with MS who have ever regularly smoked and the corresponding smoking attributable fraction (AF) over calendar year of birth by five-year birth-cohort.

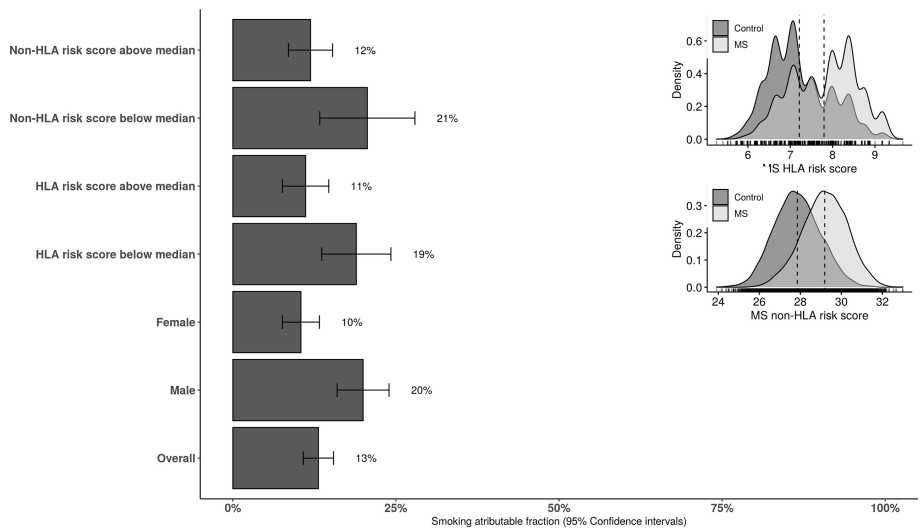

FIGURE 2 | The overall and stratified smoking attributable fraction (95% confidence intervals) for risk of MS. HLA and non-HLA risk scores are categorized to below and above the median score in the population-based controls. HLA, human leucocyte antigen.

HLA DRB1*15:01 status (6.8%, 95%CI: 5.5 to 7.9) while the AF due to HLA independent of smoking was 23% (95%CI: 22.1 to 23.8). The risk (OR) associated with both DRB1*15:01 carriage and smoking was 4.9 (95%CI: 4.4 to 5.4) compared to 3.4 (95% CI: 3.1 to 3.8) and 1.5 (95%CI: 1.4 to1.7) for only DRB1*15:01 carriage and smoking, respectively. AF in MS cases due to having both exposures was 20% (95%CI: 19.4 to 20.5).

CONCLUSION

There is strong evidence for a link between tobacco smoking and the risk of MS, and data support the concept that this link is most likely causal. (13) Based on this concept, this study indicates that at least 13% of cases of MS could be prevented through the avoidance of tobacco smoking. Considering the prevalence of the disease, this

represents a very large group of people in absolute numbers who would never develop MS. We believe that, from a global perspective, these numbers may represent an underestimate. The study was based on a well-characterized sample of Swedish population. In Sweden, the prevalence of MS is high, and the prevalence of smoking is low. (14) As the AF increases with the prevalence of smoking, we believe that the AF in a country with a high prevalence of smoking could be considerably higher.

Our previous estimates of smoking AF in MS was 20% (active or passive) which was further increased to 41% in subjects who had carriage of HLA-DRB1*15 and absence of HLA-A*02. (15) This study includes a much greater number of cases and controls compared to our previous study. We also used polygenic risk scores and specific risk alleles (HLA DRB1*15:01) which provide additional information with regard to the interaction between smoking and MS genetics. We previously estimated that about 5% of the MS cases could be attributed to passive smoking. (15) Passive smoking (for example, exposure to parental smoking in childhood) has not been included in the present analysis. However, we could confirm that smoking cessation and to lesser extent reducing smoking intensity could be potentially beneficial in reducing the impact of smoking in MS. We also observed a decline in smoking AF in a more contemporary cohorts of MS as the smoking prevalence declines in the general population. Given that the majority of environmental risk factors for MS tend to exert their most powerful effect in childhood and adolescent years and that parental smoking is a risk factor for MS, it is possible that a further fraction of MS could be prevented in the offspring of smokers. Although an AF of MS due to passive smoking cannot be calculated in the current study, this consideration reiterates the possibility that the 13% of MS attributable to smoking is underestimated.

Smoking remains a major modifiable risk factor for many morbidities, including MS. The association of smoking with a worsened outcome in multiple conditions, including MS, (16) has incentivized smoking cessation. In this regard, several smoking cessation campaigns have been effective in reducing smoking in the population, encouraging cessation. (17) If a smoking avoidance campaign has similar rates of success, an important proportion may avoid MS.

Our previous studies addressed the interaction between MS risk factors such as smoking, HLA DRB1*15:01, obesity, sun exposure, and EBV seropositivity. The interaction of HLA DRB1*15:01 with smoking increased the combined AF for MS. Here, we used a combined genetic risk scores which does not take only HLA DRB1*15:01 into account, and find a slightly less multiplicative increase when population was stratified by their MS genetic susceptibility. We consider a smoking AF of 13% an accurate overall estimate, but are aware that the interaction of smoking with other risk factors is complex. For example, smoking has complex, stimulatory effects on the immune system (4) and can enhance inflammatory contributors such as EBV and counteract protective factors such as sun exposure. Smoking has also shown to be associated with the worsening of disability in MS (1) and smoking cessation to be beneficial (2, 18). To date, no study has investigated the smoking AF with regard to disability accumulation in MS. Such study could be highly valuable in informing persons with MS and healthcare providers about benefits of smoking cessation.

Our AF for smoking in MS of 10% to 15% is lower than for other conditions, including lung cancer. (6) However, this proportion, as well as absolute numbers behind it, is nevertheless substantial. Taking the conservative AF of 13%, a minimum 364,000 of the 2.8 million MS cases worldwide could potentially have been prevented. Considering that fear of long-term disability associated with neurological disease is for many people more prominent than fear of cancer, (19) MS is probably a strong deterrent to smoking initiation. Avoidance and prevention-focused educational campaigns may be more successful than cessation campaigns.

This, and the fact that smoking preventability is more achievable than prevention of other risk factors such as EBV infection or its consequence, infectious mononucleosis, qualifies smoking prevention as a key strategy in preventing MS.

In addition, many more MS cases, even if unpreventable, would be likely to have a milder course, given the contribution of smoking to disease severity and progression.

In conclusion, integrated efforts need to be aimed not only at smoking cessation but crucially also at smoking prevention. The former will make relapses and disability progression in MS in part preventable, while the latter will make a substantial proportion of MS a preventable disease.

DATA AVAILABILITY STATEMENT

Data related to the current article are available from Ingrid Kockum, Karolinska Institutet. To be able to share data, a data transfer agreement needs to be completed between Karolinska Institutet and the institution requesting data access. This is in accordance with the data protection legislation in Europe (General Data Protection Regulation [GDPR]). Persons interested in obtaining access to the data should contact AM (ali.manouchehrinia@ki.se).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stockholm ethical regional board at Karolinska Institutet. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM analyzed and interpreted data and wrote and revised the manuscript. JHu analyzed and interpreted data and wrote and revised the manuscript. JHi generated and interpreted data and revised the manuscript. TO generated and interpreted data and revised the manuscript. LA generated and interpreted data and revised the manuscript. IK generated and interpreted data and revised the manuscript. CC wrote and revised

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.840158/full#supplementary-material

REFERENCES

1. Manouchehrinia A, Tench C, Maxted J. Tobacco Smoking and Disability Progression in Multiple Sclerosis: United Kingdom Cohort Study. *Brain* (2013) 136:2298–304. doi: 10.1093/brain/awt139
2. Ramanujam R, Hedström A-K, Manouchehrinia A, Alfredsson L, Olsson T, Bottai M, et al. Effect of Smoking Cessation on Multiple Sclerosis Prognosis. *JAMA Neurol* (2015) 72:1117–23. doi: 10.1001/jamaneurol.2015.1788
3. Olsson T, Barcellos LF, Alfredsson L. Interactions Between Genetic, Lifestyle and Environmental Risk Factors for Multiple Sclerosis. *Nat Rev Neurol* (2017) 13:25–36. doi: 10.1038/nrneurol.2016.187
4. Alrouji M, Manouchehrinia A, Gran B, Constantinescu CS. Effects of Cigarette Smoke on Immunity, Neuroinflammation and Multiple Sclerosis. *J Neuroimmunol* (2019) 329:24–34. doi: 10.1016/j.jneuroim.2018.10.004
5. Poorolajal J, Bahrami M, Karami M, Hooshmand E. Effect of Smoking on Multiple Sclerosis: A Meta-Analysis. *J Public Health (Bangkok)* (2017) 39:312–20. doi: 10.1093/PUBMED/FDW030
6. Hansen MS, Licaj I, Braaten T, Lund E, Gram IT. The Fraction of Lung Cancer Attributable to Smoking in the Norwegian Women and Cancer (NOWAC) Study. *Br J Cancer* (2020) 1243(124):658–62. doi: 10.1038/s41416-020-01131-w
7. Hedström A, Hillert J, Olsson T, Alfredsson L. Smoking and Multiple Sclerosis Susceptibility. *Eur J* (2013) 28:867–74. doi: 10.1007/s10654-013-9853-4
8. Manouchehrinia A, Stridh P, Khademi M, Leppert D, Barro C, Michalak Z, et al. Plasma Neurofilament Light Levels Are Associated With Risk of Disability in Multiple Sclerosis. *Neurology* (2020) 94:e2457–67. doi: 10.1212/WNL.0000000000009571
9. Hillert J, Stawiarz L. The Swedish MS Registry - Clinical Support Tool and Scientific Resource. *Acta Neurol Scand* (2015) 132:11–9. doi: 10.1111/ane.12425
10. Patsopoulos NA, Baranzini SE, Santaniello A, Shoostari P, Cotsapas C, Wong G, et al. Multiple Sclerosis Genomic Map Implicates Peripheral Immune Cells and Microglia in Susceptibility. *Science* (2019) 365. doi: 10.1126/SCIENCE.AAV7188
11. Moutsianas L, Jostins L, Beecham AH, Dilthey AT, Xifara DK, Ban M, et al. Class II HLA Interactions Modulate Genetic Risk for Multiple Sclerosis. *Nat Genet* (2015) 47:1107–13. doi: 10.1038/NG.3395
12. Dahlqwist E, Zetterqvist J, Pawitan Y, Sjölander A. Model-Based Estimation of the Attributable Fraction for Cross-Sectional, Case–Control and Cohort Studies Using the R Package AF. *Eur J Epidemiol* (2016) 316(31):575–82. doi: 10.1007/S10654-016-0137-7
13. Hedström AK, Hillert J, Olsson T, Alfredsson L. Smoking and Multiple Sclerosis Susceptibility. *Eur J Epidemiol* (2013) 28:867–74. doi: 10.1007/s10654-013-9853-4
14. Ramström L, Borland R, Wikmans T. Patterns of Smoking and Snus Use in Sweden: Implications for Public Health. *Int J Environ Res Public Health* (2016) 13:1–14. doi: 10.3390/IJERPH13111110
15. Hedström AK, Olsson T, Alfredsson L. Smoking Is a Major Preventable Risk Factor for Multiple Sclerosis. *Mult Scler* (2016) 22:1021–6. doi: 10.1177/1352458515609794
16. Manouchehrinia A, Tench CR, Maxted J, Bibani RH, Britton J, Constantinescu CS. Tobacco Smoking and Disability Progression in Multiple Sclerosis: United Kingdom Cohort Study. *Brain* (2013) 136:2298–304. doi: 10.1093/brain/awt139
17. Papadakis S, Pipe A, Kelly S, Pritchard G, Wells GA. Strategies to Improve the Delivery of Tobacco Use Treatment in Primary Care Practice. *Cochrane Database Syst Rev* (2015) 2015:1–9. doi: 10.1002/14651858.CD011556
18. Tanasescu R, Constantinescu CS, Tench CR, Manouchehrinia A. Smoking Cessation and the Reduction of Disability Progression in Multiple Sclerosis: A Cohort Study. *Nicotine Tob Res* (2018) 20:585–95. doi: 10.1093/ntr/ntx084
19. Doward J. *Call to End the Stigma of Having a Brain Disorder | Disability | The Guardian*. Available at: https://www.theguardian.com/society/2016/jun/25/disability-victims-suffer-bullying-and-teasing?CMP=gu_com (Accessed December 6, 2021).

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REVIEW
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Secondary Prevention in Radiologically Isolated Syndromes and Prodromal Stages of Multiple Sclerosis

Maria Pia Amato[1,2], Nicola De Stefano[3], Matilde Inglese[4,5], Emanuele Morena[6], Giovanni Ristori[6,7], Marco Salvetti[6,8] and Maria Trojano[9] on behalf of the Italian Study Group for Radiologically Isolated Syndromes and Prodromal Stages of MS*

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Following the extraordinary progress in the treatment of multiple sclerosis (MS), two major unmet needs remain: understanding the etiology of the disease and, hence, designing definitive cures (this perspective is neither at hand, nor it can be taken for granted that the etiologic targets will be readily treatable); the prevention of an overt and disabling disease, which seems to be a more realistic and pragmatic perspective, as the integration of genetic data with endophenotypes, MRI, and other biomarkers ameliorates our ability to identify early neuroinflammation. Radiologically isolated syndrome (RIS; diagnosed when the unanticipated MRI finding of brain spatial dissemination of focal white matter lesions highly suggestive of MS occurs in subjects without symptoms of MS, and with normal neurological examinations) and the recently focused "prodromal MS" are conditions at risk of conversion toward overt disease. Here, we explore the possibility of secondary prevention approaches in these early stages of neuroinflammation. RIS and prodromal MS are rare conditions, which suggest the importance of Study Groups and Disease Registry to implement informative clinical trials. We summarize ongoing preventive approaches in the early stages of the demyelinating process, especially in RIS conditions. Moreover, we highlight the importance of the biomarkers and the predictors of evolution to overt disease, which may be useful to select the individuals at risk of conversion to clinically isolated syndrome (CIS) and/or clinically definite MS. Finally, we illustrate the importance of the endophenotypes to test the frontline immunomodulatory approach for preventive strategies. Future investigations, especially in relatives of patients, based on MRI techniques and biological studies (better with integrated approaches) may provide opportunities to understand the MS early causal cascade and may help to identify a "therapeutic window" to potentially reverse early disease processes.

Keywords: clinically silent demyelination, radiologically isolated syndrome, prodromal multiple sclerosis, endophenotype, preventive approaches clinically silent demyelination, BCG—Bacille Calmette-Guérin vaccine, vaccine, preventive approach

INTRODUCTION

The occurrence of incidental brain white matter lesions suggestive of multiple sclerosis (MS) in subjects who did not have symptoms or signs of MS during their lifetime is well-documented, as it was described several decades ago in several postmortem studies. The widespread use of MRI, as the standard *in vivo* study of central nervous system (CNS) demyelination, has greatly increased the detection of the asymptomatic brain and spinal cord abnormalities of uncertain clinical significance. In 2009, Okuda et al. neologized formally this entity, using the term radiologically isolated syndrome (RIS) (1). More recently, the concept of prodrome (an early set of signs, symptoms, or other findings that occur before the onset of the typical disease features) has begun to be considered in MS (2), thanks to several investigations based on population-based studies and biomarkers of early CNS damage (refer below).

The present review will focus on these conditions potentially leading to overt MS, to consider current attempts of secondary prevention, in the absence of an etiologic therapy. The possible integration of genetic data with endophenotypes, MRI data, and other biomarkers seems to promise fruitful approaches to the aim of counteracting the development of the overt disease. To provide a survey on these topics, we searched PubMed for all articles published from database inception to September 1, 2021, with no language limitations. Keywords included clinically silent demyelination, prodromal MS, RIS, subclinical MS, endophenotype of MS, MS prevention.

CLINICALLY SILENT DEMYELINATION

Neuropathological studies demonstrated that brain demyelination might remain clinically silent for the whole lifetime in a significant proportion of people (about 0.1–0.3% of the autopsies in those studies) (1). The location of lesions in clinically silent areas, the low degree of inflammation, or even a particularly effective individual response to injury (e.g., functional compensatory adaptation, neuronal plasticity, and repair) might explain the absence of clinically relevant signs of MS in those subjects. However, caution is needed when interpreting these data, as it is difficult to ascertain whether these subjects were truly asymptomatic with normal neurological examination during their life (3). A recent study demonstrated, for example, that 33% of patients consulting for a first demyelinating event had prior symptoms suggestive of central nervous system (CNS) demyelination that had gone unnoticed (4). Moreover, the samples included in the studies were not representative of the general population due to selection bias toward those who were subjected to autopsy. Finally, such figures are probably underestimated nowadays, in view of an increasing prevalence and incidence of MS.

The advent of MRI and its development as the most sensitive and prominent paraclinical tool for the evaluation of morphologic brain abnormalities has modified our perspective on the occurrence of incidental brain findings. In a large meta-analysis (more than 15,000 subjects from 16 studies), the prevalence of neoplastic and non-neoplastic incidental findings on brain MRI was 2.7%, with the observed incidence increasing with age (5). Among those, only <0.1% could be interpreted as inflammatory–demyelinating lesions if white matter hyperintensities of suspected cerebrovascular origin were excluded. Similar prevalence for an MRI pattern suggestive of MS was found in a recent study that performs a systematic revision of the MRI scans and related clinical charts (1,907 individuals) in a high-incidence region for MS (6). These figures are higher, however, in asymptomatic first-degree relatives of both patients with sporadic MS (4%) and families with members affected by MS (10%) (7). In a recent prospective population-based study, incidental findings on brain MRI necessitating further diagnostic evaluation, but mostly without direct clinical consequences, were found in over 3% of the general middle-aged and elderly population, although no case with demyelinating lesions was reported (8).

According to *ex vivo* and *in vivo* data, the occurrence of silent demyelination should be, therefore, considered uncommon in clinical practice, with a higher occurrence in specific conditions such as family members of patients with MS. However, it must be stressed that the growing use of MRI has significantly increased the probability to find asymptomatic intracranial abnormalities of potential clinical significance, which includes silent demyelination, in current prospective studies in comparison with previous retrospective or neuropathological studies.

EMERGING EVIDENCE FOR A PRODROMAL PHASE OF MS

The precise etiology of MS is not yet known, although the evidence pertaining to different research fields indicates that genetic and environmental factors interact with each other in a complex manner, which eventually determines an abnormal autoimmune response (9). In particular, the evidence that environmental factors can play a role long before the clinical onset of MS is well-established and suggests the existence of a prodromal phase for the disease. The possibility of a prodrome indicates a window of opportunity to potentially act on early disease processes before the clinical disease becomes evident.

The concept of a prodrome is defined as the time period between the onset of a decline in a baseline level of functioning until criteria for disease diagnosis are met (10). The question of whether there is a prodrome in MS has not been extensively studied so far. Other neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, and other inflammatory autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease, in which several biomarkers are better established, have a more advanced understanding of their prodromal phase than we currently have in MS (11). However, the last 10 years have provided increasing evidence that also MS may have a prodromal phase. Recently, population-based studies have demonstrated that it is possible to objectively measure a symptomatic prodromal period in MS, which may last 5–10 years or perhaps long before the occurrence of classical "MS symptom onset." In one case-control study conducted in Canada

(12), the analysis of health administrative data linked with MS revealed that the use of healthcare services by patients was higher in the 5 years preceding their first clinical demyelinating event than that of controls. In the year before the first clinical demyelinating event, hospitalizations and physician visits were 78 and 88% higher, respectively, for people with MS than for matched controls. Similarly, dispensed prescription medications were 49% higher among patients who went on to develop MS. A subsequent case-control study conducted in the UK revealed a significantly higher number of visits to general practitioners among patients with MS, considering a time window up to 10 years before the first MS record (13).

Regarding the clinical profile of the prodromes, an elevated mental health burden is evident, with more visits to psychiatrists and diagnoses of depression and anxiety. Other issues include pain and headache, gastrointestinal complaints, bladder issues, sleep disturbances, and cognitive problems (11). Low cognitive performance before the onset of typical MS symptoms has been reported in a nested case-control study in Norway in men who entered the mandatory national military service at the age of 18–19 years (2). Moreover, fewer pregnancies and greater use of hormonal contraceptives have also been observed in the 5-year prodromal period, particularly in the year before MS onset, relative to a matched population without MS (12).

However, the possibility of identifying a prodromal syndrome exclusively on a clinical base has to be interpreted critically, as the above observations rely mostly on symptoms that are non-specific and common in the general population as well. To reliably identify a prodromal phase of MS, further research is needed that focuses on the identification of biomarkers. Indeed, biological markers of inflammation or neurodegeneration that indicate preexisting disease provide support to a pathogenic process underlying the prodromal period in MS: serum levels of neurofilament light chain (NfL) were increased up to 6 years before MS onset in 30 MS cases relative to 30 healthy controls (14). Such biomarkers are likely to be critical to distinguish whether or not non-specific symptoms such as fatigue represent the prodromal phase of neurodegenerative disease.

Radiologically Isolated Syndrome as a Rare Condition: The Importance of Study Groups and Disease Registry

The radiologically isolated syndrome is a rare condition, although the exact prevalence of RIS is still unknown. One large hospital-based study in Sweden indicated a prevalence of 0.05% (0.15% among those aged 15–40 years) among 2,105 individuals who underwent MRI for any reason during a 1-year period (15). A meta-analysis that includes about 16,000 individuals with no history of neurological symptoms reported that 0.06% had MRI findings that were suggestive of demyelination (5). It is well-known that subjects with RIS can evolve toward relapsing-remitting (RR) or progressive (PP) MS, as in detail reported in the next paragraph.

The current available national and international MS databases and registries—"big MS data"—constitute the key tools to develop clinical research in the field of rare conditions, to improve healthcare planning and new clinical perspectives based on real-world data (16). By collecting longitudinal data on clinical and MRI disease activity over time, MS registries become crucial in the study of the natural history of patients with RIS, to assess the risk factors associated with the conversion to clinically definite MS, and to identify candidates for possible preventive or therapeutic approaches. MS clinical data sharing initiative has a longstanding tradition in Italy for over 20 years. In 2014, the Italian MS Foundation, in collaboration with the University of Bari, promoted the creation of the Italian MS Register (17). Currently, it is one of the largest registers in Europe, with 118 MS centers, that provided data of 72,202 patients (about 50,000 of them with a longitudinal follow-up >5 years) in different phases of the disease, which include RIS subject (**Figure 1**).

Registries can also offer the opportunity of including longitudinal evaluation of neuropsychological testing, quantitative MR metrics, and biological markers in subjects with RIS suggestive of MS. An Italian study on patients recruited from 5 MS centers highlighted that cognitive impairment of the same profile as that of RRMS was found in 27.6% of subjects with RIS, and comparable levels of MRI lesion loads and brain atrophy were found in RIS and RRMS (18). In a more recent analysis of prospectively collected data from a population-based registry of the MS Center of Tel Aviv (19), cognitive performance was relatively preserved in RIS subjects, although all cognitive measurements, in particular those related to information processing speed, were below the mean performance of age- and education-matched healthy population. The crucial assumption of this article was that the cognitive performance of RIS subjects should be followed closely to identify any changes that may indicate conversion to MS.

Predictors of Evolution in RIS

There is an ever-growing effort for improving the characterization of RIS and identifying predictors of clinical and radiological evolution. This is important not only to prevent diagnosis but also to improve knowledge on prognosis and provide guidelines for surveillance or prophylactic treatment.

The most important risk factors for an initial clinical event have been identified by the collective effort of the Radiologically Isolated Syndrome Consortium (RISC), which led to two main reports related to the 5- and 10-year risk of developing a first clinical event. Specifically, the estimated 5-year risk of developing a first clinical event was 34% in a cohort of 451 RIS subjects (86% women) with a mean age at RIS diagnosis of 37.2 years. The independent predictors were male gender, young age (age $\leq$37 years), and the presence of MRI spinal cord lesions at baseline (20). Fifteen patients from the same cohort evolved to primary progressive MS (PPMS) in a median time of 3.5 years. Male patients with older age and a higher number of spinal cord lesions were at higher risk of PPMS evolution (21).

The cumulative probability of a first clinical event at 10 years was 51.2% in the same cohort. The independent predictors of a subsequent clinical event were age, the presence of cerebrospinal fluid (CSF)-restricted oligoclonal bands (OB), MRI infratentorial lesions, and spinal cord lesions at baseline and gadolinium-enhancing lesions during follow-up (22). The same group

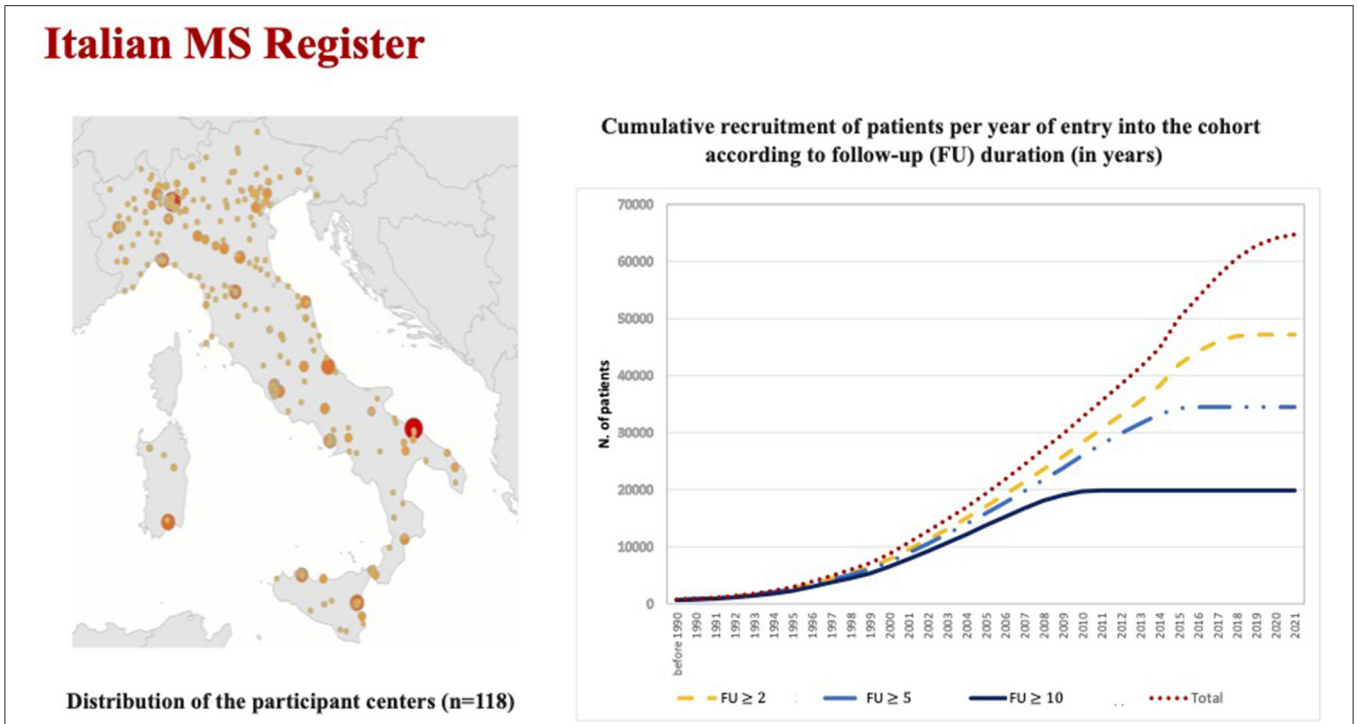

FIGURE 1 | The figure on the right reports the increasing temporal trends of the total cohort and sub-cohorts with different follow-up duration (≥ 2.0 years: n = 47,161, ≥ 5.0 years: n = 34,488, and ≥ 10 years: n = 19,873).

recently confirmed the predictors of 10-year conversion to MS and reported data relevant to the number of enrolled patients needed to detect a potential treatment effect (23).

Furthermore, a study conducted in an international historical cohort of 61 children with RIS who were followed longitudinally for a mean of 4.2 ± 4.7 years further enforced the importance of CSF OB whose presence increased the specificity of MRI criteria to predict MS in children with RIS (24).

A recent study employed optical coherence tomography (OCT), a non-invasive imaging technique that uses light waves to take cross-section pictures of the retina, to investigate whether it plays a role as a predictor of evolution in individuals with RIS. A total of 36 RIS subjects were followed up for a mean of 46 [26–58] months; the eight RIS subjects who converted to MS showed a thinning of the peripapillary retinal nerve fiber layer (pRNFL). Specifically, subjects with a pRNFL of 99 μm or lower were at a 7.5-fold risk for MS conversion compared to individuals with higher pRFNL measures. The Cox proportional hazards regression revealed a hazard ratio of 1.08 for conversion to MS for each 1 μm decline in pRNFL, which suggests that OCT might be useful for risk stratification in RIS subjects (25). Similar results were reported in a work showing that OCT can be potentially useful for predicting prognosis in RIS, being OCT measurements associated with brain volumetrics and clinical conversion to MS (26).

Among biomarkers explored as the potential predictors of RIS evolution, levels of CSF IL8, a marker of diffuse intrathecal inflammation, and CSF NfL levels have shown some promising results. A small study that includes 18 RIS subjects showed higher CSF IL8 levels in MS converters than in non-converters (p = 0.03). Moreover, in the multivariate regression model including known predictors such as age, gender, and the presence of spinal cord lesions, a high level of CSF IL8 was an independent predictor of MS conversion in RIS (p = 0.02) (27). A larger study investigated the prognostic role of chitinase 3-like 1 (CHI3L1), NfL, and OB for conversion to clinically isolated syndrome (CIS) and MS in 75 RIS subjects. In contrast to CHI3L1, which did not show any influence on clinical conversion, NfL levels and OB were the independent risk factors for the development of CIS and MS. Fixing the best cutoff at a CSF NfL level of 619 ng/l, higher values were associated with a trend to shorter time to CIS (p = 0.079) and a significantly shorter time to MS (p = 0.017), which supports the importance of CSF analysis in individuals with RIS (28).

The available data provide evidence that a meaningful number of RIS subjects evolve to MS and support the need for standardized biomarkers to identify those subjects at greatest risk for conversion to MS who need appropriate clinical and treatment management.

Ongoing Preventive and Therapeutic Approaches in RIS

Following the extraordinary progress in the treatment of overt MS, a major unmet need remains for translational research: preventing or significantly slowing the disease onset or the progression of the neuroinflammatory process to aim at the

"dream" of a world free of MS. We can hope to make the dream come true by understanding the etiology of the disease and hence designing definitive cures. Unfortunately, this perspective is neither at hand, nor it can be taken for granted that the etiologic targets, once discovered, will be readily treatable. A more realistic and pragmatic perspective may be the prevention of the clinical onset of the disease, a research field that promises to become increasingly important as the integration of genetic data with endophenotypes, MRI, and other biomarkers ameliorates our ability to act before the development of the overt disease (refer to next paragraph).

Radiologically isolated syndrome falls within the endophenotypes and thus offers the opportunity to try to prevent the onset of the clinical demyelinating disorder. The best approach to this aim remains an object of controversy. In particular, whether or not to treat the RIS remains currently a clinical conundrum: several recommendations and guidelines have been published (29–31). To summarize the various standpoints, the absence of clinical disturbances suggests caution against interventions; on the other hand, the presence of OB in the CSF or signals of progression at MRI (besides the above-reported predictors of conversion to clinical disease) prompt MS specialists to consider disease-modifying therapies (DMTs) presently used for CIS or MS.

Three therapeutic approaches are currently registered at ClinicalTrials.gov for RIS (whereas no trial comes out for "prodromal MS"): NCT03122652 with teriflunomide, NCT02739542 with dimethyl fumarate, and the recently proposed NCT04877457 with ocrelizumab. Epidemiological data support the view that vitamin D supplementation, prevention of metabolic disorders, and smoking avoidance are candidate approaches for primary and secondary prevention of MS (32). In a recent study, which shows the prevalence of RIS and white matter abnormalities in healthy relatives of patients with MS, smoking was associated with the presence of multiple altered signals in white matter and obesity with the fulfillment of RIS pattern (33). The study, together with evidence coming from numerous epidemiologic investigations in MS people, would suggest preventive attempts in an early condition such as RIS. However, rather unexpectedly, preventive approaches based on vitamin D supplementation, reduction of metabolic pressure by diet, and smoking avoidance are not currently tried in RIS; rather, ongoing registered trials are largely oriented toward DMT used in MS management (see above).

Among other approaches that may have characteristics compatible with a preventive intervention, BCG vaccination has been tested with encouraging results in early MS and CIS (34–36). Italian groups proposed this approach as a sort of secondary prevention (rather than as a treatment) against RIS progression, also given the characteristics of the BCG vaccine, which is safe, cheap, and handy. A phase II, double-blind, randomized, controlled, multicenter study with two parallel groups of subjects (one arm will be vaccinated with a single dose of BCG vaccine and the other with placebo) is currently ongoing (NCT03888924). The rationale includes, among the others, the fact that the BCG vaccine may prevent the development of neuroinflammation by antagonizing the effects of "Westernization." This approach could in fact somehow compensate for the deprivation of benign exposure to microbes and the changes of lifestyle habits that occurred over the last decades of the twentieth century in developed countries. The "Westernization" seems to be associated with the increased incidence of complex diseases, such as cancer, metabolic disorders, and neurodegenerative diseases, and immunopathological conditions, such as autoimmune and atopic disorders (37–39). Recent evidence on the possible preventive effects of the BCG vaccine against the development of neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases, which are supposed not to be of primary inflammatory nature; **Figure 2**) contribute to support this view (40–43).

INCIDENTAL MRI FINDINGS OR SUBCLINICAL MS? THE CONTRIBUTION OF BIOLOGICAL ENDOPHENOTYPES

Since its first description, RIS has been widely debated and the risk of RIS evolving into MS has been investigated. According to existing data (20, 22), several RIS subjects evolve to MS over time, which demonstrates that RIS, at least in some cases, represents a preclinical stage of MS (refer to the paragraph "predictors of evolution in RIS"). The first issue related to this lies in what still needs to be done to provide a more specific characterization of these asymptomatic subjects and accurately discriminate subjects who can have a subclinical form of MS. In this context, assuming a carefully collected clinical history and a meticulous clinical examination, the first step of the management of these asymptomatic subjects is to consider an appropriate differential diagnosis and assess the extent to which MRI lesions fulfilling the RIS criteria in asymptomatic subjects may be related to disorders other than MS. Moreover, the reason for the initial brain MRI should always be carefully considered. Whereas, there are subjects whose brain MRI is performed for reasons which have no relation with CNS or MS, on many occasions, MRI is performed due to symptoms that might be somehow related to MS. Headache is by far the most common reason for performing an MRI (about 50% of cases with RIS; 20), but other relatively less frequent indications for an MRI are also seizures, paroxysmal symptoms, anxiety, depression, and other psychiatric disorders. Whereas, it is not possible to establish whether these conditions are related to the MRI findings, it is also true that they might represent unusual clinical symptoms associated with MS (refer above: prodromal MS).

Another important contribution may come from laboratory studies, which start to delineate biological signatures capable of integrating MRI data to refine the condition of subclinical neuroinflammation. The potential utility of the NfL levels was already reported in the context of the prodromal MS and RIS (refer above). However, this biomarker, though sensitive and able to peripherally mirror CNS tissue damage, is not specific, as demonstrated by studies in other CNS diseases, especially of primary neurodegenerative nature (44).

As anticipated according to MRI data, studies in relatives of MS patients are especially informative to identify MS endophenotypes. A recent system biology approach on

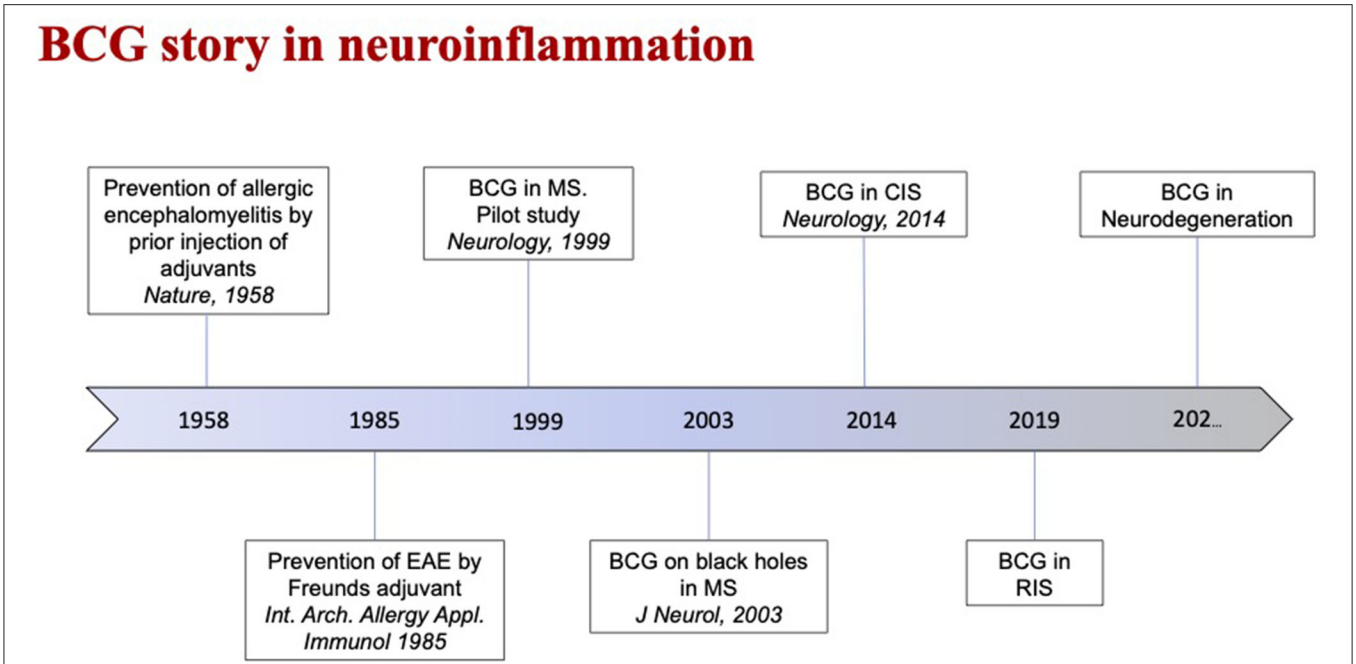

FIGURE 2 | This time-line highlights the evolution of the role of Bacille Calmette-Guérin (BCG) vaccine in neuroinflammatory diseases as Multiple Sclerosis (MS).

peripheral immune signatures in identical twin pairs discordant for MS showed remarkably similar patterns; however, distinct traits in effector CD4+ T-cells in clinically healthy twins, with signs of prodromal MS, were comparable with those of the overtly affected co-twins, suggesting the importance of these immune traits in subclinical neuroinflammation (45). On the same line, increased CSF sulfatide levels and serum autoantibody against glycosphingolipids were reported in healthy siblings of patients MS compared to unrelated healthy donors (46).

Concerning the identification of endophenotypes due to genetic risk, glutamate biology seems to contain relevant biomarkers that pose a risk for disease development. Associations of at-risk single nucleotide polymorphisms (SNPs) with high glutamate concentration in CNS (47) or with brain volume changes in MS (48) were described, and they may contribute to clarifying the MS genetic risk in the "target" organ. Recent development in multiomics approaches have demonstrated alterations in easily accessible fluids: correlating scores of genetic risk and blood analytes, Wainberg et al. showed changes in clinically healthy individuals that mirrored those seen in people with complex diseases, including MS, and that represented early signs of dysfunctions preceding the clinically overt disease (49). Considering the infectious mononucleosis (IM) as a non-genetic risk factor for MS, Jons et al. assayed MS-relevant CSF cyto- or chemokines from non-MS individuals with or without previous IM and MS people as a reference group. They found a stepwise inflammation from IM sequelae to an MS endophenotype in a subgroup of IM patients, which shows CSF changes comparable to those of the MS reference group (50).

CONCLUSION

Extreme caution is needed in classifying RIS subjects. Indeed, it should be stressed that only when these subjects are expertly diagnosed, the stratification of risk can be accurate, and we can thus have sufficient information to be able to differentiate subjects with a form of subclinical MS at low or high risk of developing the disease (22, 23, 29).

Even more, caution is needed to identify a prodromal MS syndrome, which is largely based on symptoms that are non-specific and common in the general population, and that currently lacks objective supports to disentangle a "real" condition preluding to MS. Future advances in MRI techniques, biological studies, and especially integrated approaches to identify and follow individuals at high disease risk are a concrete hope for the identification of the initial phases of the demyelinating process and better MS management. This kind of investigation may provide a powerful opportunity to understand the MS early causal cascade, and, more importantly, may help to identify a "therapeutic window" to potentially reverse early disease processes.

AUTHOR CONTRIBUTIONS

MPA has focused on prodromes, predictors of evolution, and silent demyelination. NDS contributed describing prodromal

phase of MS and silent demyelination. MI reported the role of predictors of evolution in RIS. EM, GR, and MS contributed in treatment and biological endophenotypes description. MT has focused on Study Groups and Disease Registry information. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Okuda DT, Mowry EM, Beheshtian A, Waubant SE E, Baranzini, Goodin DS, et al. Incidental MRI anomalies suggestive of multiple sclerosis: the radiologically isolated syndrome. *Neurology*. (2009) 72:800–5. doi: 10.1212/01.wnl.0000335764.14513.1a
2. Cortese M, Riise T, Bjørnevik K, Bhan A, Farbu E, Grytten N, et al. Preclinical disease activity in multiple sclerosis: a prospective study of cognitive performance prior to first symptom. *Ann Neurol*. (2016) 80:616–24. doi: 10.1002/ana.24769
3. Giovannoni G. How long is the presymptomatic phase of multiple sclerosis? *Mult Scler Relat Disord*. (2016) 7:12–3. doi: 10.1016/j.msard.2016.02.010
4. Gout O, Lebrun-Frenay C, Labauge P, Page GEL, Clavelou P, Allouche S, et al. Prior suggestive symptoms in one-third of patients consulting for a "first" demyelinating event. *J Neurol Neurosurg Psychiatry*. (2011) 82:323–5. doi: 10.1136/jnnp.2008.166421
5. Morris Z, Whiteley WN, Longstreth WT, Weber F, Lee Y-C, Tsushima Y, et al. Incidental findings on brain magnetic resonance imaging: systematic review and meta-analysis. *BMJ*. (2009) 339:b3016. doi: 10.1136/bmj.b3016
6. Forslin Y, Granberg T, Jumah AA, Shams S, Aspelin P, Kristoffersen-Wiberg M, et al. Incidence of radiologically isolated syndrome: a population-based study. *AJNR Am J Neuroradiol*. (2016) 37:1017–22. doi: 10.3174/ajnr.A4660
7. De Stefano N, Cocco E, Lai M, Battaglini M, Spissu A, Marchi P, et al. Imaging brain damage in first-degree relatives of sporadic and familial multiple sclerosis. *Ann Neurol*. (2006) 59:634–9. doi: 10.1002/ana.20767
8. Bos D, Poels MMF, Adams HHH, Akoudad S, Cremers LGM, Zonneveld HI, et al. Prevalence, clinical management, and natural course of incidental findings on brain mr images: the population-based rotterdam scan study. *Radiology*. (2016) 281:507–15. doi: 10.1148/radiol.2016160218
9. Amato MP, Derfuss T, Hemmer B, Liblau R, Montalban X, Soelberg Sørensen P, et al. Environmental modifiable risk factors for multiple sclerosis: report from the 2016 ECTRIMS focused workshop. *Mult Scler*. (2018) 24:590–603. doi: 10.1177/1352458516686847
10. (8) (PDF) The Schizophrenia Prodrome. Available online at: https://www.researchgate.net/publication/7263816_The_Schizophrenia_Prodrome (accessed September 09, 2021).
11. Makhani N, Tremlett H. The multiple sclerosis prodrome. *Nat Rev Neurol*. (2021) 17:515–21. doi: 10.1038/s41582-021-00519-3
12. Wijnands JMA, Kingwell E, Zhu F, Zhao Y, Högg T, Stadnyk K, et al. Health-care use before a first demyelinating event suggestive of a multiple sclerosis prodrome: a matched cohort study. *Lancet Neurol*. (2017) 16:445–51. doi: 10.1016/S1474-4422(17)30076-5
13. Disanto G, Zecca C, MacLachlan S, Sacco R, Handunnetthi L, Meier UC, et al. Prodromal symptoms of multiple sclerosis in primary care. *Ann Neurol*. (2018) 83:1162–73. doi: 10.1002/ana.25247
14. Bjornevik K, Munger KL, Cortese M, Barro C, Healy BC, Niebuhr DW, et al. Serum neurofilament light chain levels in patients with presymptomatic multiple sclerosis. *JAMA Neurol*. (2020) 77:58–64. doi: 10.1001/jamaneurol.2019.3238
15. Granberg T, Martola J, Kristoffersen-Wiberg M, Aspelin P, Fredrikson S. Radiologically isolated syndrome–incidental magnetic resonance imaging findings suggestive of multiple sclerosis, a systematic review. *Mult Scler*. (2013) 19:271–80. doi: 10.1177/1352458512451943
16. Trojano M, Tintore M, Montalban X, Hillert J, Kalincik T, Iaffaldano P, et al. Treatment decisions in multiple sclerosis - insights from real-world observational studies. *Nat Rev Neurol*. (2017) 13:105–18. doi: 10.1038/nrneurol.2016.188
17. Trojano M, Bergamaschi R, Amato MP, Comi G, Ghezzi A, Lepore V, et al. The Italian multiple sclerosis register. *Neurol Sci*. (2019) 40:155–65. doi: 10.1007/s10072-018-3610-0
18. Amato MP, Hakiki B, Goretti B, Rossi F, Stromillo ML, Giorgio A, et al. Association of MRI metrics and cognitive impairment in radiologically isolated syndromes. *Neurology*. (2012) 78:309–14. doi: 10.1212/WNL.0b013e31824528c9
19. Menascu S, Stern M, Aloni R, Kalron A, Magalshvili D, Achiron A. Assessing cognitive performance in radiologically isolated syndrome. *Mult Scler Relat Disord*. (2019) 32:70–3. doi: 10.1016/j.msard.2019.04.030
20. Okuda DT, Siva A, Kantarci O, Inglese M, Katz I, Tutuncu M, et al. Radiologically isolated syndrome: 5-year risk for an initial clinical event. *PLoS ONE*. (2014) 9:e90509. doi: 10.1371/journal.pone.0090509
21. Kantarci OH, Lebrun C, Siva A, Keegan MB, Azevedo CJ, Inglese M, et al. Primary progressive multiple sclerosis evolving from radiologically isolated syndrome. *Ann Neurol*. (2016) 79:288–94. doi: 10.1002/ana.24564
22. Lebrun-Frenay C, Kantarci O, Siva A, Sormani MP, Pelletier D, Okuda DT. Radiologically isolated syndrome: 10-year risk estimate of a clinical event. *Ann Neurol*. (2020) 88:407–17. doi: 10.1002/ana.25799
23. Lebrun-Frénay C, Rollot F, Mondot L, Zephir H, Louapre C, Le Page E, et al. Risk factors and time to clinical symptoms of multiple sclerosis among patients with radiologically isolated syndrome. *JAMA Network Open*. (2021) 4:e2128271. doi: 10.1001/jamanetworkopen.2021.28271
24. Makhani N, Lebrun C, Siva A, Narula S, Wassmer E, Brassat D, et al. Oligoclonal bands increase the specificity of MRI criteria to predict multiple sclerosis in children with radiologically isolated syndrome. *Mult Scler J Exp Transl Clin*. (2019) 5:2055217319836664. doi: 10.1177/2055217319836664
25. Aly L, Havla J, Lepennetier G, Andlauer TFM, Sie C, Strauß E-M, et al. Inner retinal layer thinning in radiologically isolated syndrome predicts conversion to multiple sclerosis. *Eur J Neurol*. (2020) 27:2217–24. doi: 10.1111/ene.14416
26. *Retinal degeneration is associated with brain volume reduction and prognosis in radiologically isolated syndrome - Atay Vural, Serhat Okar, Asli Kurne, Güliz Sayat-Gürel, Nazire Pinar Acar, Erdem Karabulut, Kader Karli Oguz, Sibel Kadayifçilar, Rana Karabudak*. (2020). Available online at: https://journals.sagepub.com/doi/10.1177/1352458518817987?icid=int.sj-full-text.similar-articles.2& (accessed December 29, 2021).
27. Rossi S, Motta C, Studer V, Macchiarulo G, Germani G, Finardi A, et al. Subclinical central inflammation is risk for RIS and CIS conversion to MS. *Mult Scler*. (2015) 21:1443–52. doi: 10.1177/1352458514564482
28. Matute-Blanch C, Villar LM, Álvarez-Cermeño JC, Rejdak K, Evdoshenko E, Makshakov G, et al. Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. *Brain*. (2018) 141:1085–93. doi: 10.1093/brain/awy021
29. De Stefano N, Giorgio A, Tintoré M, Pia Amato M, Kappos L, Palace J, et al. Radiologically isolated syndrome or subclinical multiple sclerosis: MAGNIMS consensus recommendations. *Mult Scler*. (2018) 24:214–21. doi: 10.1177/1352458517717808
30. Yamout B, Sahraian M, Bohlega S, Al-Jumah M, Goueider R, Dahdaleh M, et al. Consensus recommendations for the diagnosis and treatment of multiple sclerosis: 2019 revisions to the MENACTRIMS guidelines. *Mult Scler Relat Disord*. (2020) 37:101459. doi: 10.1016/j.msard.2019.101459
31. Barboza AG, Carnero Contentti E, Curbelo MC, Halfon MJ, Rojas JI, Silva BA, et al. Radiologically isolated syndrome: from biological bases to practical management. *Neurol Sci*. (2021) 42:1335–44. doi: 10.1007/s10072-021-05069-6
32. Belbasis L, Bellou V, Evangelou E, Tzoulaki I. Environmental factors and risk of multiple sclerosis: findings from meta-analyses and Mendelian randomization studies. *Mult Scler*. (2020) 26:397–404. doi: 10.1177/1352458519872664
33. Gabelic T, Ramasamy DP, Weinstock-Guttman B, Hagemeier J, Kennedy C, Melia R, et al. Prevalence of radiologically isolated syndrome and white matter signal abnormalities in healthy relatives of patients with multiple sclerosis. *AJNR Am J Neuroradiol*. (2014) 35:106–12. doi: 10.3174/ajnr.A3653

34. Ristori G, Buzzi MG, Sabatini U, Giugni E, Bastianello S, Viselli F, et al. Use of Bacille Calmette-Guèrin (BCG) in multiple sclerosis. *Neurology*. (1999) 53:1588–9. doi: 10.1212/WNL.53.7.1588
35. Paolillo A, Buzzi MG, Giugni E, Sabatini U, Bastianello S, Pozzilli C, et al. The effect of Bacille Calmette-Guérin on the evolution of new enhancing lesions to hypointense T1 lesions in relapsing remitting MS. *J Neurol.* (2003) 250:247–8. doi: 10.1007/s00415-003-0967-6
36. Ristori G, Romano S, Cannoni S, Visconti A, Tinelli E, Mendozzi L, et al. Effects of Bacille Calmette-Guerin after the first demyelinating event in the CNS. *Neurology*. (2014) 82:41–8. doi: 10.1212/01.wnl.0000438216.93319.ab
37. Rook GA, Ristori G, Salvetti M, Giovannoni G, Thompson EJ, Stanford JL. Bacterial vaccines for the treatment of multiple sclerosis and other autoimmune disorders. *Immunol Today*. (2000) 21:503–8. doi: 10.1016/S0167-5699(00)01700-X
38. Rook GA. Regulation of the immune system by biodiversity from the natural environment: an ecosystem service essential to health. *Proc Natl Acad Sci USA*. (2013) 110:18360–7. doi: 10.1073/pnas.1313731110
39. Bove RM. Why monkeys do not get multiple sclerosis (spontaneously): an evolutionary approach. *Evol Med Public Health*. (2018) 2018:43–59. doi: 10.1093/emph/eoy002
40. Yong J, Lacan G, Dang H, Hsieh T, Middleton B, Wasserfall C, et al. BCG vaccine-induced neuroprotection in a mouse model of Parkinson's disease. *PLoS ONE*. (2011) 6:e16610. doi: 10.1371/journal.pone.0016610
41. Laćan G, Dang H, Middleton B, Horwitz MA, Tian J, Melega WP, et al. Bacillus Calmette-Guerin vaccine-mediated neuroprotection is associated with regulatory T-cell induction in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *J Neurosci Res*. (2013) 91:1292–302. doi: 10.1002/jnr.23253
42. Chang SS. Re: Bacillus Calmette-Guérin (BCG) therapy lowers the incidence of Alzheimer's disease in bladder cancer patients. *J Urol*. (2020) 204:1379. doi: 10.1097/JU.0000000000001282.02
43. Gofrit ON, Klein BY, Cohen IR, Ben-Hur T, Greenblatt CL, Bercovier H. Bacillus Calmette-Guérin (BCG) therapy lowers the incidence of Alzheimer's disease in bladder cancer patients. *PLoS ONE*. (2019) 14:e0224433. doi: 10.1371/journal.pone.0224433
44. Gaetani L, Parnetti L, Calabresi P, Di Filippo M. Tracing neurological diseases in the presymptomatic phase: insights from neurofilament light chain. *Front Neurosci*. (2021) 15:549. doi: 10.3389/fnins.2021.672954
45. Gerdes LA, Janoschka C, Eveslage M, Mannig B, Wirth T, Schulte-Mecklenbeck A, et al. Immune signatures of prodromal multiple sclerosis in monozygotic twins. *Proc Natl Acad Sci USA*. (2020) 117:21546–56 doi: 10.1073/pnas.2003339117
46. Haghighi S, Lekman A, Nilsson S, Blomqvist M, Andersen O. Increased CSF sulfatide levels and serum glycosphingolipid antibody levels in healthy siblings of multiple sclerosis patients. *J Neurol Sci*. (2013) 326:35–9. doi: 10.1016/j.jns.2013.01.007
47. Baranzini SE, Srinivasan R, Khankhanian P, Okuda DT, Nelson SJ, Matthews PM, et al. Genetic variation influences glutamate concentrations in brains of patients with multiple sclerosis. *Brain*. (2010) 133:2603–11. doi: 10.1093/brain/awq192
48. Strijbis EMM, Inkster B, Vounou M, Naegelin Y, Kappos L, Radue E-W, et al. Glutamate gene polymorphisms predict brain volumes in multiple sclerosis. *Mult Scler*. (2013) 19:281–8. doi: 10.1177/1352458512454345
49. Wainberg M, Magis AT, Earls JC, Lovejoy JC, Sinnott-Armstrong N, Omenn GS, et al. Multiomic blood correlates of genetic risk identify presymptomatic disease alterations. *Proc Natl Acad Sci USA*. (2020) 117:21813–20. doi: 10.1073/pnas.2001429117
50. Jons D, Zetterberg H, Malmeström C, Bergström T, Axelsson M, Blennow K, et al. Intrathecal immunoreactivity in people with or without previous infectious mononucleosis. *Acta Neurol Scand*. (2020) 161–8. doi: 10.1111/ane.13280

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REVIEW
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Vitamin D as a Risk Factor for Multiple Sclerosis: Immunoregulatory or Neuroprotective?

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Vitamin D insufficiency during childhood has been linked to the development of multiple sclerosis (MS), typically an adult-onset inflammatory demyelinating disease of the central nervous system (CNS). Since vitamin D was known to have immunoregulatory properties on both innate and adaptive immunity, it was hypothesized that low vitamin D resulted in aberrant immune responses and the development of MS. However, vitamin D receptors are present on many cell types, including neurons, oligodendrocytes, astrocytes and microglia, and vitamin D has profound effects on development and function of the CNS. This leads to the possibility that low vitamin D may alter the CNS in a manner that makes it vulnerable to inflammation and the development of MS. This review analysis the role of vitamin D in the immune and nervous system, and how vitamin D insufficiency in children may contribute to the development of MS.

Keywords: multiple sclerosis, vitamin D, neuroprotection, immune regulation, oxidative stress

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INTRODUCTION

The importance of vitamin D (VitD) in health was formally recognized in 1922 when it was determined that cod liver oil and sunlight cured rickets (1, 2). Our understanding of the role of VitD has expanded well beyond bone health with the observation that VitD receptors (VDR) are widely expressed on many cell types in many tissues. VitD is a fat-soluble vitamin with limited availability in foods. Thus, the predominant source of VitD is synthesis in the skin after sun exposure. VitD is a steroid hormone that regulates numerous genes important in cell differentiation, proliferation and homeostasis. Unfortunately, VitD deficiency is prevalent worldwide, with infants, pregnant women, the elderly, and dark-skinned individuals being the most affected (3). There is substantial literature describing the importance of VitD as an immune regulator with a growing body of evidence on the importance of VitD on the development and function of the nervous system. While VitD deficiency has been correlated with a variety of human diseases, there is substantial evidence that VitD deficiency, particularly in children, may be a major risk factor for the development of multiple sclerosis (MS), which is typically diagnosed in young adults. Historically, it has been postulated that low VitD may be promoting a dysregulated and/or hyperactivated immune system that leads to CNS inflammation. However, the fact that low VitD in children appears to be more closely associated with MS than other autoimmune diseases suggests that VitD insufficiency may be playing an important role in the central nervous system (CNS), making it more vulnerable to inflammation. Thus, VitD deficiency in children may be contributing to the risk of developing MS as an adult by dysregulation of genes in both the immune system and CNS.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an inflammatory, demyelinating disease affecting an estimated 2.8 million people worldwide (4). Clinically, MS is characterized by relapsing and progressive neurological dysfunction. In most patients, the disease begins with episodes of neurological dysfunction followed by complete or partial remission— the relapsing/remitting form of the disease (RRMS). In some RRMS patients, the disease is later transformed into uninterrupted progression of neurological deficits — the secondary progressive phase of the disease (SPMS). Other patients' disease initiates with a slow, progressive accumulation of neurological dysfunction — primary progressive multiple sclerosis (PPMS) (5). Pathologically, MS is characterized by focal plaques of demyelination with activated microglia and abundant peripheral inflammatory cells in the CNS. The cause of the disease is unknown and therapies are limited to disease modifying medications that reduce the number inflammatory lesions and slow disease progression.

Geographical Distribution of MS

Many decades ago it was found that MS has the lowest frequency along the equator, and increases prevalence with increasing latitude (6). The relationship between latitude and MS risks has been observed in several studies. MS frequency among French farmers displayed a north-south gradient and was inversely correlated with exposure to sunlight, though the gene pools and life styles of individuals were broadly comparable (7). UK migrants who live in Tasmania in the south had greater MS frequency than those that migrated to tropical Queensland (8). Although MS risks seem to decrease with migration from high to low latitudes (9), the timing of migration has critical effects on this change. Migration studies have shown that people who are younger than 15 years at the time of migration tend to adopt MS risks of the country to which they migrate, whereas those who are older than 15 years retain similar incidence as their country of origin. A recent study in New Zealand confirmed the latitude gradient, but also found that the prevalence gradient was strongest at birth (10). A comprehensive meta-analysis of 94 studies published through 2018 confirmed the latitude gradient in MS (11). Analysis of sun exposure based on age found that living in an area with high UV-B before MS onset was associated with a 45% lower risk of MS, and a 51% reduction in risk when living in a medium to high UV-B area from 5-15 year of age (12). Overall, the risk of developing MS is largely determined before the age of 15 years (13–17) or at least within the first two decades (18), suggesting a role for the environment in modifying MS risks during childhood and adolescence.

Genetic and Environmental Risk Factors in MS

The cause of MS is unknown. It remains unclear what triggers the immune system to attack the myelin sheath. Twin studies reveal that genetic factors have important roles in MS risk. The rate of concordance for MS among monozygotic twins is 25–40%, which is much higher than the 5% concordance rate among dizygotic twins (19). Genome-wide sequencing studies have further identified human leukocyte antigen (HLA) class II exerting the strongest association with MS risks (20, 21). On the other hand, the concordance rate among monozygotic twins is not 100%, which means MS risks are not fully determined by genetics.

Unquestionably, the environment is also influential on disease susceptibility. Although the identity of environmental factors involved in MS is not yet unequivocally known, accumulating evidence lends strong supports to several candidates: Epstein-Barr virus (EBV) infection, cigarette smoking and VitD. The relationship between EBV seropositivity and MS risks is now firmly established ($p < 10–23$). Virtually all (99.5%) patients with MS are seropositive for antibodies directed against EBV (22). A recent study analyzed multiple environmental factors that may contribute to MS risk, including VitD levels and EBV antibody titers (23). EBV antibody titers were significantly higher in MS patients and there was an inverse relationship between VitD levels and EDSS, yet no correlation between VitD levels and EBV antibody titers. The prevalence of EBV infection is high (94%) in age/gender-matched controls, so the vast majority of infected individuals do not develop MS, which suggests that EBV infection may be a necessary contributing factor to MS risk but not a cause of MS. For cigarette smoking, a positive association between smoking before age of onset and MS risks is found in some case-control studies (24, 25). Individuals with RRMS have an increased risk of developing SPMS if they have ever smoked, compared with non-smokers (26). These factors— genetics, EBV infection and smoking— may work interactively to determine MS susceptibility, but none of them can fully explain the geographic variations in MS frequency and the changes in risk that occur with migration.

Mouse Model of MS

Much of our fundamental understanding of MS is based on observations in rodent models of MS such as experimental autoimmune encephalomyelitis (EAE). EAE resembles MS in both clinical and pathological aspects (27, 28). Susceptibility to EAE and clinical course vary among strains of mice. For instance, B10.PL and SJL/J mouse strains are two of the more commonly used susceptible strains, whereas BALB/c is much less susceptible (29). SJL/J mice develop a relapsing-remitting disease that can transition into a progressive disease over time, closely resembling RRMS and the transition to the SPMS form (28). C57B/6 mice have become the most utilized EAE model due to the availability of genetically modified mice on the C57B/6 background that allows for defining the role of specific molecules in CNS autoimmunity. The downside of using the C57B/6 mouse model is that disease course and inflammatory components of the lesion have distinct differences from the human condition. Instead, C57B/6 mice develop a rapid-onset, chronic neurological disease without relapses. Furthermore, antibodies and neutrophils contribute significantly to lesion formation which is not typical of MS (30, 31). Some have speculated that C57B/6 EAE may actually be a better model for neuromyelitis optica (NMO), a rare autoimmune neurodegenerative disease very similar in phenotype to MS in which antibodies to aquaporin 4 and neutrophils are known to

contribute to the formation of demyelinating lesions (32). These different EAE models have contributed to our understanding of CNS autoimmunity, yet we should be cognizant of how they may or may not reflect MS.

EAE can be induced by several methods. Active induction of EAE is done by direct immunization with myelin proteins or peptides emulsified in complete Freund's adjuvant. The myelin protein and/or peptide used differs because of variations in MHC between strains of mice. Passive induction of EAE is done by transfer of activated myelin-specific CD4 T cells into a naïve mouse. The myelin-specific CD4 T cells can be generated by immunization with a myelin protein/peptide, followed by removal of the draining lymph nodes, reactivation of the myelin-specific T cells *in vitro*, and injection of the myelin-specific T cells into naïve mice resulting in EAE. Alternatively, T cell receptor transgenic T cells specific for a myelin peptide can be used. For example, CD4 T cells from a T cell receptor transgenic B10.PL mouse in which all the CD4 T cells recognize myelin basic protein (MBP) Ac1-11 peptide can be activated *in vitro* and injected into naïve B10.PL mice, resulting in classical EAE (33–35). In EAE, both myelin-specific Th1 and Th17 cells contribute to pathogenesis, and both cell types have been implicated in MS (35, 36). While myelin-specific T cells can be found in both healthy individuals and MS patients, myelin-reactive CD4 T cells from MS patients have an activated and/or memory phenotype, whereas those cells are naïve in healthy individuals (37–41), supporting the idea that myelin-specific CD4 T cells are contributing to disease pathology in MS.

PHYSIOLOGY OF VITAMIN D

For most people, exposure to sunlight is their major source of VitD. Ultraviolet B (UVB) photolyses 7-dehydrocholesterol to pre-vitamin D3 in the epidermis, which then isomerizes to vitamin D3 (**Figure 1**). VitD can be also obtained from diet through ingestion of vitamin D2 (ergocalciferol) derived from plants, colecalciferol supplements/fortified foods and oily fish. Vitamin D3 in the body then undergoes a series of hydroxylations, first to 25-hydroxyvitamin D3 (25(OH)D3) in the liver, the main circulating form of the vitD with relatively long half-life, and then to biologically active hormone 1,25-dihydroxyvitamin D3 (1,25(OH)2D3, also known as calcitriol) in the kidney (42). 1,25-dihydroxyvitamin D3 is the ligand for VitD receptor (VDR), a member of the nuclear receptor family of transcription factors which activates or represses the expression of many genes (43), and exerts rapid non-genomic effects via the membrane VDR (44).

VitD primarily acts as a hormone that regulates gene transcription. VitD enters cells using carrier proteins or diffusion where it can bind VDR in the cytoplasm (**Figure 2**). VitD/VDR complexes are translocated to the nucleus where VDR dimerizes with retinoid X receptors (RXR). VDR/RXR complexes bind to VitD response elements which are present in nearly 1,000 genes, thus playing a major transcriptional role in many cell types. There are non-genomic roles for VitD which occur within minutes, far too soon to be mediated by altered gene expression. The most noteworthy non-genomic effect of VitD is calcium regulation. VitD binds to protein disulfide isomerase family A, member 3 (PDIA3), resulting in the upregulation of PKA, pI3K and p38MAPK which contribute to the intracellular flux of calcium (**Figure 2**). While changes in intracellular calcium may be independent of VDR engagement, calcium homeostasis is affected by VDR signaling as seen in people with type II genetic rickets and VDR-deficient mice which have severe hypocalcemia (45–48).

Although the best-known function of VitD is to regulate calcium physiology, it also has important effects on the development and function of CNS. Neurons and microglia express VDR. In addition, they can directly metabolize 25(OH)D3 because they express 1-α hydroxylase (49). 1,25(OH)2D3 has been shown to regulate glial cell line-derived neurotrophic factor (GDNF) (50) and nerve growth factor (NGF) (51) expression. The ability of 1,25(OH)2D3 to regulate certain neurotrophic factors and influence inflammation has led to the hypothesis that 1,25(OH)2D3 is neuroprotective (52). In fact, it has shown a reduction in ROS induced cell death and increased anti-oxidant species in glia cell by 1,25(OH)2D3 (53). VitD insufficiency is associated with several other neurological disorders beside MS, including Parkinson disease, schizophrenia, depression and cognitive decline (54), suggesting its essential role in maintaining normal CNS function.

How much VitD is optimal is somewhat controversial. The most commonly published normal range for blood VitD levels is 20–40 ng/mL (50–100 nmol/L) with levels below 20 ng/mL (50 nmol/L) considered VitD deficient. The Endocrine Society considers VitD levels of 20–29 ng/mL (50–74 nmol/L) to indicate VitD insufficiency, and that VitD levels should be 30-100 ng/mL (75–250 nmol/L) for optimal health benefit (55). Based on these guidelines, it is estimated that 30–50% of Americans may be VitD deficient. A New England Journal of Medicine article suggested that VitD deficiency may be overstated, and perhaps our current metrics for VitD-deficiency are incorrect, because of misinterpretation of the Institute of Medicine's reference values (56). It should be noted that most of the studies evaluating VitD levels in health are based on intestinal uptake of calcium and bone health, so it remains unclear what the optimal dose may be for optimal overall health.

VITAMIN D AND MS

One of the strongest correlates of latitude is the duration and intensity of sunlight, and the synthesis of ViD is subsequently affected by ultraviolet B (UVB) radiation. The incidence gradient according to latitude and the effect of migration within genetically uniform groups can be explained by VitD— as the link between latitude and MS risk. The VitD hypothesis is supported by studies of sunlight exposure history. The seasonal fluctuations in VitD levels resulted in decreased VitD concentrations in *utero*, which may contribute the month-of birth effect in MS (57). While not all studies are in agreement, a large meta-analysis found that individuals born in the Spring have a significantly higher risks of developing MS compared to individuals born in the fall

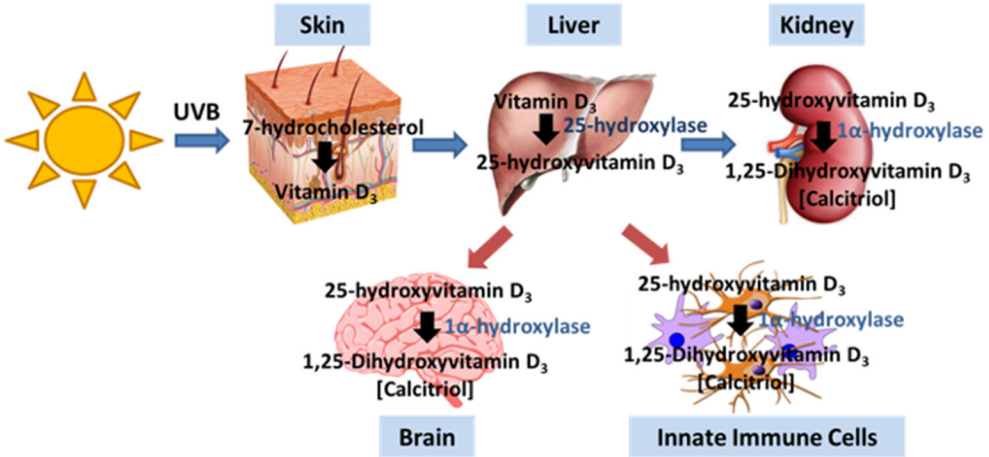

FIGURE 1 | Vitamin D production pathway. Vitamin D3 is produced in the skin by UVB irradiation. Typically, the liver and kidney generate intermediates that ultimately generate calcitriol, the active form of VitD. The brain and immune cells also express the enzyme that allows for the generation of calcitriol.

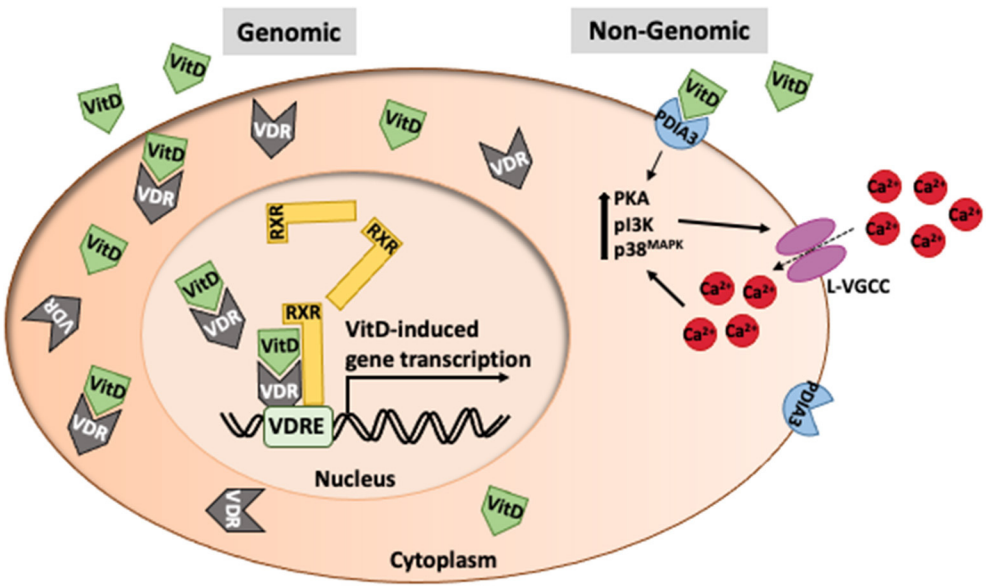

FIGURE 2 | Intracellular function of vitamin D. VitD typically acts as a transcription factor in association with retinoid-X-receptors (RXR) to mediate gene transcription at VitD response elements in promoter regions of genes. However, VitD can have immediate effects on cell function (non-genomic) via interaction with PDIA3 that leads to changes in calcium transport.

(58–60). Insufficient maternal 25-hydroxyvitamin D during early pregnancy is associated with a 2-fold increased risk of MS in offspring (61). Similarly, a Danish study used dried blood spot samples collected near the time of birth to measure VitD in individuals who later developed MS and matched controls (62). Neonatal VitD levels were inversely associated risk of developing MS, supporting the notion that maternal VitD levels may be important to prevent MS in children. Higher sun exposure during childhood (age of 6–15 years) was shown associated with reduced MS risks (63). Time spent on outdoor activities during childhood and adolescence (significant for age of 6–20 years) in the summer was inversely related to the risks, whereas there was no such effect in the winter (64). A study of monozygotic twins who were discordant with MS has shown that twins with MS reported significantly lower levels of childhood sun exposure than their healthy sibling (65). However, sunlight may have benefits to prevent CNS autoimmunity beyond VitD. A study in EAE found that UV light suppressed EAE independent of VitD and VDR (66).

Further evidence for the VitD hypothesis comes from the studies of dietary VitD intake. At high latitudes, prevalence of MS was lower than expected in populations with high

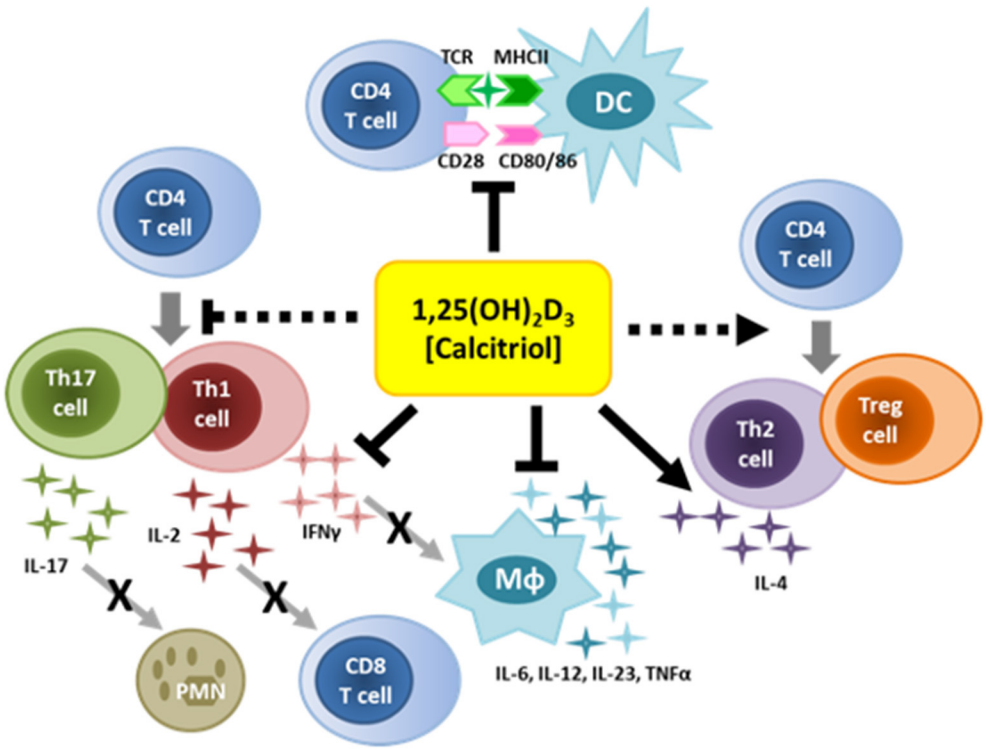

FIGURE 3 | Immunoregulatory functions of vitamin D. VitD can regulate the function of numerous immune cells. VitD suppresses inflammatory cytokines and antigen-presentation by innate immune cells. VitD also suppresses T cell activation, and favors the generation of Th2 cells and Tregs.

consumption of VitD-rich oily fish (67). A 40% reduction in MS risks was found among women who used supplemental VitD, compared with women who did not use supplements (68). Lastly, a study directly measured the circulating 25(OH)D3 (the circulating form of VitD) concentrations in individuals who served in the US military, and concluded that serum level of 25(OH)D3 in healthy young white adults is an important predictor of their risk of developing MS (69). These epidemiology studies (latitude, migration, history of sunlight exposure, VitD intake and serum concentration of VitD) give credibility to the hypothesis that VitD, especially in early life, has protective effect against MS development. Nevertheless, due to often confounding variables in epidemiology studies, prospective experimental studies are needed to validate the effect of VitD in determining MS risks. A mendelian randomization study in which single nucleotide polymorphisms associated with 25-hydroxyvitamin D were identified and analyzed in the International Multiple Sclerosis Genetics Consortium found that there was a significant increased susceptibility to MS in individuals with a genetically lowered level of 25-hydroxyvitamin D (70). This genetic study supports the epidemiology data that optimal VitD levels are protective against the development of MS. Interestingly, a study of polymorphisms in the VitD-binding protein found an association with MS risk in whites, but not blacks or Hispanics, indicating that VitD may not be a significant risk factor in all ethnicities (71).

After disease onset, VitD also acts in modulating MS clinical course. Serum concentrations of 25-hydroxyvitamin D3 in MS patients were lower during relapses than during remissions (72), and correlated inversely with disease severity (73) and frequency of relapse (74, 75). Although these results might indicate lower sun exposure in patients with severe MS rather than a beneficial effect of VitD, convincing studies with EAE have demonstrated the immunomodulatory effect of VitD in inflammatory CNS disease. Expression of VDR has been described in immune cells, including dendritic cells, macrophages and activated T and B cells (76). VitD supplementation clearly suppressed EAE preventively (77, 78) and therapeutically (79). Moreover, the therapeutic effects of VitD required VDR function in T cells (80), and were through promoting IL-4, TGF-β (81) and IL-10 (82) production, and inhibiting TH1 cells differentiation (83, 84). With these results established from EAE, experimental basis supports the beneficial role of VitD in modulating disease progression. Yet, there are numerous studies that indicate that VitD supplementation in MS patients has little, if any benefit to reducing symptoms (85). The SOLAR trial found that VitD supplementation (14,000 IU/d) in MS patients on interferon beta-1a was beneficial in reducing new lesions, but no change in progression of disability or annualized relapse rate was observed (86). In the 2-year CHOLINE trial, MS patients on interferon beta-1a were given 100,000 IU of oral cholecalciferol or placebo biweekly. The end point (changed in annualized relapse rate) was

not met, yet there were positive benefits observed on imaging and the average EDSS score was significantly lower in the treatment group (87). Analysis of 12 random controlled trials evaluating VitD supplementation concluded that VitD supplementation had no significant benefit on relapse rates, progression of disability or MRI lesions (88). Thus, the benefit of VitD supplementation in MS patients is unclear, but given that most MS patients are VitD deficient, supplementation is prudent and likely benefits that overall health of the patients.

VITAMIN D AND THE IMMUNE SYSTEM

The first evidence that VitD may affect the immune system came from a study in 1983 in which VitD promoted the fusion of macrophages (89). In 1986, it was shown that VitD inhibited IL-2 production and proliferation by T cells (90), providing solid evidence that VitD had the capacity to regulate T cells. There is now substantial evidence that VitD is a major regulator of both innate and adaptive immune cells and influences the outcomes of infections, cancer and autoimmunity.

Innate Immunity

Activated macrophages and monocytes upregulate expression of CYP27B1, the gene that encodes 1α-hydroxylase, the enzyme that converts 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 (calcitriol), the active form of VitD (**Figure 1**), indicating that macrophages/monocytes have the capacity to increase VitD at site of inflammation (91). The local expression of VitD by activated macrophages/monocytes sets up an autocrine pathway since macrophages/monocytes express VDR, resulting in the production of anti-microbial products such as cathelicidin and defensins. Cathelicidin is particularly important against infections by destablilizing microbial membranes and disrupting viral envelopes (92–94). Equally important, VitD regulates the maturation and activation of macrophages and dendritic cells, compromising their ability to be effective antigen presenting cells (**Figure 3**). Upon TLR engagement, macrophages and dendritic cells upregulate MHCII, CD80/CD8, CD40 and cytokines which are critical to antigen presentation to T cells. VitD suppresses these molecules, promoting macrophages and dendritic cells that are immature and somewhat tolerogenic (95, 96). This suppression of macrophages and dendritic cells may be due to the suppression of toll-like receptors (97–99) or inhibition of IL-12 via NF-κB (100) mediated by VitD.

VitD affects the function of microglia which are known to secrete inflammatory mediators that contribute to myelin damage during CNS autoimmunity. Mice with EAE treated with calcitriol immediately following EAE induction have reduced microglia activation and oxidative stress, and less blood brain barrier permeability (101). Partial deletion of VDR in young mice attenuated microglia activation and reduced the incidence and severity of EAE (102). In various models of CNS diseases and injury, VitD has been shown to regulate microglia phenotype and oxidative stress (103–106). Thus, vitD appears to effectively skew microglia from a pro-inflammatory M1 phenotype to a reparative M2 phenotype, reducing inflammation and limiting demyelination.

Adaptive Immunity

Both T cells and B cells can express VDR and respond to VitD (**Figure 3**). There is actually very little, if any, VDR expression on resting human T and B cells; however, VDR is rapidly upregulated upon activation (107–109). Similar to innate immune cells, activated T cells express CYP27B1 and can make active VitD. VitD suppresses T cell proliferation via reduced IL-2. In addition, VitD alters the differentiation of CD4 T cells by skewing CD4 T cells toward Th2 and away from Th1 and Th17, the phenotypes associated with MS (110, 111). Also of particular relevance to MS, VitD promotes the differentiation of Tregs (112), which are known to be defective in MS patients (113–118). The mechanism by which VitD promotes Treg development appears to be via altered APCs, since VitD added to human dendritic cells alters glucose metabolism favoring the differentiation of Tregs over Teff cells (119). VitD status in MS patients positively correlates with Treg function, supporting the observation the VitD promotes Treg development (120). VitD in association with CD46 was shown to promote Type I regulatory T cells (Tr1) cells in MS patients (121), indicating that optimal VitD may be an important component of immune regulation.

B cell differentiation and maturation into plasma cells is also regulated by VDR, thus affecting antibody production. In addition, VitD downregulates co-stimulatory molecules on B cells, similar to what is observed for macrophages and dendritic cells (122). Thus, optimal VitD may compromise the ability of B cells to act as antigen presenting cells. B cell –depletion therapies have been very beneficial in the treatment of MS. Given that the beneficial affects appear to be independent of antibody production, there is speculation and evidence that B cells are critical antigen-presenting cells in MS (123–125). An immune profile study on MS patients on B cell depletion therapy indicated that the T cell profile showed a favorable change, reflected by a reduction in memory T cells and an increase in Tregs (124, 125), consistent with the role of B cells as antigen-presenting cells. Thus, low VitD may enhance the ability of B cells to drive the activation and differentiation of T cells, increasing the probability developing MS.

VITAMIN D AND THE CENTRAL NERVOUS SYSTEM

Substantial evidence indicates that VitD acts as a neurosteroid. VDR is expressed throughout the developing and mature brain, including the hippocampus, amygdala, hypothalamus, cortex and cerebellum (49, 126, 127), implicating VitD as an important modulator of gene expression throughout the CNS. Furthermore, 1α-hydroxylase and 25-hydroxylase are both expressed in the brain providing the critical enzymes to generate VitD locally (49, 128). While a major role of VitD is gene regulation, it also has non-genomic functions, particularly regulation of calcium signaling which is critical in normal cellular function.

Vitamin D and Neurogenesis

VitD promotes cell differentiation and apoptosis which are critical for embryonic development. When VitD is removed

during gestation in model systems, multiple regions of the brain have increased cell proliferation and decreased apoptosis, as well as enhance cell proliferation, leading to CNS anomalies (129–131). The increased proliferation was mediated by increased expression of cyclin genes which were regulated by VDR signaling (132), while the reduction in apoptosis may have been due to increased levels of BAX and Bcl-2 (131). Low VitD also leads to more neural stem cells which may be due to a loss of regulation of cell proliferation or a failure in neural stems to efficiently differentiate into neural cell progenitors (133). Changes in brain morphology have been observed in rodents with VitD deficiency (129, 134). In humans, VitD deficiency is associated with decreased brain volume and enlarged ventricles in older adults (135). *Ex vivo* studies illustrated that VitD inhibits the proliferation of hippocampal neurons, while promoting neurite outgrowth (130). Analysis of dopaminergic neurons found that VitD-deficiency reduced Nurr1 and P57kip2 gene expression during embryogenesis which are critical to the development and homeostasis of dopaminergic neurons (136). In addition, VitD has been found to regulate the expression of genes essential in the normal function of dopaminergic neurons (137, 138). Interestingly, while it appears that VitD promotes the differentiation of neurons, astrocyte differentiation appears to be impaired by VitD when using adult neural stem cells (139). VitD also promotes the differentiation of neural stem cells into oligodendrocytes (139), the myelinating cells of the CNS. Oligodendrocyte precursor cells fail to differentiate into oligodendrocytes when VDR signaling is blocked (140). These studies implicate VitD as an essential regulator of neuron and oligodendrocyte development. The signaling between neurons and oligodendrocytes is essential to the development of a properly myelinated CNS during early life, as well as remyelination that occurs following CNS damage.

Functional Consequences of Low VitD in the CNS

In addition to the development and differentiation of CNS cells, VitD plays a role in their ability to function properly. The release of several neurotransmitters is affected by VitD. In dopaminergic neurons, VitD promotes the release of dopamine (141). VitD appears to regulate neurotransmitter synthesis, for example, VitD regulates the inhibitory neurotransmitter GABA, via upregulation of GAD65 and GAD67 (142–144). Neurotrophic factors, which are essential for CNS homeostasis and communication between cells in the CNS, are also regulated by VitD. VitD appears to induce nerve growth factor (NGF) expression in neurons (129, 135). Neural stem cells upregulate brain-derived growth factor (BDNF), Glia cell line-derived nerve factor (GDNF), and ciliary neurotropic factor (CNTF) in the presence of VitD (139). In astrocytes, VitD appears to regulate the expression of neurotrophin receptors, as well GDNF, NT-3 and NT-4 (145–147).

VitD also plays a role in neuronal plasticity. In cultured cortical neurons, VitD increased the expression of microtubule associated protein-2, growth-associated protein-43, and synapsin 1 which are important in synaptic vesicle transport and axonal growth (148). Low VitD during embryogenesis resulted in altered expression of proteins important in cytoskeletal integrity, organelle transport, and synaptic plasticity (149, 150), suggesting that VitD is critical to the normal development and function of the CNS.

Calcium Regulation

VitD plays a vital role in regulating calcium levels in the CNS which is particularly important given that high levels of calcium are neurotoxic. In neurons, VitD modulates L-type voltage-gated calcium channels by downregulation of A1C subunits (151). Mice lacking VitD have upregulated L-type voltage-gated calcium channels and elevated calcium influx in neurons (152) (**Figure 2**). *In vitro* treatment of neurons with VitD downregulated L-type voltage-gated calcium channels and protected neurons from excitotoxicity (153). VitD was shown to very rapidly increase the uptake of extracellular calcium via L-type voltage gated calcium channels (154). Since this occurred within a few minutes, it was clear that the effect was independent of gene transcription. The increase in calcium influx was dependent on the PKA, pI3K, and $p38^{MAPK}$. The non-genomic effects of VitD have largely been attributed to VitD interaction with PDIA3 (also known as $1,25D_3$-Marrs) on the plasma membrane (155, 156). In addition, VitD regulates the expression of numerous genes associated with calcium homeostasis vital to the proper regulation of calcium in the CNS and other tissues (150, 154, 157).

Vitamin D and Neuroprotection

Epidemiological data indicate that VitD has neuroprotective properties. Optimal VitD levels during early life appear to be important to minimize the risk of several psychiatric and neurodegenerative diseases. Schizophrenia, depression and autism spectrum disorders have all been associated with low VitD, particularly during embryogenesis and infancy (158–162). There is an inverse correlation between Parkinson's disease risk and VitD levels (163, 164). Given that VitD protects dopaminergic neurons by upregulating genes numerous genes associated with the function of dopaminergic neurons (137, 138), it is logical that VitD may be a critical factor in minimizing the risk of developing Parkinson's disease. Similarly, Alzheimer's disease patients tend to have low serum VitD levels compared to matched healthy controls (165). The risk of dementia and symptoms of neurodegenerative diseases, such as cognitive and memory impairments and impaired motor function, increases with low serum VitD levels (166–169). Serum VitD deficiency is linked to greater infarct volumes, increased overall stroke severity, and worse long-term outcomes in stroke patients (170–172). Impacts on the risks and outcomes in these neurological conditions are clearly multifactorial, but it stands to reason that VitD plays a role in susceptibility and outcome.

The neuroprotective properties of VitD take effect though several mechanisms. Direct neuroprotective action of VitD is associated with the regulation of neurotrophic factors and reduction in oxidative stress. Neurotrophic factors are critical for the differentiation, survival and maintenance of neural and glial cells. VitD stimulates expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell

line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT3) (173). Neurotrophic factors downregulate their expression into adulthood, therefore remaining levels have critical cell maintenance functions. Reduction in VitD-assisted neurotrophic factor expression due to deficiency may leave neurons more vulnerable to insult.

Neurons are particularly susceptible to oxidative damage because of increased oxygen consumption, bi-products of neurotransmitter production, excitotoxicity and high overall lipid content (174). Additional neuroinflammation will increase the reactive oxygen species (ROS) load. Adequate VitD levels downregulate intracellular oxidative-stress related activities, while suboptimal levels result in increased oxidative damage and neuronal apoptosis (175). Increased reactive oxygen production has been implicated in the pathogenesis of multiple neurodegenerative conditions, including Parkinson's disease (176), Alzheimer's disease (177), Huntington's disease (178), stroke (179) and Multiple Sclerosis (180), and suboptimal serum VitD levels have been linked to these conditions. VitD is a potent regulator of the nuclear factor erythroid-2-related factor 2 (Nrf2) antioxidant pathway in neurons and glial cells, and intracellular Nrf2 levels are inversely correlated with the accumulation of mitochondrial ROS. Within the CNS, upregulation of Nrf2 target genes superoxide dismutase (SOD), catalase (CAT) and heme oxygenase-1 (HMOX1) to make neurons more resistant to oxidative insults (181). Furthermore, neurotrophic signaling pathways, such as the BDNF-TrkB pathway that is essential for mature neuron survival and normal function, also signal Nrf2 activation (182, 183). VitD then has double the influence on neuronal survival – first in neurotrophic action by increasing levels of BDNF, and second in oxidative defense by direct activation of Nrf2. The neuroprotective properties of VitD center around its antioxidant function, and in conjunction with neurotrophic factor expression, likely enhances neural defense and repair mechanisms.

Vitamin D as a Neuroprotective Agent in MS Through Antioxidant Pathways

Oxidative stress and mitochondrial dysfunction are prominent features of MS. T cells can produce ROS and T cell activity and proliferation are influenced by ROS, adding an increased level of complexity to the impact of ROS in MS (184). Activated microglia and macrophages are the major contributors to the elevated ROS observed in the disease. These cells produce oxidating radicals such as superoxide, hydrogen peroxide and nitric oxide with the help of ROS-generating enzymes, such as myeloperoxidase, NADPH oxidase and nitric oxide synthase. ROS have shown to mediate demyelination in both MS and its animal models (184, 185). Studies in postmortem brains of MS patients have identified myeloperoxidase expression in activated macrophages and microglia near lesions. Elevated expression of myeloperoxidase was detected in demyelinated regions of postmortem MS brain homogenates when compared to unaffected regions from the same individual (186, 187). Other markers of oxidative damage, such as 4-hydroxy-2-noneal (4-HNE), produced by lipid peroxidation of cell membranes, and nitrotyrosine, the product of nitric oxide and superoxide reactions, increase and accumulate in macrophages and astrocytes in MS lesions (188–190).

ROS in later stages of MS stems from mitochondrial dysfunction within neurons themselves. Mitochondrial dysfunction and associated ROS have been implicated in non-inflammatory mediated axonal degeneration that occurs with chronic demyelination. It is believed that mitochondria become taxed after sodium channel redistribution in response to demyelination. Sodium channel redistribution causes large influxes of sodium, taxing the ATP dependent sodium-potassium pump (191). Increased ATP needs trigger mitochondria production and proliferation, resulting in increased ROS (192, 193). Notably, increased mitochondrial heat shock protein 70, a marker of mitochondrial stress, has been observed in astrocytes and axons within MS lesions (192). There is some controversy regarding whether increased ROS from mitochondria exists primarily due to mitochondrial proliferation and ATP production after demyelination (chronic injury) or if mitochondria actually acquire oxidative damage during the inflammatory stage of the disease (acute injury) (194).

Antioxidant enzymes are the endogenous ROS defense system in the CNS. ROS exposure activates Nrf2, which then translocates to the nucleus and activates antioxidant response element promoters (ARE) for antioxidant enzyme production. Expression of hundreds Nrf2 responsive antioxidant genes have already been identified (195). Numerous studies have suggested a role for Nrf2 inactivity in the pathogenesis of MS. Nrf2 knock-out EAE mice experience more rapid disease onset, a more severe clinical course, increased glial activation, increased pro-inflammatory cytokine expression and increased axonal degeneration compared to Nrf2 inclusive controls (196–198). Conversely, increasing the activity of Nrf2 in the CNS of EAE mice lessened the clinical severity (199). In postmortem brain tissue of MS patients, NRF2 is strongly upregulated in active MS lesions and expression is most pronounced in degenerating neurons and glial cells, including oligodendrocytes (200, 201).

NRF2 activity is already relevant in MS clinical treatment. Both VitD and dimethyl fumerate (DMF or Tecfidera^{TM}) are NRF2 activators. DMF treatment is an approved oral RRMS therapy known to reduce disease activity and progression, and accomplishes these outcomes via immunomodulatory and neuroprotective mechanisms (202–207). VitD and DMF both signal through the NRF2/KEAP1 pathway to generate antioxidant action and specifically increase glutathione signaling for neuroprotection. DMF and VitD can also exert protective effects by reducing proinflammatory cytokine expression and increasing neurotrophic factor expression (208). DMF and VitD derivatives have demonstrated a cooperative effect on increased VDR expression and Nrf2 activity that limit leukemia progression (209). Although mechanisms of action overlap, DMF is a safer therapeutic option for avoiding calcemic toxicity that can occur with long-term use of high levels of VitD. In fact, excess VitD can exacerbate EAE, emphasizing the point that a measured approach to VitD-based therapies is critical (210). It should also be noted that melatonin which is produced during the dark has similar anti-oxidant properties as VitD in

EAE (211, 212). There is contradicting data on the relationship between melatonin and VitD, yet both appear to be beneficial in reducing CNS inflammation. Thus, the interplay between appropriate sunlight to optimize VitD levels and appropriate darkness to optimize melatonin to maintain circadium rhythm should be considered in strategies to prevent and/or treat MS. It is unlikely that DMF mimics every function of VitD, but similarities in function between DMF and VitD suggest that VitD has a critical role as an endogenous neuroprotective mediator in MS.

An important consideration is the temporal influence of VitD, NRF2 activity, and the endogenous antioxidant system during the course of MS. Upregulated expression of NRF2 in MS brain lesions suggests that the NRF2 pathway is already highly active in distressed cells (200, 201) and it appears that endogenous antioxidant mechanisms are not enough to halt demyelination and axonal degeneration at end stages of disease. However early in the disease, VitD and NRF2 signaling may have increased potential for neuroprotection because less neuroinflammatory-induced oxidative damage has occurred. Understanding the neuroprotective potential of VitD in early stages of MS is complicated by the striking frequency of VitD deficiency in patients at the time of diagnosis. Therefore, whether preventing VitD deficiency prior to disease onset can enhance neuroprotection and alter disease progression warrants further investigation.

IMMUNOREGULATION VS. NEUROPROTECTION

MS is a complex disease in which immune and nervous system components interact to form and sometimes, resolve CNS inflammatory, demyelinating lesions. Genetic studies have largely implicated immune genes as susceptibility factors, supporting the hypothesis that MS is an immune-mediated disease and that the CNS is the unfortunate target of the aberrant immune response. There is significant data to support that VitD has a profound immunoregulatory role on both innate and adaptive immune cells, indicating that low VitD may alter normal immune regulation leading to autoimmunity. Given that the CNS is the sole target of the aberrant immune response in MS, it is important to consider if the CNS is somehow more vulnerable to inflammation in some individuals that may make them at an increased risk of developing MS. While the answer is still unknown, the literature clearly indicates that VitD impacts CNS development and function.

The epidemiology studies indicate that optimal VitD is particularly important during embryogenesis and childhood in determining risk of developing MS. During childhood, our immune system is repeatedly being challenged by pathogens, most of which are cleared with minimal clinical consequences. Some childhood viruses, such as Epstein-Barr, varicella zoster, and some strains of influenza, can infect neurons, yet they typically do not cause clinical CNS manifestations. Even in the absence of CNS clinical signs of illness, do these viruses affect the CNS differently in children with low VitD? Our recent study in which partial VDR deletion was induced in young mice specifically in neurons resulted in an enhanced susceptibility to EAE in adult mice, suggesting that low VitD signaling in neurons in early life may increase the vulnerability of the CNS to inflammation (102). There is substantial evidence that viral infections are affected by VitD levels. Of particular interest in MS is EBV which has been long speculated to be a vital risk factor for the onset of disease and this has been confirmed in a new study of >10 million young adults (213). While many theories have been explored, a recent study identified EBV infection as a precipitating factor that may be driving molecular mimicry (214–217). EBV antibody titers are negatively correlated with VitD levels in MS patients (218) suggesting that these two environmental factors may be synergistic. EBV is also associated with activating endogenous retroviruses (ERVs) and ERV levels in MS plaques correlates with disease activity (219). These ERVs may act as novel antigens that drive CNS inflammation. VitD supplementation mitigates EBV reactivation (220), which may in turn limit ERV activation and the associated inflammation. In a humanized mouse model, it was shown that HLA-DR15-restricted T cells fail to control EBV infection, suggesting that there is potential relationship between the strongest genetic factor (HLA-DR15) and EBV with respect to MS risk (221). The relationship between ERVs, VitD and MS has become of increasing interest and some speculate that EBV may be the missing link between ERVs and VitD that trigger the development of MS (222). While many autoimmune diseases have been associated with low VitD to some extent, the epidemiology data in MS is far more convincing suggesting that low VitD is likely increasing the risk of developing MS due to the negative impact on immune regulation and CNS homeostasis.

A recent study of >1,900 subjects demonstrated that sun exposure negatively correlated with development of MS, and high VitD levels (>30.31 ng/mL) in MS patients reduced the risk of relapses and accumulation of disability (223). The evidence that VitD level is important in risk of developing MS and disease severity appears well established, yet questions still remain as to whether VitD supplementation is beneficial to patients with MS. Since most MS patients have low VitD levels, supplementation is now common practice. Perhaps the more important question is how do we prevent low VitD? Although VitD is currently a common food supplement in many countries, it is unclear whether we are doing enough to ensure that children are getting sufficient VitD. Rickets is rare in countries in which food is supplemented with VitD, indicating that the levels of VitD provided via food supplementation is sufficient for bone health. However, it is unclear whether these levels of VitD are adequate for optimal neuroprotection, given that countries like the United States still have a high incidence of MS. Additional VitD supplementation may be essential for children in higher latitudes where sunlight is very limited for several months each year, and perhaps in children with a family history of MS in which genetic risks are highest. We should also balance pros and cons of sunscreen which

reduces UVB induced VitD synthesis by 95% and may negatively impact health of our immune and nervous systems. Sunlight remains the best source of VitD so ensuring that children play outside daily may be the best solution to the epidemic of low VitD.

AUTHOR CONTRIBUTIONS

AL-R, SG, PL, and ES each reviewed the literature and wrote sections of the manuscripts. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Chick H, Dalyell EJH, Hume EM, Mackay HMM, Henderson-Smith H. The aetiology of rickets in infants: prophylactic and curative observations at the Vienna University Kinderklinik. *Lancet*. (1922) ii:7–11. doi: 10.1016/S0140-6736(01)00835-2
2. McCollum EV, Simmonds N, Becker JE, Shipley PG. Studies on experimental rickets. XXI An experimental demonstration of the existence of a vitamin which promotes calcium deposition. *J Biol Chem.* (1922) 53:293–312. doi: 10.1016/S0021-9258(18)85783-0
3. Lips P. Worldwide status of vitamin D nutrition. *J Steroid Biochem Mol Biol*. (2010) 124:197–300. doi: 10.1016/j.jsbmb.2010.02.021
4. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the atlas of MS, third edition. *Mult Scler*. (2020) 26:1816–21. doi: 10.1177/1352458520970841
5. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on clinical trials of new agents in multiple sclerosis. *Neurology.* (1996) 46:907–11. doi: 10.1212/WNL.46.4.907
6. Archeson ED. Bachrach CA. The distribution of multiple sclerosis in US veterans by birthplace. *Am J Hyg.* (1960) 72:88–99. doi: 10.1093/oxfordjournals.aje.a120137
7. Vukusic S, Van Bockstael V, Gosselin S, Confavreux C. Regional variations in the prevalence of multiple sclerosis in French farmers. *J Neurol Neurosurg Psychiatry.* (2007) 78:707–9. doi: 10.1136/jnnp.2006.101196
8. Hammond SR, de Wytt C, Maxwell IC, Landy PJ, English E, McLeod G, et al. The epidemiology of multiple sclerosis in Queensland, Australia. *J Neurol Sci.* (1987) 80:185–204. doi: 10.1016/0022-510X(87)90154-7
9. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. *Prog Neurobiol.* (1995) 47:425–48. doi: 10.1016/0301-0082(95)80008-V
10. Sabel CE, Pearson JF, Mason DF, Willoughby E, Abernathy DA, Taylor BV. The latitude gradient for multiple sclerosis prevalence is established in the early life course. *Brain.* (2021) 144:2038–46. doi: 10.1093/brain/awab104
11. Simpson S, Wang W, Otahal P, Blizzard L, van der Mei IAF, Taylor BV. Latitude continues to be significantly associated with the prevalence of multiple sclerosis: an updated meta-analysis. *J Neurol Neurosurg Psychiatry.* (2019) 90:1193–200. doi: 10.1136/jnnp-2018-320189
12. Tremlett H, Zhu F, Ascherio A, Munger KL. Sun exposure over the life course and associations with multiple sclerosis. *Neurol.* (2018) 90:e1191–9. doi: 10.1212/WNL.0000000000005257
13. Alter M, Leibowtz U, Speer J. Risk of multiple sclerosis related to age at immigration to Israel. *Arch Neurol.* (1966) 15:234–7. doi: 10.1001/archneur.1966.00470150012002
14. Dean G, Elian M. Age at immigration to England of Asian and Caribbean immigrants and the risk of developing multiple sclerosis. *J Neurol Neurosurg Psychiatry.* (1997) 63:565–8. doi: 10.1136/jnnp.63.5.565
15. Hammond SR, English DR, McLeod JG. The age-range of risk of developing multiple sclerosis: evidence from a migrant population in Australia. *Brain.* (2000) 123:968–74. doi: 10.1093/brain/123.5.968
16. McLeod JG, Hammond SR, Kurtzke JF. Migration and multiple sclerosis in immigrants to Australia from United Kingdom and Ireland: a reassessment. I Risk of MS by age at immigration. *J Neurol.* (2011) 258:1140–9. doi: 10.1007/s00415-010-5898-4
17. McLeod JG, Hammond SR, Kurtzke JF. Migration and multiple sclerosis in United Kingdom and Ireland immigrants to Australia: a reassessment. II. Characteristics of early (pre-1947) compared to later migrants. *J Neurol.* (2012) 259:684–93. doi: 10.1007/s00415-011-6244-1
18. Kurtzke JF, Beebe GW, Norman JE. Epidemiology of multiple sclerosis in US veterans III. Migration and the risk of MIS. *Neurology.* (1985) 35:672. doi: 10.1212/WNL.35.5.672
19. Willer CJ, Dyment DA, Rsch NJ, Sadovnick AD, Ebers GC. Canadian Collaborative Study Group. *Proc Natl Acad Sci USA*. (2003) 100:12877–82. doi: 10.1073/pnas.1932604100
20. International Multiple Sclerosis Genetics Consortium, Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, et al. Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med*. (2007) 357:851–62. doi: 10.1056/NEJMoa073493
21. Australia and New Zealand Multiple Sclerosis Genetics Consortium, Bahlo M, Booth DR, Broadley SA, Brown MA, Foote SJ, et al. Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. *Nat Genet*. (2009) 41:824–8. doi: 10.1038/ng.396
22. Goodin DS. The Causal Cascade to Multiple Sclerosis: a model for MS pathogenesis. *PLoS ONE.* (2009) 4:e4565. doi: 10.1371/journal.pone.0004565
23. Dominguez-Mozo N, Perez-Perez S, Villarrubia N, Costa-Frossard L, Fernandez-Velasco JI, Ortega-Madueno I, et al. Herpesvirus antibodies, vitamin d and short-chain fatty acids: their correlation with cell subsets in multiple sclerosis patients and healthy controls. *Cells.* (2021) 10:119. doi: 10.3390/cells10010119
24. Riise T, Nortvedt MW, Ascherio A. Smoking is a risk factor for multiple sclerosis. *Neurology.* (2003) 61:1122–4. doi: 10.1212/01.WNL.0000081305.66687.D2
25. Hedstrom AK, Baarnhielm M, Olsson T, Alfredsson L. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. *Neurology.* (2009) 73:696–701. doi: 10.1212/WNL.0b013e3181b59c40
26. Hernán MA, Jick SS, Logroscino G, Olek MJ, Ascherio A, Jick H. Cigarette smoking and the progression of multiple sclerosis. *Brain.* (2005) 128:1461–5. doi: 10.1093/brain/awh471
27. Bjelobaba I, Begovic-Kupresanin V, Pekovic S, Lavrnja I. Animal models of multiple sclerosis: focus on experimental autoimmune encephalomyelitis. *Neurosci Res*. (2018) 96:1021–42. doi: 10.1002/jnr.24224
28. Kipp M, Nyamoya S, Hochstrasser T, Amor S. Multiple sclerosis models: a clinical and histopathological perspective. *Brain Pathol.* (2016) 27:123–37. doi: 10.1111/bpa.12454
29. Lindsey JW. Characteristics of initial and reinduced experimental autoimmune encephalomyelitis. *Immunogenetics.* (1996) 44:292–7. doi: 10.1007/BF02602559
30. Wu F, Cao W, Yang Y, Liu A. Extensive infiltration of neutrophils in the acute phase of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Histochem Cell Biol.* (2010) 133:313–22. doi: 10.1007/s00418-009-0673-2
31. Cross AH, Trotter JL, Lyons JA. B cells and antibodies in CNS demyelinating disease. *J Neuroimmunol.* (2001) 112:1–14. doi: 10.1016/S0165-5728(00)00409-4
32. Carnero Contentti E, Correale J. Neuromyelitis optica spectrum disorders: from pathophysiology to therapeutic strategies. *J Neuroinflammation.* (2021) 18:208. doi: 10.1186/s12974-021-02249-1
33. Yang Y, Weiner J, Liu Y, Smith AJ, Huss DJ, Winger R, et al. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J Exp Med.* (2009) 206:1549–64. doi: 10.1084/jem.20082584
34. Huss DJ, Winger RC, Peng H, Yang Y, Racke MK, Lovett-Racke AE. TGF-beta enhances effector Th1 cell activation but promotes self-regulation via Il-10. *J Immunol.* (2010) 184:5628–36. doi: 10.4049/jimmunol.1000288
35. Huss DJ, Winger RC, Mavrikis Cox G. Guerau-de-Arellano, Yang Y, Racke MK, et al. TGF-β signaling via Smad4 drives IL-10 production in effector Th1 cells and reduces T-cell trafficking in EAE. *Eur J Immunol.* (2011) 41:2987–96. doi: 10.1002/eji.201141666

36. Lovett-Racke AE, Yang Y, Racke MK. Th1 versus Th17: are T cell cytokines relevant in multiple sclerosis? *Biochim Biophys Acta.* (2011) 1812:246–51. doi: 10.1016/j.bbadis.2010.05.012
37. Allegretta M, Nicklas JA, Sriram S, Albertini RJ. T cell responsive to myelin basic protein in patients with multiple sclerosis. *Science.* (1990) 247:718–21. doi: 10.1126/science.1689076
38. Lovett-Racke AE, Trotter JL, Lauber J, Perrin PJ, June CH, Racke MK. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *J Clin Invest.* (1998) 101:725–30. doi: 10.1016/S0165-5728(98)91630-7
39. Perrin PJ, Lovett-Racke A, Phillip SM, Racke MK. Differential requirements of naïve and memory T cells for CD28 costimulation in autoimmune pathogenesis. *Histol Histopathol.* (1999) 14:1269–76. doi: 10.14670/HH-14.1269
40. Burns J, Bartholomew B, Lobo S. Isolation of myelin basic protein-specific T cells predominantly from the memory T-cell compartment in multiple sclerosis. *Ann Neurol.* (1999) 45:33–9.
41. Racke MK, Ratts RB, Arredondo L, Perrin PJ, Lovett-Racke A. The role of costimulation in autoimmune demyelination. *J Neuroimmunol.* (2000) 107:205–15. doi: 10.1016/S0165-5728(00)00230-7
42. Holick MF. The vitamin D epidemic and its health consequences. *J Nutr.* (2005) 135:2739S–48S. doi: 10.1093/jn/135.11.2739S
43. Wang TT. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol Endocrinol.* (2005) 19:2685–95. doi: 10.1210/me.2005-0106
44. Norman AW. Minireview: vitamin D receptor: new assignments for an already busy receptor. *Endocrinology.* (2006) 147:5542–8. doi: 10.1210/en.2006-0946
45. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, et al. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci USA.* (1997) 94:9831–5. doi: 10.1073/pnas.94.18.9831
46. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet.* (1997) 16:391–6. doi: 10.1038/ng0897-391
47. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herch E, Kato S, et al. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci USA.* (2001) 98:13324–9. doi: 10.1073/pnas.231474698
48. Tiosano D, Hada S, Chen Z, Nemirovsky A, Gepstin V, Militianu D, et al. Calcium absorption, kinetics, bone density, and bone structure in patients with hereditary vitamin D-resistant rickets. *J Clin Endocrinol Metab.* (2011) 96:3701–9. doi: 10.1210/jc.2011-1432
49. Eyles DW, Smith S, Kinobe R, Hewison M, McGrath JJ. Distribution of the Vitamin D receptor and 1α-hydroxylase in human brain. *J Chem Neuroanat.* (2005) 29:21–30. doi: 10.1016/j.jchemneu.2004.08.006
50. Sanchez B, Relova JL, Gallego R, Ben-Batalla I, Perez-Fernandez R. 1,25-Dihydroxyvitamin D 3administration to 6-hydroxydopamine-lesioned rats increases glial cell line-derived neurotrophic factor and partially restores tyrosine hydroxylase expression in substantia nigra and striatum. *J Neurosci Res.* (2009) 87:723–32. doi: 10.1002/jnr.21878
51. Neveu I, Naveilhan P, Jehan FR, Baudet C, Wion D, De Luca HF, et al. 1,25-Dihydroxyvitamin D3 regulates the synthesis of nerve growth factor in primary cultures of glial cells. *Mol Brain Res.* (1994) 24:70–6. doi: 10.1016/0169-328X(94)90119-8
52. Garcion E, Wion-Barbot N, Montero-Menei CN, Berger F, Wion D. New clues about vitamin D functions in the nervous system. *Trends Endocrinol and Metab.* (2002) 13:100–5. doi: 10.1016/S1043-2760(01)00547-1
53. Garcion E, Sindji L, Montero-Menei C, Andre C, Brachet P, Darcy F. Expression of inducible nitric oxide synthase during rat brain inflammation: regulation by 1,25-dihydroxyvitamin D3. *Glia.* (1998) 22:282–94.
54. Kesby JP, Eyles DW, Burne THJ, McGrath JJ. The effects of vitamin D on brain development and adult brain function. *Mol Cell Endocrinol.* (2011) 347:121–7. doi: 10.1016/j.mce.2011.05.014
55. Holick MF, Binkley NC, Bischoll-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* (2011) 96:1911–30. doi: 10.1210/jc.2011-0385
56. Manson JE, Brannon PM, Rosen CJ, Taylor CL. Vitamin D deficiency – is there really a pandemic? *N Engl J Med.* (2016) 375:1817–20. doi: 10.1056/NEJMp1608005
57. Willer CJ, Dyment DA, Sadovnick AD, Rothwell PM, Murray TJ, Ebers GC. Timing of birth and risk of multiple sclerosis: population based study. *BMJ.* (2005) 330:120–120. doi: 10.1136/bmj.38301.686030.63
58. Dobson R, Giovannoni G, Ramagopalan S. The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude. *J Neurol Neurosurg Psychiatry.* (2013) 84:427–32. doi: 10.1136/jnnp-2012-303934
59. Fiddes B, Wason J, Sawcer S. Confounding in association studies: month of birth and multiple sclerosis. *Neurol.* (2014) 261:1851–6. doi: 10.1007/s00415-014-7241-y
60. Walleczek NK, Frommlet F, Bsteh G, Eggers C, Rauschka H, Koppi S, et al. Month-of-birth-effect in multiple sclerosis in Austria. *Mult Scler.* (2019) 25:1870–7. doi: 10.1177/1352458518810924
61. Munger KL, Aivo H, Hongell K, Soilu-Hanninen M, Surcel HM, Ascherio A. Vitamin D status during pregnancy and risk of multiple sclerosis in offspring of women in the Finnish Maternity Cohort. *JAMA Neurol.* (2016) 73:515–9. doi: 10.1001/jamaneurol.2015.4800
62. Nielsen NM, Munger KL, Koch-Henriksen N, Hougaard DM, Magyari M, Jorgensen KT, et al. Neonatal vitamin D status and risk of multiple sclerosis: a population-based case-control study. *Neurol.* (2017) 88:44–51. doi: 10.1212/WNL.0000000000003454
63. Van der Mei IA, Ponsonby AL, Dwyer T, Blizzard L, Simmons R, Taylor BV, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *BMJ.* (2003) 327:316. doi: 10.1136/bmj.327.7410.316
64. Kampman MT, Wilsgaard T, Mellgren SI. Outdoor activities and diet in childhood and adolescence relate to MS risk above the arctic circle. *J Neurol.* (2007) 254:471–7. doi: 10.1007/s00415-006-0395-5
65. Islam T, Gauderman WJ, Cozen W, Mack TM. Childhood sun exposure influences risk of multiple sclerosis in monozygotic twins. *Neurology.* (2007) 69:381–8. doi: 10.1212/01.wnl.0000268266.50850.48
66. Irving AA, Marling SJ, Seeman J, Plum LA, DeLuca HF. UV light suppression of EAE (a mouse model of multiple sclerosis) is independent of vitamin D and its receptor. *Proc Natl Acad Sci U S A.* (2019) 116:22552–5. doi: 10.1073/pnas.1913294116
67. Swank RL, Lerstad O, Strøm A, Backer J. Multiple Sclerosis in Rural Norway – its geographic and occupational incidence in relation to nutrition. *N Engl J Med.* (1952) 246:721–8. doi: 10.1056/NEJM195205082461901
68. Munger KL, Zhang SM, O'Reilly E, Hernán MA, Olek MJ, Willett WC, et al. Vitamin D intake and incidence of multiple sclerosis. *Neurology.* (2004) 62:60–5. doi: 10.1212/01.WNL.0000101723.79681.38
69. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA Neurol.* (2006) 296:2832–8. doi: 10.1001/jama.296.23.2832
70. Mokry LE, Ross S, Ahmad OS, Forgetta V, Smith GD, Goltzman D, et al. Vitamin D and risk of multiple sclerosis: a Mendelian randomization study. *PLoS Med.* (2015) 12:e1001866. doi: 10.1371/journal.pmed.1001866
71. Langer-Gould A, Lucas RM, Xiang AH, Wu J, Chen LH, Gonzales E, et al. Vitamin D-binding protein polymorphisms, 25-hydroxyvitamin D, sunshine and multiple sclerosis. *Nutrients.* (2018) 10:184. doi: 10.3390/nu10020184
72. Correale J, Ysrraelit MC, Gaitan MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain.* (2009) 132:1146–60. doi: 10.1093/brain/awp033
73. van der Mei IAF, Ponsonby AL, Dwyer T, Blizzard L, Taylor BV, Kilpatrick T, et al. Vitamin D levels in people with multiple sclerosis and community controls in Tasmania, Australia. *J Neurol.* (2007) 254:581–90. doi: 10.1007/s00415-006-0315-8
74. Simpson S, Taylor B, Blizzard L, Ponsonby AL, Pittas F, Tremlett H, et al. Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS. *Ann Neurol.* (2010) 68:193–203. doi: 10.1002/ana.22043
75. Runia TF, Hop WCJ, de Rijke YB, Buljevac D, Hintzen RQ. Lower serum vitamin D levels are associated with a higher relapse risk in multiple sclerosis. *Neurology.* (2012) 79:261–6. doi: 10.1212/WNL.0b013e31825fdec7

76. Provvedini D, Tsoukas C, Deftos L, Manolagas S. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science.* (1983) 221:1181–3. doi: 10.1126/science.6310748
77. Lemire JM, Archer DC. 1,25-dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. *J Clin Invest.* (1991) 87:1103–7. doi: 10.1172/JCI115072
78. Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proc Natl Acad Sci USA.* (1996) 93:7861–4. doi: 10.1073/pnas.93.15.7861
79. Nashold FE, Miller DJ, Hayes CE. 1,25-dihydroxyvitamin D3 treatment decreases macrophage accumulation in the CNS of mice with experimental autoimmune encephalomyelitis. *J Neuroimmunol.* (2000) 103:171–9. doi: 10.1016/S0165-5728(99)00247-7
80. Mayne CG, Spanier JA, Relland LM, Williams CB, Hayes CE. 1,25-Dihydroxyvitamin D3 acts directly on the T lymphocyte vitamin D receptor to inhibit experimental autoimmune encephalomyelitis. *Eur J Immunol.* (2011) 41:822–32. doi: 10.1002/eji.201040632
81. Cantorna MT, Woodward WD, Hayes CE, DeLuca HF. 1,25-dihydroxyvitamin D3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-beta 1 and IL-4. *J Immunol.* (1998) 160:5314–9.
82. Spach KM, Nashold FE, Dittel BN, Hayes CE. IL-10 Signaling Is Essential for 1,25-Dihydroxyvitamin D3-Mediated Inhibition of Experimental Autoimmune Encephalomyelitis. *J Immunol.* (2006) 177:6030–7. doi: 10.4049/jimmunol.177.9.6030
83. Mattner F, Smiroldo S, Galbiati F. Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D3. *Eur J Immunol.* (2000) 30:498–508. doi: 10.1002/1521-4141(200002)30:2<498::AID-IMMU498>3.0.CO;2-Q
84. Muthian G, Raikwar HP, Rajasingh J, Bright JJ. 1,25 dihydroxyvitamin-D3 modulates JAK–STAT pathway in IL-12/IFNγ axis leading to Th1 response in experimental allergic encephalomyelitis. *J Neurosci Res.* (2006) 83:1299–309. doi: 10.1002/jnr.20826
85. Miclea A, Bagnoud M, Chan A, Hoepner R. A brief review of the effects of vitamin d on multiple sclerosis. *Front Immunol.* (2020) 11:781. doi: 10.3389/fimmu.2020.00781
86. Hupperts R, Smolders J, Vieth R, Holmoy T, Marhardt K, Schluep M, et al. Randomized trial of daily high-dose vitamin D3 in patients with RRMS receiving subcutaneous interferon beta-1a. *Neurol.* (2019) 93:e1906–16. doi: 10.1212/WNL.0000000000008445
87. Camu W, Lehert P, Pierrot-Deseilligny C, Hautecoeur P, Besserve A, Deleglise ASJ, et al. Cholecalciferol in relapsing-remitting MS: a randomized clinical trial (CHOLINE). *Neurol Neuroimmunol Neuroinflamm.* (2019) 6:e597. doi: 10.1212/NXI.0000000000000597
88. Jagannath VA, Filippini G, Di Pietrantonj C, Asokan GV, Robak EW, Whamond L, et al. Vitamin D for the management of multiple sclerosis. *Cochrane Database Syst Rev.* (2018) 9:CD008422. doi: 10.1002/14651858.CD008422.pub3
89. Abe, E. Miyaura C, Tanaka H, Shiina Y, Kuribayashi T, Suda S, et al. 1 alpha,25-diyhdroxyvitamin D3 promotes fusin of mouse alveolar macrophages both by a direct mechanism and by a spleen-mediated indirect mechanism. *Proc Natl Acad Sci USA.* (1983) 80:5583–7. doi: 10.1073/pnas.80.18.5583
90. Haq AU. 1,25-dihydroxyvitamin D3 (calcitriol) suppresses IL-2 induced murine thymocyte proliferation. *Thymus.* (1986) 8:295–306.
91. Adams JS, Ren S, Liu PT, Chun RF, Lagishetty V, Gombart AF, et al. Vitamin D-directed rheostatic regulation of monocyte antibacterial responses. *J Immunol.* (2009) 182:4289–95. doi: 10.4049/jimmunol.0803736
92. Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J, et al. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE.* (2011) 6:e25333. doi: 10.1371/journal.pone.0025333
93. Shahmiri M, Enciso M, Adda CG, Smith BJ, Perugini MA, Mechler A. Membrane core-specific antimicrogial action of cathelicidin LL-37 peptide switches between pore and nanofiber formation. *Sci Rep.* (2016) 6:38184. doi: 10.1038/srep38184
94. Sousa FH, Casanova V, Findlay F, Stevens C, Svoboda P, Pohl J, et al. Cthelicidins display conserved direct antiviral activity towards rhinovirus. *Peptides.* (2017) 95:76–83. doi: 10.1016/j.peptides.2017.07.013
95. Piemonti L, Monti P, Sironi M, Fraticelli P, Leone BE, Dal Cin E, et al. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol.* (2000) 164:4443–51. doi: 10.4049/jimmunol.164.9.4443
96. Szeles L, Keresztes G, Torocsik D, Balajthy Z, Krenacs L, Poliska S, et al. 1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype. *J Immunol.* (2009) 182:2074–83. doi: 10.4049/jimmunol.0803345
97. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science.* (2006) 311:1770–3. doi: 10.1126/science.1123933
98. Li P, Xu X, Cao E, Yu B, Li W, Fan M, et al. Vitamin D deficiency causes defective resistant to Aspergillus fumigatus in mice via aggravated and sustained inflammation. *PLoS ONE.* (2014) 9:e99805. doi: 10.1371/journal.pone.0099805
99. Martinez-Moreno J, Hernandez JC, Urcuqui-Inchima S. Effect of high doses of vitamin D supplementation on dengue virus replication, toll-like receptor expression, and cytokine profiles on dendritic cells. *Mol Cell Biochem.* (2020) 464:169–80. doi: 10.1007/s11010-019-03658-w
100. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest.* (1998) 101:252–62. doi: 10.1172/JCI1050
101. de Oliveira RC, Mimura LAN, Fraga-Silva TFC, Ishikawa LLW, Fernandes AAH, Zorzella-Pezavento SFG, et al. Calcitriol prevents neuroinflammation and reduces blood-brain barrier disruption and local macrophage/microglia activation. *Front Pharmacol.* (2020) 11:161. doi: 10.3389/fphar.2020.00161
102. Lee PW, Selhorst A, Lampe SG, Liu Y, Yang Y, Lovett-Racke AE. Neuron-specific vitamin D signaling attenuates microglia activation and CNS autoimmunity. *Front Neurol.* (2020) 11:19. doi: 10.3389/fneur.2020.00019
103. Evans MA, Kim HA, Ling YH, Uong S, Vinh A, De Silva TM, et al. Vitamin D(3) supplementation reduces subsequent brain injury and inflammation with ischemic stroke. *Neuromolecular Med.* (2018) 20:147–59. doi: 10.1007/s12017-018-8484-z
104. Calvello R, Cianciulli A, Nicolardi G, De Nuccio F, Giannotti L, Salvatore R, et al. Vitamin D treatment attenuates nueroinflammation and dopaminergic neurodegeneration in an animal model of Parkinson's disease, shifting M1 to M2 microglia. *J Neuroimmune Pharmacol.* (2017) 12:327–39. doi: 10.1007/s11481-016-9720-7
105. Cui C, Xu P, Li G, Qiao Y, Han W, Geng C, et al. Vitamin D receptor activation regulates microglia polarization and oxidative stress in spontaneously hypertensive rats and angiotensin II-exposed microglial cells: role of renen-angiotensis system. *Redox Biol.* (2019) 26:101295. doi: 10.1016/j.redox.2019.101295
106. He MC, Shi Z, Sha NN, Chen N, Peng SY, Liao DF, et al. Paricalcitol alleviates lipopolysaccharide-induced depressive-like behavior by suppressing hypothalamic microglia activation and neuroinflammation. *Biochem Pharmacol.* (2019) 163:1–8. doi: 10.1016/j.bcp.2019.01.021
107. Bhalla AD, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab.* (1983) 57:1308–10. doi: 10.1210/jcem-57-6-1308
108. Amento EP, Bhalla AK, Kurnick JT, Kradin RL, Clemens TL, Holick SA, et al. 1 alpha,25-dihydroxyvitamin D3 induces maturation of the human monocyte cell line U937, and, in association with a factor from human T lymphocytes, augments production of the monokine, mononuclear cell factor. *J Clin Invest.* (1984) 73:731–9. doi: 10.1172/JCI111266
109. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol.* (2007) 179:1634–47. doi: 10.4049/jimmunol.179.3.1634
110. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-dihydroxyvitamin D3 has a direct effect on naïve CD4(+) T cells

to enhance the development of Th2 cells. *J Immunol.* (2001) 167:4974–80. doi: 10.4049/jimmunol.167.9.4974
111. Tang J, Zhou R, Luger D, Zhu W, Silver PB, Grajewski RS, et al. Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response. *J Immunol.* (2009) 182:4624–32. doi: 10.4049/jimmunol.0801543
112. Gregori S, Giarratana N, Smiroldo S, Uskokovic M, Adorini L. A 1alpha,25-diydroxyvitamin D(3) analog enhances regulatory T cells and arrests autoimmune diabetes in NOD mice. *Diabetes.* (2002) 51:1367–74. doi: 10.2337/diabetes.51.5.1367
113. Viglietta V, Baecher-Allan C, Weiner HL. Hafler DA Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med.* (2004) 199:971–9. doi: 10.1084/jem.20031579
114. Haas J, Hug A, Viehöver A, Fritzsching B, Falk CS, Filser A, et al. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol.* (2005) 35:3343–52. doi: 10.1002/eji.200526065
115. Kumar M, Putzki N, Limmroth V, Remus R, Lindemann M, Knop D et al. CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. *J Neuroimmunol.* (2006) 180:178–84. doi: 10.1016/j.jneuroim.2006.08.003
116. Huan J, Culbertson N, Spencer L, Bartholomew R, Burrows GG, Chou YK et al. Decreased FOXP3 levels in multiple sclerosis patients. *J Neurosci Res.* (2005) 81:45–52. doi: 10.1002/jnr.20522
117. Haas J, Fritzsching B, Trübswetter P, Korporal M, Milkova L, Fritz B et al. Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol.* (2007) 179:1322–30. doi: 10.4049/jimmunol.179.2.1322
118. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL et al. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology.* (2008) 123:79–89. doi: 10.1111/j.1365-2567.2007.02690.x
119. Vanherwegen AS, Eelen G, Ferreira GB, Ghesquiere B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. *J Steroid Biochem Mol Biol.* (2019) 187:134–45. doi: 10.1016/j.jsbmb.2018.11.011
120. Smolders J, Thewissen M, Peelen E, Menheere P, Tervaert JW, Kamoiseaux J, et al. Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. *PLoS ONE.* (2009) 4:e6635. doi: 10.1371/journal.pone.0006635
121. Killick J, Hay J, Morandi E, Vermeren S, Kari S, Angles T, et al. Vitamin D/CD46 crosstalk in human T cells in multiple sclerosis. *Front Immunol.* (2020) 11:598727. doi: 10.3389/fimmu.2020.598727
122. Drozdenko G, Scheel T, Heine G, Baumgrass R, Worm M. Impaired T cell activation and cytokine production by calcitriol-primed human B cells. *Clin Exp Immunol.* (2014) 178:364–72. doi: 10.1111/cei.12406
123. Cross AH, Stark JL, Lauber J, Ramsbottom MJ, Lyons JA. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol.* (2006) 180:63–70. doi: 10.1016/j.jneuroim.2006.06.029
124. Lovett-Racke AE, Gormley M, Liu Y, Yang Y, Graham C, Wray S, et al. B cell depletion with ublituximab reshapes the T cell profile in multiple sclerosis patients. *J Neuroimmunol.* (2019) 332:187–97. doi: 10.1016/j.jneuroim.2019.04.017
125. Lovett-Racke AE, Yang Y, Liu Y, Gormley M, Kraus E, Graham C, et al. B cell depletion changes the immune cell profile in multiple sclerosis patients: one-year report. *J Neuroimmunol.* (2021) 359:577676. doi: 10.1016/j.jneuroim.2021.577676
126. Stumpf WE, Sar M, Clark SA, DeLuca HF. Brain target sites for 1,25-kihydroxyvitamin D3. *Science.* (1982) 215:1403–5. doi: 10.1126/science.6977846
127. McGrath JJ, Feron FP, Burne TH, Mackay-Sim A, Eyles DW. Vitamin D3-implications for brain development. *J Steroid Biochem Mol Biol.* (2004) 89–90:557–60. doi: 10.1016/j.jsbmb.2004.03.070
128. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, et al. Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. *J Clin Endocrinol Metab.* (2001) 86:888–94. doi: 10.1210/jc.86.2.888
129. Eyles D, Brown J, Mackay-Sim A, McGrath JJ, Feron F. Vitamin D3 and brain development. *Neuroscience.* (2003) 118:641–53. doi: 10.1016/S0306-4522(03)00040-X
130. Brown J, Bianco JI, McGrath JJ, Eyles DW. 1,25-dihydroxyvitamin D3 induces nerve growth factor, promotes neurite outgrowth and inhibits mitosis in embryonic rat hippocampal neurons. *Neurosci Lett.* (2003) 343:139–43. doi: 10.1016/S0304-3940(03)00303-3
131. Ko P, Burkert R, McGrath J, Eyles D. Maternal vitamin D3 deprivation and the regulation of apoptosis and cell cycle during rat brain development. *Brain Res Dev Brain Res.* (2004) 153:61–8. doi: 10.1016/j.devbrainres.2004.07.013
132. Sinkkonen L, Malinen M, Saavalainen K, Vaisanen S, Carlberg C. Regulation of the human cyclin C gene via multiple vitamin D3-repsonsive regions in its promoter. *Nucleic Acids Res.* (2005) 33:2440–51. doi: 10.1093/nar/gki502
133. Cui X, McGrath JJ, Burne TH, Mackay-Sim A, Eyles DW. Maternal vitamin D depletion alters neurogenesis in the developing rat brain. *Int J Dev Neurosci.* (2007) 25:227–32. doi: 10.1016/j.ijdevneu.2007.03.006
134. Al-Amin MM, Sullivan RKP, Kurniawan ND, Burne THJ. Adult vitamin D deficiency disrupts hippocampal-dependent learning and structural brain connectivity in BALB/c mice. *Brain Struct Funct.* (2019) 224:1315–29. doi: 10.1007/s00429-019-01840-w
135. Annweiler C, Bartha R, Karras SN, Gautier J, Roche F, Beauchet O. Vitamin D and white matter abnormalities in older adults: a quantitative volumetric analysis of brain MRI. *Exp Gerontol.* (2015) 63:41–7. doi: 10.1016/j.exger.2015.01.049
136. Cui X, Pelekanos M, Burne TH, McGrath JJ, Eyles DW. Maternal vitamin D deficiency alters the expression of genes involved in dopamine specification in the developing rat mesencephalon. *Neurosci Lett.* (2010) 486:220–3. doi: 10.1016/j.neulet.2010.09.057
137. Orme RP, Bhangal MS, Fricker RA. Calcitriol imparts neuroprotection in vitro to midbrain dopaminergic neurons by upregulating GDNF expression. *PLoS ONE.* (2013) 8:e62040. doi: 10.1371/journal.pone.0062040
138. Pertile RAN, Cui X, Eyles DW. Vitamin D signaling and the differentiation of developing dopamine systems. *Neuroscience.* (2016) 333:193–203. doi: 10.1016/j.neuroscience.2016.07.020
139. Shirazi HA, Rasouli J, Ciric B, Rostami A, Zhang GX. 1,25-dihydroxyvitamin D3 enhances neural stem cell proliferation and oligodendrocyte differentiation. *Exp Mol Pathol.* (2015) 98:240–5. doi: 10.1016/j.yexmp.2015.02.004
140. De la Fuenta AG, Errea O, van Wijngaarden P, Gonzalez GA, Kerninon C, Jarjour AA, et al. Vitamin D receptor-retinoid X receptor heterodimer signaling regulates oligodendrocyte progenitor cell differentiation. *J Cell Biol.* (2015) 211:975–85. doi: 10.1083/jcb.201505119
141. Cass WA, Peters LE, Fletcher AM, Yurek DM. Evoked dopamine overflow is augmented in the striatum of calcitriol treated rats. *Neurochem Int.* (2012) 60:186–91. doi: 10.1016/j.neuint.2011.11.010
142. Groves NJ, Kesby JP, Eyles DW, McGrath JJ, Mackay-Sin A, Burne TH. Adult vitamin D deficiency leads to beavioural and brain neurochemical alterations in C57BL/6J and BALB/c mice. *Behav Brain Res.* (2013) 241:120–31. doi: 10.1016/j.bbr.2012.12.001
143. Jiang P, Zhang LH, Cai HL, Li HD, Liu YP, Tang MM, et al. Neurochemical effects of chronic administration of calcitriol in rats. *Nutrients.* (2014) 6:6048–59. doi: 10.3390/nu6126048
144. Kesby JP, Turner KM, Alexander S, Eyles DW, McGrath JJ, Burne THJ. Developmental vitamin D deficiency alters multiple neurotransmitter systems in the neonatal rat brain. *Int J Dev Neurosci.* (2017) 62:1–7. doi: 10.1016/j.ijdevneu.2017.07.002
145. Neveu I, Naveilhan P, Baudet C, Brachet P, Metsis M. 1,25-dihydroxyvitamin D3 regulates NT-3, NT-4 but not BDNF mRNA in astrocytes. *Neuroreport.* (1994) 6:124–6. doi: 10.1097/00001756-199412300-00032
146. Naveilhan P, Neveu I, Wion D, Brachet P. 1,25-kihydroxyvitamin D3, an inducer of glial cell line-derived neurotrophic factor. *Neuroreport.* (1996) 7:171–5. doi: 10.1097/00001756-199609020-00023
147. Naveilhan P, Neveu I, Baudet C, Funakoshi H, Wion D, Brachet P, et al. 1,25-kihydroxyvitamin D3 regulates the expression of the low-affinity neurotrophin receptor. *Brain Res Mol Brain Res.* (1996) 41:259–68. doi: 10.1016/0169-328X(96)00103-9
148. Taniura H, Ito M, Sanada N, Kuramoto N, Ohno Y, Nakamichi N, et al. chronic vitamin D3 treatment protects against neurotoxicity by

glutamate in association with upregulation of vitamin D receptor mRNA expression in cultured rat corical neurons. *J Neurosci Res.* (2006) 83:1179–89. doi: 10.1002/jnr.20824
149. Almeras L, Eyles D, Benech P, Laffite D, Villard C, Patatian A, et al. Developmental vitamin D deficiency alters brain protein expression in the adult rat: implications for neuropsychiatric disorders. *Proteomics.* (2007) 7:769–80. doi: 10.1002/pmic.200600392
150. Eyles D, Almeras L, Benech P, Patatian A, Mackay-Sim A, McGrath J, Feron F. Developmental vitamin D deficiency alters the expression of genes encoding mitochondrial, cytosketetal and synaptic proteins in the adult rat brain. *J Steroid Biochem Mol Biol.* (2007) 103:538–45. doi: 10.1016/j.jsbmb.2006.12.096
151. Gezen-Ak D, Dursun E, Yilmazer S. The effects of vitamin D receptor silencing on the expression of LVSCC-A1C and LVSCC-A1D and the release of NGF in cortical neurons. *PLoS ONE.* (2011) 6:e17553. doi: 10.1371/journal.pone.0017553
152. Zhu Y, Zhou R, Yang R, Zhang Z, Bai Y, Chang F, et al. Abnormal neurogenesis in the dentate gyrus of adult mice lacking 1,25-dihydroxy vitamin D3 (1,25-(OH)2 D3). *Hippocampus.* (2012) 22:421–33. doi: 10.1002/hipo.20908
153. Brewer LD, Thibault V, Chen KC, Langub MC, Landfield PW, Porter NM. Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. *J Neurosci.* (2001) 21:98–108. doi: 10.1523/JNEUROSCI.21-01-00098.2001
154. Zanatta L, Goulart PB, Goncalves R, Pierozan P, Winkelmann-Duarte EC, Woehl VM, et al. 1alpha,25-kihydroxyvitamin D(3) mechanism of action: modulation of L-type calcium channels leading to calcium uptake and intermediate filament phosphorylation in cerebral cortex of young rats. *Biochim biophys Acta.* (2012) 1823:1708–19. doi: 10.1016/j.bbamcr.2012.06.023
155. Boyan BD, Sylvia VL, Dean DD, Schwartz Z. Membrane mediated signaling mechanisms are used differentially by metabolites of vitamin D(3) in musculoskeletal cells. *Steroids.* (2002) 67:421–7. doi: 10.1016/S0039-128X(01)00178-7
156. Chen J, Doroudi M, Cheung H, Grozier AL, Schwartz Z, Boyan BD. Plasma membrane Pdia3 and VDR interact to elicit rapid responses to 1alpha,25(OH)(2)D(3). *Cell Signal.* (2013) 25:2362–73. doi: 10.1016/j.cellsig.2013.07.020
157. Nemere I, Garbi N, Hammerling G, Hintze KJ. Role of the 1,25D3-MARRS receptor in the 125(OH)2D3-stimulated uptake of calcium and phosphate in intestinal cells. *Steroids.* (2012) 77:897–902. doi: 10.1016/j.steroids.2012.04.002
158. Jozefowicz O, Rabe-Jablonska J, Wozniacka A, Strzelecki D. Analysis of vitamin D status in major depression. *J Psychiatr Pract.* (2014) 20:329–37. doi: 10.1097/01.pra.0000454777.21810.15
159. Kerr DC, Zava DT, Piper WT, Saturn SR, Frei B, Gombart AF. Associations between vitamin D levels and depressive symptoms in healthy young adult women. *Psychiatry Res.* (2015) 227:46–51. doi: 10.1016/j.psychres.2015.02.016
160. Coskun S, Simsek S, Camkurt MA, Cim A, Celik SB. Association of polymorphisms in the vitamin D receptor gene and serum 25-hydroxyvitamin D levels in children with autism spectrum disorder. *Gene.* (2016) 588:109–14. doi: 10.1016/j.gene.2016.05.004
161. Sourander A, Upadhyaya S, Surcel HM, Hinkka-Yli-Salomaki S, Cheslack-Postava K, Silwal S, et al. Maternal vitamin D levels during pregnancy and offspring autism spectrum disorder. *Biol Psychiatry*. (2021) 90:790–7. doi: 10.1016/j.biopsych.2021.07.012
162. Cui X, McGrath JJ, Burne THJ, Eyles DW. Vitamin D and schizophrenia: 20 years on. *Mol Psychiatry.* (2021) 26:2708–20. doi: 10.1038/s41380-021-01025-0
163. Evatt ML, Delong MR, Khazai N, Rosen A, Triche S, Tangpricha V. Prevalence of vitamin d insufficiency in patients with Parkinson disease and Alzheimer disease. *Arch Neurol.* (2008) 65:1348–52. doi: 10.1001/archneur.65.10.1348
164. Lv L, Tan X, Peng X, Bai R, Xiao Q, Tan J, et al. The relationships of vitamin D, vitamin D receptor gene polymorphisms, and vitamin D supplementation with Parkinson's disease. *Transl Neurodegener.* (2020) 9:34. doi: 10.1186/s40035-020-00213-2
165. Annweiler C, Llewellyn DF, Beauchet O. Low serum vitamin D concentrations in Alzheimer's disease: a systemic review and meta-analysis. *J Alzheimers Dis.* (2013) 33:659–74. doi: 10.3233/JAD-2012-121432
166. Balion C, Griffith LE, Strifler L, Henderson M, Patterson C, Heckman G, et al. Vitamin D, cognition, and dementia: a systematic review and meta-analysis. *Neurology.* (2012) 79:1397–405. doi: 10.1212/WNL.0b013e31826c197f
167. Peterson AL, Mancini M, Horak FB. The relationship between balance control and vitamin D in Parkinson's disease-a pilot study. *Mov Disord.* (2013) 28:1133–7. doi: 10.1002/mds.25405
168. Peterson AL, Murchison C, Zabetian C, Leverenz JB, Watson GS, Montine T, et al. Memory, mood, and vitamin D in persons with Parkinson's disease. *J Park Dis.* (2013) 3:547–55. doi: 10.3233/JPD-130206
169. Littlejohns TJ, Henley WE, Lang IA, Annweiler C, Beauchet O, Chaves PHM, et al. Vitamin D and the risk of dementia and Alzheimer disease. *Neurology.* (2014) 83:920–8. doi: 10.1212/WNL.0000000000000755
170. Turetsky A, Goddeau RP, Henninger N. Low serum vitamin D is independently associated with larger lesion volumes after ischemic stroke. *J Stroke Cerebrovasc Dis.* (2015) 24:1555–63. doi: 10.1016/j.jstrokecerebrovasdis.2015.03.051
171. Huang H, Zheng T, Wang S, Wei L, Wang Q, Sun Z. Serum 25-hydroxyvitamin D predicts early recurrent stroke in ischemic stroke patients. *Nutr Metab Cardiovasc Dis.* (2016) 26:908–14. doi: 10.1016/j.numecd.2016.06.009
172. Nie Z, Ji XC, Wang J, Zhang HX. Serum levels of 25-hydroxyvitamin D predicts infarct volume and mortality in ischemic stroke patients. *J Neuroimmunol.* (2017) 313:41–5. doi: 10.1016/j.jneuroim.2017.10.002
173. Di Somma C, Scarano E, Barrea L, Zhukouskaya VV, Savastano S, Mele C, et al. Vitamin D and neurological diseases: an endocrine view. *Int J Mol Sci.* (2017) 18:11. doi: 10.3390/ijms18112482
174. Sivandzade F, Prasad S, Bhalerao A, Cucullo L. NRF2 and NF-κB interplay in cerebrovascular and neurodegenerative disorders: molecular mechanisms and possible therapeutic approaches. *Redox Biol.* (2019) 21:101059. doi: 10.1016/j.redox.2018.11.017
175. Wimalawansa SJ. Vitamin D deficiency: effects on oxidative stress, epigenetics, gene regulation, and aging. *Biology*. (2019) 8:30. doi: 10.3390/biology8020030
176. Trist BG, Hare DJ, Double KL. Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. *Aging Cell.* (2019) 18:e13031. doi: 10.1111/acel.13031
177. Niedzielska E, Smaga I, Gawlik M, Moniczewski A, Stankowicz P, Pera J, et al. Oxidative stress in neurodegenerative diseases. *Mol Neurobiol.* (2016) 53:4094–125. doi: 10.1007/s12035-015-9337-5
178. Browne SE, Beal MF. Oxidative damage in Huntington's disease pathogenesis. *Antioxid Redox Signal.* (2006) 8:2061–73. doi: 10.1089/ars.2006.8.2061
179. Rodrigo R, Fernandez-Gajardo R, Guierrez R, Matamala JM, Carrasco R, Miranda-Merchak A, et al. Oxidative stress and pathophysiology of ischemic stroke: novel therapeutic opportunities. *CNS Neurol Disord Drug Targets.* (2013) 12:698–714. doi: 10.2174/18715273113120500 15
180. Di Filippo M, Chiasserini D, Tozzi A, Picconi B, Calabresi, P. Mitochondria and the link between neuroinflammation and neurodegeneration. *J Alzheimers Dis.* (2010) 20:S369–79. doi: 10.3233/JAD-2010-100543
181. Brandes MS, Gray NE. NRF2 as a Therapeutic Target in Neurodegenerative Diseases. *ASN Neuro.* (2020) 12:1759091419899782. doi: 10.1177/1759091419899782
182. Khairy EY, Attia MM. Protective effects of vitamin D on neurophysiologic alterations in brain aging: role of brain-derived neurotrophic factor (BDNF). *Nutr Neurosci.* (2021) 24:650–9. doi: 10.1080/1028415X.2019.1665854
183. Hannan A, Dash R, Al Mamun Sohag A, Haque N, Soo Moon I. Neuroprotection Against Oxidative Stress: Phytochemicals Targeting TrkB Signalaing and the Nrf2-ARE Antioxidant System. *Fronter Mol Neurosci.* (2020). doi: 10.3389/fnmol.2020.00116
184. Ohl K, Tenbrock K, Kipp M. Oxidative stress in multiple sclerosis: Central and peripheral mode of action. *Exp Neurol.* (2016) 277:58–67. doi: 10.1016/j.expneurol.2015.11.010

185. Genestra M. Oxyl radicals, redox-sensitive signaling cascades and antioxidants. *Cell Signal*. (2007) 19:1807–19. doi: 10.1016/j.cellsig.2007.04.009
186. Gray E, Thomas TL, Betmouni S, Scolding N, Love S. Elevated activity and microglial expression of myeloperoxidase in demyelinated cerebral cortex in multiple sclerosis. *Brain Path*. (2008) 18:86–95. doi: 10.1111/j.1750-3639.2007.00110.x
187. Gray E, Thomas TL, Betmouni S, Scolding N, Love S. Elevated myeloperoxidase activity in white matter in multiple sclerosis. *Neurosci Lett*. (2008) 444:195–8. doi: 10.1016/j.neulet.2008.08.035
188. Cross AH, Manning PT, Keeling RM, Schmidt RE, Misko, TP. Peroxynitrite formation within the central nervous system in active multiple sclerosis. *J Neuroimmunol*. (1998) 88:45–56. doi: 10.1016/S0165-5728(98)00078-2
189. Liu JS, Zhao ML, Brosnan CF, Lee SC. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Amer J Pathol*. (2001) 158:2057–66. doi: 10.1016/S0002-9440(10)64677-9
190. van Horssen J, Schreibelt G, Drexhage J, Hazes T, Dijkstra CD, van der Valk P, et al. Severe oxidative damage in multiple sclerosis lesions coincides with enhanced antioxidant enzyme expression. *Free Radical Biol Med*. (2008) 45:1729–37. doi: 10.1016/j.freeradbiomed.2008.09.023
191. Craner MJ, Newcombe J, Black JA, Hartle C, Cuzner ML, Waxman SG. Molecular changes in neurons in multiple sclerosis: altered axonal expression of Nav1.2 and Nav1.6 sodium channels and Na+/Ca2+ exchanger. *Proc Natl Acad Sci USA*. (2004) 101:8168–73. doi: 10.1073/pnas.0402765101
192. Witte ME, Bø L, Rodenburg RJ, Belien JA, Musters R, Hazes T. Enhanced number and activity of mitochondria in multiple sclerosis lesions. *J Pathol*. (2009) 219:193–204. doi: 10.1002/path.2582
193. Campbell G, Mahad DJ. Mitochondrial dysfunction and axon degeneration in progressive multiple sclerosis. *FEBS Lett*. (2018) 592:1113–21. doi: 10.1002/1873-3468.13013
194. Waxman SG. Ions, energy and axonal injury: towards a molecular neurology of multiple sclerosis. *Trends Mol Med*. (2006) 12:192–5. doi: 10.1016/j.molmed.2006.03.001
195. Hybertson BM, Gao B, Bose SK, McCord JM. Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. *Mol Aspects Med*. (2011) 32:234–46. doi: 10.1016/j.mam.2011.10.006
196. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. *Toxicological Sci*. (2010) 114:237–46. doi: 10.1093/toxsci/kfp274
197. Larabee CM, Desai S, Agasing A, Georgescu C, Wren JD, Axtell RC, et al. Loss of Nrf2 exacerbates the visual deficits and optic neuritis elicited by experimental autoimmune encephalomyelitis. *Molecular Vision*. (2016) 22:1503–13.
198. Morales Pantoja IE, Hu CL, Perrone-Bizzozero NI, Zheng J, Bizzozero OA. Nrf2-dysregulation correlates with reduced synthesis and low glutathione levels in experimental autoimmune encephalomyelitis. *J Neurochem*. (2016) 139:640–50. doi: 10.1111/jnc.13837
199. Linker RA, Lee DH, RyanS, van Dam AM, Conrad R, Bista P, et al. Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain*. (2011) 134:678–92. doi: 10.1093/brain/awq386
200. van Horssen J, Drexhage JA, Flor T, Gerritsen W, van der Valk P, de Vries HE. Nrf2 and DJ1 are consistently upregulated in inflammatory multiple sclerosis lesions. *Free Radical Biol Med*. (2010) 49:1283–9. doi: 10.1016/j.freeradbiomed.2010.07.013
201. Licht-Mayer S, Wimmer I, Traffehn S, Metz I, Brück W, Bauer J, et al. Cell type-specific Nrf2 expression in multiple sclerosis lesions. *Acta neuropathological*. (2015) 130:263–77. doi: 10.1007/s00401-015-1452-x
202. Ghoreschi K, Bruck J, Kellerer C, Deng C, Peng H, Rothfuss O, et al. Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells. *J Exp Med*. (2011) 208:2291–303. doi: 10.1084/jem.20100977
203. Kappos L, Gold R, Miller DH, Macmanus DG, Havrdova E, Limmroth V, et al. Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study. *Lancet*. (2008) 372:1463–72. doi: 10.1016/S0140-6736(08)61619-0
204. Albrecht P, Bouchachia I, Goebels N, Henke N, Hofstetter HH, Issberner A, et al. Effects of dimethyl fumarate on neuroprotection and immunomodulation. *J neuroinflammation*. (2012) 9:163. doi: 10.1186/1742-2094-9-163
205. Fox RJ, Miller DH, Phillips JT, Hutchinson M, Havrdova E, Kita M, et al. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. *N Engl J Med*. (2012) 367:1087–97. doi: 10.1056/NEJMoa1206328
206. Gold R, Kappos L, Arnold DL, Bar-Or A, Giovannoni G, Selmaj K, et al. Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *N Engl J Med*. (2012) 367:1098–107. doi: 10.1056/NEJMoa1114287
207. Peng H. Guerau-de-Arellano M, Mehta VB, Yang Y, Huss DJ, Papenfuss TL, et al. Dimethyl fumarate inhibits dendritic cell maturation via nuclear factor kB (NF-kB)and extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mitogen stress-activated kinase 1 (MSK1) signaling. *J Biol Chem*. (2012) 287:28017–26. doi: 10.1074/jbc.M112.383380
208. Salehi MS, Borhani-Haghighi A, Pandamooz S, Safari A, Dargahi L, Dianatpour M, et al. Dimethyl fumarate up-regulates expression of major neurotrophic factors in the epidermal neural crest stem cells. *Tissue cell*. (2019) 56:114–20. doi: 10.1016/j.tice.2019.01.004
209. Nachliely M, Trachtenberg A, Khalfin B, Nalbandyan K, Cohen-Lahav M, Yasuda K, et al. Dimethyl fumarate and vitamin D derivatives cooperatively enhance VDR and Nrf2 signaling in differentiating AML cells in vitro and inhibit leukemia progression in a xenograft mouse model. J Steroid Biochem Mol Biol. (2019) 188:8–16. doi: 10.1016/j.jsbmb.2018.11.017
210. Hausler D, Torke S, Peelen E, Bertsch T, Djukic M, Nau R, et al. High dose vitamin D exacerbates central nervous system autoimmunity by raising T-cell excitatory calcium. *Brain*. (2019) 142:2737–55. doi: 10.1093/brain/awz190
211. Long T, Yang Y, Peng L, Li Z. Neuroprotective effects of melatonin on experimental allergic encephalomyelitis mice via anti-oxidative stress activity. *J Mol Neurosci*. (2018) 64:233–41. doi: 10.1007/s12031-017-1022-x
212. Ghareghani M, Reiter RJ, Zibara K, Farhadi N. Latitude, vitamin D, melatonin, and gut microbiota act in concert to initiate multiple sclerosis: a new mechanistic pathway. *Front Immunol*. (2018) 9:2484. doi: 10.3389/fimmu.2018.02484
213. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science*. (2022) 375:296–301. doi: 10.1126/science.abj8222
214. Lang HLE, Jacobsen H, Ikemizu S, Andersson C, Harlos K, Madsen L, et al. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat Immunol*. (2002) 3:940–3. doi: 10.1038/ni835
215. Lindsey JW. Antibodies to the Epstein-Barr virus proteins BFRF3 and BRRF2 cross-react with human proteins. *J Neuroimmunol*. (2017) 10:131–4. doi: 10.1016/j.jneuroim.2017.07.013
216. Tengvall K, Huang J, Hellstrom C, Kammer P, Bistrom M, Ayoglu B, et al. Molecular mimicry between anoctamin 2 and Epstein-Barr virus nuclear antigen 1 associates with multiple sclerosis risk. *Proc Natl Acad Sci U S A*. (2019) 116:16955–60. doi: 10.1073/pnas.1902623116
217. Lanz TV, Brewer RC, Ho PP, Moon JS, Jude KM, Fernandez D, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. *Nature*. (2022) doi: 10.21203/rs.3.rs-1239863/v1
218. Perez-Perez S, Dominguez-Mozo MI, Garcia-Martinez MA, Aladro Y, Martinez-Gines M, Garcia-Dominguez JM, et al. Study of the possible link of 25-hydroxyvitamin D with Epstain-Barr virus and human herpesvirus 6 in patients with multiple sclerosis. *Eur J Neurol*. (2018) 25:1446–53. doi: 10.1111/ene.13749
219. Mameli G, Madeddu G, Mei A, Uleri E, Poddighe L, Delogu LG, et al. Activation of MSRV-type endogenous retroviruses during infectious mononucleosis and Epstein-Barr virus latency: the missing link with multiple sclerosis. *PLoS ONE*. (2013) 8:e78474. doi: 10.1371/journal.pone.0078474
220. Zwart SR, Mehta S, Ploutz-Snyder R, Bourbeau Y, Locke JP, Pierson DL, et al. Response to vitamin D supplementation during Antarctic winter is related to BMI, and supplementation can mitigate Epstein-Barr virus reactivation. *J Nutr*. (2011) 141:692–7. doi: 10.3945/jn.110.134742
221. Zdimerova H, Murer A, Engelmann C, Raykova A, Deng Y, Gujer C, et al. Attenuated immune control of Epstein-Barr virus in humanized mice is

associated with the multiple sclerosis risk factor HLA-DR15. *Eur J Immunol.* (2021) 51:64–75. doi: 10.1002/eji.202048655

222. Brutting C, Stangl GI, Staege MS. Vitamin D, Epstein-Barr virus, and endogenous retroviruses in multiple sclerosis – facts and hypothesis. *J Integr Neurosci.* (2021) 20:233–8. doi: 10.31083/j.jin.2021.01.392
223. Ostkamp P, Salmen A, Pignolet B, Gorlich D, Andlauer TFM, Schulte-Mecklenbeck A, et al. Sunlight exposure exerts immunomolulatory effects to reduce multiple sclerosis severity. *Proc Natl Acad Sci U S A.* (2021) 18:e2018457118. doi: 10.1073/pnas.2018457118

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The Potential for EBV Vaccines to Prevent Multiple Sclerosis

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There is increasing evidence suggesting that Epstein-Barr virus infection is a causative factor of multiple sclerosis (MS). Epstein-Barr virus (EBV) is a human herpesvirus, Human Gammaherpesvirus 4. EBV infection shows two peaks: firstly, during early childhood and, secondly during the teenage years. Approximately, 90–95% of adults have been infected with EBV and for many this will have been a subclinical event. EBV infection can be associated with significant morbidity and mortality; for example, primary infection in older children or adults is the leading cause of infectious mononucleosis (IM). A disrupted immune response either iatrogenically induced or through genetic defects can result in lymphoproliferative disease. Finally, EBV is oncogenic and is associated with several malignancies. For these reasons, vaccination to prevent the damaging aspects of EBV infection is an attractive intervention. No EBV vaccines have been licensed and the prophylactic vaccine furthest along in clinical trials contains the major virus glycoprotein gp350. In a phase 2 study, the vaccine reduced the rate of IM by 78% but did not prevent EBV infection. An EBV vaccine to prevent IM in adolescence or young adulthood is the most likely population-based vaccine strategy to be tested and adopted. National registry studies will need to be done to track the incidence of MS in EBV-vaccinated and unvaccinated people to see an effect of the vaccine on MS. Assessment of vaccine efficacy with MS being a delayed consequence of EBV infection with the average age of onset being approximately 30 years of age represents multiple challenges.

Keywords: Epstein-Barr virus (EBV), prophylactic vaccination, epidemiological evidence, vaccine evaluation, multiple sclerosis

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INTRODUCTION

Epstein-Barr virus (EBV) is a human herpesvirus, *Human Gammaherpesvirus 4* (1) first isolated during the early 1960s (2). Human herpesviruses have a common structure comprising double-stranded DNA contained within an icosahedral nucleocapsid which is surrounded by a tegument and a host cell-derived outer membrane containing virus glycoproteins. The EBV genome is approximately 170 kilobases long and expresses over 80 different proteins (3–5). These include

TABLE 1 | EBV proteins and associated antibodies commonly used as infection markers and in investigations of associations with multiple sclerosis[a].

EBV protein	Function	Corresponding antibody
Virus Capsid (VCA)	A lytic phase protein which is a complex comprising major, minor and smallest capsid proteins. Structural proteins which encapsidate the virus genome (11).	VCA IgM is a marker of acute infection. Initial detection of VCA IgG corresponds also with acute infection; however, following seroconversion VCA IgG usually remains detectable for life (10–14).
Early Antigen (EA)	Protein complexes produced early in the lytic cycle. Two forms identified based on characteristic immunofluorescence patterns. Early antigen diffuse (EA-D) and Early antigen restricted (EA-R). Essential for viral DNA polymerase activity.	EA IgG responses vary with different populations limiting its utility as a general marker of acute infection. High levels of EA-R IgG are associated with Burkitt lymphoma and high levels of EA-D IgG with nasopharyngeal carcinoma. Seropositivity in general populations is approximately 20–30% (10, 14).
Nuclear Antigen 1 (EBNA1)	A DNA binding protein, expressed in virus infected cells, that is produced during latency. It is essential for the persistence and replication of the viral genome (5, 7, 9, 11).	EBNA1 IgG is present in convalescent or past infection; however, a few individuals fail to produce this antibody following infection. Higher levels compared to controls are associated with multiple sclerosis (10–14).
Nuclear Antigen 2 (EBNA2)	Forms complex with DNA-binding cellular transcription factors. Activates viral and cellular promoters as heterodimer. Promotes cell proliferation (5, 7, 9, 11).	EBNA2 IgG has a limited role in EBV diagnosis as it appears earlier than EBNA1 IgG and is present in healthy controls. High levels are reported to be strongly associated with an increased risk of development of multiple sclerosis (10, 11, 13).

[a]*Numbers in parentheses list relevant references.*

several viral glycoproteins present on the surface of the virus, including gp350, gH, gL, and gp42 which are important for the attachment and fusion of the virus to the cell and are the targets of prophylactic vaccines (6). EBV is adapted to maintain a long-term existence with the human host as a result of highly effective virus mechanisms capable of evading the human immune response (7, 8). Latency (9) is established following the virus expression of several proteins including the EBV nuclear antigens (EBNAs 1, 2, 3, LP) and latent membrane proteins (LMPs). Antibodies to some of these latent proteins (10), especially EBNA1 have been implicated in the pathogenesis of MS (**Table 1**).

EBV infection typically occurs during early childhood and in the great majority of young children it is asymptomatic or subclinical; however, in older children and adults EBV infection is a major cause of infectious mononucleosis (12, 13). The most frequent signs and symptoms of infectious mononucleosis (IM) in young adults are sore throat (95–98%), cervical lymphadenopathy (80–88%), and fatigue (70–78%) of variable duration (median 15 days). The reported rates of presentation of IM following primary EBV infection in young adults are variable e.g. 25–74% (12, 13). Other frequent symptoms of IM include fever, headache, loss of appetite, myalgia and upper respiratory tract symptoms and rarer clinical findings include abdominal pain, hepatomegaly, splenomegaly, nausea, and vomiting (12, 13). In cases when the immune system is compromised, severe EBV-mediated disease may occur such as post-transplant lymphoproliferative disorders following immunosuppression (15). Chronic active EBV infection is a rare lymphoproliferative disease associated with high morbidity and mortality in which an ineffective T-cell response enables the clonal proliferation of infected cells in patients without an apparent immune defect (14). Defective immune responses due to inherited genetic conditions may also result in failure to contain EBV replication such as occurs with X-linked lymphoproliferative syndrome (16). EBV is an oncogenic virus, and it is associated with the development of several lymphomas and carcinomas (17). Finally, EBV infection is associated with the development of several autoimmune diseases (11).

EBV is frequently shed in oral fluid (18) and transfer of the virus via sharing saliva is the main route of virus transmission. EBV can also be transmitted via sexual activity and transplantation (12, 19). Epithelial cells and B-lymphocytes are the primary sites of infection (20). Latency is established in memory B cells and the expression of latent proteins are key for immune avoidance and persistence of the virus within the host (7, 9, 21).

In England and Wales, EBV seroprevalence studies show two peaks of infection in children less than 5 years old (seroprevalence of 35% in 1–4 year-olds) and in young adults (seroprevalence of 72% in 15–19 year-olds) (22). Significant disparities exist with the age of acquisition of infection with a trend toward older children experiencing infection in certain population groups and geographical regions (23). EBV infection occurs worldwide with 90–95% of adults having serological evidence of EBV infection (24).

MULTIPLE SCLEROSIS

MS is the most common chronic inflammatory demyelinating disease of the central nervous system (CNS), affecting at least 2.5 million people worldwide. It is one of the most frequent causes of disability in young adults (25).

The etiology of MS remains unknown, however, EBV may be causally related (26).The pathogenesis of MS is thought to be mediated by the immune system. Evidence for immune-mediated mechanisms (27) comes from the pathology of disease (28), the contribution of genes of the immune response to disease susceptibility (29), experimental observations in relevant animal models (30), and from the efficacy of immunotherapies in those affected by MS (31).

TABLE 2 | Epstein-Barr virus prophylactic vaccine clinical trials: past and present[a].

Vaccine	Manufacturer	Clinical trial/publication	Outcome
Recombinant gp350	GlaxoSmithKline Biologicals, Rixensart, Belgium	Phase 1 and phase 2 studies to evaluate safety and immunogenicity of a recombinant gp350 EBV vaccine in healthy adults (58)	Phase 1 (59 subjects evaluated). One severe adverse event. Phase 1 and 2 studies (79 subjects evaluated). One severe adverse event.
Recombinant gp350 and ASO_4 adjuvant system	GlaxoSmithKline Biologicals, Rixensart, Belgium	Recombinant gp350 vaccine for infectious mononucleosis: a phase 2, randomized, double-blind, placebo-controlled trial to evaluate the safety, immunogenicity, and efficacy of an EBV vaccine in healthy young adults. (59)	A total of 178 EBV seronegative subjects were evaluated. No subject discontinued medication for reasons of safety or reactogenicity. In an intention to treat analysis, IM cases were distributed as follows: 9 cases (1 probable and 8 definite) were found in the placebo group and 2 cases (both definite) were found in the vaccine group ($p = 0.03$; $\alpha = 0.05$, by 1-sided Fisher's exact test).
CD8+ T-cell synthetic peptide HLA B*0801-restricted epitope and tetanus toxoid vaccine	Queensland Institute of Medical Research, Australia	Phase 1 trial of a CD8+ T-cell peptide epitope-based vaccine for infectious mononucleosis. (60)	A total of 14 subjects were evaluated. No serious adverse events were reported. Trial too small to estimate vaccine efficacy. Vaccine immunogenic in most individuals.
mRNA-1189	Moderna TX, Inc.	A phase 1, randomized, observer-blind, placebo-controlled, dose-ranging study of an EBV candidate vaccine, mRNA-1189, in 18- to 30-year-old healthy adults. Trial ongoing. Estimated completion date June 2023. NCT05164094	Main outcome is to evaluate the safety and reactogenicity of mRNA-1189 in 18- to 30-year-old healthy adults. Secondary outcome is to evaluate vaccine immunogenicity.
EBV gp350-Ferritin nanoparticle vaccine adjuvanted with Matrix M1	National Institute of Allergy and Infectious Diseases, USA	A phase 1 study of the safety and immunogenicity of an EBV gp350-Ferritin nanoparticle vaccine in healthy adults with or without EBV infection. Trial ongoing. Estimated completion date July 2025. NCT04645147	Main outcome is to evaluate the safety and reactogenicity of gp350-Ferritin nanoparticle vaccine in 18- to 29-year-old healthy adults.

[a]*Numbers in parentheses list relevant references.*

MS lesions are characterized by loss of CNS myelin, axonal damage, activated microglia, and inflammatory infiltrates of peripheral immune cells including T and B lymphocytes and plasma cells (32), as well as gliosis in more advanced disease. Both white matter and gray matter myelin are affected in the brain and spinal cord. The pathology of MS in its different stages is reviewed in detail elsewhere (33).

Over 200 gene polymorphisms contribute to MS susceptibility (34). The strongest association is with genes of the immune response, primarily certain class I and II alleles of the human leucocyte antigen / major histocompatibility complex (29). Alleles of the HLA-DRB1 locus confer a higher risk of MS and interact with environmental factors known to influence susceptibility such as smoking and solvent exposure, EBV infection (35), childhood and adolescent obesity (36) and low vitamin D levels (37). Other non-HLA genes of the immune response have a more modest effect (34).

Environmental factors, both infectious and non-infectious, influence the risk of developing MS by interacting with the genetic variation that predisposes to autoimmune responses (38, 39). The incidence and prevalence of MS vary geographically, with the prevalence increasing with geographic latitude. A number of studies have found an inverse relationship between sun exposure, ultraviolet radiation exposure, or serum vitamin D levels, and the prevalence of MS (40). Smoking increases the risk of developing MS and may also be a risk factor for disease progression (41).

Among infectious factors, viral infections have been reported to be associated with MS, particularly when they occur in adolescence. Evidence for the role of EBV infection in the development of MS is compelling to the extent that it is now considered a putative causative agent (see below).

Understanding the factors that set off MS can potentially enable prevention. Lifestyle and environmental factors can be subject to possible interventions, in particular in individuals at risk for developing MS, such as relatives of people with MS. EBV infection is an MS trigger (26) and preventing it could prevent MS.

Epidemiology of EBV in Relation to MS

A link between EBV infection and MS was first suspected over 40 years ago based on the similarity between the epidemiology of MS and infectious mononucleosis (IM), which is a common manifestation of primary EBV infection occurring during adolescence or later in life (42). Further support for this hypothesis came from the observation that individuals with a

history of IM are 2–3-fold more likely to develop MS than individuals with asymptomatic EBV infection (43–45). This association could be explained by confounding by hygiene – a proposition known as the "hygiene hypothesis": IM is a marker of a high level of hygiene during childhood, which predisposes to MS by priming the immune system toward pro-inflammatory responses (46). Such a hypothesis could be tested by determining the MS risk in young adults who are EBV negative. Since EBV negativity is a reliable marker of a hygienic upbringing, if high levels of hygiene were causally related to MS, these individuals should have an increased MS risk. Paradoxically, the results of several cross-sectional studies have suggested that not only do individuals with high levels of hygiene have a greater risk of MS, but, as long as they remain EBV negative, they have a markedly lower risk than EBV infected individuals (44, 47). Similar results were obtained in pediatric MS (48, 49). These findings, together with evidence from several longitudinal studies that circulating IgG antibody titres to EBNA1 give a robust prediction of future MS risk (50–54), suggest that EBV plays a direct causal role in MS. Alternative explanations, however, remain possible. On one hand, the low risk of MS in EBV negative individuals has been inferred from case-control studies that included individuals recruited several years after MS diagnosis – by design, these studies could not exclude the possibility that EBV infection occurred soon after MS. On the other, it has been argued that higher anti-EBNA1 titres could result from an immune dysregulation preceding the onset of neurological symptoms of MS.

Only recently have these alternative explanations been refuted and compelling evidence has been provided that EBV causes MS. This demonstration relied on the longitudinal investigation of a cohort comprising over 10 million young adults (26). At the time of recruitment, about 5% of these individuals were EBV seronegative, thus providing a unique setting to investigate the temporal relation between EBV infection and MS risk. The results were striking – MS risk remained close to zero in EBV negative individuals but increased 32-fold after infection with EBV. In contrast, no increase in MS risk was found after infection with cytomegalovirus, which, like EBV, is mostly transmitted by saliva. Further, serum levels of neurofilament light chain (NfL), a sensitive, albeit not disease-specific, biomarker of neuroaxonal degeneration, were used to examine the temporal relation between EBV infection and the beginning of the putative pathological process leading to MS. Serum NfL levels increased up to 6 years before the clinical onset of MS and can thus be used as a marker of the time of potential disease initiation (26). Serum NfL levels in individuals who went on to develop MS were similar to those of individuals who remained healthy before and around the time of EBV seroconversion but increased after EBV seroconversion. This finding demonstrates that EBV infection precedes the earliest sign of the probable pathological process leading to MS. In the same investigation, the concern that non-specific immune dysregulation could explain the association between EBV, and MS was further examined by conducting a comprehensive agnostic search of the anti-virome antibody response using VirScan, a phage-based immunoprecipitation and sequencing technology (55). This search, conducted using pre- and post-onset serum samples from 30 MS cases and 30 closely matched controls, revealed that only EBV-derived peptides elicited stronger responses in MS cases than controls.

The biological plausibility (56), temporal sequence, and particularly the strength of the EBV-MS association, which virtually exclude confounding by any known or hypothetical risk factor, support the conclusion that EBV is the leading cause of MS (57).

Current Status of EBV Vaccines

Two types of EBV vaccines are under development; a prophylactic vaccine to prevent infection or disease, and a therapeutic vaccine to treat persons with EBV-associated cancers (**Table 2**). The prophylactic vaccine furthest along in clinical trials contains the major viral glycoprotein gp350 (58), which is important for virus attachment to B cells, in alum/monophosphoryl lipid A adjuvant. In a phase 2 study the vaccine reduced the rate of infectious mononucleosis by 78% but did not prevent EBV infection (59). A phase 1 study of an EBV peptide, derived from EBNA3A, in tetanus toxoid and oil and water emulsion, showed a trend in reduction of infectious mononucleosis (60). More recently, several other vaccines have been tested in small animal models and some in non-human primates. These vaccines contain gp350 or other viral glycoproteins including gH/gL and/or gB, which are required for fusion of the virus to B cells and epithelial cells, and gp42, which is essential for the virus to fuse to B cells. Vaccine formats have included display of gp350 or gH/gL/gp42 on ferritin nanoparticles in Sigma Adjuvant System (61, 62), trimeric recombinant gB and gH/gL in alum and CpG (63), Newcastle disease virus-like particles with gp350, gH/gL/gp42 or gB in alum/monophosphoryl lipid A (64), and EBV virus-like particles deleted for certain EBV latent and lytic genes (65). Vaccination of small animals with each of these vaccines induced EBV neutralizing antibodies to B cells and/or epithelial cells, and antibodies elicited by some of these vaccines inhibited EBV-mediated glycoprotein fusion to B cells and epithelial cells. In January 2022, Moderna initiated a clinical trial of an mRNA vaccine containing EBV gp350, gH/gL, and gp42 [clinicaltrials.gov NCT05164094 (66)]. A clinical trial of a gp350 ferritin nanoparticle vaccine in Matrix-M1 began in 2022 at the NIH [clinicaltrails.gov NCT04645147 (67)]. While the ultimate goal of a prophylactic vaccine would be to prevent infection with the virus, the vaccine might not completely block infection but instead, reduce EBV associated diseases including malignancies and autoimmune diseases associated with the virus.

To prevent the development of EBV-associated diseases, vaccines designed to also harness T cell responses to the viral proteins could be employed to generate antiviral T cells alongside the neutralizing antibodies. T cells that target EBV structural proteins that are delivered into the cells as pre-formed virion components could eradicate the newly infected cells before they express growth transforming viral latency proteins; thus, such vaccines might prevent the establishment of a permanent latent infection. Indeed, T cells have been identified that recognize epitopes from structural proteins in newly infected B cells,

including gp350, gH, gL and gB (68) as well as tegument and capsid proteins (68, 69).

Importantly, therapeutic vaccine strategies can also be employed to treat individuals with EBV-associated diseases by boosting the existing antiviral T cell responses or even inducing novel antiviral responses. The advantage to such strategies is their exquisite tumor-specificity that allows the cancer to be targeted with minimal risk to normal tissue. In such trials, EBV proteins expressed in latently infected tumor cells such as EBV nuclear antigens (EBNAs) or latent membrane proteins (LMPs) have been used as vaccine targets. The initial therapeutic vaccine trials in nasopharyngeal carcinoma (NPC) patients employed autologous monocyte-derived dendritic cells (DCs) loaded with LMP2 CD8+ T cell epitope peptides. The first trial showed that 9 out of 18 vaccinated patients exhibited an increase in circulating LMP2-specific T cells, of whom 2 had partial clinical responses (70); similarly, the second trial showed increased circulating LMP2-specific T cells in 7 out of 16 vaccinated patients but no clinical responses (71). Importantly, these trials only used a limited number of pre-defined LMP2 CD8+ T cell epitopes, so to expand the range of T cell specificities an alternative approach using autologous DCs transduced with an adenovirus expressing truncated LMP1 and full-length LMP2 was employed. However, patients on this trial had been heavily pre-treated with cytotoxic chemotherapy and no expansions in T cell responses were observed, yet out of 12 patients, one partial clinical response and two instances of stable disease were achieved (72). Since these DC-based vaccination trials were initiated, non-cell based therapeutic vaccines have been developed that employ recombinant viral vectors expressing EBNA1, LMP1 and/or LMP2 and have undergone clinical trials in NPC patients. An initial phase 1 trial using an adenoviral vector expressing LMP2 induced a dose-dependent increase in the number of LMP2-specific CD3+ CD4+ T cells (73). In phase 1 trials of a modified vaccinia Ankara (MVA) virus expressing the carboxyl terminus of EBNA1 fused to full-length LMP2 designed to induce both CD4+ and CD8+ T cell responses respectively, a two-fold increase in the T cell response to one or both of the EBV proteins was observed in NPC patients from Hong Kong (74) and the UK (75). Furthermore, the vaccination boosted a broad range of CD4+ and CD8+ T cell responses against EBNA1 and LMP2. Since portions of EBNA1 have been reported to mimic cellular proteins resulting in cross-reacting antibodies that could affect the nervous system (76), these portions of EBNA1 might be deleted from a vaccine to reduce the risk of inducing an immune response to these cellular proteins. To summarize, while therapeutic vaccines have had modest clinical activity, relatively few studies have been performed and many of the patients had previously received cytotoxic chemotherapy which would likely impair their response to vaccines.

Potential Benefits of EBV Vaccines in the Prevention of MS

EBV is not only causally linked to MS, but is considered to play a potentially causal role in other autoimmune disease such as systemic lupus erythematosus (SLE) (77) and is associated with several malignancies including nasopharyngeal carcinoma, gastric carcinoma, and Burkitt, Hodgkin, and other lymphomas (78). EBV's oncogenic potential has resulted in an emphasis on developing vaccines that induce sterilizing immunity with the objective of preventing EBV infection (79, 80). However, it is debatable whether sterilizing immunity is necessary for preventing MS. EBV-associated infectious mononucleosis (IM) is a stronger risk factor than asymptomatic EBV infection for MS (43, 81, 82). High titres of anti-EBNA EBV antibodies at least in part represent a risk factor different from IM (83). High antibody titres to EBNA1 are associated with a greater MS risk and may indicate an inability to control EBV viral loads. This may be due to the MS-associated HLA DR15 haplotype, which may be associated with reduced control of EBV. In a humanized mouse model of EBV infection, the MS-associated HLA DR15 haplotype was associated with higher EBV viral loads (84). Therefore, non-sterilizing immunity from a vaccine that protects against IM and reduces immune responses to EBNA1, but not EBV infection, may be sufficient to reduce the incidence of MS and other autoimmune diseases.

CONCLUSIONS

An EBV vaccine to prevent IM in adolescence or young adulthood is the most likely population-based vaccine strategy to be tested and adopted. Based on the recent experience with COVID-19 vaccines the general population is likely to be risk-averse when it comes to the potential uptake of a new EBV vaccine (85). Therefore, it is likely that an EBV vaccine will be targeted at 12–13-year-olds, piggybacking on the HPV vaccine program. Although seroprevalence rates vary, over 50% of children in the general population will have already seroconverted by the age of 5 (86). This is arguably why an EBV vaccine will have to target a younger age group, e.g., 2–3 years of age before children are exposed to much horizontal transfer of EBV. For vertical transmission, i.e., mother-to-child transmission that occurs in lower-income environments, vaccination may have to occur even earlier. This is relevant for using an EBV vaccine to prevent some cancers such as Burkitt lymphoma and nasopharyngeal cancer.

Once a population-based primary EBV vaccination has been adopted, national registry studies will need to be done to track the incidence of MS, other autoimmune disease and EBV-associated lymphomas and cancers in EBV-vaccinated and unvaccinated people to see a delayed effect of the vaccine. With MS being a delayed consequence of EBV infection with the average age of onset being approximately 30 years of age this study will take a decade or more to deliver an answer.

In high-prevalence countries where MS has an annual incidence of 7.5 per 100,000 person-years, for a vaccine to reduce the incidence by two-thirds, preliminary estimates are that over 500,000 subjects would be needed (87). A registry of vaccinated individuals could be used for a matched cohort study, in which the exposure is EBV vaccination, and the outcome is MS. A two-sided test of whether the hazard ratio is one with an overall sample size of 512,138 subjects (of which 256,069 are

in the control group and 256,069 are in the treatment group) achieves 80% power at a 0.05 significance level when the hazard ratio is actually 0.33 (a 67% reduction). The number of events required to achieve this power is 25.5. It is anticipated that proportions of subjects having the event during the study is 0.000075 for the control group and 0.00002475 for the vaccine group (rate 1/3 of the 7.5 per 100,000 incidence). These results assume that the hazard ratio is constant throughout the study and that Cox proportional hazards regression is used to analyze the data.

Targeting EBV-negative older people at high risk of getting MS would potentially be quicker. However, making it through the early lifespan and not being infected may enrich for a selection bias that reduces the incidence. By being older the lag time to expected to MS development is shorter. While the duration of the study might be shortened, the sample size to establish the reduced incidence would remain essentially at 500,000 and since 80 to 95% of individuals are EBV-positive by age 20, the amount of screening necessary would involve over 3 million subjects to obtain the eligible randomized cohort assuming 100% consent to enroll. One proposal is to recruit first and second-degree relatives of people who have MS as these people will be more likely to volunteer for the study. As with the general population study above, the majority of older relatives >16 years of age would have already been infected with EBV and those destined to get MS may already have subclinical disease. However, just as older and immunosuppressed people benefit from the VZV vaccine in preventing shingles, EBV-seropositive people may benefit from the EBV vaccine as well.

Immunological data suggest that people with MS have a problem controlling EBV and have elevated antibody titres against EBNA1 (52). They have more EBNA1 reactive CD4+ T-cells (88), which respond to a larger repertoire of epitopes distributed across the EBNA1 protein (88). In comparison, T-cells from healthy controls only react to the immunodominant portion of the protein (89). It has also been shown that the poor control of EBV in persons with MS is due to cytotoxic CD8+ T-cells being exhausted and poorly responsive to EBV (90, 91). This is the rationale for the use of autologous and HLA-restricted allogeneic CTLs as a treatment for MS (92, 93). It is, therefore, possible that an EBV vaccine may stimulate immunity to overcome this T-cell exhaustion and reduce the chances of someone developing MS by improved control of EBV. Therefore, there is a strong argument to vaccinate all-comers. In reality, a study of older EBV seronegative high-risk individuals is not feasible. Based on vaccinating all-comers with an assumption that the familial incidence of MS is 300 per 100,000, for 90% power and a 3-fold reduction in the incidence from 300 to 100 per 100,000 a sample size of about 43,000 is required.

In summary, the more pragmatic approach would be doing a matched case-control study using a registry of EBV vaccinated individuals with the outcome being MS and in parallel an all-comer trial, agnostic of EBV status, in high-risk first- and second-degree family members using first clinical event compatible with demyelination as the primary outcome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PM, AA, JC, GG, CS-L, and RT wrote sections of the manuscript and reviewed it. GC reviewed the manuscript and performed several statistical calculations. BG planned and organized the manuscript and contributed to the writing of the manuscript and reviewed it. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Adriaenssens EM, Alfenas-Zerbini P, et al. Changes to virus taxonomy and to the international code of virus classification and nomenclature ratified by the international committee on taxonomy of viruses (2021). *Arch Virol.* (2021) 166:2633–48. doi: 10.1007/s00705-021-05156-1
2. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from burkitt's lymphoma. *Lancet.* (1964) 1:702–3. doi: 10.1016/S0140-6736(64)91524-7
3. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, et al. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature.* (1984) 310:207–11. doi: 10.1038/310207a0
4. Dolan A, Addison C, Gatherer D, Davison AJ, Mcgeoch DJ. The genome of Epstein-Barr virus type 2 strain AG876. *Virology.* (2006) 350:164–70. doi: 10.1016/j.virol.2006.01.015
5. Young LS, Yap LF, Murray PG. Epstein-Barr virus: more than 50 years old and still providing surprises. *Nat Rev Cancer.* (2016) 16:789–802. doi: 10.1038/nrc.2016.92
6. Balfour HHJr, Schmeling DO, Grimm-Geris JM. The promise of a prophylactic Epstein-Barr virus vaccine. *Pediatr Res.* (2020) 87:345–52. doi: 10.1038/s41390-019-0591-5
7. Cohen JI. The biology of Epstein-Barr virus: lessons learned from the virus and the host. *Curr Opin Immunol.* (1999) 11:365–70. doi: 10.1016/S0952-7915(99)80062-4
8. Murata T, Sugimoto A, Inagaki T, Yanagi Y, Watanabe T, Sato Y, et al. Molecular basis of epstein-barr virus latency establishment and lytic reactivation. *Viruses.* (2021) 13:2344. doi: 10.3390/v13122344
9. Young LS, Dawson CW, Eliopoulos AG. The expression and function of Epstein-Barr virus encoded latent genes. *Mol Pathol.* (2000) 53:238–47. doi: 10.1136/mp.53.5.238
10. Almohmeed YH, Avenell A, Aucott L, Vickers MA. Systematic review and meta-analysis of the sero-epidemiological association

between Epstein Barr virus and multiple sclerosis. *PLoS ONE.* (2013) 8:e61110. doi: 10.1371/journal.pone.0061110
11. Houen G, Trier NH. Epstein-Barr virus and systemic autoimmune diseases. *Front Immunol.* (2021) 11:587380. doi: 10.3389/fimmu.2020.587380
12. Dunmire SK, Verghese PS, Balfour HHJr. Primary Epstein-Barr virus infection. *J Clin Virol.* (2018) 102:84–92. doi: 10.1016/j.jcv.2018.03.001
13. Balfour HHJr, Dunmire SK, Hogquist KA. Infectious mononucleosis. *Clin Transl Immunol.* (2015) 4:e33. doi: 10.1038/cti.2015.1
14. Odumade OA, Hogquist KA, Balfour HHJr. Progress and problems in understanding and managing primary Epstein-Barr virus infections. *Clin Microbiol Rev.* (2011) 24:193–209. doi: 10.1128/CMR.00044-10
15. Lindsay J, Othman J, Heldman MR, Slavin MA. Epstein-Barr virus posttransplant lymphoproliferative disorder: update on management and outcomes. *Curr Opin Infect Dis.* (2021) 34:635–45. doi: 10.1097/QCO.0000000000000787
16. Rezaei N, Mahmoudi E, Aghamohammadi A, Das R, Nichols KE. X-linked lymphoproliferative syndrome: a genetic condition typified by the triad of infection, immunodeficiency and lymphoma. *Br J Haematol.* (2011) 152:13–30. doi: 10.1111/j.1365-2141.2010.08442.x
17. Farrell PJ. Epstein-Barr Virus and Cancer. *Annu Rev Pathol.* (2019) 14:29–53. doi: 10.1146/annurev-pathmechdis-012418-013023
18. Johnson KH, Webb CH, Schmeling DO, Brundage RC, Balfour HHJr. Epstein-Barr virus dynamics in asymptomatic immunocompetent adults: an intensive 6-month study. *Clin Transl Immunol.* (2016) 5:e81. doi: 10.1038/cti.2016.28
19. Higgins CD, Swerdlow AJ, Macsween KF, Harrison N, Williams H, Mcaulay K, et al. A study of risk factors for acquisition of Epstein-Barr virus and its subtypes. *J Infect Dis.* (2007) 195:474–82. doi: 10.1086/510854
20. Chen J, Longnecker R. Epithelial cell infection by Epstein-Barr virus. *FEMS Microbiol Rev.* (2019) 43:674–83. doi: 10.1093/femsre/fuz023
21. Guo R, Gewurz BE. Epigenetic control of the Epstein-Barr lifecycle. *Curr Opin Virol.* (2021) 52:78–88. doi: 10.1016/j.coviro.2021.11.013
22. Morris MC, Edmunds WJ, Hesketh LM, Vyse AJ, Miller E, Morgan-Capner P, et al. Sero-epidemiological patterns of Epstein-Barr and herpes simplex (HSV-1 and HSV-2) viruses in England and Wales. *J Med Virol.* (2002) 67:522–7. doi: 10.1002/jmv.10132
23. Dowd JB, Palermo T, Brite J, McDade TW, Aiello A. Seroprevalence of Epstein-Barr virus infection in U.S. children ages 6-19, 2003-2010. *PLoS ONE.* (2013) 8:e64921. doi: 10.1371/journal.pone.0064921
24. Winter JR, Jackson C, Lewis JE, Taylor GS, Thomas OG, Stagg HR. Predictors of Epstein-Barr virus serostatus and implications for vaccine policy: A systematic review of the literature. *J Glob Health.* (2020) 10:010404. doi: 10.7189/jogh.10.010404
25. Dobson R, Giovannoni G. Multiple sclerosis - a review. *Eur J Neurol.* (2019) 26:27–40. doi: 10.1111/ene.13819
26. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science.* (2022) 375:296–301. doi: 10.1126/science.abj8222
27. Murua SF, Farez MF, Quintana FJ. The immune response in multiple sclerosis. *Annu Rev Pathol.* (2022) 17:121–39. doi: 10.1146/annurev-pathol-052920-040318
28. Tobin WO, Kalinowska-Lyszczarz A, Weigand SD, Guo Y, Tosakulwong N, Parisi JE, et al. Clinical correlation of multiple sclerosis immunopathologic subtypes. *Neurology.* (2021) 97:e1906–13. doi: 10.1212/WNL.0000000000012782
29. Kim W, Patsopoulos NA. Genetics and functional genomics of multiple sclerosis. *Semin Immunopathol.* (2022) 44:63–79. doi: 10.1007/s00281-021-00907-3
30. T Hart, B. A., and Kap, Y.S. (2017). An essential role of virus-infected B cells in the marmoset experimental autoimmune encephalomyelitis model. *Mult Scler J Exp Transl Clin.* 3:2055217317690184. doi: 10.1177/2055217317690184
31. Martin R, Sospedra M, Rosito M, Engelhardt B. Current multiple sclerosis treatments have improved our understanding of MS autoimmune pathogenesis. *Eur J Immunol.* (2016) 46:2078–90. doi: 10.1002/eji.201646485
32. Machado-Santos J, Saji E, Troscher AR, Paunovic M, Liblau R, Gabriely G, et al. The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain.* (2018) 141:2066–82. doi: 10.1093/brain/awy151
33. Lassmann H. Multiple Sclerosis Pathology. *Cold Spring Harb Perspect Med.* (2018) 8a028936. doi: 10.1101/cshperspect.a028936
34. Baranzini SE, Oksenberg JR. The genetics of multiple sclerosis: from 0 to 200 in 50 years. *Trends Genet.* (2017) 33:960–70. doi: 10.1016/j.tig.2017.09.004
35. Munz C. Kissing genetic MS risk loci to life. *EBioMed.* (2021) 72:103594. doi: 10.1016/j.ebiom.2021.103594
36. Hedstrom AK, Hillert J, Brenner N, Butt J, Waterboer T, Strid P, et al. DRB1-environment interactions in multiple sclerosis etiology: results from two Swedish case-control studies. *J Neurol Neurosurg Psychiatry.* (2021) 92:717–22. doi: 10.1136/jnnp-2020-325676
37. Cocco E, Meloni A, Murru MR, Corongiu D, Tranquilli S, Fadda E, et al. Vitamin D responsive elements within the HLA-DRB1 promoter region in Sardinian multiple sclerosis associated alleles. *PLoS ONE.* (2012) 7:e41678. doi: 10.1371/journal.pone.0041678
38. Morandi E, Tanasescu R, Tarlinton RE, Constantinescu CS, Zhang W, Tench C, et al. The association between human endogenous retroviruses and multiple sclerosis: a systematic review and meta-analysis. *PLoS ONE.* (2017) 12:e0172415. doi: 10.1371/journal.pone.0172415
39. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. *N Engl J Med.* (2018) 378:169–80. doi: 10.1056/NEJMra1401483
40. Zarghami A, Li Y, Claflin SB, Van Der Mei I, Taylor BV. Role of environmental factors in multiple sclerosis. *Expert Rev Neurother.* (2021) 21:1389–408. doi: 10.1080/14737175.2021.1978843
41. Tanasescu R, Constantinescu CS, Tench CR, Manouchehrinia A. Smoking cessation and the reduction of disability progression in multiple sclerosis: a cohort study. *Nicotine Tob Res.* (2018) 20:589–95. doi: 10.1093/ntr/ntx084
42. Wergeland S, Riise T, Torkildsen O. Response to 'Seasonal variation of vitamin D and Epstein-Barr virus antibody in multiple sclerosis patients', a comment letter regarding 'Vitamin D, HLA-DRB1 and Epstein-Barr virus antibody levels in a prospective cohort of multiple sclerosis patients'. *Eur J Neurol.* (2018) 25:e104. doi: 10.1111/ene.13719
43. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann Neurol.* (2006) 59:499–503. doi: 10.1002/ana.20820
44. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol.* (2007) 61:288–99. doi: 10.1002/ana.21117
45. Nielsen TR, Rostgaard K, Nielsen NM, Koch-Henriksen N, Haahr S, Sorensen PS, et al. Multiple sclerosis after infectious mononucleosis. *Arch Neurol.* (2007) 64:72–5. doi: 10.1001/archneur.64.1.72
46. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med.* (2002) 347:911–20. doi: 10.1056/NEJMra020100
47. Ascherio A, Munch M. Epstein-Barr virus and multiple sclerosis. *Epidemiology.* (2000) 11:220–4. doi: 10.1097/00001648-200003000-00023
48. Alotaibi S, Kennedy J, Tellier R, Stephens D, Banwell B. Epstein-Barr virus in pediatric multiple sclerosis. *JAMA.* (2004) 291:1875–9. doi: 10.1001/jama.291.15.1875
49. Pohl D, Krone B, Rostasy K, Kahler E, Brunner E, Lehnert M, et al. High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. *Neurology.* (2006) 67:2063–5. doi: 10.1212/01.wnl.0000247665.94088.8d
50. Ascherio A, Munger KL, Lennette ET, Spiegelman D, Hernan MA, Olek MJ, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA.* (2001) 286:3083–8. doi: 10.1001/jama.286.24.3083
51. Sundstrom P, Juto P, Wadell G, Hallmans G, Svenningsson A, Nystrom L, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. *Neurology.* (2004) 62:2277–82. doi: 10.1212/01.WNL.0000130496.51156.D7
52. Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. *JAMA.* (2005) 293:2496–500. doi: 10.1001/jama.293.20.2496
53. Delorenze GN, Munger KL, Lennette ET, Orentreich N, Vogelman JH, Ascherio A. Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. *Arch Neurol.* (2006) 63:839–44. doi: 10.1001/archneur.63.6.noc50328
54. Munger KL, Levin LI, O'Reilly EJ, Falk KI, Ascherio A. Anti-Epstein-Barr virus antibodies as serological markers of multiple sclerosis: a prospective

study among United States military personnel. *Mult Scler.* (2011) 17:1185–1193. doi: 10.1177/1352458511408991
55. Xu GJ, Kula T, Xu Q, Li MZ, Vernon SD, Ndung'u T, et al. Comprehensive serological profiling of human populations using a synthetic human virome. *Science.* (2015) 348:aaa0698. doi: 10.1126/science.aaa0698
56. Kuchroo VK, Weiner HL. How does Epstein-Barr virus trigger MS? *Immunity.* (2022) 55:390–2. doi: 10.1016/j.immuni.2022.02.008
57. Giovannoni G, Hawkes CH, Lechner-Scott J, Levy M, Yeh EA, Gold J. Is EBV the cause of multiple sclerosis? *Mult Scler Relat Disord.* (2022) 58:103636. doi: 10.1016/j.msard.2022.103636
58. Moutschen M, Leonard P, Sokal EM, Smets F, Haumont M, Mazzu P, et al. Phase I/II studies to evaluate safety and immunogenicity of a recombinant gp350 Epstein-Barr virus vaccine in healthy adults. *Vaccine.* (2007) 25:4697–705. doi: 10.1016/j.vaccine.2007.04.008
59. Sokal EM, Hoppenbrouwers K, Vandermeulen C, Moutschen M, Leonard P, Moreels A, et al. Recombinant gp350 vaccine for infectious mononucleosis: a phase 2, randomized, double-blind, placebo-controlled trial to evaluate the safety, immunogenicity, and efficacy of an Epstein-Barr virus vaccine in healthy young adults. *J Infect Dis.* (2007) 196:1749–53. doi: 10.1086/523813
60. Elliott SL, Suhrbier A, Miles JJ, Lawrence G, Pye SJ, Le TT, et al. Phase I trial of a CD8+ T-cell peptide epitope-based vaccine for infectious mononucleosis. *J Virol.* (2008) 82:1448–57. doi: 10.1128/JVI.01409-07
61. Kanekiyo M, Bu W, Joyce MG, Meng G, Whittle JR, Baxa U, et al. Rational design of an Epstein-Barr virus vaccine targeting the receptor-binding site. *Cell.* (2015) 162:1090–100. doi: 10.1016/j.cell.2015.07.043
62. Bu, W., Joyce, M. G., Nguyen, H., Banh, D. V., Aguilar, F., Tariq, Z., et al. (2019). Immunization with components of the viral fusion apparatus elicits antibodies that neutralize Epstein-Barr virus in b cells and epithelial cells. *Immunity.* 50:1305–1316.e1306. doi: 10.1016/j.immuni.2019.03.010
63. Cui X, Cao Z, Ishikawa Y, Cui S, Imadome KI, Snapper CM. Immunization with Epstein-Barr virus core fusion machinery envelope proteins elicit high titers of neutralizing activities and protect humanized mice from lethal dose EBV challenge. *Vaccines (Basel).* (2021) 9. doi: 10.3390/vaccines9030285
64. Escalante GM, Foley J, Mutsvunguma LZ, Rodriguez E, Mulama DH, Muniraju M, et al. A pentavalent epstein-barr virus-like particle vaccine elicits high titers of neutralizing antibodies against Epstein-Barr virus infection in immunized rabbits. *Vaccines (Basel).* (2020) 8169. doi: 10.3390/vaccines8020169
65. Ruiss R, Jochum S, Wanner G, Reisbach G, Hammerschmidt W, Zeidler R. A virus-like particle-based Epstein-Barr virus vaccine. *J Virol.* (2011) 85:13105–13. doi: 10.1128/JVI.05598-11
66. NCT05164094. A Study of an Epstein-Barr Virus (EBV) Candidate Vaccine, mRNA-1189, in 18- to 30-Year-Old Healthy Adults. Available onine at: https://clinicaltrials.gov/ct2/show/NCT05164094 [Accessed].
67. NCT04645147. Safety and Immunogenicity of an Epstein-Barr Virus (EBV) gp350-Ferritin Nanoparticle Vaccine in Healthy Adults With or Without EBV Infection [Online]. Available online at: https://clinicaltrials.gov/ct2/show/NCT04645147 [Accessed].
68. Brooks JM, Long HM, Tierney RJ, Shannon-Lowe C, Leese AM, Fitzpatrick M, et al. Early T cell recognition of B cells following epstein-barr virus infection: identifying potential targets for prophylactic vaccination. *PLoS Pathog.* (2016) 12:e1005549. doi: 10.1371/journal.ppat.1005549
69. Dowell AC, Haigh TA, Ryan GB, Turner JE, Long HM, Taylor GS. Cytotoxic CD4+ T-cells specific for EBV capsid antigen BORF1 are maintained in long-term latently infected healthy donors. *PLoS Pathog.* (2021) 17:e1010137. doi: 10.1371/journal.ppat.1010137
70. Lin CL, Lo WF, Lee TH, Ren Y, Hwang SL, Cheng YF, et al. Immunization with Epstein-Barr Virus (EBV) peptide-pulsed dendritic cells induces functional CD8+ T-cell immunity and may lead to tumor regression in patients with EBV-positive nasopharyngeal carcinoma. *Cancer Res.* (2002) 62:6952–8.
71. Li J, Chen QY, He J, Li ZL, Tang XF, Chen SP, et al. Phase I trial of adoptively transferred tumor-infiltrating lymphocyte immunotherapy following concurrent chemoradiotherapy in patients with locoregionally advanced nasopharyngeal carcinoma. *Oncoimmunology.* (2015) 4:e976507. doi: 10.4161/23723556.2014.976507
72. Chia WK, Wang WW, Teo M, Tai WM, Lim WT, Tan EH, et al. A phase II study evaluating the safety and efficacy of an adenovirus-DeltaLMP1-LMP2 transduced dendritic cell vaccine in patients with advanced metastatic nasopharyngeal carcinoma. *Ann Oncol.* (2012) 23:997–1005. doi: 10.1093/annonc/mdr341
73. Si Y, Deng Z, Lan G, Du H, Wang Y, Si J, et al. The Safety and Immunological Effects of rAd5-EBV-LMP2 vaccine in nasopharyngeal carcinoma patients: a phase I clinical trial and two-year follow-up. *Chem Pharm Bull (Tokyo).* (2016) 64:1118–23. doi: 10.1248/cpb.c16-00114
74. Hui EP, Taylor GS, Jia H, Ma BB, Chan SL, Ho R, et al. Phase I trial of recombinant modified vaccinia ankara encoding Epstein-Barr viral tumor antigens in nasopharyngeal carcinoma patients. *Cancer Res.* (2013) 73:1676–88. doi: 10.1158/0008-5472.CAN-12-2448
75. Taylor GS, Jia H, Harrington K, Lee LW, Turner J, Ladell K, et al. A recombinant modified vaccinia ankara vaccine encoding Epstein-Barr Virus (EBV) target antigens: a phase I trial in UK patients with EBV-positive cancer. *Clin Cancer Res.* (2014) 20:5009–22. doi: 10.1158/1078-0432.CCR-14-1122-T
76. Robinson WH, Steinman L. Epstein-Barr virus and multiple sclerosis. *Science.* (2022) 375:264–5. doi: 10.1126/science.abm7930
77. Nelson P, Rylance P, Roden D, Trela M, Tugnet N. Viruses as potential pathogenic agents in systemic lupus erythematosus. *Lupus.* (2014) 23:596–605. doi: 10.1177/0961203314531637
78. Bakkalci D, Jia Y, Winter JR, Lewis JE, Taylor GS, Stagg HR. Risk factors for Epstein Barr virus-associated cancers: a systematic review, critical appraisal, and mapping of the epidemiological evidence. *J Glob Health.* (2020) 10:010405. doi: 10.7189/jogh.10.010405
79. Van Zyl DG, Mautner J, Delecluse HJ. Progress in EBV Vaccines. *Front Oncol.* (2019) 9:104. doi: 10.3389/fonc.2019.00104
80. Ruhl J, Leung CS, Munz C. Vaccination against the Epstein-Barr virus. *Cell Mol Life Sci.* (2020) 77:4315–24. doi: 10.1007/s00018-020-03538-3
81. Handel AE, Giovannoni G, Ebers GC, Ramagopalan SV. Environmental factors and their timing in adult-onset multiple sclerosis. *Nat Rev Neurol.* (2010) 6:156–66. doi: 10.1038/nrneurol.2010.1
82. Levin LI, Munger KL, O'Reilly EJ, Falk KI, Ascherio A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol.* (2010) 67:824–830. doi: 10.1002/ana.21978
83. Hedstrom AK, Huang J, Michel A, Butt J, Brenner N, Hillert J, et al. High levels of Epstein-Barr virus nuclear antigen-1-specific antibodies and infectious mononucleosis act both independently and synergistically to increase multiple sclerosis risk. *Front Neurol.* (2019) 10:1368. doi: 10.3389/fneur.2019.01368
84. Zdimerova H, Murer A, Engelmann C, Raykova A, Deng Y, Gujer C, et al. Attenuated immune control of Epstein-Barr virus in humanized mice is associated with the multiple sclerosis risk factor HLA-DR15. *Eur J Immunol.* (2021) 51:64–75. doi: 10.1002/eji.202048655
85. Cata-Preta BO, Santos TM, Mengistu T, Hogan DR, Barros AJD, Victora CG. Zero-dose children and the immunisation cascade: understanding immunisation pathways in low and middle-income countries. *Vaccine.* (2021) 39:4564–70. doi: 10.1016/j.vaccine.2021.02.072
86. Kuri A, Jacobs BM, Vickaryous N, Pakpoor J, Middeldorp J, Giovannoni G, et al. Epidemiology of Epstein-Barr virus infection and infectious mononucleosis in the United Kingdom. *BMC Public Health.* (2020) 20:912. doi: 10.1186/s12889-020-09049-x
87. Power Analysis and Sample Size Software. *Tests for Two Survival Curves Using Cox's Proportional Hazards Mode*. Kaysville, UT: NCSS; LLC (2021). Available online at: http:ncss.com/software/pass
88. Lunemann JD, Edwards N, Muraro PA, Hayashi S, Cohen JI, Munz C, et al. Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. *Brain.* (2006) 129:1493–506. doi: 10.1093/brain/awl067
89. Ruprecht K, Wunderlich B, Giess R, Meyer P, Loebel M, Lenz K, et al. Multiple sclerosis: the elevated antibody response to Epstein-Barr virus primarily targets, but is not confined to, the glycine-alanine repeat of Epstein-Barr nuclear antigen-1. *J Neuroimmunol.* (2014) 272:56–61. doi: 10.1016/j.jneuroim.2014.04.005
90. Cencioni MT, Magliozzi R, Nicholas R, Ali R, Malik O, Reynolds R, et al. Programmed death 1 is highly expressed on CD8(+) CD57(+) T cells in patients with stable multiple sclerosis and inhibits their cytotoxic response to Epstein-Barr virus. *Immunology.* (2017) 152:660–76. doi: 10.1111/imm.12808
91. Pender MP, Csurhes PA, Burrows JM, Burrows SR. Defective T-cell control of Epstein-Barr virus infection in multiple sclerosis. *Clin Transl Immunology.* (2017) 6:e126. doi: 10.1038/cti.2016.87

92. Pender MP, Csurhes PA, Smith C, Douglas NL, Neller MA, Matthews KK, et al. Epstein-Barr virus-specific T cell therapy for progressive multiple sclerosis. *JCI Insight*. (2018) 3:e124714. doi: 10.1172/jci.insight.124714
93. Ioannides ZA, Csurhes PA, Douglas NL, Mackenroth G, Swayne A, Thompson KM, et al. Sustained clinical improvement in a subset of patients with progressive multiple sclerosis treated with epstein-barr virus-specific T cell therapy. *Front Neurol*. (2021) 12:652811. doi: 10.3389/fneur.2021.652811

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