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## EDITED BY

Manu Rangachari,  
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## REVIEWED BY

Shalina Ousman,  
University of Calgary, Canada  
Alexey N. Boyko,  
Pirogov Russian National Research  
Medical University, Russia

## \*CORRESPONDENCE

Nikolaos Grigoriadis  
✉ ngrigoriadis@auth.gr

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# Sex, aging and immunity in multiple sclerosis and experimental autoimmune encephalomyelitis: An intriguing interaction

Marina Boziki<sup>1</sup>, Paschalis Theotokis<sup>1</sup>, Evangelia Kesidou<sup>1</sup>,  
Eleni Karafoulidou<sup>1</sup>, Chrystalla Konstantinou<sup>1</sup>,  
Iliana Michailidou<sup>1</sup>, Yasemin Bahar <sup>2</sup>, Ayse Altintas<sup>3</sup> and  
Nikolaos Grigoriadis<sup>1\*</sup>

<sup>1</sup>Laboratory of Experimental Neurology and Neuroimmunology and Multiple Sclerosis Center, 2nd Neurological University Department, AHEPA General Hospital of Thessaloniki, School of Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece, <sup>2</sup>Hacettepe University Medical School, Ankara, Turkey, <sup>3</sup>School of Medicine, Koç University, Istanbul, Turkey

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with a profound neurodegenerative component early in the disease pathogenesis. Age is a factor with a well-described effect on the primary disease phenotype, namely, the relapsing-remitting vs. the primary progressive disease. Moreover, aging is a prominent factor contributing to the transition from relapsing-remitting MS (RRMS) to secondary progressive disease. However, sex also seems to, at least in part, dictate disease phenotype and evolution, as evidenced in humans and in animal models of the disease. Sex-specific gene expression profiles have recently elucidated an association with differential immunological signatures in the context of experimental disease. This review aims to summarize current knowledge stemming from experimental autoimmune encephalomyelitis (EAE) models regarding the effects of sex, either independently or as a factor combined with aging, on disease phenotype, with relevance to the immune system and the CNS.

## KEYWORDS

sex, relapsing-remitting multiple sclerosis, progressive multiple sclerosis, experimental autoimmune encephalomyelitis, aging

## 1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) with its neurodegenerative component being increasingly recognized and studied over the last years (1). Similar to the majority of autoimmune diseases (2), a female predilection is evident, based on large epidemiological data from several countries (3). Moreover, MS is a highly heterogeneous disease with a very diverse range of clinical manifestations, radiology, course, and prognosis. In addition to sex, age of onset, as

well as aging are also considered significant factors for the overall disease course and prognosis. Aging is defined as the biological processes that occur over time affecting all organs on a tissue and cellular level. Aging is characterized by profound alterations in an organism's physiology and homeostasis, thus affecting several systems and functions, such as the metabolism, endocrine function, and the immune system (4). Recent advances with respect to “omic” technologies have allowed detailed fingerprinting of disease phenotypes, as well as of the underlying pathological processes, on a tissue and cellular level. It is prominent that sex variations account for specific differences in aging processes, thus being responsible for sex disparities in disease phenotype and progression (5).

The sex variation and aging in CNS autoimmunity have been extensively studied in experimental models of MS, prominently in the rodent model of experimental autoimmune encephalomyelitis (EAE). Although not entirely similar to MS, EAE recapitulates several aspects of disease pathology and is considered the most widely used experimental model for MS (6, 7). In the induced model of EAE, CNS autoimmunity is triggered by active immunization of rats or mice with an encephalitogen protein and/or peptide factor, together with an adjuvant. EAE is typically induced in female laboratory animals of young age, specifically 4–6 weeks of age, in order to investigate sex and age-related differences that would induce significant heterogeneity in the model. Moreover, experimental evidence highlights alterations in disease processes under various effects of sex and age. This review aims to summarize current knowledge stemming from EAE models regarding the effects of sex, either independently or as a factor combined with aging, on disease phenotype, with particular relevance to the immune system and the CNS.

## 2. Sex and aging in MS

### 2.1. Sex and MS phenotype: Clinical evidence

In most epidemiological studies addressing the incidence and prevalence of MS, there is a consistently higher predilection for women in relation to MS frequency, varying from 2:1 to 3:1 with respect to the female-to-male ratio (8). This pattern appears universal and is not affected by latitude within the countries studied (3). Moreover, the female-to-male ratio for MS appears to increase over the last decades in several nationwide studies, an observation possibly linked with alterations in environmental factors (9–15). Evidence on the effect of environmental factors on MS incidence has long been described in epidemiological studies (16), with Epstein–Barr virus suspected to be related to the events preceding the disease onset for a long time. However, knowledge of the exact mechanisms by which EBV infection may contribute to MS incidence is lacking. Epidemiological

evidence has recently identified EBV infection as a trigger of MS (17). Interestingly, the expression levels of genetic risk loci linked with MS were shown to differ between males and females, possibly due to epigenetic modifications (18). Furthermore, in EBV-infected B cells *in vitro*, the expression levels of estradiol receptors were shown to correlate with EBV infection traits, such as EBV latency III genes, thus providing evidence toward the sex-associated pathways of pathogenicity in MS and EBV-related pathology (18).

In addition to the effects of sex on MS incidence, extensive research focuses on whether sex is related to different clinical and/or radiological outcomes, as well as disease prognosis. The annual relapse rate has been reported to be higher for female patients, although a profound positive association between increased annual relapse rate and younger age has also been described (19–21). These findings indicate a higher inflammatory component for female patients with relapsing-remitting MS. However, these findings do not coincide with faster disability accumulation for female patients as the male sex is reportedly linked with faster disability outcome deterioration over the disease course than the female sex (21, 22). Of note, these differences were balanced for men and women after the age of 50 years, thus indicating sex-hormone-dependent mechanisms in relation to disease course (21). A beneficial effect of estrogens on MS outcomes has been described in studies addressing alterations in the disease course in menopausal patients with MS compared with patients before menopause onset (23, 24). Moreover, disability accumulation reportedly occurs faster in female patients with later-onset MS, i.e., >40 years old at disease onset, than in male patients with MS, and this sex-associated difference has been related to differential gene expression profiles in men and women (25). Conversely, male patients with relapsing-remitting MS have been correlated with faster conversion toward progressive disease, namely, secondary progressive MS (SPMS) (26, 27). This observation has been further supported by transcriptomic profile analysis in female and male patients with MS, revealing sex-specific molecular mechanisms in disease evolution (25, 28).

### 2.2. Aging and MS phenotype: Clinical evidence

In addition to the chronological age, defined by the date of birth, biological age is depicted by the relative assessment of molecular markers linked with basic biological processes of aging. Over the last years, through the development of multi-omics, defined as an integrative fingerprinting analysis approach that encompasses datasets generated from genomics, epigenomics, transcriptomics, proteomics, and metabolomics (29), considerable knowledge has been acquired in the field of systems immunology and the complex interactions with

other basic biological mechanisms, such as aging. Aging represents a paradox of immunodeficiency and inflammation (inflammaging), with profound implications for autoimmunity. Upon aging processes, genetic and epigenetic changes confer alterations in pathways of innate and acquired immunity, thus differentially shaping the antigen receptor repertoires and dysregulating the complex interactions between cellular and molecular components of immune response (30). More specifically, several basic biological mechanisms are considered the pillars underlying aging processes, including but not restricted to, for instance, genomic instability, epigenetic changes, cellular senescence, and changes in intercellular communication (30). Moreover, aging processes result in age-related phenotypes with implications for the overall function and wellbeing, such as loss of muscle mass, age-related diseases, and frailty (30). Immune alterations in the elderly and extremely old or over-aged (age >85 years) have long been studied and recognized as alterations in the circulating immune cell types and the lymphocyte subpopulations, as well as diminished responses against the antigen (25). Additionally, in aging organisms, deficient clearance of senescent cells results in the accumulation of cellular and molecular debris, with further harmful effects on tissue homeostasis (30).

Immune alterations in the context of aging have been implicated in MS phenotype with advancing age, as well as the several comorbidities that accumulate in older patients with MS. Inflammaging is characterized by low-grade, chronic, and systemic inflammation in the elderly and is associated with the predominance of pro-inflammatory phenotypes in cellular components of the innate and adaptive immunity, the proinflammatory cytokine and chemokine production, and the expansion of senescent cellular phenotypes (5). Nevertheless, individual aging and inflammaging mechanisms appear to be diverse among individuals (31), and this heterogeneity may account for the varying burden of inflammaging in autoimmune diseases of the elderly and the age-related phenotype alterations in MS (32, 33).

In MS, neuroinflammation and neurodegeneration are pathological processes that coexist in the CNS and their relative contribution across the disease course is a primary factor for the disease phenotype, assessed by clinical outcomes that depict relapse activity and/or disability accumulation (34). The age of disease onset is a determinant factor for the disease phenotype. Patients with younger onset ages develop a relapsing-remitting form with a high neuroinflammatory pathological component, whereas patients with older onset ages at onset more frequently display progressive disease. For patients of older age at onset, progression is present either from the onset, thus signifying the primary progressive disease form (PPMS), or appears faster in the disease course, due to a more rapid conversion from RRMS to SPMS, than patients of younger age at onset (35). Thus, the disease phenotype and the transition from RRMS toward SPMS appear to be primarily an age-dependent phenomenon

(36). Moreover, age progression signifies a reduction in the annualized relapse rate, most likely linked to a reduction in the effectiveness of the disease-modifying treatments (DMTs), as these factors primarily target the neuroimmune aspects of disease activity (16, 37). In line with these epidemiological observations, clinical MRI data confirm accelerated cortical atrophy and brain volume loss in patients with progressive disease compared with RRMS, and these MRI alterations are enhanced with advanced age (38) and correlate with clinical outcomes of disability accumulation (39). Other biological markers known to correlate with age, such as the serum neurofilament light chain, also appear to correspond, at least in part, to clinical and MRI markers of increased MS pathology, and their concentration increases over time in patients with MS (40, 41).

### 3. Sex and EAE phenotypes

Experimental autoimmune encephalomyelitis is one of the most widely used animal models for MS, where neuropathological mechanisms and evaluation of miscellaneous therapeutic compounds can be studied (42–44). In addition to EAE, Theiler's murine encephalomyelitis virus infection and chemically induced demyelination (cuprizone and lyssolecithin) reflect axonal impairment and remyelination processes in MS, respectively. Although no experimental model has established all aspects of human MS, EAE is considered to be the most suitable (45). There are several EAE models, and each one can bring to life different hallmarks of the disease (46). EAE models fundamentally mimic the immune aspect of MS; active lesions, CNS infiltration of peripheral macrophages, relapsing-remitting events, and microglia and astroglia accumulation. Besides neuroinflammation, EAE progression can resemble chronicity and include neurodegeneration, axonal loss, demyelination, and even remyelination to a lesser extent. This dual nature of EAE can potentially become a tripartite, an event trifecta considering one more key player, which is the sex. Sex-dependent differences are quite prominent, as seen through experimental approaches primarily in rodents, and will be further reviewed in the following sections.

#### 3.1. EAE models recapitulate different aspects of the MS disease pathology

Experimental autoimmune encephalomyelitis can be induced either passively or actively. Passive induction of EAE through adoptive transfer of activated encephalitogenic T-cell clones allows the study of differentiated Th1 or Th17 populations and their cell trafficking in the recipient mice (47). Active immunization and induction of EAE are achieved by the administration of encephalitogenic peptides such as proteolipid

protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and spinal cord homogenate. In most cases, pertussis toxin is co-administered to increase both the incidence and severity of the disease. Pathogenesis of EAE exhibits ascending paralysis, and the typical assessment tool is a clinical score resembling the Expanded Disability Status Scale score in human disease. Despite the similarities, each model can provide different insights into the MS-like progression and pathogenesis (48).

One of the first EAE models used was the active immunization of Swiss Jim Lambert (SJL) mice with PLP139–151, which fairly recapitulates a relapsing/remitting disease phenotype (49). Moreover, as occurs in humans, it is intriguing that the establishment of EAE is more severe in female mice. The initial phase begins on day 10 post-immunization, culminating around day 25 and the relapse is evident on day 40. Relapse arises from the expansion of epitope spreading, in which, due to secondary endogenous peptides, reactive T cells emerge as a consequence of the initial phase of myelin destruction. Remission is associated with a temporary loss of inflammatory cells. This particular model is used to study autoimmune T-cell-mediated responses, the compromise of the blood–brain barrier (BBB), relapse mechanisms in epitope spreading, antibody-mediated demyelination, and the evaluation of many anti-inflammatory therapeutic compounds for RRMS (50).

MOG35–55 EAE in C57BL/6 mice initiates a chronic–progressive form of EAE (51). On the contrary, if Biozzi ABH mice are immunized with the same encephalitogenic peptide, animals will display a more RRMS clinical outcome (52). In either case, this phenomenon implies that MOG peptides are unique in that they trigger an encephalitogenic T-cell response and a demyelinating autoantibody-mediated response to certain mouse strains (53). Moreover, mice exhibit microglial and astrocyte activation both in white and gray matter, as well as neuronal and synaptic loss in gray matter. Axonal damages contribute to a self-sustained chronic neurodegenerative process due to the presence of outgrowth inhibitory factors (54), which is established even in the absence of continued peripheral cell infiltration. In addition to paw paralysis, mice show evidence of CNS demyelination both in the spinal cord and the brain. This model has been used to investigate neurodegenerative mechanisms, axonal loss, T-cell priming, Th1/Th17 CD4+ T-cell-mediated CNS damage, and T-cell self-tolerance. Moreover, it is considered the most suitable for compound profiling and preclinical evaluation of cellular therapies and restorative agents (55).

The least employed model is the spontaneous EAE model, which offers the advantage of studying autoimmune mechanisms developing in a genetically controlled background and eliminating the effect of exogenous manipulations (56). Numerous strains are suitable such as C57BL/6, SJL, and B10.PL, and some are even genetically modified for specific susceptibility factors such as humanized Tg(HLA-DR2) and

humanized Tg(HLA A3) (57). Spontaneous EAE models exhibit paralysis, optic neuritis, ataxia, and present sparse levels of progression. They are used to study spontaneous T-cell activation mechanisms and innate immune mechanisms. Spontaneous EAE models are excellent tools to study B-cell responses in EAE.

## 3.2. Sex-specific aspects of the immune system: Lessons from EAE

There is accumulating evidence that males and females exhibit different immunological responses throughout life, whereas others are only present after puberty and before reproductive senescence, suggesting that both genes and hormones are involved. EAE can be a useful tool to unravel those sex-specific differences in immune responses (58). Notably, different strains display different susceptibility to EAE. For example, C57BL/6, SJL, ASW, and NZW mice demonstrate an increased tendency for EAE in females than males, but B10.PL and PL/J are more prone to diseases in males than females (59). Moreover, androgens such as testosterone are considered an ameliorative factor in SJL/J EAE, and gonadectomy of male mice makes them more vulnerable to EAE severity (60). On the contrary, *in vivo* administration of testosterone ameliorates EAE severity and favors T helper 2 proliferation in an MBP immunization model (61), whereas administration of exogenous testosterone on female splenocytes *in vitro* minimizes the ratio of IFN- $\gamma$ :IL-10 (62). Taken together, experimental data denote that female mice are prone to worsened EAE with low levels of testosterone being a potential factor. A more detailed view of the specific aspects of this sexual dimorphism is presented in the following sections.

### 3.2.1. Chromosome-based aspects

The relative contribution of sex and chromosomes to immunological processes can be investigated with the four-core genotype (FCG) mouse model (63). The FCG model provides valuable information regarding genital determination that can be separated from the inheritance of the Y chromosome (64). Double transgenic mice can have four different genotypes and can be either XX gonadal males or females and XY gonadal males or females, respectively. This is due to Sry knockout of the Y chromosome (YSryKO), which can be ectopically expressed on chromosome 3 (Tg-Sry) (65). In the absence of Sry, animals undergo the female hormonal pathway and can be hormonally and chromosomally female (XX) or hormonally female and chromosomally male (XYSryKO). On the contrary, in the presence of Sry animals become hormonally male and can be hormonally and chromosomally male (Tg-Sry XYSryKO) or hormonally male and chromosomally female (Tg-Sry XX).

A complementary model called the XY\* model, in which a male produces XX, XO, XY, and XXY gametes, can be used to identify the precise mechanism underlying a sex difference observed by the FCG model (66). Hormonal influences and sexual chromosomal interactions can sometimes counteract each other's effects, whereas this method has been occasionally used to identify particular X or Y genes that either worsen or protect against a disease (67). For example, double transgenic FCG mice have revealed sex and chromosome-related findings upon active immunization in EAE on the SJL/J background. In this scenario, XX SJL/J mice and Tg-Sry XX, both chromosomally female mice, develop EAE of greater severity than chromosomally male mice XYSryKO and Tg-Sry XYSryKO. In addition, passive induction of EAE with adoptive transfer of XX T-cells triggered EAE of greater severity than XYSryKO T-cells. These findings were indicative of the T-cell compartment, which makes female mice prone to develop EAE of greater severity (68).

Different series of experiments explored neurodegeneration, and severe clinical disease was associated with the expression of the X gene Toll-like receptor 7 (TLR7), known to induce neuronal damage. These findings may indicate differential expression of TLR7 in the male and female CNS and could explain the increased susceptibility in women (69). This also could explain why female SJL/J mice develop EAE of higher severeness in contrast to males upon adoptive transfer (68). Parental imprinting, an effect in which a single locus will entirely shape one's phenotype, although two alleles are inherited, has also been studied under such circumstances (70). Teuscher and colleagues crossed XX C57BL6/J female mice with B6 males that carried Y chromosome variants known to cause susceptibility to autoimmune diseases and revealed that the EAE severeness of female progeny was dependent on the Y chromosome of their male siblings. Moreover, susceptibility in several clinical subtypes of EAE in both males and females was related to autosomal EAE loci on chromosome 13 (71). This locus was positively linked to susceptibility of monophasic remitting/non-relapsing EAE in males but not females; therefore, it is possible that endogenous androgens may be EAE-protective in a given strain depending on the allele 13 inheritance (72).

### 3.2.2. Hormone-based aspects

An intriguing topic is the effect of male vs. female hormones in immune responses and how they affect the establishment or even the progress of EAE as most terminally differentiated immune cells express sex hormone receptors (73). Estrogens include estrone (E1), estradiol (E2), and estriol (E3) – produced only during pregnancy – and they act through estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ ). Androgens, in contrast, bind to androgen receptors (AR), respectively, and these were found in neutrophils, macrophages, B cells, and T cells. Studies revealed that CD4 T helper cells express more ER $\alpha$

than ER $\beta$ , and CD8 T cells and monocytes express low amounts of both ERs. On the contrary, B cells express significantly increased amounts of ER $\beta$  than ER $\alpha$ , and antigen-presenting cells express both ER $\alpha$  and ER $\beta$  (74).

As mentioned earlier, both androgens and estrogens can modulate EAE progression and severity; however, estrogens seem to operate in a more diverse way. The leading hypothesis relies on a threshold effect through which sex-specific hormones can reflect their dynamic through a protective vs. harmful equilibrium. For estrogens specifically, an accumulating body of evidence implies the negative contribution in the thymus and T-cell maturation (75, 76). This peculiar action may be mediated by distinct ER types, affecting the T-cell maturation pathway, either directly or indirectly. Estrogens can directly influence the developing T cells or indirectly can affect thymic epithelial cells to inhibit secretion of pivotal elements for T cells or generation of signaling important for thymocyte survival (75). Moreover, E2 is far more vigorous than testosterone in accelerating thymic atrophy. Estrogens, at least partly, can also induce thymocyte apoptosis and finally cause thymic atrophy (76, 77). This detrimental effect of estrogens has been mediated again *via* both ER receptors (78, 79) and GPR30-mediated mechanisms (77).

Continuing with the deleterious effect of estrogens, a study on thymic involution in pregnant mice implies that increased estrogen and progesterone levels during this period affect the proliferation of T-cell repertoire (80). More specifically, pregnancy does not directly affect thymocyte precursor populations in the bone marrow, but instead triggers a detrimental loss of early thymic progenitors in the thymus as early as day 12.5 of pregnancy. The similarities between estrogen-mediated involution and pregnancy-mediated involution suggest that estrogen is a pivotal regulator of loss of thymocyte cellularity during pregnancy, and probably functions primarily by reducing thymocyte proliferation (80). The same research team showed an injection of 17 $\beta$ -estradiol into mice causes excess loss of early thymocyte precursors and inhibits the proliferation of developing thymocytes (81). In addition, exogenous E2 supply may minimize levels of CD4+CD25+FoxP3+ T regulatory cells (Tregs) that are responsible for maintaining immunosurveillance, and loss of function of foxp3 gene in those cell populations is associated with immune-mediated inflammatory lesions (82). Collectively, the aforementioned data support an increased incidence of EAE in female rodents; however, for a complete understanding of the complex estrogen effects on T-cell development, miscellaneous details are still lacking.

In contrast, protective effects of estrogens have also been revealed and numerous studies show that estrogen treatment (with estriol and estradiol) ameliorates both active and adoptive EAE in different mice strains such as SJL, C57BL/6, B10.PL, and B10.RIII (83–89). Furthermore, estrogen treatment has been associated with reduced chemokine levels in the CNS of mice



with EAE and affects the expression of matrix metalloproteinase-9 (MMP-9), each leading to impaired recruitment of cells to the CNS (87, 90). Interestingly, estrogens modulate astrocytic response to injury (91) and exhibit anti-inflammatory effects on microglial activation (92). Estradiol administration may also mediate neuroprotective action in both white and gray matter pathology in spinal cords of mice with EAE by downregulation of microglial/monocyte (Mac 3+) cells on gray matter (93). Thus, apart from the anti-inflammatory effects of peripheral immune cells, estradiol treatment also suppresses CNS white matter inflammation and demyelination.

Estrogens can also regulate the equilibrium of anti-inflammatory T- and B-cell production in favor of homeostatic maintenance. It has been shown that they can promote the production of Tregs by upregulating the expression of FoxP3 (94, 95), an X-chromosome gene whose expression is higher in males than in females (96), along with other Treg subsets such as B regulatory cells (Bregs), CD8+ CD122+ Treg cells, and CD11b+ CD206+ ARG-1+ M2 such as macrophages (82). Furthermore, estrogens can directly modulate adaptive immune responses of B cells and T cells mediating changes in lymphopoiesis and can more specifically affect the expression of autoimmune regulator (AIRE) protein, which is a major regulator in the thymic expression of self-antigens (97). Finally, an interesting study in pregnant mice revealed that increased levels of E2 during pregnancy ameliorate B-cell number and activity of B lymphocyte precursors in the bone marrow, a finding that can imply that estrogen can act as a pivotal regulator of B-cell lymphopoiesis (98).

Of utmost importance is that these sex-specific differences in hormone levels can be reflected in their receptor expression, which, in turn, can affect treatment responses in EAE. For instance, an ER $\alpha$ -dependent increased dose requirement for the effective treatment of female vs. male mice using partial MHC (pMHC) class II constructs in chronic EAE was found (99). Results suggested that the divergence in effective dose for the treatment of chronic EAE with DR $\alpha$ 1-mMOG-35-55 is sex dependent, and the DR $\alpha$ 1-mMOG-35-55 treatment efficacy of female mice depends on estrogen signaling through ER $\alpha$ . In addition, DR $\alpha$ 1-mMOG-35-55 treatment can upregulate the levels of CD206+ CD11b+ M2-like macrophages/microglia found in spinal cord with a significant reduction in the expression of proinflammatory genes and enhancement of genes involved in neurosurvival and regeneration (100). A similar experiment has demonstrated that RTL401, an I-As /PLP-139–151 construct, could reverse clinical disease and ongoing CNS damage in male SJL/J mice with relapsing-remitting EAE even when administered on day 20 post-disease induction (101). Elucidation of sex-specific differences may lead to insights into the evaluation of which elements are hormonally regulated and may allow design of better therapies for both sexes.

Finally, the mechanisms underlying the protective effects of androgens have also been studied. Testosterone treatment

ameliorates EAE severity *in vitro* mediating its effect through Th2 bias. More specifically, androgen-treated T-cell cultures secrete lower amount of IFN- $\gamma$  than IL-10 relative to untreated controls (102). These results are in total agreement with experiments in T-cell cultures from PLP139–151 immunized mice that were stimulated with PLP139–151 peptides and treated with 5 $\alpha$ -dihydrotestosterone (DHT). Results revealed increased IL-10 and decreased IFN-g production in cultures treated with DHT (62). Similar results with increase in IL-10 and decrease in IFN-g were observed *in vivo* too (61). Ultimately, testosterone was associated with improved structure and function of dendrites and synapses in the cerebral cortex in cognitive neurodegenerative models (103), findings quite encouraging considering that synaptic stripping and loss are observed in MS cortex (104).

### 3.2.3. Other sex-specific aspects

Sex hormones can also indirectly regulate CNS functions. For example, estrogen reduces BBB inflammation through annexin A1 (ANXA1), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (105). Maggioli and colleagues showed that estrogen binding to ERs promotes ANXA1 phosphorylation, which efficiently recruits formyl peptide receptor 2 (FPR2) and stabilizes BBB tight junctions *via* actin reorganization (106). Another molecule implicated in BBB integrity is sphingosine-1-phosphate receptor 2 (S1PR2), while being validated as a sex-specific mechanism that disproportionately affects women. S1PR2 was highly expressed in female EAE mice relative to male EAE mice or healthy controls, directly correlated to disorganized BBB tight junctions (105). Furthermore, extracellular matrix composition in EAE mouse cortical tissue was modified, with an increased stiffness of the female cohort, showcasing again sexual dimorphism in specific collagen genes (107). Finally, brain energy metabolism is particularly interconnected to immunity. A key molecule that is highly expressed in male T cells is peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), providing an advantage to males over the deleterious effects of EAE (108, 109).

It is already established that C57BL/6 female mice after active immunization with MOG35–55 peptide exhibit increased spinal cord infiltration and demyelination than males. On the contrary, males demonstrate increased inflammatory response but elevated regulatory cell types than females (110). Experiments on MOG EAE mice after engraftment of microglia-like cells demonstrate increased expression of CNS inflammatory factors in female but not in male mice accompanied by upregulated major histocompatibility complex class II expression of infiltrating Ly6C-hi monocytes during EAE peak and cytokine production in the female CNS (111). Complement activation *via* astrocyte compartment may also be a key player in chronic EAE establishment. More specifically,

striking upregulation of C3 expressing marker for astrocytes (A1 phenotype) was found in EAE females with increased axonal loss, whereas EAE males expressed the THBS1 astrocyte marker (A2 phenotype) manifesting as a neuroprotective potential (112).

### 3.2.4. Sex-specific aspects in relation to aging: Epigenetic modifications

The low concordance rates of MS in monozygotic twins (25), the missing heritability (27, 31), the parent-of-origin effect on disease transmission (30), and the implication of environmental factors (32, 33) in the pathogenesis suggest an effect of epigenetic mechanisms on the predisposition to MS. Changes in the levels of DNA methylation (31) and histone acetylation (15), and altered expression of micro (mi)RNAs (16) were identified in patients with MS. The possible impact of such modifications on myelin and immune gene expression is a topic of interest as certain epigenetic signatures were functionally related to oligodendrocytes (17), B (29, 34), and T cells (35, 37).

The age and sex are two variables associated with epigenetic modifications in MS and related experimental models. In cuprizone-induced demyelination, chronological age influences the mechanisms of histone acetylation affecting the intrinsic capacity of oligodendrocyte progenitors to remyelinate (36). In MS, chronological age was negatively correlated with methylation at the VMP1/MIR21 locus and expression levels of miR-21 in CD4+ T cells (16). Estimation of biological age based on DNA methylation showed accelerated aging of glial cells in patients with MS compared with controls (38). Notably, females had a reduced level of global methylation in leukocytes (39) and often showed dissimilar ratio between MS cases and controls in genome-wide DNA methylation studies [reviewed in Zheleznyakova et al. (41)] than men. Overall, these data suggest that the age and sex should be accounted for when analyzing epigenetics data and further studied experimentally for their role in MS in relation to epigenetic modifications.

## 4. Sex and aging-related neuroinflammation

A wide range of molecular pathways cooperate to buffer homeostasis as we age, which inevitably results in a functional decline and illness trajectory. Although the hypothalamus, which controls reproductive function, was once the only part of the brain thought to be responsive to sex hormones, it is now widely acknowledged that the whole brain is both a target and a source of sex hormones (113). Primarily in the adult brain, an arsenal of sex hormones has a variety of protective and antioxidant effects that ensures neural cell protection and prosperity. However, sex-based hormonal decline can be spotted

in both sexes; ovarian hormones are lost very quickly after menopause, whereas testosterone dramatically diminish in an aging organism, findings that could potentially lead to age-related neurodegenerative diseases such as PPMS (114) and Alzheimer's disease (AD) (115). This reproductive senescence effect will be further discussed on the basis of the key immune cell regulator of the CNS, microglia, which is primarily affected by aging and adversely orchestrate acute and chronic reactions in EAE.

### 4.1. Do males and females age differently? Microglia implication for the neuroimmune axis

As mentioned earlier, a wide variety of profound alterations occur between sexes in the immunocompromised brain. Microglia modulate the microenvironment in physiological conditions maintaining homeostasis toward a healthy brain. Impaired surveillance of this resident immune population has been partly held accountable for aging in both sexes (116). More specifically, it is the neurodegenerative senescent state of microglia, and to a somewhat extent astroglia, that drives this sex-specific motif (117). However, as microglia constitute the sole immunocompetent cells of the CNS and these cells are dysregulated by aging, current research is being focused on the key sex differences in multiple levels such as the hormonal (118–120), chromosomal (121, 122), epigenetic-driven mechanisms (123), microRNAs (124), and miscellaneous exogenous stimuli (125).

Sex-related differences can be initially distinguished in the gene expression level in different brain areas. For instance, Berchtold and colleagues identified profound sex-based genetic changes in hippocampus and entorhinal cortex in a human cohort between 20 and 99 years of age (126). Notably, hippocampal alterations in gene expression that were detected in the aged brain were primarily originated in the complement pathway of microglial populations (117). Although there is paucity of data pertaining sex hormones and DNA repair mechanism, a system that progressively worsens due to loss of genomic maintenance in the aging brain, various investigations have linked estrogen antiaging neuroprotective mechanisms to different DNA repair enzymes (127). Additionally, epigenetic-mediated mechanisms such as immune training and tolerance lead to differential epigenetic reprogramming of microglia, as seen in a mouse model of Alzheimer's pathology, suggesting a tool to take advantage of aging (128).

Aging fosters a slow yet significant microglia dysregulation over time. A marked upregulation in female genes associated with inflammation and immune function was observed compared with the male gene pool, highlighted in

ovariectomized rat experiments after studying microglia activation (129). This increase in neuroinflammation mostly seen with TNF $\alpha$  and IL-1 $\beta$  was attributed to the lack of ovarian function in aged mice (130). It is widely established that a variety of physiological and pathological events can cause activated microglia to polarize either toward a proinflammatory/cytotoxic M1 or an anti-inflammatory/neuroprotective M2 phenotype (119, 131). Growing evidence in aging and neurodegenerative diseases suggests that the polarization toward the neuroprotective status can be triggered by estrogens (132, 133), and this has been proven by estrogen replacement treatment through microglial ER subtypes, ER $\alpha$  and ER $\beta$  (134–136).

Clusters of genes and their relative messenger RNAs (mRNAs) that regulate microglia's sensing functions are referred to as the "sosome" (137). Those, among other genes, have been involved in microglia priming, which is an exaggerated or heightened response, yet ineffective in a senescent CNS and differs between sexes (116). The differential expression of multiple microglial genes such as *Spp1*, *ApoE*, *Ccl3*, *Clec7a*, and *Ccl4* in female mice may explain sex-related differences in aging and AD (117, 138). One of the most interesting microglia mediators is *Tyrobp*, also known as *TREM2*; however, its involvement in AD is still under investigation (139). Clusters of homeostatic genes, but more importantly, another group of genes, including *Ms4a7*, *Klra2*, *Clec12a*, and *Mrc1*, were found to be upregulated in female EAE mice with single-cell sequencing (scRNA-seq) of CD45+ cells (140). The authors of this study concluded that the cells implicated in antigen presentation such as DCs and monocyte-derived microglial cells are purposeful targets during EAE.

Another age-related, female-predominant pattern of gene expression in a plethora of genes of the complement system such as *C1qa*, *C1qc*, and *Ccl4* was also identified (117). Similarly, complement cascade genes and interleukin 1 receptor-like 1 (*IL1RL1*) were increased in women in a human cohort (141). Finally, the cellular population equally affected by age-related female traits, such as menopause, are astroglial cells, producing dystrophic astrocytes. With aging, these cells exhibit increased expression of the intermediate glial fibrillary acidic protein (GFAP) and filamentous vimentin thereby accumulating as toxic aggregates (142). Taken together, experimental and clinical studies reveal that menopause and aging both promote neuroinflammation, which may explain the sex disparities in age-related neurological diseases such as AD and MS.

## 4.2. Implications for immune-mediated neurodegeneration

As discussed thus far in neuroinflammatory conditions, which differ between males and females with age,

escalating toxicity and neuronal death may contribute to neurodegeneration, however, to a lesser extent. The molecular cross-talk of autoimmunity variations and resulting neurodegeneration may be based on different imprinting of X chromosome genes, as proved by studies of T-lymphocyte DNA methylation of the X chromosome gene *Foxp3*, as well as by differential expression of neuronal Toll-like receptor 7 (TLR7), which is another X chromosome gene (67). A study showed that progesterone levels in the post-reproductive ages may lead to an increase in Tregs in lymphoid tissues and blood in males than females (143). CD4+CD25+ Treg and CD4+FoxP3+ Tregs functional capacity has been suggested to be enhanced with aging in men and male mice than women and female mice, respectively, because *FoxP3* expression upregulates Treg functionality (95). In support of this finding, the greater potency of CD4+FoxP3+ T cells in old males than females could be correlated with changes in gonadal steroid levels as estrogens and androgens also influence *FoxP3* expression (96). Conclusively, not many studies have pointed out the neurodegenerative effects of EAE; however, Tregs seem to be of significance as immune key players in the diversity of male and female neurodegeneration.

## 5. Conclusion

As with several autoimmune diseases, MS is increasingly and universally recognized to be more frequent in female than male patients. An increasing body of evidence points toward sex-specific differences in the immune system and CNS caused by effects of chromosomes, hormones, and aspects of the immune system associated with barrier function, cell migration, and effector phenotype activation of immune cellular components. Age is also a contributing factor in disease pathology and evolution, as evidenced by clinical, epidemiological, and molecular data derived from single-cell techniques, high-throughput applications, and systems immunology analyses. Several experimental models have been used in order to depict aspects of MS and/or CNS neuroinflammation, with EAE being the most widely used murine model for MS. Although EAE exhibits discrete differences from MS in terms of phenotype and pathology, it is a useful model that recapitulates specific aspects of MS evolution and thus has been extensively studied in order to offer mechanistic insights regarding the complex interaction of biological pathways implicated in the disease ontogeny, such as sex and aging processes. In this study, we summarize current knowledge stemming from EAE models regarding the effects of sex and aging on disease phenotype with relevance to the immune system and the CNS. Sex and aging associations in MS are increasingly recognized as factors determining, at least in part, disease outcomes, a knowledge with profound implications for disease management and novel treatment development.



## Author contributions

MB and NG: conceptualization. CK, EKa, EKe, MB, and PT: original drafting and writing. AA, YB, MB, NG, CK, EKa, EKe, PT, and IM: revising. NG: supervising. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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