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The Importance of Studying Sex Differences in Disease: The Example of Multiple Sclerosis

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Abstract

To date, scientific research has often focused on one sex, with assumptions that study of the other sex would yield similar results. However, many diseases affect males and females differently. The sex of a patient can affect the risk for disease susceptibility or progression by fold differences. Such differences can be brought to the laboratory bench to be investigated, potentially bringing new treatments back to the clinic. This method of research, known as a “bedside to bench to bedside” approach has been applied to studying sex differences in multiple sclerosis (MS). Females have greater susceptibility to MS, while males have worse disease progression. These two characteristics of the disease are influenced by the immune system and the nervous system, respectively. Thus, sex differences in each system must be studied. Personalized medicine has been at the for-front of research recently, and studying sex differences in disease fits with this initiative. This review will discuss the known sex differences in MS and highlight how investigating them can lead to new insights and potential treatments for both men and women.

Keywords

estrogen; testosterone; four core genotypes; sex chromosomes; experimental autoimmune encephalomyelitis

The Importance of Studying Both Sexes; an Overview of Sex Differences in Neurological Diseases

Understanding mechanisms underlying sex differences in disease can lead to the discovery of natural disease modifiers. It is important to study both sexes for many reasons. The sex of a patient often changes the risk for disease susceptibility or progression by fold differences, not mere percentages. In addition, optimal dosing of treatments, considering efficacy and side effects, can differ between the two sexes. Optimizing the dose for one sex and assuming

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findings are no different in the other sex is underserving a large portion of patients. The risk:benefit ratio for a given dose must be determined for each of the two sexes. Finally, the study of both sexes may lead to the discovery of unexpected natural disease modifiers.

Researchers studying the MS model, experimental autoimmune encephalomyelitis (EAE), found that female mice, rats, rabbits and guinea pigs exhibited protection from disease when they were pregnant (Abramsky 1994, Langer-Gould, Garren et al. 2002). Such an observation would never have been made if only the male population were studied. Since it had been anecdotally observed that there were protective effects for women with MS when they were pregnant, this observation in animals suggested that the improvement of disease in pregnant MS women was due to an important biological disease modifier of pregnancy, not merely an effect of differences in reporting or other circumstances of women during pregnancy.

When researchers included both females and males in adoptive transfer EAE experiments, they found that females, as compared to males, were more susceptible to disease in the relapsing remitting model (Voskuhl, Pitchekian-Halabi et al. 1996). Since epidemiologic studies showed that men were less susceptible to MS than women, these preclinical studies again supported a biologic mechanism for the sex difference in MS, not mere differences in MS reporting or perception of symptoms.

Multiple Sclerosis: An Autoimmune Disease of the Central Nervous System

Multiple sclerosis (MS) is a complex disease involving both the immune system and the central nervous system (CNS). In MS, T lymphocytes are activated and migrate through the blood brain barrier (BBB). These T-cells release cytokines that activate microglia and astrocytes, as well as recruit macrophages and other lymphocytes leading to demyelination and irreversible neurodegeneration (Brosnan and Raine 1996). There are several different types of MS. Relapsing-remitting MS (RRMS) is characterized by episodes of autoimmune infiltration inducing a new symptom or significantly worsening a pre-existing symptom, each known as a relapse. Relapses are followed by a period of autoimmune quiescence and clinical stability, or, remission. Over decades, RRMS often transitions into secondary progressive MS (SPMS). MS patients that no longer exhibit relapses and remissions but instead slowly accumulate worse neurodegeneration, have SPMS. Approximately 10% of MS patients do not have an initial relapsing phase that precedes the neurodegenerative phase, and this is termed primary progressive MS (PPMS)

MS exhibits sex differences in both the immune response and neurodegeneration, affecting disease susceptibility and progression, respectively. However, sex differences in the immune system appear to be different than sex differences in the CNS; women are more susceptible to MS (Whitacre, Reingold et al. 1999), but men are more likely to have disease progression (Tomassini and Pozzilli 2009, Koch, Kingwell et al. 2010, Shirani, Zhao et al. 2012). This poses the question, if the incidence of disease is higher, and peripheral immune responses are more robust in women, then why isn't disability progression faster in women? If anything, there is faster progression in men. We have hypothesized that sex related factors

could play different roles in the immune system versus the CNS, and thus different roles in susceptibility versus disability progression (Voskuhl and Gold 2012).

Sex Differences in MS Susceptibility (Female > Male)

Disease onset

There is a distinct female preponderance to a variety of autoimmune diseases including MS, systemic lupus erythematosus, and rheumatoid arthritis (Whitacre, Reingold et al. 1999). Consistent with increased susceptibility in females are reports that immune responses are more robust in healthy females compared to males, including autoantigen specific responses in MS (Pelfrey, Coteleur et al. 2002, Kantarci, Hebrink et al. 2008, Moldovan, Coteleur et al. 2008, Libert, Dejager et al. 2010). The female to male ratio in MS varies somewhat by geographic region from 2:1 to 3:1, and has increased in the past decades with a few notable exceptions (Koch-Henriksen and Sorensen 2010). This increase in the female to male ratio is driven by increased incidence in women, not by decreased incidence in men. The reasons for the increased incidence in women remain speculative. However, the short time span over which it is occurring excludes a purely genetic cause (Orton, Herrera et al. 2006). Instead, it has been proposed to be due to gene-environment interactions or epigenetic factors (Greer and McCombe 2011, Voskuhl and Gold 2012).

Relapsing-Remitting MS

While the female preponderance for RRMS has been established for many years (Runmarker and Andersen 1993), a recent study demonstrated a 17.7% higher relapse rate in females compared with males who have RRMS throughout the entire duration of disease and at any age (Kalincik, Vivek et al. 2013). Also, RRMS patients have a greater female to male ratio when they have at least 4 relapses per year in the first five years of disease, (3.3:1) than patients who experience no relapses in the first five years (2.3:1) (Kalincik, Vivek et al. 2013). Females also have a higher cumulative hazard risk of relapses than males 40 years after disease onset (Kalincik, Vivek et al. 2013). Conversely, there is no distinct sex difference in primary progressive MS patients (1.2:1), the type of MS without relapses (Kalincik, Vivek et al. 2013).

Pre-clinical model for relapsing-relmitting MS

EAE in the SJL strain of mouse exhibits a relapsing-relmitting pattern (Brown, McFarlin et al. 1982). Therefore, this model has been used extensively to study immunological mechanisms of relapses in MS by focusing on myelin specific immune responses in lymph node and spleen, inflammation and demyelination in spinal cord, and clinical EAE score that principally reflects walking disability. Females as compared to males are more susceptible to EAE in the SJL strain (Voskuhl, Pitchekian-Halabi et al. 1996).

Sex Differences in MS Disability Progression (Male > Female)

Disease Progression in RRMS Patients

For years it was reported that early predictors of future permanent disability in MS included male sex, age of onset, and degree of recovery from the first episode, (Runmarker,

Andersson et al. 1994). A large natural history study of all MS subtypes showed that the median time between disease onset and reaching a given disability level was shorter in men (Confavreux, Vukusic et al. 2003). Other studies also showed that being male was associated with shorter time to disability progression in RRMS (Weinshenker, Rice et al. 1991, Weinshenker 1994, Savettieri, Messina et al. 2004). These publications addressing changes in long term disability progression were in contrast to findings of no sex difference in disability progression during short term trials testing anti-inflammatory treatments for effects on relapses. A natural history study of untreated MS patients found that the male sex was associated with a shorter time to, and a younger age for, conversion to SPMS (Koch, Kingwell et al. 2010). Another study showed that males have a more severe disease phenotype with faster accumulation of disability (Tomassini and Pozzilli 2009), with yet another showing that male sex and older age at onset were predictive of more rapid progression from disease onset of RRMS (Shirani, Zhao et al. 2012). Finally, A very large registry-based study of over 14,000 patients revealed that male relapse-onset progressive patients accumulated disability faster than female relapse-onset progressive patients (Ribbons, McElduff et al. 2015).

While the above summarizes sex differences in several MS disabilities as measured by composites, cognition is a specific disability that appears to be worse in men. An early study showed that male MS patients performed worse on several cognitive subtests as compared to female MS patients who were matched for age, education, and other neurologic and emotional measures (Beatty and Aupperle 2002). Later, a large cohort of MS subjects (n = 533) were categorized as cognitively normal or impaired based on their performance on a battery of neuropsychological tests. These subjects also underwent genotyping for the APOE-epsilon4 allele polymorphisms. Cognitive decline was more prominent in men and was associated with the presence of the epsilon4 allele of the APOE gene (Savettieri, Messina et al. 2004). To investigate the relationship between subcortical GM atrophy and cognition in early relapsing onset MS, structural MRI and neuropsychological testing were performed in 80 MS women and 40 MS men, as well as in 30 healthy women and 20 healthy men. Several deep GM structures showed volume loss in MS compared to healthy controls, with larger effects in MS men (-11%) than in MS women (-6.3%). Also, more cognitive domains were affected in men than in women (Schoonheim, Popescu et al. 2012). Among the subcortical GM structures assessed, it was thalamic atrophy which showed the closest association with cognitive impairment.

Primary Progressive MS

It has long been known that the characteristic profile of primary progressive MS (PPMS) is being male, being older and having spinal cord lesions (Thompson 2004). A recent study aimed to identify predictors to develop PPMS in a population of subjects with radiologically isolated syndrome (RIS). RIS is the term used to describe the presence of white matter lesions not associated with other clinical symptoms (Granberg, Martola et al. 2013). It was found that male sex, older age, and lesions predominantly in spinal cord were the three predictors of evolution from RIS to PPMS (Kantarci, Lebrun et al. 2016). However, during the primary progressive disease course, males do not have significantly faster disease

accumulation than women, contrary to their faster disease accumulation in RRMS disease course (Ribbons, McElduff et al. 2015).

Pre-clinical model for neurodegeneration

As stated above, EAE has historically been used to study immune responses, walking disability, and spinal cord pathology. However, over the past decade, EAE has been shown to also include neurodegenerative pathology beyond spinal cord, particularly in the chronic EAE model in the C57BL/6 strain. The cerebellum has white matter inflammation, demyelination and axonal loss as well as Purkinje cell loss (MacKenzie-Graham, Tiwari-Woodruff et al. 2009, Du, Itoh et al. 2014). The hippocampus is also affected during EAE, displaying CA1 atrophy and loss of synaptic integrity, the latter of which correlated with altered functional synaptic transmission using electrophysiology of hippocampal slices (Ziehn, Avedisian et al. 2010, Ziehn, Avedisian et al. 2012, Nistico, Mango et al. 2013). Cerebral cortical structures in EAE show synaptic loss, transected axons, and loss of callosal projecting neurons and pyramidal neurons (Rasmussen, Wang et al. 2007, Du, Itoh et al. 2014, Spence, Kurth et al. 2014), while the corpus callosum has areas of demyelination, with decreased axon conduction by electrophysiology (Rasmussen, Wang et al. 2007, Crawford, Mangiardi et al. 2010). Additionally, the striatum is affected by altered excitatory transmission, again associated with synaptic degeneration and dendritic spine loss (Centonze, Muzio et al. 2009). Studies of optic neuritis also use EAE to show loss of retinal ganglion cell bodies and optic nerve axons. In addition to pathological abnormalities in EAE brain tissue, whole brain atrophy (MacKenzie-Graham, Rinek et al. 2012, Lepore, Waiczies et al. 2013, Spence, Kurth et al. 2014) and gray matter atrophy in cerebellum and cortex have been seen (MacKenzie-Graham, Tinsley et al. 2006, MacKenzie-Graham, Rinek et al. 2012). These neuroimaging abnormalities in EAE occur in a setting of T2 lesion hyperintensities in brainstem and cerebellum (MacKenzie-Graham, Tinsley et al. 2006, MacKenzie-Graham, Tiwari-Woodruff et al. 2009, Waiczies, Millward et al. 2012).

Unraveling Mechanisms Underlying Sex Differences in Disease

Bedside to Bench to Bedside approach

Sex differences in disease are widespread. Many of these differences were first observed at the clinical bedside, and then brought to the laboratory bench to be mechanistically dissected. These findings can then lead to novel treatments for clinical trials. This is the “bedside to bench to bedside” approach (Voskuhl and Gold 2012). Using this approach, sex differences in disease can serve as clinical observations that provide a foundation known to be physiologically relevant in humans, as opposed to starting with a molecule of interest in preclinical models without known physiological relevance. The “bedside to bench to bedside” approach in drug development is a conceptual shift, since usually the molecule comes first, with questions of physiologic relevance coming second.

Sex Hormones and Sex Chromosomes in Sex Differences

Sex differences in any disease may be due to sex hormones, sex chromosomes or both since a role for one is not mutually exclusive of a role for the other (Voskuhl 2011). Regarding sex hormones, physiologic levels of cycling estradiol and progesterone could play a role in

females. Likewise, physiologic levels of testosterone in males could play a role. Regarding sex chromosomes, the XX sex chromosome complement could play a role in females or the XY complement in males. These factors may act individually, or in conjunction with each other. Sophisticated approaches can now discern between these possibilities.

The Role of Sex Hormones in Sex Differences in MS Testosterone

Clinical observations

As previously stated, men have demonstrated worse disease progression compared to females (Runmarker, Andersson et al. 1994, Weinshenker 1994, Confavreux, Vukusic et al. 2003, Savettieri, Messina et al. 2004, Tomassini and Pozzilli 2009, Koch, Kingwell et al. 2010, Shirani, Zhao et al. 2012, Ribbons, McElduff et al. 2015). This is likely due to factors other than testosterone since clinical data from converging lines of evidence from independent sources suggests that testosterone is protective in men with MS. A retrospective cohort study was conducted using analysis of English National Hospital records from 1999-2011. It found that men with testicular hypofunction, a proxy for low testosterone levels, were at increased risk to develop MS, suggesting an immunosuppressive role of testosterone (Pakpoor, Goldacre et al. 2014, Pakpoor, Goldacre et al. 2014). In line with these results, patient reports show that men with MS have relatively low testosterone levels compared to healthy men (Wei and Lightman 1997, Foster, Daniels et al. 2003, Bove, Musallam et al. 2014). Among men with MS, those with lower testosterone levels had worse EDSS scores, and when followed longitudinally, those with lower testosterone levels showed more decline on longitudinal cognitive testing (as assessed by the SDMT) (Bove, Musallam et al. 2014). These results suggest possible neuroprotective effects of higher levels of endogenous testosterone. While it is possible that low testosterone levels are an effect of disease, the above study reporting testicular hypofunction in patients prior to their development of MS suggested that low testosterone in MS men was not merely a consequence of disease (Pakpoor, Goldacre et al. 2014).

Pre-Clinical Studies

Preclinical data has revealed a protective effect of testosterone in two different MS models, EAE (Dalal, Kim et al. 1997, Bebo, Schuster et al. 1999, Palaszynski, Loo et al. 2004, Maccio, Calfa et al. 2005) and cuprizone (Hussain, Ghomari et al. 2013, Patel, Moore et al. 2013). IN EAE, testosterone treatment decreased EAE scores of walking disability, reduced inflammation and demyelination, and spared axons (Dalal, Kim et al. 1997, Bebo, Schuster et al. 1999, Palaszynski, Loo et al. 2004). As reviewed above, in addition to walking disability, EAE in C57BL/6 mice involved cognitive dysfunction. Testosterone treatment has been shown to be protective when assessing cognitive outcomes during EAE. Specifically, testosterone treatment induced an increase in synapses by neuropathology and improved synaptic transmission by electrophysiology (Ziehn, Avedisian et al. 2012). Synaptic stripping and loss is observed in MS cortex (Trapp, Wujek et al. 2007, Spence, Kurth et al. 2014), and testosterone treatment in cognitive neurodegenerative models improves structure and function of dendrites and synapses in cerebral cortex (Dutta, Chang et al. 2011). Together these reports suggest a mechanistic hypothesis whereby testosterone treatment in MS may increase synapses and their function in cortical gray matter. Effects of testosterone

treatment may be mediated through direct binding of testosterone to the androgen receptor. Alternatively, testosterone can be converted to estrogen in the brain by aromatase to mediate neuroprotection (Remage-Healey, Saldanha et al. 2011, Saldanha, Remage-Healey et al. 2011, Rensel, Salwiczek et al. 2013). This hypothesis is consistent with the ability of estrogen treatment to increase dendritic spines, synapses and cognitive function in preclinical models of cognitive decline (Yankova, Hart et al. 2001, Waters, Mitterling et al. 2009, Smejkalova and Woolley 2010, Inagaki, Frankfurt et al. 2012, Ziehn, Avedisian et al. 2012, Kramar, Babayan et al. 2013). Beneficial effects on synapses are not mutually exclusive of additional potential effects of testosterone treatment in MS on remyelination of white matter (Hussain, Ghomari et al. 2013) or immunomodulation (Bebo, Schuster et al. 1999, Liva and Voskuhl 2001, Palaszynski, Loo et al. 2004). Indeed, many complementary pathways may ultimately be involved in testosterone-mediated neuroprotection.

Testosterone mediated neuroprotection has also been shown in other preclinical cognitive neurodegenerative models including mild cognitive impairment, Alzheimer's Disease, and Apolipoprotein E induced cognitive impairment (Raber, Bongers et al. 2002, McAllister, Long et al. 2010, Rosario, Carroll et al. 2010, Rosario, Carroll et al. 2012, Li, Kang et al. 2013, Kang, Li et al. 2014)

Clinical Trials

A single arm crossover pilot clinical trial of testosterone treatment in men with MS revealed beneficial effects of treatment on brain atrophy, muscle mass, and immune markers. Ten men with RRMS participated in an open-label phase 2 trial. Subjects were observed prior to treatment for 6 months, followed by testosterone treatment for another 12 months. This treatment increased serum testosterone levels from the low-normal to high-normal range (Sicotte, Giesser et al. 2007). Brain MRI's were obtained every month. Compared to the pretreatment phase, there was slowing in the rate of whole brain atrophy by 67% during testosterone treatment (Sicotte, Giesser et al. 2007). Whole brain atrophy in MS is driven by gray matter atrophy, and gray matter atrophy has been used as a biomarker for permanent disability (Pirko, Lucchinetti et al. 2007, Fisniku, Chard et al. 2008, Rudick, Lee et al. 2009, Arnold and De Stefano 2013, Hofstetter, Naegelin et al. 2014, Lavorgna, Bonavita et al. 2014, Sormani, Arnold et al. 2014). Thus, further analysis of the same MRIs by other investigators focused on gray matter. It was found that testosterone treatment significantly reduced gray matter atrophy as compared to pretreatment (Kurth, Luders et al. 2014). In vivo cell-mediated immune responses were decreased, as measured by delayed type hypersensitivity (DTH) (Sicotte, Giesser et al. 2007), and peripheral blood mononuclear cell (PBMC) analyses revealed modest immunomodulatory properties with interesting increases in neurotrophic growth factor production during testosterone treatment (Gold, Chalifoux et al. 2008). Cognition was also improved with testosterone treatment as seen by increased PASAT scores as well as increased spatial memory (Sicotte, Giesser et al. 2007). However, this could have been confounded by a practice effect of repeated testing.

While any one of these observations in isolation could be considered merely suggestive, consideration of all supports the hypothesis that testosterone supplementation in men with MS, boosting them from the low- normal range to the high-normal range, may be protective

(Sicotte, Giesser et al. 2007, Gold, Chalifoux et al. 2008, Kurth, Luders et al. 2014). Thus, a larger phase 2, multicenter, placebo-controlled trial of testosterone is now warranted in men with MS.

Estradiol and Progesterone

Clinical Observations

The onset of puberty correlates with an increased onset of disease in women, but not men (Ramagopalan, Valdar et al. 2009). An MRI study of relapsing-remitting MS patients revealed an increase in number and volume of enhancing lesions during the week leading up to the first day of the menstrual period (Pozzilli, Falaschi et al. 1999). However, no increase in clinical relapses has been noted during menopause or in low estrogen times in the menstrual cycle. A study on the effects of oral contraceptives on disease incidence in women showed no significant relationship (Hernan, Hohol et al. 2000). These studies suggest that physiological cycling levels of estradiol and progesterone have unclear effects on disease onset and relapses in RRMS patients.

Circulating levels of ovarian hormones during a woman's menstrual cycle have correlated with changes in pre-existing symptoms prior to the menstrual period, a time when estradiol and progesterone levels are low (Smith and Studd 1992, Zorgdrager and De Keyser 1997). Patients using oral contraceptives, containing both estrogen and progesterone, no longer had worsening of symptoms during the pre-menstrual period (Zorgdrager and De Keyser 1997). Additionally, near 50% of post-menopausal women reported a worsening of symptoms (Smith and Studd 1992). Post-menopausal women who were taking hormone replacement therapies reported an improvement in their symptoms (Smith and Studd 1992). Notably, along with biological changes, many women undergo social and environmental changes once they are post-menopausal, which complicates direct correlations with hormones in observational studies (Bove 2016). One report showed that patients who started using oral contraceptives after disease onset experienced a milder disease course as compared to those who used oral contraceptives before disease onset or never used them (Sena, Couderc et al. 2012). In another study, RRMS women who used oral contraceptives were less likely to develop secondary progressive MS (Gava, Bartolomei et al. 2014). However, a different study showed that those who used oral contraceptives had a higher risk of reaching EDSS score of 6 (D'Hooghe M, Haentjens et al. 2012). Overall, the effects of physiological levels of circulating ovarian hormones on MS symptoms and disability progression remains unclear.

Pre-Clinical Studies

Pre-clinical studies ovariectomize (OVX) rodent MS models to study the effects of adult sex hormones on disease. The results from several studies leave the effects of ovarian hormones on EAE disease course controversial. Some studies show that OVX leads to earlier onset and worse clinical disease compared to sham controls (Matejuk, Adlard et al. 2001, Lelu, Delpy et al. 2010), while another group reported no difference in EAE disease course between OVX and sham controls (Voskuhl and Palaszynski 2001). One study in rats also showed worse disease in OVX females (Hoffman, Le et al. 2001).

Ovarian hormone treatments in EAE showed that treatment with estradiol ameliorated EAE clinical disease severity in both intact and OVX animals (Trooster, Teelken et al. 1993, Bebo, Fyfe-Johnson et al. 2001, Ito, Bebo et al. 2001, Matejuk, Adlard et al. 2001). A dose response was shown for estradiol whereby higher doses were more protective (Bebo, Fyfe-Johnson et al. 2001). Pregnancy levels of estrogens were clearly protective, while lower levels were modest. Progesterone alone has been shown to have only mild effects on EAE clinical disease in C57BL/6 mice. Progesterone treatment decreased EAE clinical disease severity and decreased demyelinating lesions in spinal cord (Garay, Gonzalez Deniselle et al. 2007, Garay, Gonzalez Deniselle et al. 2008, Garay, Deniselle et al. 2009). However, when progesterone treatment was administered to SJL female mice, no significant effects on EAE were observed (Kim, Liva et al. 1999). Ovarian hormone treatment in rats resulted in slight alleviation of disease with estradiol treatment, and worsening of disease with progesterone treatment alone (Hoffman, Le et al. 2001). However, in mice and rats, combined treatment with estradiol and progesterone had greater amelioration of disease than estradiol alone, indicating an additive effect of progesterone to estradiol (Hoffman, Le et al. 2001, Garay, Gonzalez Deniselle et al. 2008).

Clinical trials

The effects of oral contraceptives were studied prospectively in a clinical trial. The study used the standard of care, IFN- β , in combination with either low or high doses of ethinylestradiol in combination with the progestin desogestrel in a 96-week trial. Significant decreases in the cumulative number of combined unique active lesions and Gd-enhancing lesions were seen in patients receiving the high dose, however this was not the primary outcome measure. No other MRI outcomes displayed significant results in either dosage group. No difference on relapse rate, the primary outcome, was observed (Pozzilli, De Giglio et al. 2015). Further trials of high dose estradiol containing oral contraceptives in MS are lacking. Increased risk of breast cancer must be considered in such trials since estradiol binds strongly to estrogen receptor alpha (ER α), the mediator of adverse events with oral contraceptives and hormone replacement therapy (Schairer, Lubin et al. 2000).

The Protective effects of Pregnancy in Females with Relapsing-Remitting MS

Clinical Observations

Disease is ameliorated during late pregnancy in both MS and the MS model experimental autoimmune encephalomyelitis (EAE) (Abramsky 1994, Confavreux, Hutchinson et al. 1998). A landmark study showed that there was over 70% reduction in MS relapses during the third trimester of pregnancy (Confavreux, Hutchinson et al. 1998). Several groups then began investigating which biological factor of late pregnancy might mediate this protection from relapses at the bench. Many hormones are increased during late pregnancy, including estradiol, estradiol and progesterone, that could contribute to the protective effects of pregnancy on MS. Estradiol is unique to pregnancy in that estradiol levels are absent in women during non-pregnancy, but are made by the fetal placental unit, and reach high levels during the third trimester, the time when a 70% decrease in relapse rate was reported (Confavreux,

Hutchinson et al. 1998). Estriol was therefore studied for its protective role during EAE in preclinical studies. Estriol is also known to be the safest of the estrogens for decades (Takahashi, Manabe et al. 2000, Granberg, Eurenus et al. 2002). ER α has been implicated in promoting breast cancer, while ER β has been shown to be less toxic, potentially inhibiting breast cancer (Paruthiyil, Parmar et al. 2004). Estriol binds ER β with higher affinity than ER α (Kuiper, Carlsson et al. 1997).

Pre-clinical studies

Investigation of the effects of estriol treatment in the EAE model showed amelioration of disease severity compared to those treated with placebo when treatment was given either before or after disease induction and in several EAE models (Jansson, Olsson et al. 1994, Kim, Liva et al. 1999, Bebo, Fyfe-Johnson et al. 2001). Pathology revealed less inflammation, less demyelination, and sparing of axons in spinal cord in estriol treated animals compared to placebo (Kim, Liva et al. 1999). Estriol treatment also resulted in sparing of hippocampal CA1 volume along with preservation of synapses and reduced microglial activation (Ziehn, Avedisian et al. 2012). This could be due to estriol acting on either estrogen receptor (ER) α or ER β , since both have been implicated in estrogens' protective effects in EAE (Enmark and Gustafsson 1999, Liu, Loo et al. 2003, Polanczyk, Zamora et al. 2003). ER α mediated protection effects all stages of disease, while ER β mediated protection effects the chronic stage of the disease (Tiwari-Woodruff, Morales et al. 2007). This is consistent with reports of ER β being expressed widely in the brain (Enmark and Gustafsson 1999), potentially acting through microglia, oligodendrocytes, or dendritic cells (Du, Sandoval et al. 2011, Saijo, Collier et al. 2011, Khalaj, Yoon et al. 2013, Wu, Tan et al. 2013). Protection against disease was also seen in males treated with estriol, not only females (Palaszynski, Liu et al. 2004). As described above, therapeutic effects of estradiol and progesterone were also investigated since they could also contribute to disease improvement in late pregnancy.

Pregnancy is protective for Th-1, not Th-2, mediated immune diseases, due to pregnancy skewing the immune response to Th-2 so as not to reject the fetus (Marzi, Vigano et al. 1996). Therefore, estriol may be beneficial for other Th-1 mediated immune diseases such as psoriasis and rheumatoid arthritis.

Clinical Trials

Since estriol was protective in EAE, clinical trials in women with MS began. A small pilot Phase 2 trial showed a 70-80% reduction in inflammatory lesions in white matter on brain MRI (Sicotte, Liva et al. 2002) and peripheral blood mononuclear cells showed favorable immunomodulation (Soldan, Alvarez Retuerto et al. 2003). A larger phase 2, randomized, placebo controlled trial of estriol versus placebo was conducted in relapsing remitting MS (RRMS) women taking standard of care MS treatment (glatiramer acetate, GA). The study hit its primary outcome measure of relapse reduction. Specifically, when these patients were given estriol in addition to the standard of care, there was a one-half to one-third further reduction in annualized relapse rates over and above that provided by standard of care treatment (Voskuhl, Wang et al. 2016). Promising effects on exploratory outcomes showed improved cognition and decreased fatigue, with slowing of cortical gray matter atrophy on

brain MRI (Voskuhl, Wang et al. 2016). A phase 3 trial is needed to confirm the benefit of estriol treatment in women with RRMS.

The other potential hormone of pregnancy could be progesterone. There is a remarkable increase in relapse rate post-partum, when progesterone and estrogen levels decrease dramatically (Confavreux, Hutchinson et al. 1998). A French group followed up on this observation with a clinical trial treating post-partum women with progesterone, aiming to decrease relapse rate as the primary outcome. The results from this study have not been published to date, but the study was terminated when it became clear that it would not hit its primary outcome (ClinicalTrials.gov Identifier: NCT00127075).

While a protective role of estriol in MS is not mutually exclusive of other pregnancy factors also playing a role, twenty years after the observation of the decreased relapses in women with MS during the third trimester, no pregnancy factor other than estriol has shown efficacy in a Phase 2 trial. Thus, other autoimmune diseases known to improve during pregnancy, such as psoriasis and rheumatoid arthritis (Whitacre, Reingold et al. 1999), should also be investigated for a protective effect of estriol at a dose recapitulating a pregnancy level.

The Role of Sex Chromosomes in Establishing Sexual Dimorphisms in MS

As described above, there have been extensive studies investigating the role of sex hormones in MS, but this is not exclusive of an additional role of sex chromosomes. Sex chromosome differences are inherently tied to sex hormone differences since the XY genotype is associated with testes and the XX genotype with ovaries. Elegant studies have established how to determine the effect of sex chromosome complement separate from effects of gonadal type and sex hormones (Smith-Bouvier, Divekar et al. 2008), using the “Four Core Genotype” (FCG) mice (Arnold and Chen 2009). Specifically, the *Sry* gene on the Y chromosome that encodes for testicular development has been deleted; these mice are signified as XY⁻. Since there is no *Sry* gene, these mice are, by default, ovary bearing (gonadal females). The FCG model permits comparisons between XX and XY⁻ mice that differ in sex chromosome complement while sharing a common gonadal type (both females throughout development). When *Sry* is inserted at an autosomal location, it results in XX^{*Sry*} and XY^{-*Sry*}, again differing in sex chromosome complement while sharing a common gonadal type (both male throughout development).

Sex Chromosome Effects in the Immune System of the MS model EAE

To ascertain whether sex chromosomes could have direct effects on the immune system in the MS model EAE, four core genotype (FCG) mice were back-crossed onto the SJL background, a genetic background known to exhibit a greater female susceptibility (Voskuhl 1996). Adoptive transfer of autoantigen-stimulated XX immune cells, compared to XY⁻ immune cells, into wild type females led to worse clinical disease and neuropathology, along with decreased Th2 anti-inflammatory cytokines such as IL-10 and IL-13 (Smith-Bouvier, Divekar et al. 2008). These studies demonstrated a role for sex chromosomes in the induction of the immune response in adoptive EAE, with the XX complement, as compared to XY⁻, more proinflammatory. A proinflammatory effect of XX compared to XY⁻ was also

shown in experimental (Smith-Bouvier, Divekar et al. 2008) and spontaneous (Sasidhar, Itoh et al. 2012) lupus models.

Sex chromosome effects in the CNS of the MS model EAE

An effect of sex chromosomes in the CNS response to injury during chronic EAE in C57BL/6 mice has been shown. (Du, Itoh et al. 2014) A challenge in MS models entails discerning whether effects of a treatment on neurodegeneration are merely a consequence of effects on the immune system. Therefore, bone marrow chimeras are widely used to study compartmental effects *in vivo* in EAE. This entails irradiating mice to abolish their immune system followed by reconstitution of their immune system using bone marrow from a donor mouse. Sex differences in neurodegeneration in EAE can be discerned if disease is induced in bone marrow chimeric mice that have an immune system of the same genetic sex, while having a CNS with a different genetic sex (XX or XY⁻). (Du, Itoh et al. 2014) Specifically, XX versus XY⁻ bone marrow chimeras were reconstituted with a common immune system of one sex chromosome complement. Using this model, EAE mice with XY⁻ sex chromosome complement in the CNS, compared with XX, demonstrated greater EAE clinical disease severity with more neuropathology in spinal cord (axonal and myelin loss), cerebellum (Purkinje cell and myelin loss) and cerebral cortex (synaptic loss) (Du, Itoh et al. 2014). This result is consistent with males as compared to females having faster disability accumulation. As an example of the importance of studying sex chromosomes, a candidate X-linked gene, Toll-like receptor 7 (Tlr7), was examined due to its role in mediating neuronal degeneration in cortical neurons (Lehmann, Kruger et al. 2012). Tlr7 protein expression in cortical neurons was higher in mice with XY⁻ compared to mice with XX CNS (Du, Itoh et al. 2014). This was the first demonstration of a direct effect of sex chromosome complement on neurodegeneration in a neurological disease (Du, Itoh et al. 2014).

The study of sex differences in neurodegeneration in the model of MS could reveal new targets for a treatment to halt disability progression in MS. This is important since the current MS treatments target relapses, with only a modest effect on disability progression and with no approved treatments for progressive MS. Treatments to halt disability progression in MS may also warrant consideration in other neurodegenerative diseases characterized by a sex difference, including Parkinson's Disease and ALS. Parkinson's Disease is clearly male predominant as shown in a meta analysis, where being male increased risk by 1.5 fold (Wooten, Currie et al. 2004). ALS also demonstrates a male predominance over 1.5 fold and an earlier onset of disease in men (Norris, Shepherd et al. 1993).

Conclusion

There are many factors that influence disease susceptibility and progression. One important factor is being female or male. Only recently has the importance of studying both sexes been appreciated in both basic and clinical research. Advancements in multiple sclerosis research underscore the need for investigating disease in both males and females. Increased susceptibility of females over males, but increased rate of disability progression of males over females, indicate gender effects on disease mechanisms. Therefore, treatments

discovered for one sex may behave differently in the other sex. Additionally, by addressing both sexes, potential new treatments involving sex hormones estradiol and testosterone are being pursued. Finally, addressing sex differences can reveal influences of sex chromosome genes on disease. None of these discoveries would have been possible without inclusion of both sexes in research. Examples such as these have prompted the new NIH policy requiring justification for exclusion of one sex in basic research grants (Clayton and Collins 2014), with implementation starting in January of 2016 (Clayton 2016). This new NIH policy is a win-win situation since experts not usually studying sex differences can ascertain whether their observations in one sex are also present in the other sex. If they are found in the other sex, then they will have extended their work to the other half of the population. If they are not found in the other sex, then investigators will have uncovered an interesting sex difference that could potentially lead to novel therapeutics.

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Significance

Understanding sex differences in disease is critical to proper treatment of both male and female patients. Clinical observations of multiple sclerosis (MS) patients entail increased susceptibility in women, while men have worse disease progression. Taking these observations to the laboratory bench has allowed researchers to disentangle mechanisms underlying sex differences, and to investigate therapeutics based on these insights. Studying sex differences in other diseases in the same “bedside to bench to bedside” manner may uncover novel therapeutics like those seen in MS.