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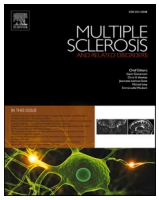

Original article

The association of menopausal hormone levels with progression-related biomarkers in multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis (MS) progression coincides temporally with menopause. However, it remains unclear whether the changes in disease course are related to the changes in reproductive hormone concentrations. We assessed the association of menopausal hormonal levels with progression-related biomarkers of MS and evaluated the changes in serum neurofilament light chain (sNfL) and glial fibrillary acidic protein (sGFAP) levels during menopausal hormone therapy (MHT) in a prospective baseline-controlled design.

Methods: The baseline serum estradiol, follicle stimulating hormone, and luteinizing hormone levels were measured from menopausal women with MS ($n = 16$) and healthy controls (HC, $n = 15$). SNfL and sGFAP were measured by single-molecule array. The associations of hormone levels with sNfL and sGFAP, and with Expanded Disability Status Scale (EDSS) and lesion load and whole brain volumes (WBV) in MRI were analyzed with Spearman's rank correlation and age-adjusted linear regression model. Changes in sNfL and sGFAP during one-year treatment with estradiol hemihydrate combined with cyclic dydrogesterone were assessed with Wilcoxon Signed Ranks Test.

Results: In MS group, baseline estradiol had a positive correlation with WBV in MRI and an inverse correlation with lesion load, sNfL and sGFAP, but no correlation with EDSS. The associations of low estradiol with high sGFAP and low WBV were independent of age. During MHT, there was no significant change in sNfL and sGFAP levels in MS group while in HC, sGFAP slightly decreased at three months but returned to baseline at 12 months.

Conclusion: Our preliminary findings suggest that low estradiol in menopausal women with MS has an age-independent association with more pronounced brain atrophy and higher sGFAP and thus advanced astrogliosis which could partially explain the more rapid progression of MS after menopause. One year of MHT did not alter the sGFAP or sNfL levels in women with MS.

1. Introduction

Multiple sclerosis (MS) affects women 2–3 times more often than men (McGinley et al., 2021). MS usually onsets during childbearing years with an inflammatory active relapsing-remitting phase. After 10 to 20 years, the neurodegenerative process typically takes over leading to relapse-independent progression of symptoms and disability accumulation (McGinley et al., 2021). Interestingly, in women with MS the transition from relapsing-remitting to a progressive disease phase overlaps with the age of menopausal transition (Bove et al., 2021; Correale and Ysrraelit, 2022; Hall, 2015).

Menopause leads to a permanent cessation of ovarian hormone secretion (Su and Freeman, 2009). Emerging evidence suggests that the depletion of ovarian estrogens and progesterone may impair brain repair mechanisms and accelerate neurodegeneration (Bassani et al., 2023; Christianson et al., 2015; Tomassini and Pozzilli, 2009). Several studies have linked menopause to disability progression in MS (Baroncini et al., 2019; Bove et al., 2016, 2015; Zeydan et al., 2020), but the data are not

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consistent (Ladeira et al., 2018; Otero-Romero et al., 2021). Menopause and ovarian aging have also been associated with greater gray matter loss in women with MS independent of chronological age and disease duration (Graves et al., 2018; Lorefice et al., 2023). Hormonal substitution could theoretically prevent these changes but the effects of menopausal hormone therapy (MHT) on MS course are mostly unknown (Bridge et al., 2023).

Along with clinical and imaging parameters, cerebrospinal fluid (CSF) and blood biomarkers could provide insight into ongoing disease activity and neurodegenerative process. Neurofilament light chain (NfL) is a neuroaxonal cytoskeletal protein that is released after axonal injury and is detected in the CSF and subsequently in low concentrations in serum (Thebault et al., 2020). In MS, NfL has been shown to reflect acute disease activity and therapy responses and to predict the disease course and brain atrophy (Bittner et al., 2021). Another novel biomarker is glial fibrillary acidic protein (GFAP) that is the major intermediate filament protein of astrocytes. GFAP is considered as a marker of astrogliosis and under pathological condition it is released to CSF and blood from injured astrocytes (Abdelhak et al., 2022). In MS, higher GFAP levels have been associated with progressive forms of disease, disease severity and disability progression (Abdelhak et al., 2018; Axelsson et al., 2011; Sun et al., 2021).

In the present study, our aim was to explore the associations of menopausal hormonal levels with the clinical and magnetic resonance imaging (MRI) measures of MS severity and with serum NfL (sNFL) and serum GFAP (sGFAP) levels in women with and without MS. Furthermore, we evaluated the changes in sNfL and sGFAP levels during one year of MHT in open-label baseline-controlled design.

2. Material and methods

2.1. Study population and procedures

Peri- and postmenopausal women with MS and healthy controls (HC) were examined at baseline and followed during one year of MHT in a prospective baseline-controlled study design. The recruitment was performed from August 2015 to September 2017 by sending an invitation letter to 45–54-year-old women with MS treated at the Neurology Outpatient Clinic of Tampere University Hospital. In addition, a shorter announcement was released through email and social media of the local and national MS organizations. The enrollment was stopped when 20 eligible women with MS were identified based on the screening questionnaire. In addition to participants with MS (PwMS), fifteen HC were recruited from the staff of Tampere University and Tampere University Hospital. The ethical approval was obtained from the Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital in Finland (code R15006M), and all participants provided a written, informed consent.

All participants were interviewed and clinically examined by neurologist and gynecologist. Menopausal status at baseline was evaluated by gynecologist based on symptoms, clinical and ultrasound examination, and serum levels of estradiol, follicle stimulating hormone (FSH), and luteinizing hormone (LH). Menopause is characterized by low estrogen levels and high FSH and LH levels. Pre-menopausal women and women with a contraindication for MHT, ongoing systemic hormonal therapy or contraception except hormonal devices were excluded. Participants were classified as peri- and postmenopausal according to an FSH value of 30 IU/l. The common definition of menopause as a complete year without menstrual bleeding was not applied because several women had other causes of amenorrhea (such as hysterectomy or hormonal intrauterine device). Other exclusion criteria included malignant, autoimmune, and neurodegenerative diseases other than MS. None of the HC had any symptoms, clinical signs, or history of neurological disease.

Inclusion criteria in the PwMS were a confirmed diagnosis of initially relapsing-remitting MS by McDonald criteria 2010 (Polman et al., 2011) and mild to moderate disability by the Expanded Disability Status Scale (EDSS) score < 6 (Kurtzke, 1983), assessed by the same neurologist (Table 1). Disease-modifying therapy (DMT) with interferon β-preparations, glatiramer acetate, teriflunomide, and dimethyl fumarate was allowed if started at least three months before the baseline visit and used steadily over the study. Patients with high-efficacy DMTs (natalizumab, fingolimod, and alemtuzumab at the time of data collection) were excluded.

At baseline, four participants with MS (PwMS) were excluded because of pre-menopausal status or a contraindication to MHT. After baseline assessments, all participants (16 PwMS and 15 HC) started per oral MHT including either 1 or 2 mg of estradiol hemihydrate combined with cyclic 10 mg dydrogesterone (Femoston®), which is widely used approved treatment in Europe for estrogen deficiency symptoms in menopausal women (Stevenson et al., 2013). Cyclical treatment was chosen to ensure better bleeding control and compliance for women in the perimenopausal and early postmenopausal period. Of the available progesterones, dydrogesterone is not an androgenic progesterone and has minimal harmful effects on lipid profile (Jiang and Tian, 2017). This is important considering the metabolic changes in menopause and the risk of cardiovascular comorbidities in MS (Palladino et al., 2020; Ryczkowska et al., 2023). The gynecologist chose the estradiol dosage based on the menopausal phase and symptoms. The efficacy and tolerability of the treatment were evaluated at a 3-month follow-up visit and the dose could be changed if the selected dose was not effective enough or caused side effects.

2.2. Laboratory measurements

Blood samples were collected at baseline and at follow-up visits at 3 and 12 months. Venous blood was collected in Vacutainer SST II Advance tubes (Becton Dickinson, US). Serum was separated by centrifugation at 1500 g for 15 min at room temperature and stored at –70 °C until further use. Baseline levels of serum estradiol, FSH, and LH were measured by the electrochemiluminescence in Fimlab Laboratories Ltd. by using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany). The lower detection limits were 18.4 pmol/l for estradiol, 0.100 IU/l for FSH, and 0.100 IU/l for LH. The analyses of sNfL and sGFAP were performed by using the single-molecule array (SIMOA) platform (Quanterix Corporation, Billerica, MA, USA). The samples were centrifuged for 5 min at 10 000 g at 22 °C and sNfL and sGFAP were

Table. 1
Baseline clinical characteristics of the participants with MS and healthy controls.

Characteristic	Participants with MS (n = 16)	Healthy controls (n = 15)	p-value[1]
Age (y), Mean (SD; range)	51.4 (2.7; 45–52)	50.6 (3.9; 45–58)	0.44
BMI (kg/m^2), Mean (SD; range)	27.7 (5.9; 18.0–41.2)	27.0 (2.9; 22.8–33.5)	0.95
Current smokers, n (%)	2 (12.5%)	1 (6.7%)	1.0
Pregnancies, Mean (SD; range)	1.6 (1.7; 0–6)	2.1 (1.2; 0–4)	0.19
Peri-/postmenopausal[2]	3/13	8/7	0.066
MS type, RRMS/SPMS	12/4		
Disease duration in years, Mean (SD; range)	15.2 (9.0; 3–34)		
EDSS, Median (IQR)	2.75 (2.5–4.5)		
Disease-modifying therapy, n			
IFN-β/GA/DMF	3/2/2		
none	9		

MS, multiple sclerosis; SD, standard deviation; BMI, body mass index; RRMS, relapsing remitting MS; SPMS, secondary progressive MS; EDSS, Expanded Disability Status Scale; IQR, interquartile range; IFN-β, interferon beta; GA, glatiramer acetate; DMF, dimethyl fumarate.

[1] Level of significance: $p < 0.05$.

[2] Participants with FSH higher than 30 IU/l were classified as postmenopausal.

quantified using a commercial kit from Quanterix following the kit instructions.

2.3. MRI metrics

Brain MRI was performed for PwMS at baseline and at 12 months by using the same 1.5 Tesla magnetic resonance scanner (GE Healthcare Signa HDxt). The MRI protocol included T1- and T2-weighted, fluid attenuation inversion recovery (FLAIR), diffusion-weighted imaging, and gadolinium (Gd) enhanced T1-weighted sequences. The neuroradiologist analyzed the scans. FLAIR images with a voxel size of 1 mm x 1 mm x 1 mm were used to quantify the whole brain volumes and the volumes of white matter hyperintensities by fully automated MSmetrix software (icometrix, Leuven, Belgium). The exact method is described elsewhere (Jain et al., 2015). Because of technical issues, volumetric analysis was unsuccessful in two scans at baseline and in three scans at 12 months.

2.4. Statistical analysis

Data were summarized as mean (standard deviation, SD) or median (interquartile range, IQR) for continuous variables and frequency (percentage) for categorical variables. Bivariate assessments of differences in demographic factors and outcome measures between study groups at baseline were conducted using the Mann-Whitney U test for continuous variables and Fisher's Exact Test for categorical variables.

The associations between hormonal levels and EDSS, MRI measures, and serum biomarkers were analyzed with Spearman's rank correlation because of the non-normal distribution of the data. Linear regressions were performed to evaluate correlation between hormonal levels and outcome measures with age or disease duration as adjustment. Only one covariate at a time was used because of small sample size. The residuals were estimated to be approximately normally distributed.

Wilcoxon Signed Ranks Test was used to test the statistical difference in the sNfL and sGFAP level within groups at 3 and 12 months compared to baseline. Nonparametric and exact tests were used because of the small sample size and skewed data distribution. Analyses were conducted with SPSS Statistics software version 26.0 and $p < 0.05$ was considered for statistical significance.

3. Results

3.1. Participants

Clinical characteristics of participants are summarized in Table 1. PwMS were more often classified as postmenopausal (FSH > 30 IU/l) and they showed lower median levels of estradiol and higher levels of FSH and LH compared to HC (Table 2). All participants had at least some menopause-related symptoms and 13/16 (81%) of PwMS and 12/15 (80%) of HC had vasomotor symptoms (hot flashes, night sweats). 14/16 (88%) of PwMS and 13/15 (87%) of HC were in perimenopausal or within 5 years of postmenopausal phase (early postmenopause). One HC has undergone surgical menopause. The levels of sNfL and sGFAP were also higher in PwMS (Table 2). However, none of these differences between study groups were statistically significant.

Table. 2
Serum hormone and biomarker levels at baseline presented as median (interquartile range).

	Participants with MS (n = 16)	Healthy controls (n = 15)	p-value [1]
E2 (nmol/l)	0.04 (0.005–0.2)	0.1 (0.0–0.8)	0.19
FSH (IU/l)	67.2 (44.9–79.3)	21.8 (6.9–74.3)	0.11
LH (IU/l)	40.0 (31.6–46.0)	19.2 (6.6–36.7)	0.07
sNfL (pg/ml)	6.8 (5.4–11.5)	6.1 (5.1–9.4)	0.36
sGFAP (pg/ml)	218.5 (182.6–310.4)	196.7 (131.9–232.9)	0.12

MS, multiple sclerosis; FSH, follicle-stimulating hormone; E2, estradiol; LH, luteinizing hormone; sNfL, serum neurofilament light chain; sGFAP, serum glial fibrillary acidic protein.
[1] Level of significance: $p < 0.05$.

As we have reported previously, disease activity in MS during the study year was mainly stable controlled by clinical assessments (relapses, EDSS) and MRI (Juutinen et al., 2022). One pwMS experienced a mild relapse during the follow-up period. No relapse treatments were needed and none of the pwMS had Gd-enhancing lesions at 12-month follow-up MRI. Used DMTs had been started five months to several years before the baseline and there were no DMT changes during the follow-up period.

3.2. Baseline associations of hormone levels with MS outcome measures

First, the associations of baseline hormone levels to clinical (EDSS) and MRI measures of MS severity were studied in PwMS. Estradiol levels correlated negatively with white matter FLAIR lesion load ($r = -0.69$, $p = 0.008$) and positively with whole brain volume ($r = 0.76$, $p = 0.003$) in MRI while there were no significant correlations between hormone levels and EDSS (Table 3).

Next, we studied the associations of hormone levels with serum biomarkers. In PwMS, estradiol correlated negatively with the levels of sNfL ($r = -0.51$, $p = 0.045$) and sGFAP ($r = -0.72$, $p = 0.002$; Table 3). There was also a positive correlation between LH levels and sNfL ($r = 0.53$, $p = 0.036$) and sGFAP ($r = 0.51$, $p = 0.043$) and between FSH with sGFAP ($r = 0.72$, $p = 0.002$). In HC, age correlated positively with sNfL ($r = 0.56$, $p = 0.030$) and sGFAP levels ($r = 0.74$, $p = 0.002$) but there was no significant correlation between hormone levels and sNfL or sGFAP (Table 3).

In multivariate regression analysis with age as a covariate, the associations of low estradiol with low whole brain volume and high sGFAP in PwMS were independent of age (Table 4). The association between FSH and sGFAP also remained significant after adjusting for age.

If MS disease duration instead of age was used as a covariate in regression analysis, low estradiol still showed an independent association with low whole brain volume ($p = 0.009$) and high sGFAP ($p = 0.043$). The association between FSH with sGFAP ($p = 0.032$) was also independent of disease duration.

3.3. Changes in MS outcome measures during one year of MHT

Fourteen (88%) PwMS and 13 (87%) HC completed the one-year follow-up with MHT. Two PwMS and one HC discontinued the treatment due to known side effects of MHT (irritability, hypertension, activation of adenomyosis), and one HC was reluctant to MHT after 3 months of use. Within the first four months, the gynecologist decreased the estradiol dose from 2 to 1 mg in three PwMS and one HC because of common side-effects (headache, breast tenderness, peripheral edema) and in one PwMS the lack of efficacy led to dose escalation. Eleven (79%) PwMS and 8 (62%) HC used the higher (2 mg) dose of estrogen for most of the follow-up period.

There was no significant change in clinical MS activity, EDSS, or white matter lesion volumes during one year of MHT as we have reported previously (Juutinen et al., 2022). Mean whole brain volume increased 1.9% ($p = 0.084$).

In PwMS, there was no significant change in sNfL or sGFAP levels during the treatment period (Table 5., Fig. 1A). In HC, sGFAP levels decreased statistically significantly at three months ($p = 0.04$), but the decrease was not sustained at 12 months (Table 5., Fig. 1B).

4. Discussion

In the present study, low baseline serum estradiol levels in menopausal women with MS correlated with the biomarkers related to disease

Table. 3
Spearman's rank correlation coefficients (rho) for studied variables at baseline in participants with MS and healthy controls.

	Participants with MS									
	E2	FSH	LH	Age	BMI	DisDur	EDSS	LV	WBV	sNfL
FSH	**−0.84****	.								
LH	**−0.50***	**0.63****	.							
Age	−0.49	0.33	0.40	.						
BMI	0.33	−0.18	0.09	−0.35	.					
DisDur	−0.41	0.28	**0.60***	**0.56***	−0.40	.				
EDSS	−0.39	0.27	0.05	0.006	−0.01	0.40	.			
LV	**−0.69****	0.35	0.31	0.38	−0.20	0.53	**0.70****	.		
WBV	**0.76****	−0.52	−0.19	−0.22	0.21	−0.17	−0.35	**−0.65***	.	
sNfL	**−0.51***	0.50	**0.53***	0.45	0.06	0.46	0.43	**0.70****	−0.46	.
sGFAP	**−0.72****	**0.72****	**0.51***	0.38	−0.36	0.44	0.13	0.38	−0.49	**0.56***
	Healthy controls									
FSH	**−0.78****	.								
LH	−0.42	**0.87****	.							
Age	−0.35	0.51	**0.52***	.						
BMI	−0.21	0.10	−0.14	−0.43	.					
sNfL	−0.19	0.43	0.45	**0.56***	−0.30					.
sGFAP	−0.14	0.08	0.08	**0.74****	−0.32					0.46

Abbreviations: MS, multiple sclerosis; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; BMI, body mass index; DisDur, MS disease duration; EDSS, Expanded Disability Status Scale; LV, white matter lesion volume; WBV, whole brain volume; sNfL, serum neurofilament light chain; sGFAP, serum glial fibrillary acidic protein.
** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table. 4
Age-adjusted associations between sex hormones and EDSS, MRI parameters, and serum NfL and GFAP levels in linear regression model.

	Participants with MS								
	E2			FSH			LH		
	B	95% CI	p	B	95% CI	p	B	95% CI	p
EDSS	−3.6	−8.8, 1.7	0.17	0.009	−0.009, 0.027	0.30	−0.003	−0.056, 0.049	0.89
LV	−54.8	−126.1, 16.5	0.12	1.1	−2.7, 4.9	0.54	1.6	−2.6, 5.8	0.41
WBV	340.8	102.4, 579.3	**0.01**	−1.0	−2.3, 0.3	0.12	−3.3	−20.8, 14.2	0.69
sNfL	−18.1	−42.9, 6.6	0.14	0.04	−0.049, 0.13	0.36	0.094	−0.15, 0.34	0.43
sGFAP	−491.4	−968.1, −13.7	**0.04**	1.7	0.13, 3.3	**0.04**	2.7	−2.3, 7.7	0.26
	Healthy controls								
sNfL	0.13	−2.8, 3.1	0.92	0.01	−0.03, 0.05	0.53	0.04	−0.04, 0.12	0.31
sGFAP	−36.4	−126.6, 53.8	0.40	−0.16	−1.4, 1.1	0.78	−0.6	−3.2, 2.0	0.62

Abbreviations: E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; B, unstandardized coefficients B; CI, confidence interval; EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging; sNfL, serum neurofilament light chain; sGFAP, serum glial fibrillary acidic protein; LV, white matter lesion volume; WBV, whole brain volume.
Significant p-values (< 0.05) are bolded.

progression, such as higher levels of sNfL and sGFAP, higher white matter lesion load and lower whole brain volume in MRI, but not with EDSS. Our main observation concerned the age-independent association of low estradiol with low whole brain volume and high sGFAP in PwMS.

The findings of this study complement several research results in which the transition phase of menopause has been related to the progression of MS (Baroncini et al., 2019; Bove et al., 2016, 2015; Graves et al., 2018; Lorefice et al., 2023). The loss of ovarian function at menopause is marked by a decline in circulating blood estradiol levels. As the anti-inflammatory and neuroprotective potential of estradiol is widely recognized (Wise et al., 2009), long-term estradiol depletion could impair brain regeneration mechanisms and promote accelerated neurodegeneration and subsequent disease progression in MS (Midaglia et al., 2020). The association between low estradiol levels and brain atrophy in PwMS seen in the present study is supported by previous findings, in which menopause and ovarian aging has been associated with lower total brain and especially gray matter volumes independent of chronological age (Graves et al., 2018; Lorefice et al., 2023). Men with MS are also more prone to brain atrophy, but this sex-specific difference is no longer evident after menopause which could be another indication of neuroprotective nature of estrogen (Jakimovski et al., 2020; Rojas et al., 2016).

One potential mechanism which could link ovarian functional decline to MS progression might be accumulating astrogliosis. We found that estradiol and FSH levels in PwMS were age-independently associated with the astrocyte damage measured by sGFAP. Astrocytes play a key role in MS disease progression as reactive astrocytes lose many of their functions in maintaining neural tissue homeostasis and gain proinflammatory and neurotoxic functions (Correale and Farez, 2015; Ponath et al., 2018). Estradiol elicits many neuroprotective actions in astrocytes either directly or indirectly by other central nervous system or immune cells expressing estrogen receptors (Acaz-Fonseca et al., 2014). Estradiol depletion might thus enhance gliosis and the secretion of pro-inflammatory and oxidative stress mediators and reduce glutamate uptake and the release of neuroprotective factors (Acaz-Fonseca et al., 2014).

Serum NfL levels showed no age-independent association with the studied hormonal levels in our data which seems to conflict with preliminary data on accelerated worsening of sNfL levels after menopause in MS (Silverman et al., 2023). This may be explained by the low levels

Table. 5
The change in EDSS, brain MRI volumes, sNfL and sGFAP during the follow-up. Data are presented as median (interquartile range) except for MRI findings as mean (standard deviation).

	Participants with MS ($n = 14$)				
	Baseline	3 months	p[1]	12 months	p[1]
EDSS	3.0 (2.5–4.5)			3.0 (2.5–4.5)	
LV (ml, $n = 10$)	15.5 (12.7)			16.5 (10.2)	0.16
WBV (ml, $n = 10$)	1413.8 (60.3)			1440.6 (70.5)	0.08
sNfL (pg/ml)	7.7 (5.9–12.2)	8.7 (6.1–12.7)	0.95	7.5 (5.8–11.9)	0.58
sGFAP (pg/ml)	233.1 (195.3–326.8)	238.6 (171.5–326.9)	0.30	258.1 (185.8–344.2)	1.0
	Healthy controls ($n = 13$)				
sNfL (pg/ml)	6.1 (4.9–10.0)	6.6 (4.6–9.7)	0.59	6.2 (5.6–10.3)	0.19
sGFAP (pg/ml)	203.5 (146.1–243.2)	198.6 (112.2–229.3)	**0.04**	201.9 (155.8–238.5)	0.31

MS, multiple sclerosis; EDSS, Expanded Disability Status Scale; LV, white matter lesion volume; WBV, whole brain volume; sNfL, serum neurofilament light chain; sGFAP, serum glial fibrillary acidic protein.
[1]Compared to baseline. Level of significance: $p < 0.05$.

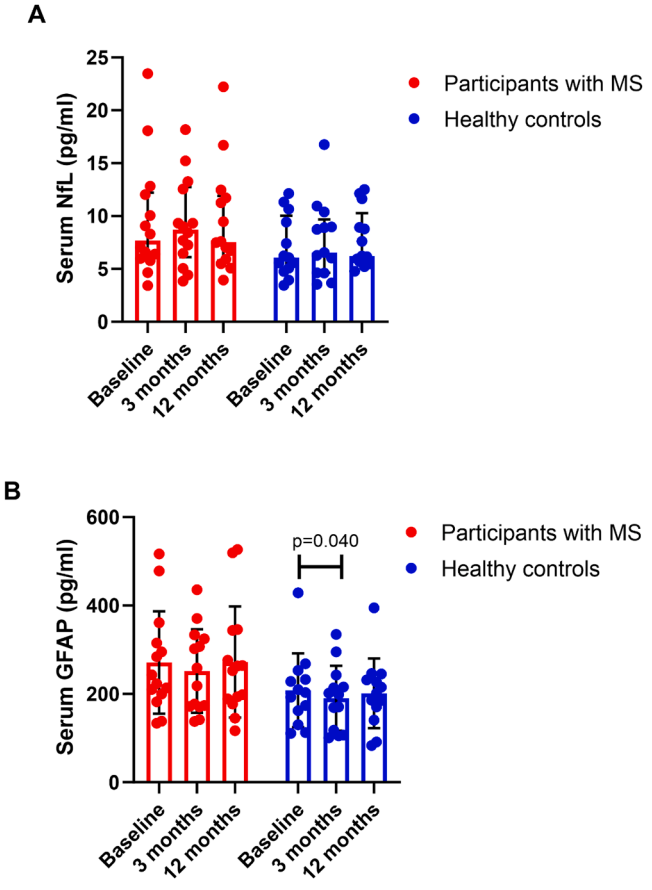

Fig.. 1. Changes in A) serum neurofilament light chain (NfL) and B) serum glial fibrillary acidic protein (GFAP) levels during one year of menopausal hormonal therapy. Bars indicate the median with interquartile range and red or blue dot indicates the individual value of each participant.

of sNfl and the stable neuroinflammatory disease activity in our MS cohort. Serum NfL mainly reflects the ongoing inflammatory-driven neuroaxonal damage in MS whereas high sGFAP have been correlated with subsequent MS progression particularly in nonactive patients (Barro et al., 2023).

Aging alone increases the sNfL and sGFAP levels (Abdelhak et al., 2022; Thebault et al., 2020). In our data, only age correlated with the serum biomarker levels in HC group and no association between hormonal levels and these biomarkers was observed. However, this does not necessarily mean that these findings are specific to MS. Although the effect of estradiol on these specific biomarkers is largely unknown, there is compelling evidence that menopausal transition and estrogen deprivation induce multiple changes also in healthy brain which might increase the risk of developing neurodegenerative diseases later in life (Brinton et al., 2015; Cheng et al., 2021). Under chronic inflammatory or neurodegenerative conditions, like in MS, neurons and glial cells are chronically exposed to inflammatory and toxic factors. Therefore, the harmful effect of decreasing estradiol levels on these cells may be more pronounced and appear earlier in patients than in healthy (Crespo-Castrillo and Arevalo, 2020). This could explain why the association of hormonal levels and serum biomarkers was seen only in PwMS in this small sample of peri- or mainly early postmenopausal women. Furthermore, transient decrease in GFAP levels during MHT in HC group may indicate the association of hormonal deprivation and reactive astrogliosis also in healthy women.

There are grounds for supposing that exogenous estrogens could act as neuroprotective factors in MS (Christianson et al., 2015). The treatment with estriol, the main estrogen in pregnancy, has shown beneficial potential in MS (Sicotte et al., 2002; Voskuhl et al., 2016) and 12 months of treatment has also been demonstrated to reduce serum NfL levels (Voskuhl et al., 2022). There are no previous reports on the effect of MHT on MS activity and progression using serum or CSF biomarkers, such as NfL and GFAP. We observed temporal stability of both sNfL and sGFAP levels in PwMS during one year of treatment with 1 mg or 2 mg estradiol hemihydrate combined with cyclic dydrogesterone. This supports our previous findings that MHT had no adverse effect on clinical or MRI-assessed MS activity (Juutinen et al., 2022). Slight, statistically non-significant increase in brain volumes during the follow-up should be interpreted with caution as it can be caused by several other factors than a real increase in brain tissue volume (Uher et al., 2021). Placebo control groups and a longer follow-up period could provide a more comprehensive insight into effects of MHT on these biomarkers. With the chosen study design, we are unable to distinguish between the treatment effect and age-related changes in brain volume and sNfL and sGFAP levels. Annual brain volume loss with healthy aging is approximately 0.4% (Fujita and Yamashita, 2019) and in stable, untreated MS patients about 0.5%–1% (Rocca et al., 2017). Aging increases sNfL on the average by 2.2% per year (Thebault et al., 2020). Thus, a small change or stabilization in these parameters may not show up among the changes brought by aging.

Baseline-controlled study design, in which PwMS and HC status on therapy was compared with status before therapy, was chosen for our small sized preliminary study given that individual differences in MS and menopausal transition are large. Possible baseline differences between the treatment and placebo groups in MS activity or treatment and menopausal phase could have complicated the interpretation of results and the control of confounders. In future studies, a larger cohort would be needed to distinguish between treatment and placebo responses. Furthermore, it will be important to assess the most potential preparations to promote beneficial actions in the brain since a variety of estrogenic compounds and selective estrogen receptor modulators (SERMs) are available. Type, dose, and route of administration all affect the actions of hormonal therapy (Dubal and Wise, 2002). Long-term estrogen deprivation also leads to downregulation of estrogen receptors, without which the treatment cannot effectively activate neuroprotective pathways (Guo et al., 2020). Previous studies strongly

suggest that estrogen only yields neuroprotection if it is applied soon after menopause to healthy neurons highlighting the importance of timing of estrogen replacement therapy (Scott et al., 2012).

Apart from the absence of placebo-control groups, the limitations of the data concern the significant fluctuation of hormone levels during menopausal transition which weakens the reliability of a single measurement at a single time point (Hall, 2015). The sample size was small which limited the statistical analysis of the data. Therefore, multiple potential confounding factors (such as age, disease duration, and BMI) could not be included in the same multivariate analysis. Subgroup analyzes according to, for example, DMT use, were not feasible. There is also evidence that vasomotor symptoms might be associated with greater white matter hyperintensity volume in midlife women (Thurston et al., 2023). Along with demyelination, this may contribute to the white matter lesion load in women with MS and should be considered as a possible confounding factor in future studies. By excluding MS patients with high efficacy DMTs, greater disability, or primary progressive MS, we wanted to control the heterogeneity of the participants and focus on those women with MS whose neurodegenerative process of the disease is probably less advanced. However, these exclusions and the stable disease activity of the participants weaken the generalizability of the findings to women with higher disability or more active MS.

In conclusion, our preliminary findings suggest that menopause characterized by low estradiol is associated with more pronounced brain atrophy and advanced astrogliosis in women with MS independent of age. The dysfunctions of astrocytes could provide one potential explanation for the more rapid progression of MS after menopause. One-year treatment with estradiol hemihydrate combined with cyclic dydrogesterone in menopause did not change the sGFAP or sNfL levels in women with MS but a definite conclusion for or against an effect of MHT on these biomarkers needs longer and larger randomized placebo-controlled studies.

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CRediT authorship contribution statement

Laura Juutinen: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Katja Ahinko:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Sanna Hagman:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Pabitra Basnyat:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Olli Jääskeläinen:** Writing – review & editing, Resources, Formal analysis, Data curation. **Sanna-Kaisa Herukka:** Writing – review & editing, Resources, Formal analysis, Data curation. **Marja-Liisa Sumelahti:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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