Increased Serum Levels of C21 Steroids in Female Patients With Multiple Sclerosis

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Summary

Multiple sclerosis (MS) is one of the most common neurological diseases. This neurodegenerative autoimmune disease manifests as inflammatory and demyelinating impairment of the central nervous system (CNS). Although some studies demonstrated associations between altered steroidogenesis and pathophysiology of MS as well as the importance of steroids in the pathophysiology of MS, the knowledge concerning the steroid metabolome in female patients is limited. Hence, 51 steroids and steroid polar conjugates were measured in the serum of 12 women with MS, untreated with steroids and 6 age-corresponding female controls with the use of gas chromatography – mass spectrometry (GC-MS). The data were processed using age adjusted ANCOVA, receiver operating characteristics (ROC) analysis and orthogonal projections to latent structures (OPLS). Our data show higher levels of circulating C21 steroids including steroid modulators of ionotropic type A γ-aminobutyric acid (GABA A) receptors and glutamate receptors. Furthermore, the levels of GABAergic androsterone and 5-androsten-3β,7α,17β-triol were also higher in the female MS patients. In conclusion, the data demonstrate higher levels of circulating C21 steroids and their polar conjugates and some bioactive C19 steroids in women with MS, which may influence neuronal activity and affect the balance between neuroprotection and excitotoxicity.

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Introduction

Steroid hormones known as neurosteroids are synthesized in the human central nervous system (CNS) (Leitner 2010). Neurosteroids have been studied in different addicting disorders (Duskova *et al.* 2012) and also in neurological diseases. Multiple sclerosis (MS) is one of the most common neurological diseases. This neurodegenerative autoimmune disease manifests as inflammatory and demyelinating impairment of the CNS. Whereas the present pharmacological treatment of the disease is limited to the application of immunomodulatory and antiinflammatory substances, which are not primarily targeted to the causation of the disease, there is a need for drugs promoting the neuronal viability (El-Etr *et al.* 2011). Beside others, some endogenous steroids like the sex hormones and GABAergic steroids as well as their artificial derivatives may be promising as targeted drugs (Melcangi *et al.* 2014). Even if some studies demonstrated associations between altered steroidogenesis and pathophysiology of MS as well as the importance of steroids in the pathophysiology of the disease, the knowledge concerning the steroid metabolome in human is limited, particularly in female patients (Bove and Chitnis 2014, Caruso *et al.* 2014, El-Etr *et al.* 2005, 2011, Noorbakhsh *et al.* 2011, Spence and Voskuhl 2012, Tomassini and Pozzilli 2009). Taking into account that a substantial part

of steroid metabolome in the CNS may reflect the peripheral steroidogenesis (Kancheva *et al.* 2011), we attempted to systematically investigate these relationships between the circulating steroids and MS using a multicomponent steroid profiling by gas chromatography – mass spectrometry (GC-MS). In addition, taking advantage of multicollinearity between circulating steroids, we tried to set up a multivariate prediction model differentiating female MS patients from the corresponding controls.

Materials and Methods

Subjects

The study was approved by the Ethics Committee of the Institute of Endocrinology. All women signed informed consent form before entering the study. Participants were 12 women with MS not treated with steroids and 6 age-corresponding female controls who were in the follicular phase of the menstrual cycle. They were 36 years of age (26, 47) (shown as median quartiles).

Chemicals

Steroids were purchased from Steraloids (Newport, RI, USA), Sylon B from Supelco (Bellefonte, PA, USA), methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and solvents from Merck (Darmstadt, Germany).

Analytical methods

The circulating levels of steroids and their polar conjugates were measured using the previously described GC-MS method (Hill *et al.* 2010).

Instruments

Measurements of steroid levels were done on GCMS-QP2010 Plus system by Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with automatic flow control, an AOC-20s autosampler, and a single quadrupole detector with an adjustable electron voltage of 10-195 V. A capillary column with a medium polarity RESTEK Rxi^{TM} – 50 (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for analyses. Electron impact ionization with electron voltage fixed at 70 V and emission current set to 160 μA was used. The temperatures of the injection port, ion source and interface were maintained at 220 °C, 300 °C, and 310 °C, respectively. Analyses were carried out in the splitless

mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set at 3 ml/min. The samples were injected using the high pressure mode (200 kPa), which was maintained for 1 min. The detector voltage was set to 1.2 kV.

Statistical analysis

To correctly explain the relationships between steroids and presence of MS in women, we used three independent statistical approaches. The first compares the steroid levels in MS and age- and gender-corresponding controls. In spite of comparable age of both groups, we used age-adjusted ANCOVA for this purpose to separate the variability in dependent variable shared with age from that explained by status of the subjects (MS vs. controls). Respecting the skewed distribution and non-constant variance in most dependent variables, these were transformed by power transformations to data symmetry and homoscedasticity prior further processing (Meloun *et al.* 2000). The homogeneity and distribution of the transformed data was checked by residual analysis as described elsewhere (Meloun *et al.* 2002, 2004). Statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for ANCOVA.

The second method used for data evaluation was the receiver operating characteristics (ROC) analysis. This method enables the finding of optimum cut-off values from individual dependent variables to differentiate between patients and controls. The positivity of the pathology may be detected at values higher or below this cut-off value. The effectiveness of individual variables for prediction of MS was assessed using area under curve (AUC) in the plot of sensitivity vs. 1-specificity and the significance of AUC in this plot was evaluated by one sided test (hypothesis that the AUC is significantly greater than 0.5). Statistical software NCSS 2007 from Number Cruncher Statistical Systems (Kaysville, Utah, USA) was used for the ROC analysis.

The third and most efficient method was simultaneous evaluation of relationships between steroids and the presence of MS by multivariate regression with a reduction of dimensionality known as orthogonal projections to latent structure (OPLS) (Madsen *et al.* 2010, Trygg and Wold 2002, Trygg *et al.* 2007), which is capable to cope with a problem of severe multicollinearity (high intercorrelations) in the matrix of predictors, whilst the ordinary multiple regression fails to

correctly evaluate such data. The multicollinearity in OPLS is favorable as it enhances a predictivity of the model.

In our model, the logarithm of the ratio of probability of pathology presence to probability of pathology absence (logarithm of likelihood ratio, LLR), was chosen as the single dependent variable, while the steroid levels represented the predictors. The variability in predictors was separated into two independent components. The first one contained the variability predictors, which was shared with probability of the pathology (the predictive component) while the orthogonal components explained the variability shared within the highly intercorrelated predictors.

The OPLS identified the best predictors as well as the best combination of predictors to estimate the probability of the pathology presence. After standardization of the variables, the OPLS model can be expressed as follows:

$$\mathbf{X} = \mathbf{T}_p\mathbf{P}_p{}^t + \mathbf{T}_0\mathbf{P}_0{}^t + \mathbf{E}$$

$$\mathbf{Y} = \mathbf{T}_p\mathbf{P}_p{}^t + \mathbf{F}$$

where $\mathbf{X}$ is the matrix with l predictors and i subjects, $\mathbf{Y}$ is the vector of dependent variable and i subjects. $\mathbf{T}_p$ represents the vector of component scores from the single predictive component and i subjects extracted from $\mathbf{Y}$, $\mathbf{T}_o$ is the vector of component scores from the single orthogonal component and i subjects extracted from $\mathbf{X}$, $\mathbf{P}_p$ represents the vector of component loadings for the predictive component extracted from $\mathbf{Y}$. $\mathbf{P}_o$ represents the vector of component loadings for the orthogonal component extracted from $\mathbf{X}$ and l independent variables, while $\mathbf{E}$ and $\mathbf{F}$ are the error terms.

The relevant predictors have been chosen using variable importance ("VIP") statistics. The statistical software SIMCA-P v.12.0 from Umetrics AB (Umeå, Sweden), which was used for OPLS analysis, enabled to find the number of relevant components, the detection of multivariate non-homogeneities and testing the multivariate normal distribution and homoscedasticity (constant variance).

The algorithm for obtaining the predictions is as follows: a) transformation of the original data to obtain the values with symmetric distribution and constant variance, b) checking the data homogeneity in predictors using Hotelling's statistics and eventual elimination of non-homogeneities, c) testing the relevance of predictors using the Variable importance statistics and elimination

of irrelevant predictors, d) calculation of component loadings for individual variables to evaluate their correlations with predictive component, e) calculation of regression coefficients for the multiple regression model to evaluate mutual independence of predictors after comparison with the corresponding component loadings from the OPLS model, f) calculation of predicted values of logarithm of the ratio of probability of pathology presence to probability of pathology absence (LLR), g) calculation of probability of the pathology presence for individual subjects, h) calculation of sensitivity and specificity of the prediction.

Results

Age-adjusted ANCOVA

The differences between steroid levels of female patients with MS and age- and gender-corresponding controls are shown in Table 1. These data indicate higher levels of most C21 steroids, androstenedione and estriol in MS patients when compared to controls.

ROC analysis

Table 2 shows a similar trend as Table 1. The levels of various C21 steroids including most 5α/β-reduced C21 steroids exhibit significant areas under curve in receiver operating characteristics (ROC) with higher levels of these substances in MS patients. In addition, estriol and some 5α-reduced C19 steroids such as androsterone and epiandrosterone are also higher in the patients. Although the levels of progesterone do not significantly differ between the female MS patients and controls, its 5α/β-reduced catabolites as well as 16α-hydroxy and 20α-dihydro-metabolites are significantly elevated in the patients.

OPLS model

Table 3 also demonstrates that most C21 steroids, two conjugated 5α/β-reduced androstanes and estriol positively correlate with the presence of MS in women. From the C21 steroids, the correlation of progesterone with MS does not reach significance but its 5α/β-reduced catabolites as well as 16α-hydroxy and 20α-dihydro-metabolites are significantly elevated in the patients. The predictivity of the OPLS model was absolute (sensitivity = 1.000 (0.758, 1.000); specificity = 1.000 (0.566, 1.000) – shown as estimates with 95 % confidence limits).

Table 1. Levels of circulating steroids (nM) in female patients with multiple sclerosis (MS) and in age- and gender-corresponding controls (C) as evaluated by age-adjusted ANCOVA (significant differences are in bold).

Variable	Mean (95 % confidence limits)		ANCOVA, MS	
	C	**MS**	**F**	**p**
Pregnenolone	**0.673 (0.49, 0.903)**	**1.47 (1.22, 1.78)**	**11.4**	**0.004**
Pregnenolone sulfate	**41.8 (33.6, 51.8)**	**102 (85.7, 121)**	**23.2**	**<0.001**
16α-Hydroxypregnenolone	0.2 (0.127, 0.293)	0.36 (0.283, 0.451)	3.8	0.07
17-Hydroxypregnenolone	0.733 (0.345, 1.42)	2.04 (1.29, 3.17)	3.6	0.078
20α-Dihydropregnenolone	2.55 (1.9, 3.39)	3.26 (2.68, 3.95)	1.1	0.312
20α-Dihydropregnenolone sulfate	**355 (299, 424)**	**685 (581, 815)**	**16.5**	**0.002**
Dehydroepiandrosterone (DHEA)	10.9 (7.38, 14.2)	15.3 (13, 17.5)	2.6	0.129
DHEA sulfate (DHEAS)	1740 (1160, 2540)	3130 (2390, 4060)	3.5	0.084
7α-Hydroxy-DHEA	0.612 (0.457, 0.784)	0.874 (0.757, 0.998)	3.6	0.079
7-oxo-DHEA	0.297 (0.215, 0.416)	0.34 (0.27, 0.431)	0.2	0.628
7β-Hydroxy-DHEA	0.592 (0.452, 0.751)	0.626 (0.523, 0.738)	0.1	0.791
16α-Hydroxy-DHEA	0.157 (0.11, 0.218)	0.207 (0.165, 0.259)	1.1	0.317
16α-Hydroxy-DHEAS	9.91 (7.08, 14.5)	11.9 (9.12, 15.9)	0.3	0.568
Androstenediol	1.72 (1.14, 2.53)	1.72 (1.29, 2.25)	0	0.995
Androstenediol sulfate	562 (324, 921)	554 (374, 797)	0	0.974
5-Androsten-3β,7α,17β-triol	**0.0724 (0.0604, 0.0852)**	**0.117 (0.106, 0.128)**	**15.9**	**0.002**
5-Androsten-3β,7β,17β-triol	0.0783 (0.058, 0.107)	0.0827 (0.0668, 0.103)	0.1	0.831
Progesterone	0.419 (0.281, 0.641)	0.741 (0.541, 1.05)	2.6	0.13
16α-Hydroxyprogesterone	0.472 (0.314, 0.735)	0.655 (0.483, 0.917)	0.8	0.375
20α-Dihydroprogesterone	**0.319 (0.263, 0.392)**	**0.626 (0.521, 0.771)**	**12.9**	**0.003**
5α-Dihydroprogesterone	0.122 (0.0893, 0.17)	0.166 (0.132, 0.213)	1.3	0.27
Androstenedione	**1.59 (1.2, 2.04)**	**2.74 (2.4, 3.12)**	**8.9**	**0.01**
Testosterone	0.803 (0.649, 1.03)	0.927 (0.785, 1.12)	0.5	0.479
5α-Dihydrotestosterone	0.552 (0.372, 0.85)	0.487 (0.37, 0.649)	0.1	0.71
Epitestosterone	0.386 (0.25, 0.556)	0.447 (0.34, 0.571)	0.2	0.647
Estrone	0.306 (0.227, 0.424)	0.213 (0.173, 0.264)	2.2	0.164
Estrone sulfate	1.38 (0.718, 2.88)	1.27 (0.789, 2.11)	0	0.881
Estradiol	0.158 (0.0759, 0.333)	0.175 (0.105, 0.294)	0	0.871
Estriol	**0.0386 (0.0328, 0.0463)**	**0.06 (0.0513, 0.072)**	**7.3**	**0.016**
Allopregnanolone	**0.176 (0.134, 0.228)**	**0.444 (0.371, 0.529)**	**20.2**	**<0.001**
Allopregnanolone sulfate	**3.74 (2.77, 5.11)**	**7.68 (5.96, 10.2)**	**6.9**	**0.02**
Isopregnanolone	**0.104 (0.0849, 0.127)**	**0.191 (0.164, 0.224)**	**12.7**	**0.003**
Isopregnanolone sulfate	**4.1 (3.39, 4.94)**	**9.21 (7.9, 10.8)**	**25.2**	**<0.001**
Pregnanolone	0.0937 (0.0745, 0.12)	0.141 (0.117, 0.176)	3.7	0.073
Conjugated pregnanolone	**8.75 (6.91, 11.2)**	**15.9 (12.9, 20.2)**	**7.2**	**0.018**
Epipregnanolone	0.0261 (0.0187, 0.0372)	0.0485 (0.0371, 0.0655)	4.2	0.058
Conjugated epipregnanolone	**0.671 (0.428, 0.994)**	**1.38 (1.06, 1.77)**	**5.2**	**0.039**
Conjugated 5α-pregnane-3β,20α-diol	**119 (78.7, 179)**	**264 (194, 368)**	**5.3**	**0.037**
Conjugated 5β-pregnane-3α,20α-diol	**14.2 (11.4, 18.3)**	**23 (18.6, 29.4)**	**4.7**	**0.049**
Androsterone	0.341 (0.275, 0.42)	0.424 (0.366, 0.488)	1.6	0.226
Androsterone sulfate	658 (362, 1000)	699 (475, 949)	0	0.88
Epiandrosterone	0.517 (0.367, 0.675)	0.749 (0.636, 0.866)	3.1	0.097
Epiandrosterone sulfate	227 (126, 362)	328 (233, 443)	0.9	0.371
Etiocholanolone	0.188 (0.143, 0.247)	0.207 (0.171, 0.252)	0.2	0.667
Conjugated etiocholanolone	46.4 (27.9, 72.2)	46.7 (32.6, 64.6)	0	0.988
Epietiocholanolone	0.0309 (0.0235, 0.0421)	0.0305 (0.0252, 0.0377)	0	0.964
Conjugated epietiocholanolone	16.4 (7.81, 30.1)	23.7 (14.9, 35.7)	0.5	0.494
5α-Androstane-3α,17β-diol	0.0497 (0.0329, 0.0723)	0.0856 (0.0679, 0.107)	3.5	0.083
Conjugated 5α-androstane-3α,17β-diol	31.8 (20.3, 45.3)	29 (20.8, 38.2)	0.1	0.785
Conjugated 5α-androstane-3β,17β-diol	38.8 (19.1, 67.8)	45.4 (28.7, 67.5)	0.1	0.758
Conjugated 5β-androstane-3α,17β-diol	6.74 (4.33, 9.27)	6.79 (5.03, 8.62)	0	0.979

Table 2. ROC analysis of steroid levels for the differentiation of female patients with multiple sclerosis (MS) from age- and gender-corresponding controls (C).

Steroid	Cut-off value (patient)	Sensitivity	Specificity	Youden's statistics	AUC	p-value (one-sided)
Pregnenolone	>1.03	83.3	100	0.833	0.903	<0.001
Pregnenolone sulfate	>56.2	90.9	100	0.909	0.909	<0.001
16α-Hydroxypregnenolone	>0.23	83.3	66.7	0.5	0.75	0.029
17-Hydroxypregnenolone	>1.32	72.7	83.3	0.561	0.773	0.011
20α-Dihydropregnenolone	>2.65	83.3	66.7	0.5	0.736	0.029
20α-Dihydropregnenolone sulfate	>480	81.8	100	0.818	0.879	<0.001
20α-Dihydroprogesterone	>0.34	100	83.3	0.833	0.958	<0.001
Estriol	>0.05	66.7	100	0.667	0.861	<0.001
Allopregnanolone	>0.24	83.3	100	0.833	0.833	0.002
Allopregnanolone sulfate	>6.57	63.6	100	0.636	0.864	<0.001
Isopregnanolone	>0.15	75	100	0.75	0.917	<0.001
Isopregnanolone sulfate	>4.75	90.9	100	0.909	0.939	<0.001
Pregnanolone	>0.08	100	50	0.5	0.819	0.002
Conjugated pregnanolone	>11.8	72.7	100	0.727	0.848	<0.001
Epipregnanolone	>0.03	75	66.7	0.417	0.736	0.035
Conjugated epipregnanolone	>0.8	90.9	66.7	0.576	0.803	0.008
Conjugated 5α-pregnane-3β,20α-diol	>254	72.7	100	0.727	0.788	0.008
Conjugated 5β-pregnane-3α,20α-diol	>21.3	63.6	100	0.636	0.818	0.002
Androsterone	>0.37	75	83.3	0.583	0.792	0.006
Epiandrosterone	>0.64	75	83.3	0.583	0.764	0.024

Table 3. Relationships between log(probability of MS presence/probability of MS absence) (matrix **Y**) and circulating steroids (matrix **X**) as evaluated by multiple regression with reduction of dimensionality (method of orthogonal predictions to latent structure, OPLS).

	Predictors	OPLS model			
		Component loading[a]	t-statistic[b]	R[c]	
	Pregnenolone	0.255	2.89	0.711	*
	Pregnenolone sulfate	0.267	9.56	0.768	**
	20α-Dihydropregnenolone	0.241	7.22	0.672	**
	20α-Dihydropregnenolone sulfate	0.248	7.47	0.713	**
	16α-Hydroxypregnenolone	0.197	1.78	0.550	
	20α-Dihydroprogesterone	0.287	7.11	0.801	**
	Estriol	0.183	2.33	0.511	*
matrix **X**	*Allopregnanolone*	0.243	9.95	0.679	**
	Allopregnanolone sulfate	0.297	5.62	0.857	**
	Isopregnanolone	0.250	3.16	0.699	**
	Isopregnanolone sulfate	0.331	12.80	0.953	**
	Pregnanolone	0.218	4.61	0.607	**
	Conjugated pregnanolone	0.246	2.78	0.710	*
	Epipregnanolone	0.227	4.00	0.633	**
	Conjugated 5β-pregnane-3α,20α-diol	0.230	2.17	0.664	*
	Conjugated 5α-pregnane-3β,20α-diol	0.288	16.65	0.830	**
matrix **Y**	*log(Probability of presence/absence of the disease) (logarithm of likelihood ratio, LLR)*	1.000	15.37	0.794	**
	Explained variability 63 % (57.9 %)				

a – component loadings for the predictive components expressed as regression coefficients; b – t-statistic is the ratio of the regression coefficient to its standard error; c – component loadings for the predictive components expressed as correlation coefficients of predictors with predictive components; *p<0.05, **p<0.01; value in parentheses represents explained variability after a cross-validation.

Discussion

This is the first study systematically investigating alterations in circulating steroids of female patients with multiple sclerosis. Three independent statistical approaches giving the same conclusions were used for the evaluation of experimental data. Our results are partly in accordance with the data of Caruso *et al.* (2014) reporting significantly increased circulating levels of pregnenolone, progesterone and isopregnanolone in male patients with MS. However, differences for progesterone levels in our data do not reach significance despite 77 % increase of progesterone in the patients. Furthermore, the levels of both allopregnanolone and allopregnanolone sulfate are even significantly elevated like the levels of their 3β-hydroxy-counterparts isopregnanolone and isopregnanolone sulfate (Table 1). In accordance with the data of Caruso *et al.* (2014), circulating androgens and estrogens do not show significant differences between controls and patients in our data except higher levels of estriol and two 5α/β-reduced androstanes in the patients. The discrepancies in allopregnanolone might be ascribed to gender differences as the female allopregnanolone is produced in both ovaries and adrenals, while the male circulating allopregnanolone is of adrenal origin (Havlikova *et al.* 2006, Kancheva *et al.* 2007).

Concerning the pathophysiological importance of systematically increased levels of circulating C21 steroids in women with MS, allopregnanolone and progesterone are known to attenuate the neuroinflammation in MS patients by numerous mechanisms (Melcangi *et al.* 2014, Noorbakhsh *et al.* 2014). Pregnenolone sulfate is known as a subtype-specific modulator of N-methyl-d-aspartate (NMDA) receptors and a negative modulator of kainate and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors while pregnanolone sulfate (the most abundant conjugated pregnenolone isomer; Havlikova *et al.* 2006) exerts inhibitory effect on both NMDA and AMPA receptors (Sedlacek *et al.* 2008). Whereas the continued overstimulation of AMPA, kainate and NMDA receptors injures oligodendrocytes *via* the cytoplasmic calcium overload; the transitory one sensitizes these cells to complement attack as a consequence of oxidative stress (Matute 2011). Some authors suggest that pharmacological impairment of NMDA receptor signaling may be a part of a neuroprotection strategy in MS (Grasselli *et al.* 2013). Nevertheless, steroid polar conjugates like pregnenolone sulfate or pregnanolone sulfate most probably do not reach sufficient concentrations for efficient modulation of ionotropic receptors in the CNS (Hill *et al.* 2014). On the other hand, circulating allopregnanolone readily penetrates through the blood-brain barrier and at the same time fortifies the blood-brain barrier against the penetration of detrimental compounds from periphery (Kancheva *et al.* 2011, Melcangi *et al.* 2014, Noorbakhsh *et al.* 2014).

From the C19 steroids the levels of GABAergic androsterone and immunoprotective 5-androsten-3β,7α,17β-triol were also higher in patients. The 7α-hydroxy-androstanes like the latter steroid compete for the active sites on the enzyme catalyzing the conversion of inactive 11-oxo-glucocorticoids to immunosuppressive 11β-hydroxy-glucocorticoids (Auci *et al.* 2009, Hennebert *et al.* 2007, 2008, Le Mee *et al.* 2008, Muller *et al.* 2006). In addition, the steroid 7α-hydroxylating enzyme CYP7B1, converts the estrogen precursors such as androstenediol (also estrogenic) and DHEA to the 7α-hydroxy-metabolites and thus hinders the formation of efficient estrogenic immunomodulators stimulating autoimmunity *via* estrogen receptors (Pettersson *et al.* 2008, 2010). More recent studies (Pettersson *et al.* 2010) show that there is no influence of DHEA to 7α-hydroxy-DHEA conversion on the function of ER-mediated response as previously indicated, but the conversion of androstenediol to 5-androstene3β,7α,17β-triol is effective.

In conclusion, our data demonstrate consistently higher levels of circulating C21 steroids and their polar conjugates and some bioactive C19 steroids in women with MS, which may influence neuronal activity and affect the balance between neuroprotection and excitotoxicity. However, further studies with a larger sample of participants are needed.

Conflict of Interest
There is no conflict of interest.

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