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Assessment of neuroactive steroids in cerebrospinal fluid comparing acute relapse and stable disease in relapsing-remitting multiple sclerosis

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ABSTRACT

Previous studies have reported an involvement of neuroactive steroids as neuroprotective and anti-inflammatory agents in neurological disorders such as multiple sclerosis (MS); an analysis of their profile during a specific clinical phase of MS is largely unknown. The pregnenolone (PREG), dehydroepiandrosterone (DHEA), and allopregnanolone (ALLO) profile was evaluated in cerebrospinal fluid (CSF) in relapsing-remitting multiple sclerosis (RR-MS) patients as well as those in patients affected by non-inflammatory neurological (control group I) and without neurological disorders (control group II). An increase of PREG and DHEA values was shown in CSF of male and female RR-MS patients compared to those observed in both control groups. The ALLO values were significantly lower in female RR-MS patients than those found in male RR-MS patients and in female without neurological disorder. During the clinical relapse, we observed female RR-MS patients showing significantly increased PREG values compared to female RR-MS patients in stable phase, while their ALLO values showed a significant decrease compared to male RR-MS patients of the same group. Male RR-MS patients with gadolinium-enhanced lesions showed PREG and DHEA values higher than those found in female RR-MS patients with gadolinium-enhanced lesions. Similary, male RR-MS patients with gadolinium-enhanced lesions showed PREG and DHEA values higher than male without gadolinium-enhanced lesions. Female RR-MS patients with gadolinium-enhanced lesions showed DHEA values higher than those found in female RR-MS patients with gadolinium-enhanced lesions. Male and female RR-MS patients with gadolinium-enhanced lesions showed ALLO values higher than those found in respective gender groups without gadolinium-enhanced lesions.

ALLO values were lower in male than in female RR-MS patients without gadolinium-enhanced lesions. Considering the pharmacological properties of neuroactive steroids and the observation that neurological disorders influence their concentrations, these endogenous compounds may have an important role as prognostic factors of the disease and used as markers of MS activity such as relapses.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the central nervous system (CNS) [1] characterized by grey and white matter lesions with infiltrating myelin-reactive lymphocytes and primary or secondary axonal *trans*-sections [2–4]. The most common clinical form of MS has a relapsing-remitting (RR-MS) course, in which the relapses depend on the formation of new demyelinating lesions or on reactivation of previously existing ones [5]. While, usually followed by a period of

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remission, residual symptoms may persist after new relapses and lead to sustained disability [6]. Recently, some clinical studies have focused on the pathophysiologic role of neuroactive steroids in the course of MS [7]. The nervous system represents a "steroidogenic organ". Indeed, both, glial cells and neurons are involved in the synthesis and metabolism of neuroactive steroids. In this synthesis cholesterol, mobilized from cytosolic lipid droplets or from lysosomes, is transported to the inner mitochondrial membrane. Cholesterol is then converted to pregnenolone (PREG) in a sequence of three reactions, all catalyzed by the side chain cleavage enzyme CYP11A. Finally, pregnenolone re-enters the cytosol and is converted to its metabolite dehydroepiandrosterone (DHEA) by CYP17 (17α-hydroxylase) [8,9]. Both of these molecules are known for their neuroprotective effects via modulation of inflammation and promotion of myelination, brain plasticity and neuronal survival in both, CNS development and the adult damaged brain [10,11]. Allopregnanolone (ALLO) is another brain endogenous neuroactive steroid that binds and exerts positive allosteric modulation on $GABA_a$ receptors. ALLO is synthesized in the brain from progesterone by the sequential action of two enzymes: (*i*) 5α-reductase (SRD5A), which reduces progesterone to 5α-DHP, and (*ii*) aldo-keto reductase 1C (AKR1C 2/3), which either converts 5α-DHP into ALLO (reductive reaction) or ALLO into 5α-DHP [8]. Recently, the alteration of neuroactive steroids has been closely related to neuroinflammation [12], and their varying levels in plasma and cerebrospinal fluid (CSF) levels compared to controls were revealed in the experimental autoimmune encephalomyelitis (EAE) model of MS [13,14]. In agreement, neuroprotective effects of these molecules have been reported in several experimental models of neurodegenerative disorders [15]. For instance, studies in adult animals after brain injury indicate that neuroactive steroids have an important role in repairing processes, enhancing myelination and reducing apoptotic processes [16]. We perform a cross-sectional study to assess the neuroactive steroid profile in CSF of RR-MS patients with relapses (active RR-MS) or remission (stable RR-MS), compared to those found in patients affected by non-inflammatory neurological and without neurological disorders.

2. Materials and methods

2.1. Study design

The study was carried out on 32 consecutive patients (20 female, 12 male; median age 38 years, range 28–48) with a diagnosis of RR-MS according to McDonald criteria [17]. All MS patients were naive to disease modifying therapy (DMT). Clinical assessment was performed by Expanded Disability Status Scale (EDSS) [18]. RR-MS patients underwent diagnostic lumbar puncture (LP) and magnetic resonance imaging (MRI) at 1.5 T for brain and spinal cord using gadolinium-based contrast agent (Gd). The clinical relapse is typically defined as a new or worsening neurological deficit lasting 24 h or more in the absence of fever or infections. Among 32 RR-MS patients, 26 were in a remitting phase and six were in clinical relapse. CSF was collected before onset of corticosteroids treatment in MS patients with relapse. Two control groups were included: control group I consisted of patients affected by non-inflammatory neurological disorders; 30 consecutive age and gender-matched patients (19 female, 11 male; median age 40 years, range 32–48 years) affected by idiopathic intracranial hypertension (IIH). The control group II included patients without neurological disorders: 14 subjects (8 female, 6 male; median age 46 years, range 30–50 years), who had undergone anesthesia for inguinal hernia repair. All women reported regular menstrual cycles (range, 21–35 days), and were medication-free. All women had normal physical findings, including gynecologic and breast examination, normal laboratory test results and a negative pregnancy test before study entry. They were fertile, did not use hormonal contraception or hormonal substitution and were in luteal phases (21st to 28th days). All patients enrolled in the study were not smokers, and we excluded patients with alcohol-use histories and were suffering from metabolic and other neurological disorders. All patients were not in treatment with influencing steroidogenesis, and antipsychotic drugs.

2.2. Ethical statement

This study was approved by the Institutional ethics committee for biomedical activities "Carlo Romano" Medical School University Federico II, Naples—(Italy). Informed written consent was obtained from all patients. The study was performed in compliance with the good clinical practice guidelines and the principles of the Declaration of Helsinki.

2.3. Sample collection

CSF samples were obtained by LP between 13:00 p.m. and 15:00 p.m. from 32 MS, 30 non-inflammatory neurological and 14 without neurological disorders patients. The samples were centrifuged immediately after collection and cell-free supernatants were stored at −80 °C for further analysis.

2.4. Gas chromatography–mass spectrometry (GC–MS)

CSF steroid analyses were performed by sensitive and specific gas chromatography/mass spectrometry method preceded by HPLC purification, as previously described [19].

CSF samples were homogenized in methanol (75% in H_2O) using a small electric pellet pestle motor (Kontes, Vernon Hills, IL, USA) on ice and then centrifuged (6000 g for 10 min). Supernatants were extracted three times with a triple amount of ethyl acetate, dried under nitrogen before HPLC and containing a trace quantity (4000 dpm) of tritiated neurosteroid (NEN Life Science Products, Wellesley, MA) to detect the HPLC fraction of interest. A constant amount of deuterated pregnenolone (D4-PREG, 400 pg) was carried throughout the entire procedure as internal standard. The steroids were isolated using solid-phase extraction with Oasis HLB cartridges (Waters). The samples were then derivatized with heptafluorobutyric acid anhydride (Sigma-Aldrich) and the resultanting derivatives analyzed by gas chromatography combined with negative ion chemical ionization mass spectrometry (Agilent 6890 gas chromatographer coupled to 5973 N mass selective detector). Standard curves were prepared for each steroid and the samples were injected in triplicate.

2.5. Statistical analysis

Data were analyzed via Generalized Linear Mixed Model (GLMM) and p-value ≤ 0.05 was considered statistically significant. Baseline variables and MRI data were analyzed by non-parametrically Kruskal–Wallis tests (with Dunn's multiple comparison test) when appropriate. The Spearman correlation coefficient was used for the correlation between neuroactive steroids profile and clinical characteristic. Statistical analysis was performed using Statistical Analysis System (SAS) software version 9.2. Clinical and demographic data of all participants presented as mean ± standard deviation (SD). Neuroactive steroids values presented as mean ± standard error of the mean (SEM).

3. Results

3.1. PREG and DHEA values higher in RR-MS patients than in control groups

Demographic, clinical and neuroimaging characteristics of RR-MS patients and control groups are reported in Table 1. No significant differences were found in age and male:female ratio between RR-MS patients and control groups. We found no significant difference of PREG values in male RR-MS patients compared to those in female RR-MS patients (Fig. 1A); the PREG values varied significantly in male RR-MS patients in respect to those found in male affected by non-inflammatory neurological and without neurological disorders (Fig. 1A). In accordance to female gender, we found a significant difference in PREG values between the RR-MS and without neurological disorders groups (Fig. 1A). We observed no significant differences in PREG values between female RR-MS and female affected by non-inflammatory neurological disorders (Fig. 1A). No significant differences were observed in DHEA values between male and female patients in the RR-MS group (Fig. 1B). A significant increase of DHEA values was found in male RR-MS patients compared to male patients without neurological disorders (Fig. 1B). Contrary, no significant difference was found in DHEA values between male RR-MS and male affected by non-inflammatory neurological disorders (Fig. 1B). Similarly, the female RR-MS patients showed a significant increase of DHEA CSF values compared to female patients without neurological disorders (Fig. 1B). No significant difference was observed in DHEA values between female RR-MS and female affected by non-inflammatory neurological disorders (Fig. 1B). We failed to find any correlations between the profile of neuroactive steroids and EDSS, age at clinical onset, and disease duration.

Table 1
Demographic, clinical and neuroimaging characteristics.

Demographic characteristics	RR-MS	CTR GROUP I	CTR GROUP II
Gender	12/M 20/F	11/M 19/F	6/M 8/F
Age	38 ± 10 y	40 ± 8 y	40 ± 14 y

Clinical and neuroimaging characteristics	
RR-MS group	N
Patients in clinical remission	
Male	11
Female	15
Patients with clinical relapse	
Male	3
Female	3
MRI-Gd+	
Male	5
Female	9
MRI-Gd-	
Male	7
Female	11
EDSS score (mean ± SD)	2.5 ± 1.5

F, female; M, male; y, years; RR-MS, relapsing-remitting multiple sclerosis; CTR GROUP I, non-inflammatory neurological disorders; CTR GROUP II, without neurological disorders; EDSS, expanded disability status scale; MRI-Gd+, gadolinium enhanced magnetic resonance imaging lesions; MRI-Gd-, gadolinium-non-enhanced magnetic resonance imaging lesions. Data expressed as mean ± standard deviation (SD).

3.2. ALLO values lower in female RR-MS patients

Differently from PREG and DHEA, ALLO values varied significantly within the RR-MS group. A significant decrease was found of ALLO values in CSF of female RR-MS compared to male RR-MS patients (Fig. 1C). In female RR-MS patients the ALLO values in CSF were lower than those observed in the female without neurological disease (Fig. 1C). No significant difference was observed in ALLO values between female RR-MS and female affected by no-inflammatory neurological disease (Fig. 1C). In accordance to male gender, we found no differences of ALLO values in CSF between RR-MS and both control groups (Fig. 1C).

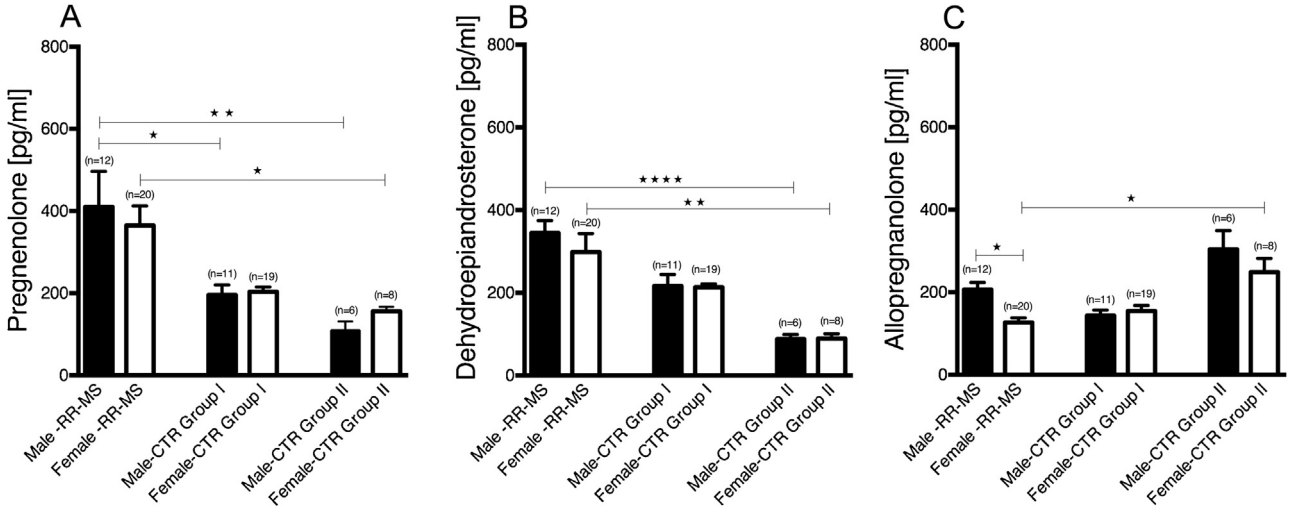

Fig. 1. Neuroactive steroids profile in relapsing-remitting multiple sclerosis compared to non-inflammatory neurological and without neurological disorders. (A) Male RR-MS patients had significantly higher concentrations of PREG in CSF compared to those in both control groups. (A) Female RR-MS patients showed significantly higher mean of PREG values than patients without neurological disorders. (A) **P < 0.01 vs male without neurological disorders, *P < 0.05 vs male affected by non-inflammatory neurological disorders; *P < 0.05 vs female without neurological disorders (B). Male and female RR-MS patients had significantly higher values of DHEA in CSF compared to patients without neurological disorders. (B) ****P < 0.0001 vs male without neurological disorders; **P< 0.01 vs female without neurological disorders. (C) Male RR-MS patients had significantly higher ALLO values in CSF compared to female RR-MS patients. (C) Values of ALLO in CSF were lower in female RR-MS than in female without neurological disorders. (C) *P < 0.05 vs female RR-MS; *P < 0.05 vs female without neurological disorders. Data was obtained by GC–MS and expressed as pg/ml, mean ± SEM.

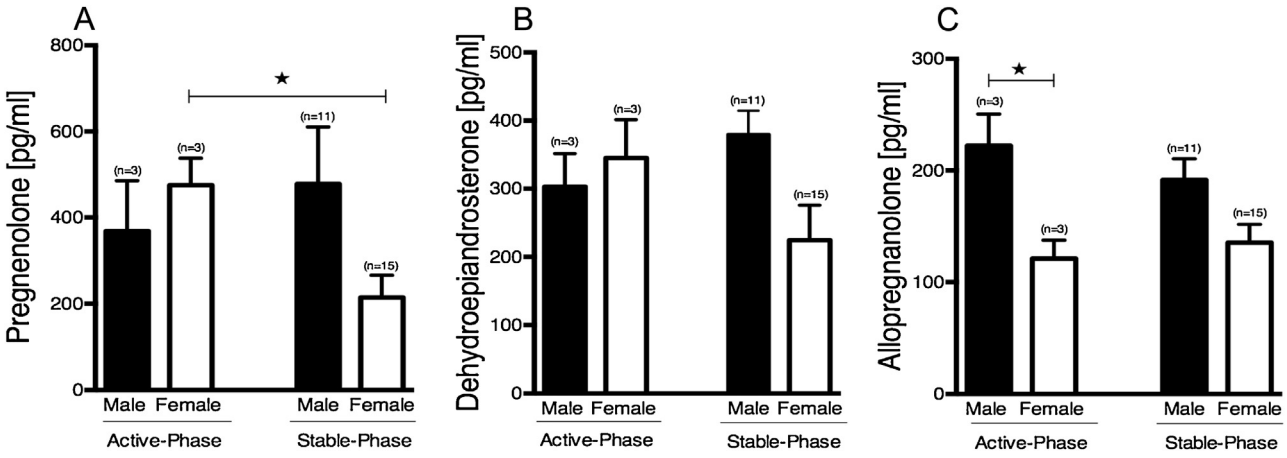

Fig. 2. Neuroactive steroids profile in MS-related disease activity. (A) Female RR-MS patients in active phase showed significantly higher mean values of PREG in CSF than those observed in female RR-MS patients in stable phase. (A) *P < 0.05 vs female in stable phase. (B) No significant differences were observed regarding DHEA values in male and female RR-MS patients in both groups. (C) Male RR-MS patients in active phase showed significantly higher mean values than those observed in female RR-MS active phase patients. (C) *P < 0.05 vs female active phase. Data obtained by GC–MS is expressed as pg/ml, mean ± SEM.

3.3. PREG and DHEA values during active and stable clinical phase

During the first neurological examination, 26 out of 32 patients were in clinical remission (stable RR-MS) while six patients experienced clinical relapses (active RR-MS). We observed no significant differences of PREG and DHEA values between male and female RR-MS patients during active and stable phase (Fig. 2A,B). Similarly, no significant difference was found in CSF of PREG and DHEA values between male RR-MS (n = 3) active phase and male RR-MS (n = 11) stable phase patients (Fig. 2A,B). Female RR-MS active phase patients (n = 3) showed PREG values higher than those revealed in female RR-MS stable phase patients (n = 15) (Fig. 2A). DHEA values were not varied between female RR-MS active phase and female RR-MS stable phase patients (Fig. 2B)

3.4. ALLO values higher in male RR-MS patients in active phase

Differently from PREG and DHEA, ALLO values were higher in CSF of male (n = 3) than those observed in female (n = 3) RR-MS active phase patients (Fig. 2C). Despite observing differing ALLO values comparing male (n = 11) and female (n = 15) RR-MS patients in stable phase, this data was not significant (Fig. 2C). We observed no difference comparing ALLO values of male and female RR-MS active phase to those found in CSF of male and female RR-MS stable phase patients (Fig. 2C).

3.5. PREG and DHEA CSF values at the presence or absence of gadolinium enhanced lesions

Interestingly, 14 out of 32 RR-MS patients had one or more gadolinium enhanced lesions (Gd +). Male RR-MS patients showing Gd+ (n = 5) coincide with a significant increase of PREG, and DHEA values compared to those found in male RR-MS with Gd- (n = 7) and in female RR-MS patients with Gd+ (n = 9) (Fig. 3A,B). No significant difference was observed in PREG and DHEA values between male RR-MS with Gd- and female RR-MS patients (n = 11) with Gd- (Fig. 3A,B). In accordance to female gender, we observed PREG values were not varied between female RR-MS patients with

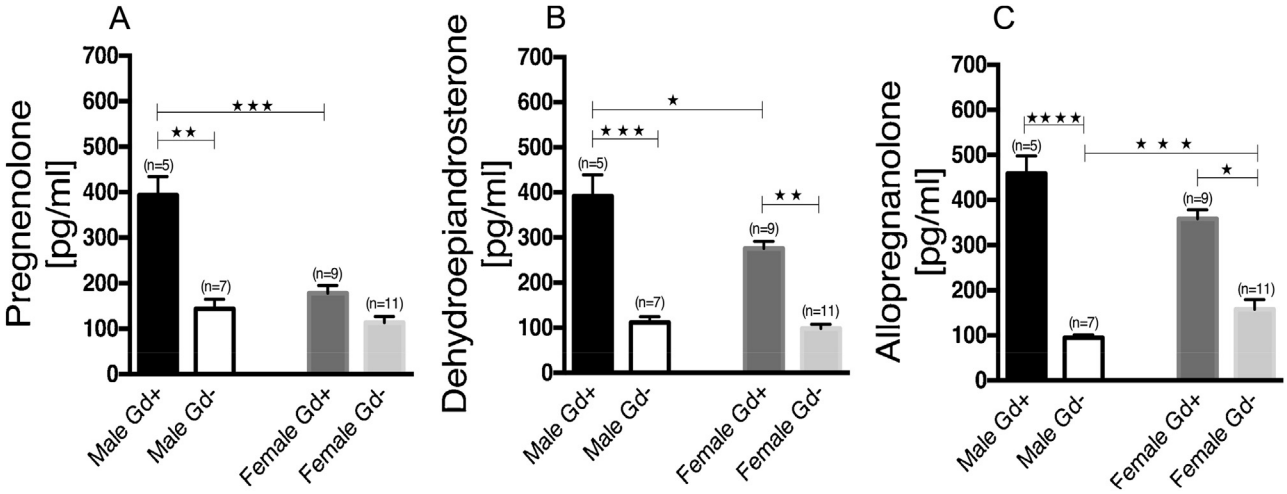

Fig. 3. Neuroactive steroids profile related to the presence (Gd +) or absence (Gd-) of MRI Gd-enhancing lesions. (A) Male RR-MS Gd+ showed higher PREG CSF values than male RR-MS Gd- and female RR-MS Gd+. (A) *** P < 0.001 vs female Gd+, **P < 0.01 vs male Gd- (B). Male RR-MS Gd+ showed higher DHEA CSF values than male Gd- and female RR-MS Gd+. (B) DHEA CSF values was higher in female RR-MS Gd+ than female Gd-. (B) *** P < 0.001 vs male Gd-; ** P < 0.01 vs female Gd-; * P < 0.05 vs female Gd+. (C) ALLO CSF values were higher in male RR-MS Gd+ than in male Gd-. (C) Male RR-MS Gd- showed ALLO CSF values lower than in female RR-MS Gd-. (C) ALLO CSF values was higher in female RR-MS Gd+ than in female Gd-. (C) **** P < 0.0001 vs male Gd-; *** P < 0.001 vs female Gd-; * P < 0.05 vs female Gd-. Data obtained by GC–MS is expressed as pg/ml, mean ± SEM.

Gd+ and with Gd- (Fig. 3A). Contrary, we observed DHEA values were higher in female RR-MS with Gd+ than those found in female with Gd- (Fig. 3B,C).

3.6. ALLO CSF values higher at the presence of gadolinium enhanced lesions

Contrary to PREG and DHEA, we observed no significant difference in ALLO CSF values between male (n = 5) and female (n = 9) RR-MS patients with Gd+ (Fig. 3C). ALLO values were higher in male RR-MS patients with Gd+ than those found in male RR-MS patients (n = 7) with Gd- (Fig. 3C). In CSF of male RR-MS patients with Gd-, the ALLO CSF values were lower than those found in CSF of female RR-MS patients (n = 11) with Gd- (Fig. 3C). A significant difference was observed in ALLO values between female RR-MS with Gd+ and Gd- (Fig. 3C).

4. Discussion

Recent evidence underlines the increasing importance of neuroactive steroids metabolism in the pathogenesis and in the course of various neurological diseases [20]. Neuroactive steroids are known to be upregulated in acute neuroinflammatory conditions such as bacterial meningitis or traumatic brain injury [21,22]. Alterations of neuroactive steroid metabolism is related to abnormal function of the hypothalamic-pituitary-adrenal (HPA) axis [23–26]. Dynamics and an increase in activity of the HPA axis are reported in the majority of MS patients. It exhibits signs ranging from increased hypothalamic corticotropin-releasing hormone (CRH) expression, adrenocorticotropic hormone (ACTH) and cortisol plasma levels [27–29], as well as from enlarged adrenal gland in MS patients [30]. Also the responsiveness of the HPA axis to the dexamethasone CRH stimulation test is enhanced in MS which is related to the clinical type of the disease (i.e., most prominently in primary-progressive, moderately in relapsing-remitting and intermediate in secondary-progressive forms of MS) [31]. This hyperactivity of the HPA axis in MS patients may be part of our protective mechanism against oncoming disease attacks or for recovery from relapses [32]. Although dexamethasone resistance suggests that steroid feedback may be implicated in the HPA axis over reactivity in MS, the exact mechanism of HPA axis activation is not known. As demonstrated in other diseases [33], [34,35], the increased HPA axis activity may be related to the presence of a polymorphism in the gene coding for hydroxysteroid (11-beta) dehydrogenase 1 (11β-HSD1), an enzyme involved in the release of cortisol for which we have observed higher values in RR-MS patients compared to control groups (data not shown) [36]. This polymorphism may have direct functional consequences on levels of 11ß-HSD1 enzyme activity leading to an accumulation of cortisol by suppressing its conversion to cortisone. We hypothesize that this accumulation leads to a reversion of cortisol synthesis driven by chemical equilibrium and therefore results in higher values of PREG and DHEA and this may limit inflammatory processes in active phase of MS. We have measured PREG and DHEA to demonstrate a more global dysregulation of neuroactive steroids in RR-MS, which is also significantly influenced by disease activity. In this study, we have also analyzed the neuroactive steroids profile in the CSF of MS patients during both relapse and stable clinical phase. To date, none of the available drugs included in first line therapy for RR-MS is totally curative. The primary aims are: (i) inducing remission after relapse, (ii) reducing the frequency of new relapses, and (iii) slowing down the progression of the disability. In this context, it is interesting to note that in our small cohort of RR-MS patients PREG and DHEA values were higher in male patients during active and stable clinical phases when compared to female patients. Considering the possible dual role for PREG and DHEA as neuroprotective and anti-inflammatory agents [37,38], our results are in line with the observation that MS prevalence in men is lower than in women. The assumption of this dual role is also supported by our neuroradiological data, which links Gd+ and Gd- to neuroactive steroids levels. Comparing values of each of the analyzed neuroactive steroids we observed that Gd+ is associated with significantly higher values of PREG in male and DHEA in male and female patients compared to Gd- (Fig. 3A,B). The administration of gadolinium contrast agent is indicative of a disrupted of blood-brain barrier (BBB) in active MS lesions, and active demyelinated lesions have been associated with inflammation and BBB leakage [39–42]. In this context, it is interesting to note that PREG, DHEA and ALLO values were higher in RR-MS patients with GD+ than to those found in patients with GD-. This would mean that neuroactive steroids are transported from brain to the circulating blood across the BBB damaged as reported by Asaba et al. [43]. Similarly to what previously observed by Caruso et al. [44], a significant increase in PREG CSF value was detected in our male RR-MS patients. However, in the present study, we have also measured and compared the levels of neuroactive steroids in CSF of male and female MS patients during clinical remission and relapse. It is interesting to note that regarding the DHEA CSF, our values are not in line with those found in male RR-MS patients reported by Caruso et al. [44]. Our data indicate that DHEA values are significantly increased in male RR-MS patients compared to those found in absence of neurological diseases. This difference of finding should be attributed to analytical methodology applied, patients' age at clinical onset (first documented sign or symptom), and/or to the site of lesions in the brain. Remains unresolved an important question about the dependence of neurosteroidogenesis on demyelinating lesions in white and grey matter. This could help to understand the differences existing among our and Caruso's observations [44], and verify whether changes in neuroactive steroids levels may be associated to the site and progression of the lesions in MS. Among neuroactive steroids, also ALLO portrays an important endogenous compound since it has been broadly investigated for its role in promoting regeneration in both central and peripheral nervous system [45,46]. Thus, ALLO may be a possible candidate as a neuroprotective neurotransmitter-modulating agent in neurodegenerative diseases. Despite having many studies supporting this neuroprotective role, most of them are based on observations in animal models [47]. Indeed, recent evidence shows that ALLO can induce neuronal generation and survival in the hippocampus of both aged mice and mice with experimental Alzheimer's disease, accompanied by restoration of associative learning and memory function [48–50]. Only few studies have investigated ALLO levels in human neuroinflammatory diseases such as MS; we assessed the ALLO profile in CSF of RR-MS patients. Our analysis indicates that, ALLO CSF values were lower in female RR-MS patients than in male RR-MS (Fig. 1C). This pattern was not present in male and female control without neurological diseases (i.e., control group II). The finding that ALLO CSF levels in control patients were not different in male vs female during the luteal phase could be surprising on the basis of observations present in literature indicating that a significative correlation between CSF and plasma levels of this neuroactive steroid was reported [51,52] and that in female during luteal phase ALLO plasma levels are higher in comparison to those observed in male patients [53]. However, in this context it is important to note that the significant correlation between CSF and plasma has been demonstrated in postmenopausal women [51,52] and not during estrous cycle. Significant lower ALLO CSF levels in female than in male patients were also observed during relapses (Fig. 2C). Similarly to PREG and DHEA values, we observed that ALLO values are higher in patients with Gd+ compared to Gd- (Fig. 3C). It has also been suggested that in MS patients a dysregulation of ALLO

synthesis in oligodendrocytes may be the first event in the etiology of disease, leading to formation of structurally altered and less stable myelin [54]. Finally, the comparison with our two different control groups raises two relevant questions. First, whether the relapse associated to change of neuroactive steroids in CSF could be considered important factors predicting of acute status in MS. Second, the difference in the vulnerability to autoimmune diseases in both sexes-related dimorphism in autoimmune disease. A large amounts of information support the fact that hormones are involved in the immunological dimorphism in males and females [55,56]. The incidence of MS is higher in female with approximately a 2:1 ratio [57]; differences in MS symptoms in relation to the menstrual cycle have been studied in small retrospective studies showing that female MS experienced more MS symptoms in the premenstrual phase [58,59] and had exacerbations of MS starting in the premenstrual period [60]. This could be correlated with the activity of cytokine-secreting cells indicating that altered sex hormones levels influence the cytokine responses to multiple myelin antigens in autoimmune patients [61–63]. Changes in disease activity on MRI scans have also been shown to be related to female steroid sex hormone variation during the menstrual cycle although the results remain partially contradictory [64–66]. Our study provides significant insights in neuroactive steroid metabolism and their presumptive role in the pathogenesis of MS between male and female. In particular, we believe that future and large studies should investigate, with a prospective controlled design, the impact of neuroactive steroids in MS disease progression.

Conflict of interest

The authors declare to have no conflict of interest.

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