



Sex and disease severity-based analysis of steroid hormones in ME/CFS

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Abstract

Purpose Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating disease characterized by persistent fatigue and decreased daily activity following physical and/or cognitive exertion. While ME/CFS affects both sexes, there is a higher prevalence in women. However, studies evaluating this sex-related bias are limited.

Methods Circulating steroid hormones, including mineralocorticoids (aldosterone), glucocorticoids (cortisol, corticosterone, 11-deoxycortisol, cortisone), androgens (androstenedione, testosterone), and progestins (progesterone, 17 α -hydroxyprogesterone), were measured in plasma samples using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Samples were obtained from mild/moderate (ME/CFSmm; females, n=20; males, n=8), severely affected patients (ME/CFSsa; females, n=24; males, n=6), and healthy controls (HC, females, n=12; males, n=17).

Results After correction for multiple testing, we observed that circulating levels of 11-deoxycortisol, 17 α -hydroxyprogesterone in females, and progesterone in males were significantly different between HC, ME/CFSmm, and ME/CFSsa. Comparing two independent groups, we found that female ME/CFSsa had higher levels of 11-deoxycortisol (vs. HC and ME/CFSmm) and 17 α -hydroxyprogesterone (vs. HC). In addition, female ME/CFSmm showed a significant increase in progesterone levels compared to HC. In contrast, our study found that male ME/CFSmm had lower circulating levels of cortisol and corticosterone, while progesterone levels were elevated compared to HC. In addition to these univariate analyses, our correlational and multivariate approaches identified differential associations between our study groups. Also, using two-component partial least squares discriminant analysis (PLS-DA), we were able to discriminate ME/CFS from HC with an accuracy of 0.712 and 0.846 for females and males, respectively.

Conclusion Our findings suggest the potential value of including steroid hormones in future studies aimed at improving stratification in ME/CFS. Additionally, our results provide new perspectives to explore the clinical relevance of these differences within specific patient subgroups.

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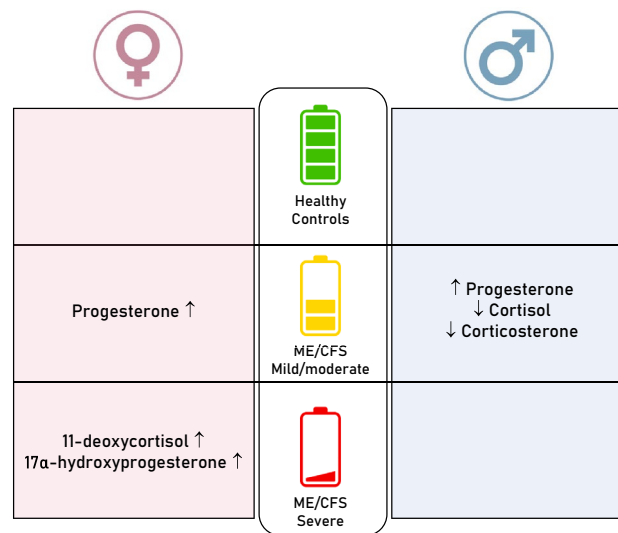
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Graphical abstract



Keywords Myalgic encephalomyelitis/chronic fatigue syndrome · Steroid hormones · Sex-related differences

Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating multisystem disease that primarily manifests as the inability to perform or participate in routine activities that were possible before the onset of the disease [1]. The decrease in activity level is accompanied by core symptoms, such as unrefreshing sleep and fatigue at rest, which persists for more than six months. Post-exertional malaise (PEM), defined as the worsening of symptoms after minimal physical or mental effort, is widely accepted as the hallmark feature for diagnosing ME/CFS [1, 2]. The clinical manifestation of ME/CFS varies from a mild form, in which patients are still able to participate in social life (e.g., work, school), to moderate and severe forms, characterized by individuals being primarily homebound and bedridden. The most extreme manifestation of the disease occurs in people who are completely dependent on assistance for basic daily needs, such as eating and repositioning in bed [1, 3]. As a result, ME/CFS not only negatively impacts the quality of life of individuals and caregivers but also places a substantial and often silent burden on the healthcare system. This is due to affected individuals often remaining undiagnosed due to a lack of standardized clinical assessment and diagnostic markers [2]. Although the etiology remains elusive, up to 75% of ME/CFS cases report an infectious episode preceding the onset of the disease [1, 2]. Current evidence strongly suggests that ME/CFS is associated with immune, metabolic, and vascular abnormalities [4]. However, despite the higher prevalence in women than in men

[1], endocrinological studies focusing on the steroid hormones that may underlie this sex bias are scarce.

Steroid hormones are synthesized from cholesterol primarily in the adrenal gland, gonads, and placenta, playing crucial regulatory roles in various physiological and pathological processes [5–7]. The adrenal gland produces mineralocorticoids to regulate blood volume and pressure, and glucocorticoids to modulate metabolism and the immune system [8, 9]. The gonads synthesize sex steroid hormones in both females and males. These hormones not only modulate sexual function but also immune responses, which can influence sex-specific susceptibility to infectious and autoimmune diseases [10]. Previous studies have reported differences in steroid hormones levels between ME/CFS patients and healthy controls (HC) using various experimental approaches [11–16]. However, there has been a lack of research aimed at analyzing potential differential patterns based on sex and disease severity. Therefore, our experimental design focused on measuring the circulating levels of nine steroid hormones using highly specific quantitative measurements by ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-MS/MS). These nine steroid hormones are crucial in biosynthetic and metabolic pathways related to steroidogenesis [5]. The set of steroid hormones was classified into four categories: mineralocorticoids (aldosterone), glucocorticoids (cortisol, corticosterone, 11-deoxycortisol, cortisone), androgens (androstenedione, testosterone), and progestins (progesterone, 17 α -hydroxyprogesterone). Plasma samples from

female and male ME/CFS patients with mild/moderate (ME/CFS_{mm}) and severe (ME/CFS_{sa}) symptoms were compared to their respective healthy controls (HC).

Materials and methods

Plasma samples and clinical data

This retrospective study utilized plasma samples collected before 10am from both female and male individuals provided by the UK ME/CFS Biobank (UKMEB) [17, 18]. The female cohort comprised 56 individuals, categorized into HC ($n=12$; 21%), ME/CFS_{mm} ($n=20$; 36%), and ME/CFS_{sa} patients ($n=24$; 43%). The male cohort included 31 individuals, distributed into HC ($n=17$; 55%), ME/CFS_{mm} ($n=8$; 26%), and ME/CFS_{sa} patients ($n=6$; 19%) (Table 1).

Individuals diagnosed with ME/CFS were evaluated by a clinician based on the Canadian Consensus [19] and/or CDC-1994 ('Fukuda') [20] criteria. The diagnosis was validated through responses on the Symptoms Assessment form, ensuring compliance with the case definition and study eligibility. Participants in the study completed a set of questionnaires aimed at evaluating disability levels, including the Fatigue Severity Scale, measuring the severity of fatigue symptoms to provide insights into their impact. Additionally, the Pain and Fatigue Analog Scale assessed the subjective experience of pain and fatigue on a visual analog scale, allowing participants to express the intensity of their symptoms. The Medical Outcomes Survey Short Form (SF-36v2) was employed as a comprehensive multidimensional instrument to evaluate various aspects of participants' physical and mental well-being, offering a holistic view of health-related quality of life. These instruments collectively contributed to a thorough assessment of participants' health status and functional components [21, 22].

Exclusion criteria for participants included individuals who, within the preceding three months, (1) had used drugs known to modify immune function (e.g., azathioprine, cyclosporine, methotrexate, steroids) or had taken antiviral medications; (2) had received any vaccinations; (3) had a history of acute or chronic infectious diseases such as hepatitis B and C, tuberculosis, or HIV (excluding infections by herpes virus or other retroviruses); (4) had another severe medical condition such as cancer, coronary heart disease, or uncontrolled diabetes; (5) had a severe mood disorder; (6) had been pregnant or breastfeeding in the prior 12 months; or (7) presented with morbid obesity (BMI ≥ 40). Home visits were conducted to recruit patients with mobility restrictions (severely affected), while healthy subjects and mild/moderate patients were invited to a recruiting center for clinical assessment and blood sampling [21, 22].

Standards and solvents

Certified reference standards of corticosterone solution (C-117-1ML; CAS: 50-22-6), cortisol solution (C-106-1ML; CAS: 50-23-7), cortisone solution (C-130-1ML; CAS: 53-06-5), and 11-deoxycortisol solution (D-061-1ML; CAS: 152-58-9) were purchased from Merck (KGaA, Darmstadt, Germany). Stable isotope-labeled standards, namely aldosterone-D₇ (CAS: 1261254-31-2), androstene-3,17-dione-¹³C₃ (CAS: 327048-86-2), corticosterone-D₄ (CAS: 2243253-91-8), cortisol-D₄ (CAS: 73565-87-4), cortisone-¹³C₃ (CAS: 2350278-95-2), 11-deoxycortisol-D₅ (CAS: 1258063-56-7), 17 α -hydroxyprogesterone-D₈ (CAS: 850023-80-2), progesterone-D₉ (CAS: 15775-74-3), and testosterone-¹³C₃ (CAS: 327048-83-9) were also obtained from Merck (KGaA, Darmstadt, Germany). Purified water was prepared in-house using a Milli-Q water system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH; 34885-1L-M), ethyl acetate (EtOAc; 1.00868), methyl-tert-butyl ether (MTBE; 34875-1L-M), LC-MS-grade acetonitrile (ACN; 1.00029.1000), 2-propanol (IPA; 1.02781.1000), formic acid (FA; 1002531000), and ammonium fluoride (338869-25G) were purchased from Merck (KGaA, Darmstadt, Germany).

Preparation of standard solutions

To prepare the stock solution of the internal standard (IS), the stable isotope-labeled compounds were combined in ACN to achieve final concentrations of 2000 ng/mL for aldosterone-D₇, cortisone-¹³C₃ and cortisol-D₄, 400 ng/mL for corticosterone-D₄ and 11-deoxycortisol-D₅, 200 ng/mL androstene-3,17-dione-¹³C₃, testosterone-¹³C₃, 17 α -hydroxyprogesterone-D₈, and 100 ng/mL for progesterone-D₉. The working solution (WSL) was obtained by diluting the stock 1:10 in MeOH. The standard stock solution mixture in MeOH with a concentration of 1000 ng/mL was prepared using the nine individual steroid hormones. A 500 μ L aliquot of the stock solution mixture was transferred to a 5 mL volumetric flask and brought up to the highest calibration point with a 50:50 (v:v) mixture of MeOH and H₂O. Nineteen additional dilutions were made using a 50:50 (v:v) MeOH:H₂O mixture, and the calibration curve samples were prepared by adding 10 μ L of the IS WSL solution to 100 μ L of the mixed standard solution to cover a calibration range from 0.00019 ng/mL to 100 ng/mL. Stock solutions were stored at -20° C and allowed to reach room temperature before use.

Sample preparation

Supported liquid extraction (SLE) was employed to mitigate the impact of the sample matrix. The SLEs were purchased from Agilent Technologies (5610-2005). Briefly, 100 μ L of

Table 1 Demographics of the female and male cohorts including continuous variables, clinical assessments, blood tests, and SF-36 questionnaire

Female	HC		ME/CFSmm		ME/CFSsa		p value
	(n) 12	(%) 21	(n) 20	(%) 36	(n) 24	(%) 43	
	Median	IQR	Median	IQR	Median	IQR	
<i>Continuous variables</i>							
Age (years)	38	28–45	43	30–48	46	31–53	0.2174
Years of disease §	-	-	5	2–12	11	5–21	0.0301*
Fatigue severity scale	16	12–22	60	53–61	60	56–62	<0.0001****
<i>Clinical assessments</i>							
Waist circumference (cm)	76	73–104	89	82–97	76	69–85	0.0150*
Pain analog scale	0.0	0.0–1.1	2.4	1.6–3.4	4.5	1.9–6.9	0.0002***
Fatigue analog scale	0.8	0.1–2.5	4.8	3.1–7.0	7.4	6.0–8.5	<0.0001****
<i>Blood tests</i>							
Inorganic phosphate (mM)	0.9	0.8–1.0	1.1	0.9–1.6	1.1	0.9–1.1.2	0.0394*
Bilirubin (µM)	11	6–11	6	3–8	6	5–7	0.0082**
Creatine phosphokinase (U/L)	83	75–233	67	56–100	59	47–73	0.0038**
Vitamin B12	380	197–488	350	259–501	521	355–689	0.0330*
<i>SF-36 questionnaire</i>							
Physical functioning	58	58–58	32	29–40	21	19–27	<0.0001****
Role physical	57	53–57	30	22–35	21	21–26	<0.0001****
Bodily pain	56	52–60	40	34–46	34	27–46	<0.0001****
General health	61	58–63	31	26–33	26	24–31	<0.0001****
Vitality	53	47–59	29	26–35	26	23–32	<0.0001****
Social functioning	57	54–57	27	18–32	17	17–22	<0.0001****
Role emotional	56	56–56	42	33–52	47	35–56	0.0070**
Mental health	52	46–58	40	36–48	48	38–53	0.0165*
Physical component summary	58	55–59	30	27–34	20	17–27	<0.0001****
Mental health component summary	54	48–57	41	32–45	46	36–49	0.0032**
Male	HC		ME/CFSmm		ME/CFSsa		p value
	(n) 17	(%) 55	(n) 8	(%) 26	(n) 6	(%) 19	
	Median	IQR	Median	IQR	Median	IQR	
<i>Continuous variables</i>							
Age (years)	33	20–47	40	36–49	35	34–40	0.3181
Years of disease §	-	-	4	1–9	9	3–23	0.3906
Fatigue severity scale	2.0	1.6–2.4	6.6	5.7–6.7	6.4	6.1–6.8	<0.0001****
<i>Clinical assessments</i>							
Body fat	16	9–22	21	15–25	27	20–38	0.0385*
Pain analog scale	0.3	0.0–1.4	2.9	1.2–5.6	3.6	1.9–6.0	0.0017**
Fatigue analog scale	0.8	0.0–1.6	5.9	3.3–7.2	6.3	4.3–7.8	0.0003***
<i>Blood tests</i>							
Glomerular filtration rate (mL/min)	84	81–90	90	86–90	77	63–90	0.0464*
<i>SF-36 questionnaire</i>							
Physical functioning	58	56–58	35	29–36	21	21–24	<0.0001****
Role physical	57	55–57	23	21–28	21	21–27	<0.0001****
Bodily pain	56	56–62	42	31–47	38	27–46	<0.0003***
General health	59	53–66	33	28–36	30	27–33	<0.0001****
Vitality	53	50–61	26	23–32	27	23–39	<0.0001****
Social functioning	57	57–57	22	17–27	17	17–29	<0.0001****
Mental health	56	49–59	40	35–51	50	48–57	0.0350*
Physical component summary	59	55–60	28	25–35	20	13–21	<0.0001****

The study population is described based on sex, age, years of disease, fatigue severity scale scores, clinical assessments, blood tests, and SF-36 questionnaire, with values reported as median and interquartile range (IQR). Participants were categorized into three groups: HC (healthy controls), ME/CFSmm (mild/moderate ME/CFS patients), and ME/CFSsa (severely affected ME/CFS patients). Statistical significance was deter-

Table 1 (continued)

mined using one-way ANOVA for normally distributed parameters or Kruskal–Wallis test for non-normally distributed parameters. The median and IQR for the variable *years of disease* (§) were calculated by comparing two independent groups (ME/CFSmm vs ME/CFSsa) using the non-parametric Mann–Whitney t-test

plasma were combined with 100 μL of H_2O and 10 μL of IS WSL. The resultant mixture was transferred to the SLE tube, placed onto the sorbent bed with gentle pressure (2–3 psi), and allowed to equilibrate for 5 min. Subsequently, 400 μL of a 1:1 mixture of methyl-tert-butyl ether (MTBE) and ethyl acetate (EtOAc) were introduced into each tube, followed by elution at a rate of 1 drop per second (2 psi). This elution process was iterated three times, and a final application of 6 psi was employed to desiccate the sorbent. The entire eluent was dried with nitrogen (N_2) flow at 40°C in a TurboVap water bath (Biotage, Sweden) and then reconstituted with 100 μL of MeOH. Samples were stored at 4 °C overnight, subjected to centrifugation at 2500 $\times g$ for 5 min, and subsequently loaded into the autosampler.

UHPLC-MS/MS measurements and conditions

All separations were performed on a 1290 Infinity UHPLC system (Agilent Technologies) equipped with an Agilent ZORBAX RRHD Eclipse Plus C_{18} column (2.1 \times 100 mm; 1.8 μm ; 821725-902) and a ZORBAX RRHD C_{18} guard column (2.1 \times 5 mm; 1.8 μm ; 821725-901). The mobile phases comprised 0.2 mM ammonium fluoride in H_2O as mobile phase A and 0.2 mM ammonium fluoride in MeOH as mobile phase B. The gradient conditions were as follows: 0–3.0 min; 50–60% B; 3.0–7.0 min, 60–86% B; 7.0–7.1 min; 86–100% B, followed by a return to the initial conditions. The total chromatographic run time was 8.5 min. The flow rate was set to 0.4 mL/min, and the column temperature was maintained at 40 °C. The injection volume was 3 μL , and a needle wash with 1:1:1:1 ACN/MeOH/IPA/ H_2O with 0.2% FA was utilized. Mass detection was carried out in dynamic multiple reaction monitoring (dMRM) mode on an Agilent 6460 triple quadrupole system using positive electrospray ionization (ESI) mode. Specific settings can be found in Tables S1 and S2, while Figure S1 provides a representative UHPLC-dMRM chromatogram.

Statistical analysis

For comparing the means of clinical variables among the three groups (HC, ME/CFSmm, and ME/CFSsa), one-way ANOVA was employed when the respective data followed a normal distribution. In cases where normal distribution was not met, the non-parametric Kruskal–Wallis test was used. The significance level for these tests was set at 5%. Central tendency and variability in the dataset were estimated using the median and interquartile range (IQR).

To assess differences in the circulating levels of steroid hormones in female and male ME/CFS patients compared to HC, ANOVA, and Kruskal–Wallis tests were utilized to determine statistical significance. Unadjusted *p* values were computed for each test. To address multiple testing, we controlled the false discovery rate (FDR) at a 5% level using the Benjamini–Hochberg (BH) and Benjamini–Yekutieli (BY) procedures.

We also conducted a predictive analysis to assess the disease status (HC versus ME/CFS) of each participant using the following classifiers and the nine steroid hormones as respective predictors: (i) linear discriminant analysis (LDA), (ii) random forest (RF), and (iii) partial least square discriminant analysis (PLS-DA). This analysis was carried out separately for male and female datasets. In PLS-DA, the analysis was performed with one and two latent components. Using a higher number of components in PLS-DA, while potentially enhancing predictive performance, was prone to overfitting the data. For LDA and PLS-DA, the probability of an individual being an ME/CFS patient was estimated based on a leave-one-out procedure. For RF, the same probability was estimated using Bootstrap with 10,000 simulated decision trees.

After obtaining the classification probability for each participant, we constructed a Receiver Operating Characteristic (ROC) curve, where 1-specificity (x-axis) was plotted against sensitivity (y-axis). The corresponding area under the curve (AUC) and the point on the curve closest to the pair (0,1) (representing perfect classification) were determined. This point served as the optimal cutoff to predict the health status of each individual. Finally, we utilized the predicted health status to estimate accuracy (proportion of individuals with correctly predicted health status), sensitivity (S_e , proportion of cases with correctly predicted health status), and specificity (S_p , proportion of controls with correctly predicted health status) associated with each classifier. This analysis was carried out in the R software using the following packages: *MASS* (for LDA) [23], *randomForest* (for RF) [24], *caret* (for PLS-DA) [25], *pROC* (for ROC curve and AUC calculation) [26], *OptimalCutpoints* (for optimal cutoff estimation, accuracy, sensitivity, and specificity) [27].

Analyses utilizing Spearman's correlation coefficient (R_{sp}) were conducted to examine the statistical relationship between data from two steroid hormones. Each steroid hormone was ranked from lowest to highest, and the correlation coefficient along with corresponding *p* values were computed using *GraphPad Prism*. Next, to compare Spearman's correlation matrices between HC and individuals with ME/

CFS (or its subgroups), with or without the inclusion of data on aldosterone (which contains missing data), a permutation variant of Jennrich's test was implemented. This test was originally designed for comparing two Pearson's correlation matrices [28]. The computational procedure involved the following steps: (i) fixing the values of the group variable; (ii) permutating the original data set; (iii) computing the Jennrich's test statistic in the permuted data set; (iv) repeating steps (ii) and (iii) until obtaining 1000 values of the test statistic; (v) estimating the p value by determining the proportion of times the observed test statistic in the original dataset was higher than the values of the test statistic based on the permuted datasets.

Principal component analysis (PCA) of the steroid hormones data was employed to evaluate the similarity of study participants concerning sex and disease severity. In this analysis, we utilized the first and second principal components, as these components explained over 90% of the data variability. Heatmaps were generated for the entire dataset and separately for the female and male cohorts using the *MetaboAnalyst* R package [29] to conduct a thorough analysis of the data. Clustering of the data was performed using Euclidean distance, aiming to unveil underlying patterns and relationships. To identify distinct subgroups, the resulting clusters were further segmented into multiple groups. Data visualization was executed using the *heatmap* R package [30].

Results

Baseline demographics

Table 1 provides an overview of the demographic and clinical characteristics of the female and male participants. Noteworthy clinical differences were primarily identified in waist circumference for the female cohort and body fat for the male cohort when comparing the three independent groups. Applying the same statistical criteria, median values of blood-related tests, such as inorganic phosphate, bilirubin, creatine phosphokinase, and vitamin B₁₂ in the female cohort, and glomerular filtration rate in the male cohort, showed differences among the three groups. Further distinctions were identified in the female cohort when comparing the aforementioned parameters between two independent groups using the non-parametric Mann–Whitney test. For instance, statistically significant differences were observed in body mass index (ME/CFSmm vs. ME/CFSsa; $p=0.0238$), body muscle (HC vs. ME/CFSsa; $p=0.0249$), and C-reactive protein (HC vs. ME/CFSsa; $p=0.0161$). Conversely, pulse oximetry (HC vs. ME/CFSmm; $p=0.0226$), albumin (HC vs. ME/CFSmm; $p=0.0135$), and thyroid stimulating hormone (HC vs. ME/CFSmm; $p=0.0439$)

demonstrated differences in the male cohort using the same criteria outlined above. Moreover, the median scores for various functional components, as assessed by the questionnaires Fatigue Severity Scale, Pain and Fatigue Analog Scale, and SF-36, revealed significant reductions in both female and male subgroups of ME/CFS patients when compared with their respective healthy counterparts (Table 1).

Univariate analysis of steroid hormone data

The concentrations of a panel of steroid hormones, including cortisone, cortisol, corticosterone, 11-deoxycortisol, aldosterone, androstenedione, testosterone, 17 α -hydroxyprogesterone, and progesterone, were measured in plasma samples by UHPLC-MS/MS (Table 2). We employed ANOVA and Kruskal–Wallis tests to assess differences in steroid hormone levels between female and male ME/CFS patients compared to HC (Fig. 1A). For each test, unadjusted, BH-adjusted, and BY-adjusted p values were calculated (Table S3). Following correction for multiple testing, only circulating levels of 11-deoxycortisol ($p=0.049$) and 17 α -hydroxyprogesterone ($p=0.049$) in females and progesterone ($p=0.005$) in males showed significant differences among our three groups (Fig. 1B–D; Table S3). Additionally, the BH procedure was utilized to determine the statistical significance of potential differences when comparing two independent groups (Table S4). In our female cohort, ME/CFSsa exhibited elevated circulating levels of 11-deoxycortisol compared to both HC ($p=0.0276$) and ME/CFSmm ($p=0.0269$), as well as higher 17 α -hydroxyprogesterone versus HC ($p=0.0129$). Furthermore, plasma progesterone levels were higher in ME/CFSmm than in HC ($p=0.0406$). Using the same statistical approach in our male cohort, ME/CFSmm displayed lower circulating levels of cortisol ($p=0.0260$) and corticosterone ($p=0.0109$) compared to HC. Conversely, ME/CFSmm exhibited higher progesterone levels than HC ($p=0.0004$). No statistical differences were found in the set of measured steroid hormones in the male cohort when comparing HC versus ME/CFSsa and ME/CFSmm versus ME/CFSsa (Table S4).

Correlation analysis of steroid hormone data

We performed non-parametric Spearman's correlation coefficient (R_{sp}) analysis to evaluate the degree of association between specific steroid hormones in both the female and male cohorts (Fig. 2). In the female HC group, we identified eight significant positive correlations: cortisol versus corticosterone ($R_{sp}=0.89$, $p=0.003$); androstenedione versus 17-hydroxyprogesterone ($R_{sp}=0.79$, $p=0.003$); testosterone versus androstenedione ($R_{sp}=0.78$, $p=0.004$); testosterone versus

Table 2 Plasma levels of steroid hormones in female and male ME/CFS patients compared to healthy controls

Female	HC		ME/CFSmm		ME/CFSsa	
	(n) 12	(%) 21	(n) 20	(%) 36	(n) 24	(%) 43
Steroid hormones (ng/mL)	Median	IQR	Median	IQR	Median	IQR
Cortisone	21.02	17.25–25.17	19.77	15.62–21.91	19.57	17.79–23.00
Cortisol	94.55	84.31–138.70	89.84	68.87–147.40	110.70	76.61–131.40
Corticosterone	1.23	1.02–1.87	1.12	0.85–3.12	1.97	0.86–4.06
11-deoxycortisol	0.12	0.08–0.25	0.13	0.09–0.18	0.22	0.14–0.56
Aldosterone	0.10	0.06–0.16	0.11	0.08–0.30	0.08	0.05–0.13
Androstenedione	0.78	0.54–1.11	0.65	0.43–0.79	0.80	0.57–1.14
Testosterone	0.23	0.13–0.30	0.20	0.15–0.26	0.23	0.16–0.32
17 α -hydroxyprogesterone	0.23	0.14–0.37	0.56	0.20–0.67	1.24	0.26–1.66
Progesterone	0.09	0.06–0.15	0.69	0.19–3.79	0.36	0.08–1.10
Male	HC		ME/CFSmm		ME/CFSsa	
Steroid hormones (ng/mL)	(n) 17	(%) 55	(n) 8	(%) 26	(n) 6	(%) 19
	Median	IQR	Median	IQR	Median	IQR
Cortisone	20.67	17.22–25.17	19.48	15.29–26.10	20.24	13.62–25.01
Cortisol	120.80	94.11–134.30	70.03	55.18–119.80	99.32	49.76–153.50
Corticosterone	1.89	1.12–3.43	0.67	0.47–2.27	1.47	0.79–10.37
11-deoxycortisol	0.16	0.12–0.26	0.13	0.08–0.24	0.23	0.11–0.84
Aldosterone	0.07	0.05–0.16	0.11	0.05–0.17	0.07	0.05–0.21
Androstenedione	0.74	0.59–0.86	0.54	0.42–0.83	0.65	0.52–0.96
Testosterone	4.78	3.07–6.75	4.47	2.81–5.13	3.32	2.57–4.89
17 α -hydroxyprogesterone	0.64	0.38–0.83	1.02	0.65–1.46	0.55	0.43–1.14
Progesterone	0.07	0.04–0.08	0.48	0.12–0.71	0.07	0.06–0.23

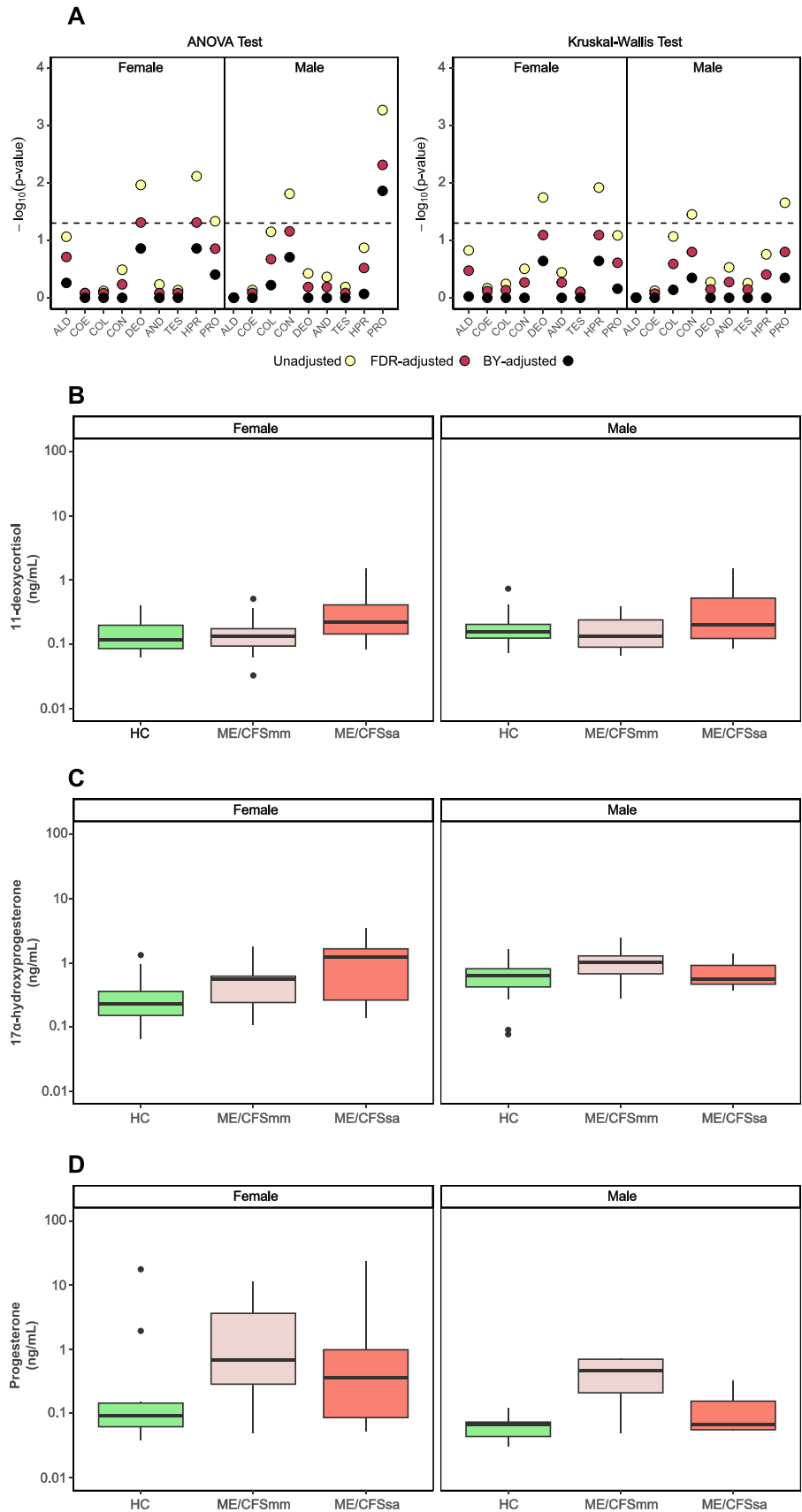
Circulating levels of cortisone, cortisol, corticosterone, 11-deoxycortisol, aldosterone, androstenedione, testosterone, 17 α -hydroxyprogesterone, and progesterone were quantified using UHPLC-MS/MS in plasma samples from healthy controls (HC), mild/moderate (ME/CFSmm), and severely affected (ME/CFSsa) female and male individuals. The table presents the number of samples (*n*), percentage (%), median, and interquartile range (IQR) values

17-hydroxyprogesterone ($R_{sp} = 0.78$, $p = 0.004$); cortisol versus cortisone ($R_{sp} = 0.69$, $p = 0.017$); androstenedione versus cortisone ($R_{sp} = 0.66$, $p = 0.022$); testosterone versus corticosterone ($R_{sp} = 0.62$, $p = 0.037$); progesterone versus 17-hydroxyprogesterone ($R_{sp} = 0.62$, $p = 0.037$). Comparing the female HC to ME/CFSmm, five of the eight positive associations were also found: cortisol versus corticosterone ($R_{sp} = 0.83$, $p = 0.001$); cortisol versus cortisone ($R_{sp} = 0.78$, $p = 0.001$); progesterone versus 17-hydroxyprogesterone ($R_{sp} = 0.77$, $p = 0.001$); testosterone versus androstenedione ($R_{sp} = 0.67$, $p = 0.001$); androstenedione versus cortisone ($R_{sp} = 0.47$, $p = 0.036$). Additionally, three positive significant correlations were observed for corticosterone versus 11-deoxycortisol ($R_{sp} = 0.69$, $p = 0.001$); corticosterone versus cortisone ($R_{sp} = 0.54$, $p = 0.013$); cortisol versus 11-deoxycortisol ($R_{sp} = 0.52$, $p = 0.021$); and one negative correlation for 17-hydroxyprogesterone versus aldosterone ($R_{sp} = -0.58$, $p = 0.016$). In the female ME/CFSsa group, only 4 positive

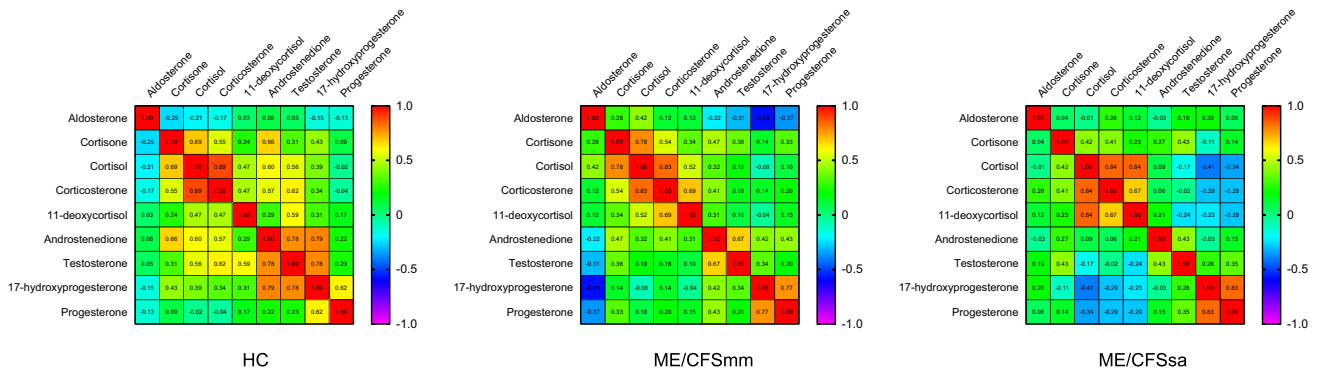
significant correlations were identified, with two overlapping with the HC group: cortisol versus corticosterone ($R_{sp} = 0.84$, $p = 0.001$); progesterone versus 17-hydroxyprogesterone ($R_{sp} = 0.83$, $p = 0.001$) and two overlapping with the ME/CFSmm group: cortisol versus 11-deoxycortisol ($R_{sp} = 0.84$, $p = 0.001$); corticosterone versus 11-deoxycortisol ($R_{sp} = 0.67$, $p = 0.001$) (Fig. 2A).

On the contrary, we identified six significantly positive significant correlations in our male HC group: progesterone versus 17-hydroxyprogesterone ($R_{sp} = 0.81$, $p = 0.001$); cortisol versus corticosterone ($R_{sp} = 0.79$, $p = 0.003$); testosterone versus progesterone ($R_{sp} = 0.79$, $p = 0.001$); testosterone versus 17-hydroxyprogesterone ($R_{sp} = 0.71$, $p = 0.002$); corticosterone versus 11-deoxycortisol ($R_{sp} = 0.60$, $p = 0.012$); androstenedione versus 17-hydroxyprogesterone ($R_{sp} = 0.57$, $p = 0.018$). Compared to the male HC group, only one of these six positive associations was found in the male ME/CFSmm counterparts: cortisol versus corticosterone ($R_{sp} = 0.83$, $p = 0.015$). However, we additionally observed

Fig. 1 Plasma levels of steroid hormones in female and male ME/CFS patients compared to healthy controls. The concentration of a steroid hormone panel, including cortisone (COE), cortisol (COL), corticosterone (CON), 11-deoxycortisol (DEO), aldosterone (ALD), androstenedione (AND), testosterone (TES), 17 α -hydroxyprogesterone (HPR), and progesterone (PRO) was assessed using UHPLC-MS/MS in plasma samples from cohorts of female and male ME/CFS patients with mild/moderate (ME/CFSmm) and severe (ME/CFSsa) presentations, in comparison to their respective healthy controls (HC). (A) ANOVA and Kruskal–Wallis tests were employed to determine p values. For each test, unadjusted (yellow circles), false discovery rate (FDR)-adjusted (light red circles), and Benjamini–Yekutieli (BY)-adjusted (black circles) p values were calculated. Each circle represents the $-\log_{10}(p)$ value of a specific statistical association test, while the dashed line represents $-\log_{10}(0.05)$, above which it was considered a statistically significant association. Benjamini–Hochberg p values were adjusted for 11-deoxycortisol (B), 17-hydroxyprogesterone (C), and progesterone (D)



A Female



B Male

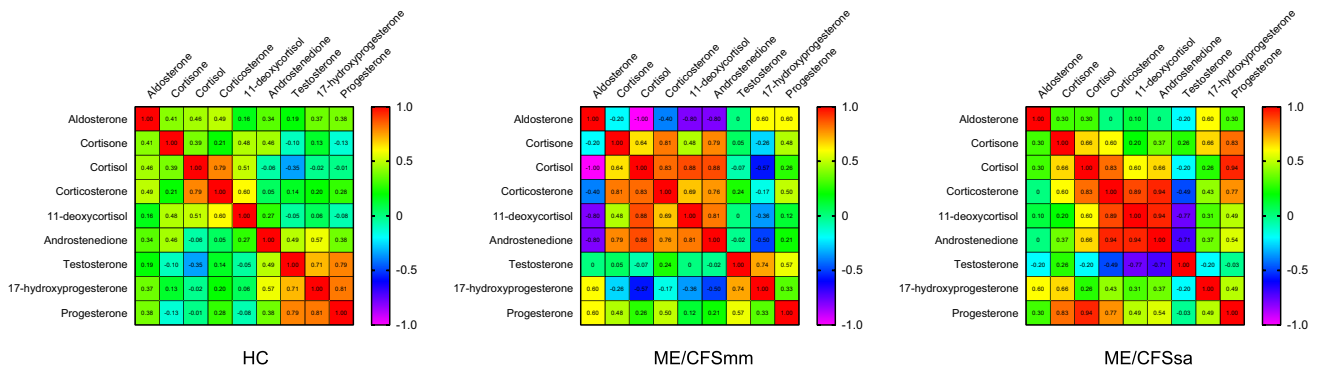


Fig. 2 Degree of association between steroid hormones in plasma from female and male healthy controls, mild/moderate and severe ME/CFS patients. The degree of association among steroid hormones (cortisone, cortisol, corticosterone, 11-deoxycortisol, aldosterone, androstenedione, testosterone, 17 α -hydroxyprogesterone, and progesterone) in female (**A**) and male (**B**) individuals was assessed using the nonparametric Spearman's test. Both female and male cohorts were categorized into: HC, representing participants recruited as healthy

controls; ME/CFSmm, denoting participants recruited as mild/moderate ME/CFS patients; and ME/CFSsa, indicating participants recruited as severe ME/CFS patients. Correlation coefficients were classified as very high, high, moderate, low, and little or no correlation, with magnitudes ranging from 0.9 to 1.0, 0.7–0.9, 0.5–0.7, 0.3–0.5, and <0.3, respectively. A positive Spearman's rank correlation coefficient indicates a direct association, while a negative coefficient indicates an inverse association

six significantly positive correlations for cortisol versus 11-deoxycortisol ($R_{sp} = 0.88, p = 0.007$); androstenedione versus cortisol ($R_{sp} = 0.88, p = 0.007$); corticosterone versus cortisone ($R_{sp} = 0.81, p = 0.022$); androstenedione versus 11-deoxycortisol ($R_{sp} = 0.81, p = 0.022$); androstenedione versus cortisone ($R_{sp} = 0.79, p = 0.028$); androstenedione versus corticosterone ($R_{sp} = 0.76, p = 0.037$). Finally, we identified four significantly positive correlations in male ME/CFSsa, one of which was also found in HC: corticosterone versus 11-deoxycortisol ($R_{sp} = 0.89, p = 0.033$), and two in ME/CFSmm: androstenedione versus corticosterone ($R_{sp} = 0.94, p = 0.017$); androstenedione versus 11-deoxycortisol ($R_{sp} = 0.94, p = 0.017$). Interestingly, a positive significant association was observed exclusively in male ME/CFSsa compared to either ME/CFSmm or HC, progesterone versus cortisol ($R_{sp} = 0.94, p = 0.017$) (Fig. 2B). To complement our analysis, we also calculated R_{sp} -based correlation matrices for the nine measured steroid hormones in each

study group. Our results showed no statistical difference between different pairs of correlation matrices associated with the study groups (Figure S2; Table S5).

Multivariate analysis of steroid hormone data

We conducted principal component analysis (PCA) to identify potential clusters within our study groups (Figure S3). This analysis aimed to visualize our cohort of individuals based on steroid hormones, sex, and disease severity in a two-dimensional plot. Using the first two principal components did not reveal any distinct clusters based on severity, sex, or a combination of both (Figure S3A). However, plotting the second and third principal components allowed us to differentiate between female and male individuals, though not individuals with varying disease severity (Figure S3B). Consequently, the steroid hormone data seemed to provide limited information about the study groups.

We further employed various statistical approaches, including linear discriminant analysis (LDA), random forest (RF), and partial least squares discriminant analysis (PLS-DA), to assess the predictive and discriminative capacities of the nine steroid hormones in distinguishing between HC and ME/CFS (Figure S4; Table 3). The two-component PLD-DA demonstrated the best predictive performance for the female cohort, with an estimated AUC of 0.712 and accuracy of 0.702. Notably, this classifier performed well in both cases and controls (Sensitivity = 0.714 vs. Specificity = 0.667). Concerning the male cohort, three out of four classifiers achieved the same level of predictive accuracy (0.818). However, the two-component PLD-DA emerged as the best classifier based on AUC. Similar to the female cohort, this classifier achieved a reasonable balance between sensitivity and specificity (0.778 vs. 0.846, respectively).

To complement our findings, we conducted a clustering analysis to categorize our study cohorts based on the similarity of their circulating steroid hormone profiles. Within the female cohort, three clusters were observed primarily driven by the concentrations of 17α -hydroxyprogesterone, progesterone, and 11-deoxycortisol in ME/CFS patients, with no clear differentiation between subgroups. While two additional patterns emerged related to aldosterone and cortisone, individuals from HC, ME/CFSmm, and ME/CFSsa were clustered together (Fig. 3A). The same analysis was applied to the male dataset, and despite a prominent cluster related to testosterone, no differences were observed between our study groups (Fig. 3B).

Discussion

Despite evidence indicating a higher prevalence of ME/CFS in women compared to men (3:1 ratio) [1], there remains a significant research gap concerning potential differences in circulating levels of steroid hormones in patients stratified by both sex and disease severity. Steroid hormones are synthesized by both males and females, displaying variations in circulating levels and effects on target tissues [5–9]. While testosterone is predominantly produced in males, and estrogen and progesterone are primarily synthesized in females [31], these differences have historically limited research on the role of testosterone in women and estrogen and progesterone in men [32]. Notably, our study revealed elevated progesterone levels in both female and male ME/CFSmm patients compared to their healthy counterparts. While the biological effects of progesterone in men are presently not fully understood, there is evidence suggesting an immunosuppressive effect of progesterone in women [33]. Our findings indicate that basal plasma progesterone is elevated in male ME/CFSmm patients, similar to previous reports in female patients [34], proving an interesting avenue for

Table 3 Predictive analysis using the nine measured steroid hormones as respective predictors to estimate the health status of each participant

Statistical method	HC versus ME/CFS			
	AUC	Accuracy	Sensitivity	Specificity
Female				
LDA	0.602	0.681	0.714	0.583
RF	0.571	0.574	0.514	0.750
PLS-DA (One component)	0.519	0.553	0.514	0.667
PLS-DA (Two components)	0.712	0.702	0.714	0.667
Male				
LDA	0.658	0.727	0.667	0.769
RF	0.641	0.818	0.556	1.000
PLS-DA (One component)	0.735	0.818	0.667	0.923
PLS-DA (Two components)	0.846	0.818	0.778	0.846

The datasets from our cohorts of female and male individuals were subjected to separate analysis to separate analyses employing linear discriminant analysis (LDA), random forest (RF), and partial least squares discriminant analysis (PLS-DA). The predictive accuracy was assessed through the area under the receiver operating characteristic curve (AUC)

further investigation. Importantly, progesterone has been associated with a hypercoagulable state [35]. This is particularly noteworthy considering recent reports of fibrinoid microclots in ME/CFS patients [36]. However, additional research is required to establish a potential link between progesterone and these immunologic and hemostasis-related effects in both female and male ME/CFS patients.

Sexual dimorphism linked to glucocorticoids significantly influences various physiological processes, encompassing both the immune response [37, 38] and metabolism [39], both of which undergo alterations in ME/CFS [40–43]. Cortisol, the principal glucocorticoid governing these processes under stress, is subject to modulation interactions with sex steroid hormones [39]. Interestingly, ME/CFS patients have been reported to exhibit reduced cortisol levels [15, 44], which is associated with documented dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis in a substantial proportion of ME/CFS cases [45]. However, existing evidence suggests that this dysfunction occurs at a central level, likely due to corticotropin-releasing hormone (CRH) deficiency rather than a primary adrenal insufficiency [46]. Consequently, our findings of elevated levels of 11-deoxycortisol in female ME/CFSsa patients and decreased levels of both cortisol and corticosterone in male ME/CFSmm patients offer novel perspectives for further investigating the intricate interplay between glucocorticoids in different ME/CFS subtypes.

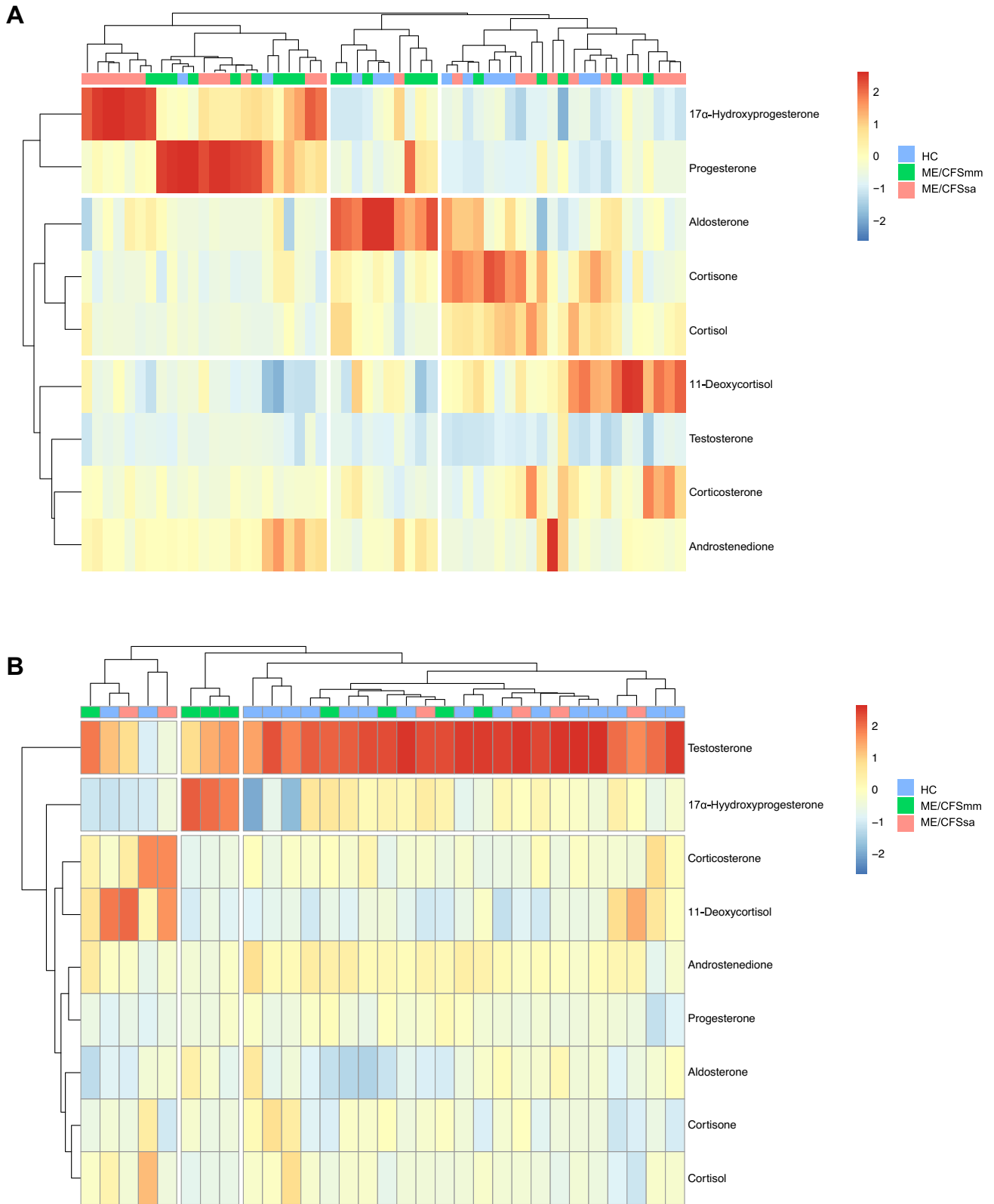


Fig. 3 Hierarchical clustering analysis based on plasma steroid hormone levels in female and male cohorts. Data obtained from cortisone, cortisol, corticosterone, 11-deoxycortisol, aldosterone, androstenedione, testosterone, 17 α -hydroxyprogesterone, and progesterone

were subjected to hierarchical clustering analysis utilizing the Euclidean distance metric. The results were visualized through heatmaps in both the female (**A**) and male (**B**) cohorts

In response to stress, the adrenal glands secrete both cortisol and progesterone in women and men [47]. Stress-induced progesterone plays a role in increasing the bioavailability of cortisol by competitively binding to corticosteroid-binding globulin (CBG), thereby reducing the amount of cortisol that can bind to CBG [48]. Additionally, it participates in the negative feedback loop of the HPA axis to regulate the magnitude of the stress response [49]. Progesterone metabolites, particularly allopregnanolone, primarily mediate this inhibitory effect. Allopregnanolone serves as a potent neuroactive steroid that inhibits hypothalamic CRH production [50]. The observed alterations in glucocorticoid and progesterone levels in male ME/CFSmm patients suggest the presence of a negative feedback mechanism. Similar patterns have been proposed in women during the luteal phase of the menstrual cycle, where elevated progesterone and decreased cortisol levels are noted, contrasting with women during the follicular phase who exhibit the opposite pattern [51]. Glucocorticoids play a central role in a broad range of normal and stress-related responses [52]. Reduced glucocorticoid production, relative to progesterone and allopregnanolone levels, may help to elucidate the characteristic dysregulation of physiological functions in ME/CFS, such as diminished physical performance leading to fatigue, pain, and cognitive impairment [45]. Nevertheless, it remains unknown whether this alteration causes the symptoms or if it represents a compensatory mechanism observed in a subset of ME/CFS patients.

The observed elevation in the progesterone/glucocorticoid ratio in ME/CFSmm patients suggests a potential dysregulation in adrenal corticosteroid pathways, considering progesterone's role as a precursor in corticosteroid biosynthesis [5–7]. Our findings point towards the adrenal enzyme P450c11 β (11 β -hydroxylase; *CYP11B1* gene) as a potential site of dysregulation. This enzyme is responsible for converting 11-deoxycorticosterone to corticosterone and 11-deoxycortisol to cortisol [5–7]. A deficiency in P450c11 β activity could contribute to the observed reduction in corticosterone and cortisol levels in male ME/CFSmm patients, as well as the accumulation of 11-deoxycortisol and 17 α -hydroxyprogesterone in female ME/CFSsa patients. However, it is important to note that the levels of steroid hormones and other parameters identified in the blood of patients in our cohort do not meet the criteria for classical P450c11 β deficiency [52]. Moreover, our findings may be influenced by sex bias due to the presence of extra-adrenal sources of progesterone and 11-deoxycorticosterone in women, specifically the ovaries [53]. While men also produce progesterone from their testicles, it occurs at lower levels [32]. This complexity suggests that the interaction between steroid hormones is more intricate than previously assumed, and consequently, no single hormone may serve as an adequate biomarker. Consistent with these

considerations, we achieved a discriminative accuracy of 0.712 and 0.846 for females and males, respectively, in distinguishing between ME/CFS and healthy controls using a two-component PLS-DA that incorporated our set of nine steroid hormones. Further studies should prioritize assessing the potential predictive value of these and other steroid hormones in stratifying ME/CFS patients based on sex and disease severity.

While we observed differences in steroid hormone levels within our ME/CFS patient cohort, stratified by both sex and severity, it is imperative to confirm our findings with a larger sample size. Although our study excluded pregnant women and those within 12 months postpartum or lactating, we recommend incorporating clinical data related to menstrual cycle, menopausal status, pregnancy history, and contraceptive use. This inclusion will enhance our understanding of the impact of ME/CFS on female reproductive health. In agreement with Pollack et al. [54], we certainly consider that this information proves value as it enables the reduction of potential selection bias and data heterogeneity, thereby positively influencing data analysis and interpretability in female ME/CFS patients. Addressing these pertinent aspects will significantly advance the comprehension of ME/CFS pathophysiology and may contribute to the development of personalized therapeutic approaches tailored to specific patient subgroups. Our findings underscore the importance of investigating variations in pathological mechanisms related to severity, with particular attention to severely affected individuals. This subgroup is often underrepresented in ME/CFS research due to limited healthcare access.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s40618-024-02334-1>.

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Data availability The dataset used in this study is available upon reasonable request to the corresponding author.

Declarations

Disclosure of potential conflicts of interest The authors declare no pertinent financial or non-financial interests to disclose.

Research involving human participants and/or animals This study, conducted in adherence to the principles outlined in the Declaration of Helsinki, received approval from two independent research ethics committees: the Medical University of Graz, Austria (reference number: 31-059 ex 18/19), and the UCL Biobank Ethical Review Committee-Royal Free (B-ERC-RF) London NHS Foundation Trust (reference number: EC2018.005).

Informed consent Informed consent was secured from all individuals participating in this study.

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