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Reference intervals for plasma concentrations of adrenal steroids measured by LC-MS/MS: Impact of gender, age, oral contraceptives, body mass index and blood pressure status

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ABSTRACT

Background: Mass spectrometric-based measurements of the steroid metabolome have been introduced to diagnose disorders featuring abnormal steroidogenesis. Defined reference intervals are important for interpreting such data.

Methods: Liquid chromatography–tandem mass spectrometry was used to establish reference intervals for 16 steroids (pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 18-oxocortisol, 18-hydroxycortisol, 17-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, dehydroepiandrosterone-sulfate, androstenedione, testosterone) measured in plasma from 525 volunteers with (n = 227) and without (n = 298) hypertension, including 68 women on oral contraceptives.

Results: Women showed variable plasma concentrations of several steroids associated with menstrual cycle phase, menopause and oral contraceptive use. Progesterone was higher in females than males, but most other steroids were higher in males than females and almost all declined with advancing age. Using models that corrected for age and gender, body mass index showed weak negative relationships with corticosterone, 21-deoxycortisol, cortisol, cortisone, testosterone, progesterone, 17-hydroxyprogesterone and 11-deoxycorticosterone, but a positive relationship with 18-hydroxycortisol. Hypertensives and normotensives showed negligible differences in plasma concentrations of steroids.

Conclusion: Age and gender are the most important variables for plasma steroid reference intervals, which have been established here according to those variables for a panel of 16 steroids primarily useful for diagnosis and subtyping of patients with endocrine hypertension.

1. Introduction

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for profiling of the steroid metabolome is increasingly showing utility for investigations of a range of disorders of steroidogenesis [1–7]. Apart from measurements of multiple steroids in a single sample of a given matrix, LC-MS/MS offers advantages of more accurate measurements than by immunoassays, which often suffer from antibody cross reactivity resulting in higher than true concentrations [8–15]. Nevertheless, LC-MS/MS is not entirely free from interference so that harmonization of methods, including agreement on reference intervals remains important [16].

As with any new measurement method it is important to establish reference intervals to enable identification of individuals within tested patient populations who return abnormal results. Reference intervals are usually established from the 95% confidence intervals or 97.5 percentiles of an appropriately sized and characterized reference population [17]. Reference intervals for steroids established by im-

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munoassays are unlikely to be valid for LC-MS/MS-based assays and there remains a general lack of published reference intervals for LC-MS/MS measurements; those that are available mostly involve mass spectrometric measurements of sex steroids, particularly in pediatric populations where clinical relevance and demand is high [10,18–23]. Since LC-MS/MS adrenal steroid profiling directed to endocrine forms of hypertension is in its infancy, reports that cover reference intervals for LC-MS/MS measurements of steroids directed to primary aldosteronism or Cushing's syndrome mostly involve single steroids or limited numbers of steroids in a panel [11,15,24–27].

Relative lack of commercial calibrators and quality control materials for LC-MS/MS multi-steroid profiling methods confounds harmonization of laboratory results and establishment of reliable reference intervals. Other considerations include biological and preanalytical influences that can affect measured steroid concentrations. Influences of age, gender, diurnal rhythms, physical or mental stress, energy status, sampling position, pubertal stage, menstrual cycle and medications may all have variable impact on sex and adrenal steroids [28]. For the adrenal steroids most useful for diagnosis or subtyping of patients with Cushing's syndrome or primary aldosteronism it is also important to establish whether measurements vary as a function of differences in blood pressure and body mass.

With the above considerations in mind the present report provides a first description of reference intervals for a panel of 16 steroids measured in plasma by an LC-MS/MS method primarily developed for diagnosis and subtyping of patients with disorders of steroidogenesis associated with endocrine hypertension. Subjects included 525 adult volunteers with and without hypertension. All blood sampling was carried out according to a standardized protocol to minimize variations due to time of day, food intake, posture and physical or mental stress. Requirements for age- and gender-specific reference intervals were examined along with influences of oral contraceptive use, blood pressure and body mass index that might confound test results in patients with primary aldosteronism and Cushing's syndrome.

2. Materials and methods

2.1. Subjects

Subjects included 525 normotensive and hypertensive volunteers, all providing written informed consent under a clinical protocol approved by the local Ethics committee that allowed for collections of data and banking of biological specimens for purposes of comparisons to patient populations and establishment of reference intervals for new diagnostic tests. Enrolment of subjects was facilitated by advertisement with a small financial reward to encourage involvement. Subjects were aged 18 to 81 years (median 42) and included 293 females and 232 males, among whom 104 (35%) of the females and 123 (53%) of the males had hypertension (Table 1).

All subjects underwent a standard medical history and physical examination that included recordings of prescribed and non-prescribed medications and dietary supplements, body weight, height, heart rate and office blood pressure, the latter recorded in triplicate. Presence of hypertension was established by systolic blood pressure above or equal to 140 mm Hg or diastolic blood pressure above or equal to 90 mm Hg and further confirmed or excluded in 378 subjects by 24 h ambulatory blood pressure monitoring (systolic blood pressure ≥ 130 and diastolic blood pressure ≥ 80). Hypertension was also defined in patients with a stated history of high blood pressure controlled by antihypertensive medications.

Among female participants 68 were taking oral contraceptives (Table 1) and 88 were identified as post-menopausal according to age-related cessation of menstruation for at least one year. Among the remaining 137 premenopausal women, 45 were identified to be in the luteal phase and 30 in the follicular phase of the menstrual cycle at the time of blood sampling. Subjects with serious medical conditions or taking steroid hormones other than oral contraceptives were excluded from the analysis.

Table 1
Demographic data for the 525 normotensive and hypertensive volunteers.

Gender	N	Age	BMI	Hypertension
Males	232	42 (18–81)	26 (19–49)	53% (123/232)
Females	293	42 (18–77)	24 (17–50)	35% (104/293)
No oral contraceptives	225	46 (19–77)	24 (17–50)	40% (89/225)
Oral contraceptives	68	26 (18–49)	23 (18–44)	22% (15/68)

Data for age (years) and body mass index (kg/m^2) are shown as medians and ranges in parentheses.

2.2. Blood sampling

All blood sampling was carried out between 8:00–10:00 AM after an overnight fast and refraining from caffeinated beverages in the morning before sampling, which was carried out after 20 min or more of supine rest. Heparinized blood samples were kept chilled and centrifuged within 2 h of collection to separate plasma, which was stored at − 80 °C until assayed.

2.3. LC-MS/MS

LC-MS/MS was performed using a QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) coupled to an Acquity® ultra performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, USA). The latter was equipped with a binary solvent manager, a sample manager, a column manager, a Phenomenex Kinetex C18 column (2.6 μm, 2.1 × 100 mm) and guard column (Phenomenex.). The method for analysis of the plasma steroid panel, including validation and assay performance characteristics has been described in detail elsewhere [29]. In brief, sample preparation involved solid phase extraction utilizing OASIS® HLB-96 well plates and a Positive-Pressure-96 Processor (all from Waters Corp.). Eluants were dried down and reconstituted in mobile phase for injection on to the LC-MS/MS system.

The steroids in the panel included pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 18-oxocortisol, 18-hydroxycortisol, 17-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEA-SO_4), androstenedione and testosterone. Over the time course of the study inter-assay coefficients of variation ranged from 3.3% to 11.8% for steroids in low to normal ranges and 5.1% to 10.0% for those in high ranges. Participation in an inter-laboratory quality assurance program (performed by the Reference Institute for Bioanalytics, Bonn, Germany) involving measurements of six steroids in the panel (cortisol, aldosterone, progesterone, 17-hydroxyprogesterone, DHEA-SO_4 and testosterone) among different European laboratories indicated measurements within limits of precision and accuracy for all six steroids.

2.4. Data analyses

The Kruskal-Wallis and the Steel Dwass all pairs methods were used for non-parametric comparisons of women in different phases of the menstrual cycle and compared to those taking oral contraceptives or of post-menopausal status. Comparisons of men with women were carried out using the Wilcoxon test, with exclusion of women taking oral contraceptives where this was established to significantly impact plasma concentrations of steroids. Influences or associations of gender, age, body mass index, blood pressure status and oral contraceptive use were examined by stepwise regression and least squares multivariate analyses. Spearman's rank correlation coefficients were used to assess

significance of relationships. The above statistical analyses utilized the JMP statistics software package (SAS Institute Inc., Cary, NC).

Age-specific reference intervals for steroids showing highly significant impacts of age were established by multivariate fractional polynomial analyses [30–32]. To derive equations for age-specific reference intervals plasma concentrations were first normalized by logarithmic, square root or x^2 transformation, with age transformed in all cases according to the formula below described by Royston and Wright [30].

$$X_{age} = e^{\frac{\log(0.01)\bullet(Age-\min(Age))}{(\max(Age)-\min(Age))}}$$

Following normalization, steroid concentrations were grouped according to age. Average values and standard deviations of the groups were then used for an independent fitting of polynomial models, which allowed estimation of best-fit polynomial coefficients [33]. Reference curves of the n^{th} percentile of each steroid were then calculated according to the below formula

$$y_n = M_n(Age) + SD_n(Age)\bullet\Phi^{-1}\left(\frac{n}{100}\right)$$

where Φ^{-1} is the inverse of the standard normal distribution, M_n(Age) is the mean and SD_n(Age) the standard deviation of the age groups. Due to the fact that both age and y_n are transformed and must be reversed to the original scale, the formulae produced by the method become overly complex. To simplify, we calculated the 2.5% and 97.5% percentiles for all patients according to the estimated y_n functions and subsequently fitted a regression line to the derived percentile values, which were then used as the reference interval curves. Fractional polynomial analysis was conducted using R Studio version 0.99.082–2016 (R version 3.2.1, R Core Team, 2013, http://www.r-project.org/). Curve fitting of percentile equations was carried out and percentile plots were prepared using the Matlab Curve fitting toolbox (3.5.1 2015) in Matlab (version R2015a).

3. Results

3.1. Menstrual cycle, menopause and oral contraceptive use

Women not taking oral contraceptives and identified to be in the luteal phase of the menstrual cycle had 24-fold higher ($P < 0.0001$) plasma concentrations of progesterone and 3-fold higher ($P < 0.0001$) concentrations of 17-hydroxyprogesterone than women in the follicular phase of the menstrual cycle (Table 2). Apart from this there were no clear differences in any of the other steroids according to luteal and follicular phases.

Older post-menopausal women had 37% to 62% lower ($P < 0.0001$) plasma concentrations of pregnenolone, DHEA and DHEA-SO_4 compared to all younger groups of women, independent of menstrual cycle phase and oral contraceptive use (Table 2). Similarly plasma concentrations of androstenedione, aldosterone and cortisone were also lower ($P < 0.05$) in postmenopausal women than in all other groups, though differences were of only a small to moderate magnitude (18% to 44%). Postmenopausal women also had 25 to 56% lower ($P < 0.005$) plasma concentrations of cortisol, corticosterone, 18-oxocortisol and testosterone, but for cortisol and testosterone these differences were confined to women in the luteal phase or taking oral contraceptives, while for 18-oxocortisol the difference was confined to women in the former group. Progesterone, 17-hydroxyprogesterone and 11-deoxycorticosterone were 55% to 98% lower ($P < 0.01$) in post-menopausal than younger women who were not taking oral contraceptives.

Oral contraceptive use was associated with divergent influences on different steroids (Table 1). Women taking oral contraceptives had 88% higher ($P < 0.0001$) plasma concentrations of cortisol and 94% higher ($P < 0.0001$) plasma concentrations of corticosterone compared to the combined group of younger premenopausal women in all phases of the menstrual cycle. In contrast, plasma concentrations of 11-deoxycorticosterone, pregnenolone, progesterone 17-hydroxyprogesterone and androstenedione were respectively 60% ($P < 0.0001$), 24% ($P = 0.0102$), 78% ($P < 0.0001$), 72% ($P < 0.0001$) and 25% ($P = 0.0040$) lower in women taking oral contraceptives compared to all premenopausal women not taking oral contraceptives. The above differences remained significant ($P < 0.0001$) for cortisol, 11-deoxycorticosterone, pregnenolone, progesterone 17-hydroxyprogesterone and androstenedione using a model that corrected for age. In contrast, for corticosterone the difference was lost ($P = 0.0602$) indicating that higher concentrations of this steroid in women taking oral contraceptives reflected their younger age.

Table 2
Plasma concentrations (medians & ranges) for the 16 steroids in post-menopausal compared to pre-menopausal females, the latter according to oral contraceptive use and follicular versus luteal phases of menstrual cycle.

Steroid	Luteal	Follicular	Oral contraceptive	Post-menopausal
N	45	30	68	88
Age	31 [18–49]**,†	38 [19–49]**,†	26 [18–49]**	57 [45–77]
Pregnenolone	8.40 [2.46–29.67]**,†	7.85 [1.54–24.77]**	5.31 [0.32–19.53]**	3.16 [0.32–9.23]
Progesterone	13.23 [0.07–83.00]**,†,§	0.56 [0.06–18.64]*,†	0.25 [0.01–1.00]	0.25 [0.03–11.42]
17-Hydroxyprogesterone	2.99 [0.37–8.28]**,†,§	1.08 [0.36–4.99]**,†	0.42 [0.11–2.00]	0.51 [0.19–3.12]
11-Deoxycorticosterone	0.27 [0.01–0.57]**,†	0.19 [0.01–0.50]*	0.07 [0.01–0.49]	0.07 [0.01–0.71]
Corticosterone	6.26 [1.88–36.1]*	5.76 [2.0–87.2]	10.20 [1.2–146.6]**	4.39 [1.5–35.8]
Aldosterone	0.19 [0.03–0.88]*	0.17 [0.02–0.65]*	0.16 [0.01–2.23]*	0.11 [0.01–0.67]
18-Oxocortisol	0.03 [0.00–0.09]*	0.03 [0.00–0.09]	0.02 [0.00–0.09]	0.02 [0.00–0.13]
18-Hydroxycortisol	1.68 [0.61–3.33]	1.71 [0.36–5.15]	1.42 [0.44–6.18]	1.22 [0.05–5.34]
21-Deoxycortisol	0.02 [0.00–0.54]	0.04 [0.00–0.22]	0.04 [0.00–0.41]	0.03 [0.00–0.35]
11-Deoxycortisol	0.45 [0.15–3.81]	0.40 [0.12–1.52]	0.29 [0.08–3.20]	0.37 [0.11–5.25]
Cortisol	295 [150–822]*,†	297 [97–979]†	509 [178–1271]**	233 [124–698]
Cortisone	63.2 [34.4–92.1]**	55.1 [28.9–87.9]*	63.0 [39.4–109.0]**	44.1 [24.7–75.2]
DHEA	13.8 [4.0–58.6]**	15.8 [3.5–41.3]**	11.2 [1.0–55.1]**	6.7 [1.4–24.0]
DHEA-S	4108 [1156–8217]**	3741 [1186–7728]**	3918 [1371–9523]**	2721 [367–12,435]
Androstenedione	4.19 [1.26–12.81]**,†	3.10 [1.80–6.91]**	2.87 [1.13–16.58]*	2.34 [1.13–9.46]
Testosterone	1.13 [0.27–2.18]*	0.93 [0.42–1.92]	1.12 [0.34–3.81]*	0.75 [0.24–2.75]

All values are shown as nmol/L. All differences according to the Steel-Dwass test for multiple comparisons.
* $P < 0.05$ different from menopause.
** $P < 0.0001$ different from menopause.
† $P < 0.005$, different from oral contraceptive.
§ $P < 0.005$, luteal different from follicular.

3.2. Gender

Plasma concentrations of pregnenolone, 11-deoxycorticosterone, corticosterone and aldosterone did not differ between males and females, whereas concentrations of progesterone were higher ($P < 0.0001$) in females than males (Table 3). The difference for progesterone reflected high concentrations in younger premenopausal women, particularly those in the luteal phase of the menstrual cycle (Table 2).

Plasma concentrations for all other steroids were higher in men than women (Table 3), with the largest difference ($P < 0.0001$) observed for testosterone, which showed no overlap between males and females. Other steroids showing strong gender differences included 17-hydroxyprogesterone, 18-oxocortisol, 18-hydroxycortisol, 11-deoxycortisol and DHEA-SO_4, which were respectively 211%, 50%, 43%, 43% and 48% higher ($P < 0.0001$) in men than women. Steroids showing smaller gender differences included 21-deoxycortisol, cortisol, cortisone, DHEA and androstenedione, which were 6% to 24% higher ($P < 0.05$) in men than women.

3.3. Multivariate analysis of gender, age and body mass index

With multivariate stepwise regression and least squares analyses the influences of gender on plasma concentrations of steroids were confirmed along with additional influences of age and body mass index (Table 4). Advancing age was associated with significant negative relationships with all steroids except 21-deoxycortisol. Strongest negative relationships ($P < 0.0001$) were observed for pregnenolone ($r_s = -0.633$), DHEA ($r_s = -0.580$), cortisone ($r_s = -0.473$), 11-deoxycorticosterone ($r_s = -0.353$) androstenedione ($r_s = -0.351$), 17-hydroxyprogesterone ($r_s = -0.344$), corticosterone ($r_s = -0.331$) and DHEA-SO_4 ($r_s = -0.311$), with significance maintained in all cases for both men and women. Negative relationships with age were weaker for cortisol ($r_s = -0.257$), aldosterone ($r_s = -0.244$), 18-oxocortisol ($r_s = -0.208$), progesterone ($r_s = -0.116$) and 18-hydroxycortisol ($r_s = -0.099$), and not always maintained for both genders. Weak negative relationships between age and 11-deoxycortisol or testosterone were only apparent with multivariate models adjusting for age and body mass index.

Body mass index, which was positively associated with age ($r_s = 0.269$, $P < 0.0001$), showed negative relationships with several steroids. These negative relationships with body mass index were generally weaker after adjusting for age and gender, and then only apparent for progesterone, 17-hydroxyprogesterone, 11-deoxcorticosterone, corticosterone, 21-deoxycortisol, cortisol, cortisone and testosterone (Table 4). In contrast, 18-hydroxycortisol showed a weak positive relationship ($r_s = -0.162$, $P = 0.0044$) with body mass index.

For testosterone, relationships with body mass index and age were gender specific (Fig. 1). Testosterone was negatively related with body mass index in males ($r_s = -0.360$, $P < 0.0001$), but not in females. In contrast, females showed a negative relationship of testosterone with advancing age ($r_s = 0.232$, $P = 0.0005$). For males the negative relationship of testosterone with age was weaker and did not remain significant after adjustment for body mass index.

3.4. Hypertensives vs normotensives

Presence of hypertension was positively associated with advancing age ($P < 0.0001$), increased body mass index ($P < 0.0001$) and male gender ($P = 0.0021$). Accounting for those variables, multivariate analyses indicated slightly higher ($P = 0.0103$) plasma concentrations 17-hydroxyprogesterone in normotensives than hypertensive and no differences for any of the other steroids except for 21-deoxycortisol, which was slightly higher in hypertensives than normotensives (Table 4). Office measurements of systolic blood pressure were also higher in males than females ($P < 0.0001$) and showed positive associations with age ($P < 0.0001$) and body mass index ($P < 0.0001$), but no clear relationships with any of the 16 steroids.

3.5. Reference intervals

Most steroids showed skewed distributions necessitating construction of reference intervals using non-parametric assessments of 2.5 and 97.5 percentiles, or when estimated as a function of age using normalization and curve fitting according to estimates of best-fit polynomial coefficients and associated variables (see Data-in-brief). For some steroids, in particular 21-deoxycortisol, 11-deoxycorticosterone and 18-oxocortisol, plasma concentrations were below detectable limits in 15%, 11% and 2% of patients respectively so that only upper cut-offs of reference intervals could be established.

As expected, testosterone and progesterone were the two steroids showing most need for gender-specific reference intervals (Table 3), with the latter also requiring consideration of post-menopausal and pre-menopausal menstrual cycle status (Table 2). Steroids such as 18-hydroxycortisol, DHEA-SO_4, 11-deoxycortisol, cortisone, androstenedione and DHEA showed higher 2.5 and 97.5 percentiles in males than

Table 3
Median plasma concentrations with 2.5 and 97.5 percentiles for the 16 steroids shown for both genders together (ALL) and separately for males and females.

Steroid	ALL		Males		Females	
Pregnenolone[a]	5.06	[1.21–21.33]	5.13	[1.45–22.88]	5.06	[1.01–20.86]
Progesterone[a]	0.28	[0.05–43.09]	0.27	[0.04–0.70][†]	0.38	[0.07–54.84]
17-Hydroxyprogesterone[a]	1.84	[0.28–6.26]	2.46	[0.87–6.24][†]	0.79	[0.24–6.84]
11-Deoxycorticosterone[a]	0.11	[< 0.49]	0.11	[< 0.54]	0.10	[< 0.47]
Corticosterone	5.54	[1.69–41.23]	5.77	[1.65–40.51]	5.20	[1.69–63.84]
Aldosterone	0.14	[0.02–0.62]	0.13	[0.01–0.45]	0.14	[0.02–0.67]
18-Oxocortisol	0.02	[< 0.09]	0.03	[< 0.10][†]	0.02	[< 0.09]
18-Hydroxycortisol	1.67	[0.45–4.33]	2.02	[0.74–4.64][†]	1.41	[0.41–3.40]
21-Deoxycortisol	0.032	[< 0.297]	0.036	[< 0.445][§]	0.029	[< 0.217]
11-Deoxycortisol	0.44	[0.12–2.18]	0.53	[0.13–2.58][†]	0.37	[0.11–1.69]
Cortisol[a]	264	[126–665]	283	[134–644][§]	247	[121–700]
Cortisone	53.0	[28.1–90.4]	54.7	[28.9–90.9][§]	51.8	[26.6–72.1]
DHEA	10.3	[1.9–40.5]	11.1	[2.5–46.7][§]	10.1	[1.7–38.3]
DHEA-SO_4	4163	[914–9390]	5333	[917–10,021][†]	3592	[850–7435]
Androstenedione[a]	3.21	[1.16–8.01]	3.33	[1.53–8.28][§]	3.04	[1.06–7.72]
Testosterone	6.34	[0.26–32.7]	16.9	[7.6–37.1][†]	0.93	[0.31–2.29]

All values are shown as nmol/L. All differences according to the Wilcoxon's rank sum test.
[a] Excludes females on oral contraceptives.
[†] $P < 0.0001$ different from females.
[§] $P < 0.05$ different from females.

Table 4
Multivariate analysis of differences in plasma concentrations of steroids according to gender, age, body mass index (BMI) and presence of hypertension.

Steroid	Gender		Age		BMI		Hypertension	
Pregnenolone[a]	0.0525		0.0001	− ve	0.1075		0.0796	
Progesterone[a]	0.0001	F > M	0.0009	− ve	0.0148	− ve	0.7093	
17-Hydroxyprogesterone[a]	0.0001	M > F	0.0001	− ve	0.0215	− ve	0.0105	N > H
11-Deoxycorticosterone[a]	0.5301		0.0001	− ve	0.0499	− ve	0.0754	
Corticosterone	0.4615		0.0001	− ve	0.0001	− ve	0.6327	
Aldosterone	0.1462		0.0001	− ve	0.1242		0.4770	
18-Oxocortisol	0.0001	M > F	0.0001	− ve	0.0743		0.5192	
18-Hydroxycortisol	0.0001	M > F	0.0030	− ve	0.0044	+ ve	0.8773	
21-Deoxycortisol	0.0197	M > F	0.2273		0.0001	− ve	0.0058	H > N
11-Deoxycortisol	0.0001	M > F	0.0253	− ve	0.6777		0.7137	
Cortisol[a]	0.0256	M > F	0.0001	− ve	0.0011	− ve	0.2700	
Cortisone	0.0002	M > F	0.0001	− ve	0.0005	− ve	0.5256	
DHEA	0.0001	M > F	0.0001	− ve	0.1227		0.4955	
DHEA-$S0_4$	0.0001	M > F	0.0001	− ve	0.3884		0.9493	
Androstenedione[a]	0.0292	M > F	0.0001	− ve	0.0524		0.3587	
Testosterone	0.0001	M > F	0.0003	− ve	0.0075	− ve	0.6823	

Data are shown as P-values and where significant (i.e., $P < 0.05$) differences are shown for gender as higher in females than males (F > M) or higher in males than females (M > F), for age and BMI according to the negative (− ve) or positive (+ ve) nature of relationships and for any differences between normotensives (N) and hypertensives (H). Data were logarithmically transformed before analyses.

[a] Excludes patients on oral contraceptives.

females that paralleled significant gender specific differences in plasma concentrations (Table 3). Larger extended upward variances in females than males, despite opposite or no significant overall differences in plasma concentrations, were observed for cortisol and aldosterone indicating respective 9% and 49% higher 97.5 percentiles for these steroids in women than men (Table 3).

Clear relationships of age with plasma concentrations of pregnenolone, androstenedione, DHEA, DHEA-SO_4, cortisone, corticosterone and 11-deoxycorticosterone indicated a striking need for age-specific reference intervals for these seven steroids, with additional needs to consider gender (Figs. 2 and 3). For some steroids (11-deoxycorticosterione and cortisone in both genders, 17-hydroxypgrogesterone and androstenedione in males and corticosterone and pregnenolone in females) there was evidence of peaks in plasma concentrations at 21 to 23 years of age, suggesting lower concentrations at ages younger than 21 years.

According to curve fitting models, DHEA, pregnenolone and corticosterone, showed the largest age related falls in plasma concentrations (Figs. 2 & 3). Upper cut-offs for plasma DHEA fell in both men and women by 69.9% from 54.1 and 48.8 nmol/L in 22-year olds to 16.3 and 14.7 nmol/L in 70-year old men and women respectively (Fig. 2, panels A & B). For pregnenolone, respective upper cut-offs in 22-year old men and women were 30.8 and 28.9 and fell by 74% and 75% to 8.0 and 7.3 nmol/L in 70-year olds (Fig. 2, panels G & H). Upper cut-offs estimated for corticosterone in 22-year old men and women were respectively 46.0 and 114.1 nmol/L and fell by 60% and 89% to 18.2 and 12.4 nmol/L in 70-year olds (Fig. 3, panels A & B). Age-related falls in upper cut-offs between 22- and 70-year olds for other steroids were also generally steeper in women than men, showing respective falls of 40% and 31% for DHEA-SO_4 (Fig. 2, panels C & D), 56% and 36% for androstenedione (Fig. 2, panels E & F), 43% and 42% for 11-deoxycorticosterone (Fig. 3, panels C & D) and 39% and 29% for cortisone

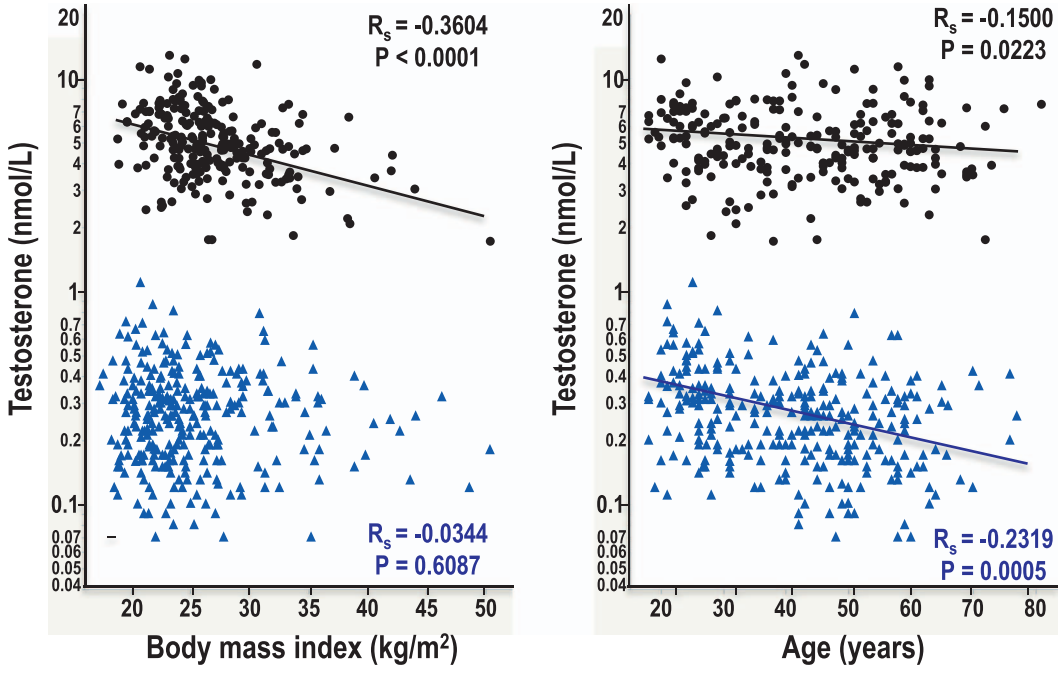

Fig. 1. Distributions of plasma concentrations of testosterone according to body mass index and age for men (•) and women (▲). Note that testosterone concentrations are presented using a logarithmic scale. Regression lines are shown for relationships that are significant ($P < 0.05$).

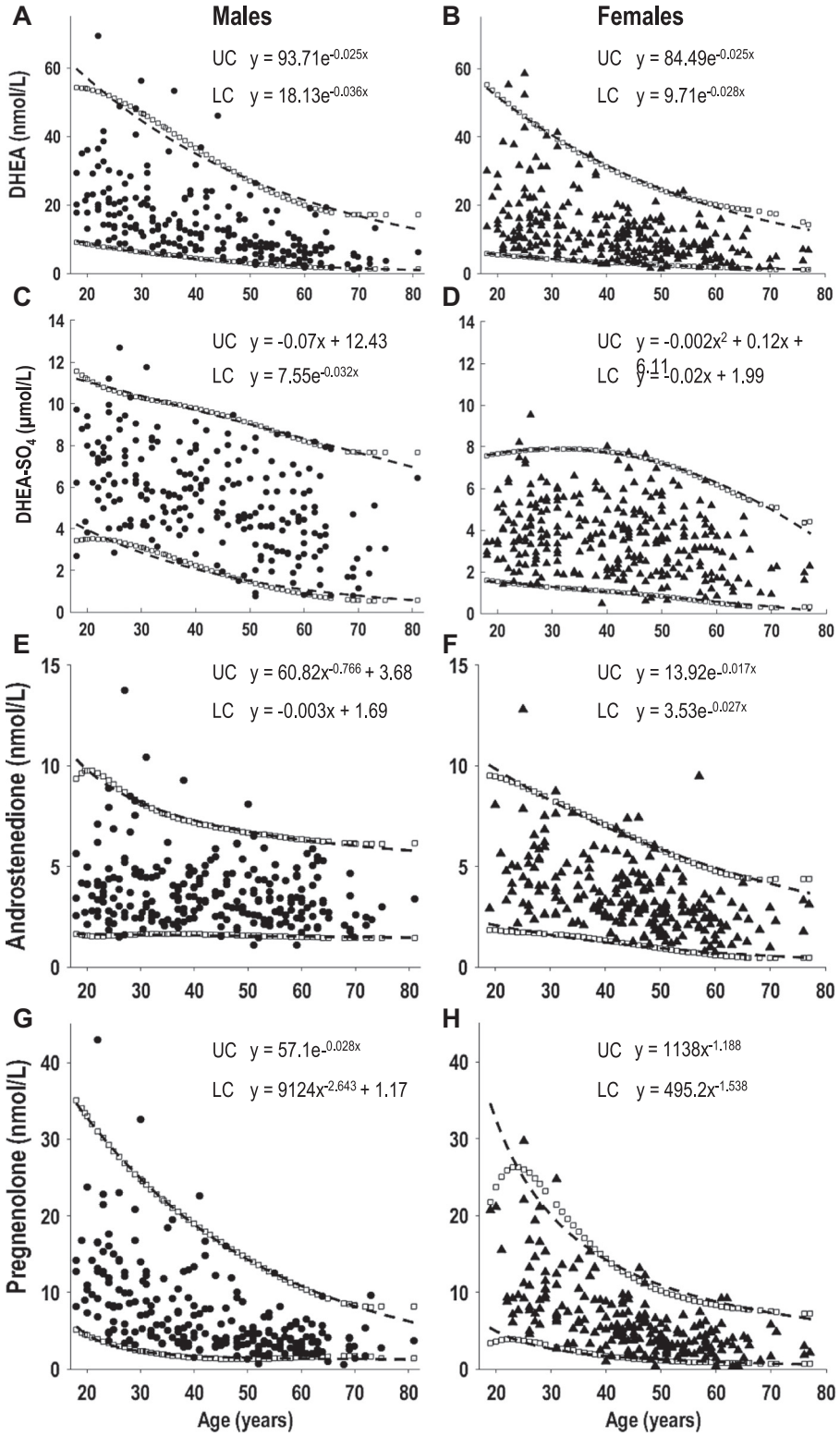

Fig. 2. Distributions of plasma concentrations of DHEA (A, B), DHEA-SO_4 (C, D), androstenedione (E, F), and pregnenolone (G, H) according to age for males (• A, C, E & G) and females (▲ B, D, F & H). Note that DHEA-SO_4 concentrations are shown in µmol/L compared to nmol/L for other steroids. Values for 97.5 and 2.5 percentiles are displayed as empty square dotted lines (□□□), whereas best-fit curves for upper and lower cut-offs are displayed by dashed lines (– – –) according to the equations shown for age specific upper cut-offs (UC) and lower cut-offs (LC).

(Fig. 3, panels E & F).

For 17-hydroxyprogesterone, a relationship with age requiring age-specific reference intervals was present for males, whereas for females there was need to consider post-menopausal and pre-menopausal status (Fig. 3 panels G & H).

From the above analyses, the reference intervals outlined in the formulae of Figs. 2 and 3, along with data in Tables 2 and 3, were compiled for all 16 steroids into a single table (see Data-in-brief) that accounted for gender and age influences as required for each steroid. For progesterone and 17-hydroxyprogesterone in women the available

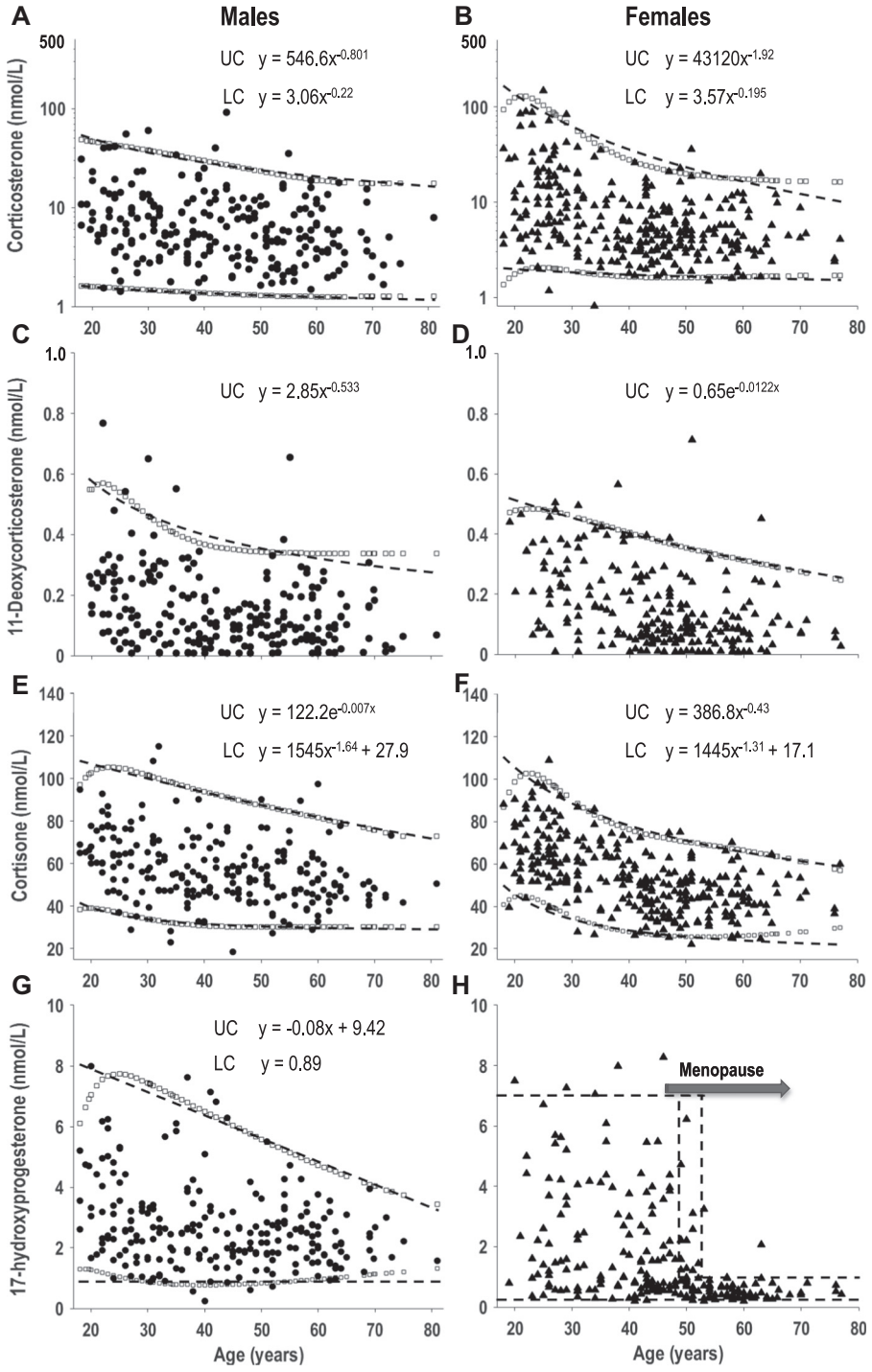

Fig. 3. Distributions of plasma concentrations of corticosterone (A, B), 11-deoxycorticosterone (C, D), cortisone (E, F), and 17-hydroxyprogesterone (G, H) according to age for males (• A, C, E & G) and females (▲ B, D, F & H). Note that corticosterone concentrations are displayed using a logarithmic scale compared to linear scales for the other steroids. Values for 97.5 and 2.5 percentiles are displayed as empty square dotted lines (□□□), whereas best-fit curves for upper and lower cut-offs are displayed by dashed lines (– – –) according to the equations shown for age specific upper cut-offs (UC) and lower cut-offs (LC). For 17-hydroxyprogesterone, age specific references are shown for males, whereas for females reference intervals are shown according pre- versus post-menopausal status.

population size did not allow calculation of 2.5 and 97.5 percentiles according to luteal and follicular phases of the menstrual cycle. Instead data are provided as lowest and highest values for those two specific groups.

4. Discussion

This study provides new data on distributions of plasma concentrations of 16 steroids in normotensive and hypertensive subjects, clarifying major steroid-specific impacts of gender and age, with minor differences according to body mass index and negligible associations with blood pressure status. The analysis also extends current knowledge about the confounding influences of oral contraceptives, which in addition to increasing plasma concentrations of total cortisol also impact several other steroids in downward directions. Most importantly the study establishes gender-specific reference intervals with additional age-specific considerations for half of the 16 steroids in the panel. Specifically for pregnenolone, DHEA, DHEA-SO_4, androstenedione, 17-

hydroxyprogesterone, cortisone, corticosterone and 11-deoxycorticosterone age-specific reference intervals are provided that can be calculated according to equations established from curve fitting. As expected for progesterone and 17-hydroxyprogesterone, there are additional requirements to consider pre- and post-menopausal age groups, including luteal and follicular phases in the former group.

Although reference intervals for commonly measured steroids, such as cortisol and testosterone, are well established for both immunoassays and LC-MS/MS measurements, for most of the other steroids reference intervals are mainly derived from immunoassays or are not well described by any method. Even where reference intervals have been described by LC-MS/MS, it remains important to establish accuracy and harmonization of methods. LC-MS/MS measurements are not immune from interferences that can manifest in different forms including ion suppression or enhancement, ionic cross talk, isobaric interferences and in source transformations [16]. For the scores of steroids present in biological fluids, some with identical mass charge ratios, isobaric interferences can be a problem for LC-MS/MS measurements when chromatographic separation of isobaric compounds is not achieved. Selection of specific product ions for steroids is particularly difficult to achieve in order to avoid isobaric interferences. Since commercial sources of most of the scores of endogenous steroids are limited, testing for such potential interferences is also problematic. Thus, even with the extended chromatographic separation achieved by our established LC-MS/MS method, interferences and biased measurements remain possible for some of the uncommonly measured steroids in the panel. As new and improved LC-MS/MS methods featuring these steroids become available it will be useful to cross validate reference intervals for those steroids.

For the more commonly measured steroids in the panel, results derived from participation in inter-laboratory quality assurance programs support accuracy of measurements and thus reliability of defined reference intervals for those particular steroids. Thus, the reference intervals described here for plasma cortisol and cortisone are similar to those described by Kushnir and colleagues [24]. Similarly median levels and reference intervals for cortisol are in reasonable agreement with those of Fanelli et al. [11], who described an LC-MS/MS method for a panel of 8 steroids. Median plasma concentrations and reference intervals for men and pre- and post-menopausal women of that study also showed generally good agreement for plasma testosterone, progesterone and 17-hydroxyprogesterone to the results of the present study. For corticosterone, 11-deoxycortisol and DHEA median plasma concentrations were measured 30% to 44% lower than in the study of Fanelli et al. [11], but reference intervals generally overlapped with no consistent difference. In contrast, median concentrations for androstenedione averaged 60% higher in the present study than in the earlier study with parallel differences in reference intervals. Differences in ages of subjects of the two studies may account for some but not all of the aforementioned differences.

In another report involving LC-MS/MS measurements of DHEA, androstenedione and testosterone by Kushnir et al. [34], median concentrations and reference intervals showed good agreement to the results of the present study for both DHEA and testosterone in both males and females, but up to 50% lower values for androstenedione. Among several LC-MS/MS methods described for plasma aldosterone [13,15,26,35–37], two outlined adult upper cut-offs of 0.62 and 0.64 nmol/L [15,36] that are almost identical to those of the present study for combined genders. Although others have described LC-MS/MS methods to quantify 18-oxocortisol and 18-hydroxycortisol for subtyping patients with primary aldosteronism [38], there appear to be few or no reports outlining reference intervals for these two steroids. The former steroid is present in plasma at particularly low and difficult to measure concentrations, so the reference intervals described here are preliminary and require further validation. Similarly for 21-deoxycortisol, plasma concentrations are low, often not detectable and the reference intervals described here require further validation using more sensitive measurements.

Estrogen-containing oral contraceptives are well established to increase plasma concentrations of cortisol by increasing corticosteroid-binding globulin, resulting in increased total levels of cortisol [39,40]. Oral contraceptives are also established to decrease plasma concentrations of several steroids, such as progesterone, 17-hydroxyprogesterone and androstenedione [19,22,39], as also confirmed here. We also show that 11-deoxycorticosterone is reduced by oral contraceptives. Oral contraceptive use is thus a major confounder for interpretation of steroid profiles.

Apart from the expected gender-associated differences in androgens, we also establish higher concentrations in males than females of most other steroids, which translated to a need for gender-specific reference intervals. In agreement with earlier work [11], for 17-hydroxyprogesterone and progesterone, gender-specific reference intervals require separate considerations for post-menopausal compared to higher and variable plasma concentrations during luteal and follicular phases of the menstrual cycle.

In addition to gender differences, negative relationships of plasma concentrations of androgens with age are well established [41,42], necessitating both gender and age-related reference intervals. In two recent reports, one involving a large population of women [19] and the other men [43], reference intervals for LC-MS/MS-based measurements of androstenedione and DHEA-SO_4 were described by reference curves that agree closely with those described here. Pregnenolone has also been established to display an age-associated negative relationship [44], with distributions for men and women in line with those described here. The present study extends the findings of these other reports by showing negative relationships of age with many other steroids, such as corticosterone and 11-deoxycorticosterone, for which age-specific reference intervals are now also established according to curve fitted equations. Negative relationships of advancing age with several additional steroids, including cortisol and aldosterone, though significant were relatively weak so that requirements of age-specific reference intervals for those steroids appear less critical.

Negative relationships of advancing age with plasma concentrations of steroids in adults contrasts with the situation in children where plasma concentrations of many steroids (e.g., corticosterone, cortisone, cortisol, androstenedione, 17-hydroxyprogesterone and DHEA) increase with advancing age during childhood and through the teenage years [10,20,21]. This is also inline with the present findings for many of the same steroids, which showed evidence of peak concentrations at 21 to 23 years of age. Thus, it is important to appreciate the highly dynamic nature of steroid profiles in both children and adults and that the reference intervals established in the present study are applicable only to adults over the age of 21 years.

Although disordered steroidogenesis is widely accepted to be associated with obesity, metabolic syndrome and hypertension [45,46], it is also clear that such associations are complex, involving other regulatory systems and differences in metabolism and clearance [47–49]. The relatively minimal and weak associations of measured steroids with body mass index and hypertension are thus not unexpected given the evidence for associations involving tissue-specific differences in metabolism and biological actions. The negative relationship of testosterone with body mass index in men is nevertheless consistent with other evidence that male obesity is associated with low testosterone [50].

In summary, this study outlines gender and age-specific reference intervals for LC-MS/MS-derived measurements of plasma concentrations for 16 steroids in a panel designed primarily for investigation of patients with endocrine forms of hypertension. For many of the commonly measured steroids results agree with literature findings, but for others there are as yet no other comparable published data and further verification will be important. For this, comparisons to other LC-MS/MS methods will be useful (see Data-in-brief). It also remains important to consider preanalytical factors [28]. Diurnal variations are

particularly important to consider for many of the steroids in the panel [5], so that it must be appreciated that the results of the present study apply only to samples collected in morning hours.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2017.05.002.

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