Steroid reference intervals in women: influence of menopause, age and metabolism

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Abstract

Objective: To investigate the impact of age, obesity and metabolic parameters on 13 circulating steroids in reproductive and menopausal age. To define reference intervals (RIs).

Design: Cross-sectional.

Methods: Three hundred and twenty five drug-free, healthy and eumenorrheic women were selected from the general population. Independent relationships of LC-MS/MS-determined steroid levels with age, BMI and metabolic parameters were estimated. Reference sub-cohorts were defined for calculating upper and lower limits in reproductive age, menstrual phases and menopause, and these were compared with limits in dysmetabolic sub-cohorts.

Results: Lower androgens, pro-androgens and estrogens, but higher cortisol and metabolites were found in menopausal compared to reproductive age women. Androgens and precursors decreased during reproductive age ($P < 0.001$ – $P = 0.002$) but not after menopause. 17OH-progesterone decreased with BMI ($P = 0.006$) and glucocorticoids with waist circumference ($P < 0.001$ – $P = 0.002$) in reproductive age, but increased with triglycerides ($P = 0.011$ – $P = 0.038$) after menopause. Inverse associations of dihydrotestosterone with BMI ($P = 0.004$) and HDL-cholesterol ($P = 0.010$), estrone with total cholesterol ($P = 0.033$) and estradiol with triglycerides ($P = 0.011$) were found in reproductive age. After menopause, estrone increased with waist circumference ($P < 0.001$) and decreased with insulin resistance ($P = 0.012$). Ovarian steroid RIs were estimated in menstrual phases and menopause. Age- and reproductive status-specific RIs were generated for androgens, precursors and corticosteroids. Lower limits for reproductive age cortisol ($P = 0.020$) and menopausal 11-deoxycortisol ($P = 0.003$) in dysmetabolic sub-cohorts were reduced and increased, respectively, compared to reference limits.

Conclusions: Obesity and dysmetabolism differently influence circulating steroids in reproductive and menopausal status. Age, menstrual and menopausal status-specific RIs were provided by LC-MS/MS for a broad steroid panel.

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Introduction

LC-MS/MS is used by an increasing number of clinical laboratories, providing reliable determinations of steroid hormones for the diagnosis and management of endocrine diseases (1, 2). By enlarging the panel of measurable precursors and metabolites, LC-MS/MS is also boosting renewed interest in understanding the steroid system derangement in several conditions. However, translating

research findings into clinical advancements require the definition of pathophysiologic states influencing the circulating steroid levels, as well as of appropriate reference intervals (RI) allowing the effective interpretation of laboratory results (3).

Recently, LC-MS/MS has been applied to study the steroid profile of adrenal tumors (4), type I diabetes (5),

or female hyperandrogenism (1, 6, 7, 8, 9). In contrast, only a few studies were purposely designed to define steroid RI. Moreover, some studies relied on healthcare-seeking subjects. This represents an intrinsic bias for RI estimation, given the adaptive nature of steroid hormones to stressful or debilitating conditions. Other studies did not report an exhaustive characterization of subjects, for example aimed at detecting subtle hyperandrogenic states manifesting as menstrual irregularities or hirsutism, while others tolerated excess weight (10).

Overweight and obesity affect half of the female population (11, 12). Steroids are involved in systemic and intracrine mechanisms that, once dysregulated, contribute to excess fat, mostly of abdominal type, hypertension, impaired glucose control and dyslipidaemia. However, the interplay among glucocorticoids, androgens and estrogens in such mechanisms varies with the menopausal transition (12, 13). Androgen excess, ovarian dysfunction and metabolic impairment represent a vicious cycle in young women, however, a variegated spectrum of manifestations is observed, for which effective hormonal markers are yet undefined (14). The androgen decline and the loss of ovarian hormones contribute to the central redistribution of body fat, the increased cardiometabolic risk, sarcopenia and bone frailty experienced after menopause. Besides, the modulation of adrenal function throughout women lifespan, and the interplay between the hypothalamus-pituitary-adrenal (HPA) axis dysregulation and obesity before and after menopause were not elucidated (15, 16).

In a recent study, we demonstrated that the circulating steroid profile in men is not only influenced by aging, but also by obesity and metabolic derangement, and we provided age-specific RI estimated in appropriate reference cohorts (17). In the present study, by using a similarly standardized procedural approach, we aimed at describing the relationships of age, obesity and metabolic status with 13 circulating steroids in women in reproductive and menopausal age. Based on the depicted associations, we generated age, menopause and menstrual phase specific RI in selected reference sub-cohorts, and investigated whether unrestricted inclusion criteria could bias steroid RI estimation.

Subjects and methods

Subjects

Women aged 18–86 years were recruited from the general population (18). The study was approved by the S.Orsola

Policlinic ethical committee (85/2008/O/Tess). All women signed the informed consent before they were interviewed and examined by a trained endocrinologist, between 08:00 h and 10:00 h. Waist circumference and BMI were recorded. systolic (SBP) and diastolic (DBP) blood pressures were measured in supine position after 3 min rest. Inclusion criteria were: BMI ≥ 18.5 kg/m², weight stability in previous 3 months and complete sexual development. Exclusion criteria included signs of clinical hyperandrogenism, history of menstrual irregularities, steroidal (including estro-progestogen) and non-steroidal drug assumption in previous 3 months (except antipyretic or non-steroidal anti-inflammatory compounds tolerated before the previous month, and thyroxine replacement in compensated hypothyroidism), present or past endocrine, hepatic, renal, oncologic, autoimmune, cardiovascular, hematologic, neurologic or psychiatric diseases, sleep disorders, shift working, frequent flying or allergies requiring treatment. Among 653 women examined, 328 were excluded because of present or previous diseases ($n = 32$), irregular sleeping ($n = 80$), glucose-lowering ($n = 3$), cholesterol-lowering ($n = 111$), anti-hypertensive ($n = 171$) and anti-depression ($n = 40$) drug assumption. Therefore, 325 women were included in the present study.

Biochemical and hormonal evaluation

Blood was withdrawn in overnight fasting condition in Vacuette Z serum beads clot activator tubes (Greiner Bio-One, Kremsmunster, Austria) after 10 min saline infusion for minimizing venepuncture stress. After 20 min settling, tubes were centrifuged (2000g, 10 min, room temperature) and serum for LC-MS/MS was stored at -80°C. Routine hormones and biochemicals were measured in fresh blood as previously reported (17). The homeostatic model assessment-insulin resistance (HOMA-IR) was computed (19).

Steroid measurement by LC-MS/MS

We applied two in-house LC-MS/MS assays (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article) including 17-hydroxypregnenolone (17OHP5), dehydroepiandrosterone (DHEA), progesterone (P4), 17-hydroxyprogesterone (17OHP4), 11-deoxycorticosterone (DOC), corticosterone (B),

11-deoxycortisol (11S), cortisol (F), androstenedione (A4), testosterone (T), dihydrotestosterone (DHT), estrone (E1) and estradiol (E2) (18, 20, 21).

Study design

Women were classified in reproductive (regular menses, age 18–54 years; $n = 186$) or menopausal (amenorrhea in previous 12 months or more, age 48–86 years; $n = 127$) age, and further subdivided in normal weight (NW, $18.5 < \text{BMI} \leq 25.0 \text{ kg/m}^2$; $n = 123$ and 63 , respectively), overweight (OW, $25.0 < \text{BMI} \leq 30.0 \text{ kg/m}^2$; $n = 35$ and 49 , respectively) and obese (OB, $\text{BMI} > 30.0 \text{ kg/m}^2$; $n = 28$ and 15 , respectively). Women in reproductive age were further stratified in early follicular (day 1–6; $n = 31$), pre-ovulatory (day 9–13; $n = 30$) and mid-luteal (day 18–24; $n = 33$) menstrual phases. Perimenopausal women were also identified (<6 menstrual bleedings in previous 6 months or more, age 48–54 years; $n = 12$). Anthropometric, metabolic and steroid values were compared between reproductive and menopausal cohorts, among BMI classes and among menstrual phases. Afterward, we assessed the independent effect of age, BMI, waist circumference, SBP, DBP, HOMA-IR, total cholesterol, HDL-cholesterol and triglycerides on each steroid in reproductive and menopausal ages. According to the results, we defined steroid-specific reference sub-cohorts by excluding women displaying alterations in metabolic parameters influencing the steroid levels. Therefore, we identified subjects with normal ($\leq 88 \text{ cm}$, norWC; $n = 216$) or elevated ($> 88 \text{ cm}$, dysWC; $n = 106$) waist circumference, normal (< 2.5 , norHOMA; $n = 194$) or elevated (≥ 2.5 , dysHOMA; $n = 36$) HOMA-IR, normal ($< 5.17 \text{ mmol/L}$, norTC; $n = 194$) or elevated ($\geq 5.17 \text{ mmol/L}$, dysTC; $n = 123$) total cholesterol, normal ($\geq 1.29 \text{ mmol/L}$, norHDL; $n = 203$) or reduced ($< 1.29 \text{ mmol/L}$, dysHDL; $n = 55$) HDL-cholesterol, and normal ($< 1.69 \text{ mmol/L}$, norTG; $n = 302$) or elevated ($\geq 1.69 \text{ mmol/L}$, norTG; $n = 15$) triglycerides. Lower (LRL) and upper (URL) reference limits defining the central 95% of steroid distribution were estimated in reference sub-cohorts. Age-specific LRL and URL were estimated when required. Finally, to evaluate whether altered metabolic parameters could influence RI estimation, LRL and URL were compared with lower and upper limits calculated in dysmetabolic sub-cohorts including subjects with alterations in the parameters influencing that particular steroid, respectively.

Statistical analysis

Box–Cox transformation was used for variables showing a significant skewness at the Kolmogorov–Smirnov test (22). Far outliers at the Tukey's test were removed (23). Variables were compared between reproductive age and menopausal cohorts by *t*-test. The ANOVA trend test was used for comparing BMI classes (SPSS package v.20, IBM Co). Comparisons among menstrual phases were performed by one-way ANOVA. The stepwise multiple regression included age, BMI, waist circumference, SBP, DBP, HOMA-IR, total-cholesterol, HDL-cholesterol and triglyceride levels as covariates, and each steroid as dependent variable. The menstrual phase was added as cofactor for steroids varying with the menstrual cycle. The effect size (f^2) was estimated as $f^2 = \frac{sr^2}{(1 - R^2_{full})}$, where sr^2 is the semipartial correlation coefficient for the predictor of interest, and R is the full correlation coefficient obtained by the multiple regression model (24).

LRL and URL were estimated as the mean – and + (1.96 $\times$ s.d.) of the transformed variables, respectively, then, values were back-transformed to the original unit (25). Age-specific RI was estimated by modeling the transformed steroid variable on age distribution, according to the fractional polynomial regression by Royston and Wright (26). Age (X) was transformed in order to stabilize the steroid variable (Y) for large values of X according to the formula: $e^X = \exp \frac{(\log(0.01) \times (X - \min(X)))}{(\max(X) - \min(X))}$. Then, we selected the optimal model providing the lowest polynomial degree (parsimony) with maximum decrease in deviance (goodness of fit). Best-fit polynomial coefficients were selected by fp syntax, and RI was visually inspected by xrigls syntax in STATA (v.13.0, StataCorp LLC).

A large number of cases showed values below the sensitivity limit for DOC in the whole cohort ($n = 276$ of 325), and P4, DHT and E2 in menopausal cohort ($n = 90$, 75 and 115 of 127 , respectively) (Supplementary Table 1). Therefore, DOC comparisons were performed by Kruskal–Wallis and Mann–Whitney tests, while no multiple regression was performed for DOC and for P4, DHT and E2 in menopausal cohort. RI for DOC and P4, and menopausal RI for DHT and E2 were estimated as the 2.5–97.5 centiles of distribution. Lower and upper limits were compared between reference and dysmetabolic sub-cohorts by *z* distribution. Two-tailed *P* values < 0.05 were considered significant. Data were analyzed by MedCalc Software (v.18.2.1, Mariakerke, Belgium) except where specified.

Results

Anthropometric, metabolic and hormonal features of the cohort

Worsening metabolic parameters at increasing BMI were observed both in reproductive age (min–max BMI: 18.5–42.9 kg/m²) and menopausal women (min–max BMI: 18.9–41.2 kg/m²). In reproductive age, levels of B ($P < 0.001$), F ($P = 0.008$) and DHT ($P = 0.006$) decreased with increasing BMI classes, with OB showing lower B, F and DHT compared to NW ($P = 0.002$, $P = 0.023$ and $P = 0.017$) and lower B and F compared to OW (both $P < 0.001$) women. In menopausal women, E1 levels increased with BMI classes ($P = 0.002$), with both OW ($P = 0.046$) and OB ($P = 0.007$) displaying higher values than NW women (Table 1). Women in different menstrual phases displayed similar anthropometric and metabolic parameters, but different levels of P4, 17OHP4, E1, E2 (all $P < 0.001$), DOC ($P = 0.043$) and 11S ($P = 0.048$). In particular, women in mid-luteal phase had higher P4 and 17OHP4 as compared to early follicular and pre-ovulatory (all $P < 0.001$), and higher DOC compared to pre-ovulatory ($P = 0.047$) women. Moreover, lower E1 and E2 levels were found in early follicular compared to pre-ovulatory and mid-luteal phases (all $P < 0.001$) (Supplementary Table 2).

Independent impact of age, anthropometric and metabolic parameters on steroid levels

Stepwise multiple regression results are detailed in Table 2. In women in reproductive age, 17OHP5, DHEA, A4, T (all $P < 0.001$) and DHT ($P = 0.002$) decreased with age, with a large effect size for A4 ($f^2 = 0.28$), DHEA and T (both $f^2 = 0.22$), and moderate for 17OHP5 ($f^2 = 0.12$) and DHT ($f^2 = 0.11$). DHT was also negatively associated with BMI ($P = 0.004$, $f^2 = 0.09$) and HDL-cholesterol ($P = 0.010$, $f^2 = 0.08$). 17OHP4 inversely associated with BMI ($P = 0.006$, $f^2 = 0.22$), while B ($P < 0.001$, $f^2 = 0.20$), F ($P < 0.001$, $f^2 = 0.17$) and 11S ($P = 0.002$, $f^2 = 0.09$) inversely associated with waist circumference. Finally, E1 and E2 negatively associated with increasing total cholesterol ($P = 0.033$, $f^2 = 0.10$) and triglycerides ($P = 0.011$, $f^2 = 0.15$), respectively. In menopausal women, no age and BMI influence were detected on steroid levels. 17OHP4 ($P = 0.026$, $f^2 = 0.06$), 11S ($P = 0.011$, $f^2 = 0.07$) and F ($P = 0.038$, $f^2 = 0.05$) directly associated with triglycerides. E1 directly associated with waist circumference ($P < 0.001$,

$f^2 = 0.16$), and inversely associated with HOMA-IR ($P = 0.012$, $f^2 = 0.08$).

Steroid reference intervals

Age- and menopause-specific RI were estimated for 17OHP5, DHEA, A4, T and DHT (Table 3). All women were included in the reference sub-cohort for 17OHP5, DHEA, A4 and T ($n = 325$). For DHT, the reference sub-cohort included women in reproductive age who were both NW and norHDL, and all menopausal women ($n = 65$ and $n = 127$, respectively). Androgens and precursors peaked around age 25 years and progressively declined. A reduction in LRL and URL was found for A4 (-83.2 and -57.5%, respectively), DHEA (-67.6 and -56.0%, respectively), 17OHP5 (-49.9 and -43.1%, respectively) and T (-24.9 and -17.2%, respectively), as well as in DHT URL (-56.3%) from age 25 to 65 years (Fig. 1).

Reproductive age RI were estimated in the whole cohort for DOC ($n = 186$), and in norWC sub-cohort for B, 11S and F ($n = 132$). Menopausal RI were estimated in the whole cohort for DOC, B, P4 and E2 ($n = 127$), in norTG sub-cohort for 17OHP4, 11S and F ($n = 112$), and in women who were both norWC and norHOMA for E1 ($n = 50$) (Table 4).

Finally, menstrual phase specific RI were estimated for P4, 17OHP4, E1 and E2. Of 32 women in mid-luteal phase, 10 (age 39.2 ± 10.3 years) exhibited P4 levels < 10 nmol/L suggestive of incorrect classification or of anovulatory cycle (27), and were therefore excluded. The reference sub-cohorts for early follicular, pre-ovulatory and mid-luteal phase included 31, 30 and 22 women for P4; 22, 26 and 17 NW women for 17OHP4; 20, 25 and 17 norTC women for E1; 25, 28 and 21 norTG women for E2, respectively (Table 5).

Impact of metabolic risk factors on the estimation of reference limits

LRL and URL of steroids influenced by metabolic parameters were compared with lower and upper limits calculated in dysmetabolic subjects. Higher 11S values were found in the lower limit calculated in dysTG ($n = 10$) compared to LRL in norTG ($n = 112$) menopausal women (+0.343 nmol/L, +48.4%, $P = 0.003$). At variance, reduced F values were found in lower limit in dysWC ($n = 52$) compared with LRL in norWC ($n = 132$) women in reproductive age (-35.5 nmol/L, -24.0%; $P = 0.020$, respectively) (Table 4).

Table 1 Anthropometric, metabolic and hormonal features of the cohort. Data are expressed as mean $\pm$ s.d., except 11-deoxycorticosterone where median (75th centile) are shown. Mean values are back transformed means of the transformed variables. Blood was withdrawn between 8:00 h and 10:00 h after overnight fasting.

	Women in reproductive age						Women in menopausal age					
	All			ANOVA trend, P			All			ANOVA trend, P		
	n	Normal weight	Overweight	Obese			Normal weight	Overweight	Obese			P ^t
Age, years	186	123	35	28			63	49	15			
BMI, kg/m ²	38.2 $\pm$ 8.4	37.1 $\pm$ 8.6	40.4 $\pm$ 8.0	41.0 $\pm$ 6.8	0.186		58.2 $\pm$ 7.4	62.4 $\pm$ 8.2	59.4 $\pm$ 6.9	0.515		<0.001
WC, cm	21.8 $\pm$ 5.3	20.4 $\pm$ 1.7	25.8 $\pm$ 1.4 ^a	33.8 $\pm$ 3.7 ^{a,b}	<0.001		21.6 $\pm$ 1.4	25.8 $\pm$ 1.4 ^a	32.2 $\pm$ 3.4 ^{a,b}	<0.001		<0.001
SBP, mmHg	81.1 $\pm$ 12.8	75.8 $\pm$ 6.7	88.2 $\pm$ 8.8 ^a	104.7 $\pm$ 9.2 ^{a,b}	<0.001		81.5 $\pm$ 7.1	90.0 $\pm$ 5.6 ^a	104.7 $\pm$ 8.2 ^{a,b}	<0.001		<0.001
DBP, mmHg	117.5 $\pm$ 14.8	112.5 $\pm$ 10.6	125.6 $\pm$ 17.2 ^a	124.6 $\pm$ 15.3 ^a	<0.001		129.1 $\pm$ 15.1	129.7 $\pm$ 17.7	140.7 $\pm$ 22.2	0.027		<0.001
Glucose, mmol/L	77.5 $\pm$ 8.6	74.9 $\pm$ 8.1	81.7 $\pm$ 7.9 ^c	80.7 $\pm$ 8.5 ^a	<0.001		81.7 $\pm$ 7.0	81.7 $\pm$ 7.8	88.8 $\pm$ 11.2 ^{c,d}	0.003		<0.001
Insulin, μ U/mL	4.53 $\pm$ 0.73	4.42 $\pm$ 0.67	4.45 $\pm$ 0.75	5.11 $\pm$ 0.74 ^d	<0.001		4.72 $\pm$ 0.79	4.93 $\pm$ 0.77	5.08 $\pm$ 0.88	0.114		<0.001
HOMA-IR	6.2 $\pm$ 4.0	5.1 $\pm$ 2.4	7.1 $\pm$ 3.2 ^a	10.0 $\pm$ 5.3 ^{a,e}	<0.001		5.9 $\pm$ 2.5	7.7 $\pm$ 4.3 ^c	9.5 $\pm$ 5.3 ^a	<0.001		0.052
TC, mmol/L	1.29 $\pm$ 0.95	1.04 $\pm$ 0.55	1.34 $\pm$ 0.68 ^a	2.24 $\pm$ 1.24 ^{a,b}	<0.001		1.27 $\pm$ 0.65	1.67 $\pm$ 1.19 ^f	2.14 $\pm$ 1.50 ^c	<0.001		0.013
HDL-C, mmol/L	4.52 $\pm$ 0.82	4.41 $\pm$ 0.80	4.79 $\pm$ 0.92	4.67 $\pm$ 0.73	0.133		5.30 $\pm$ 0.95	5.50 $\pm$ 0.76	5.37 $\pm$ 0.58	0.677		<0.001
TG, mmol/L	1.50 $\pm$ 0.37	1.57 $\pm$ 0.35	1.51 $\pm$ 0.31	1.31 $\pm$ 0.44 ^{a,d}	<0.001		1.65 $\pm$ 0.40	1.53 $\pm$ 0.39	1.46 $\pm$ 0.31	0.089		0.117
17OHP5, nmol/L	0.70 $\pm$ 0.34	0.64 $\pm$ 0.22	0.80 $\pm$ 0.38 ^a	0.86 $\pm$ 0.53 ^a	<0.001		0.81 $\pm$ 0.40	1.10 $\pm$ 0.53	0.97 $\pm$ 0.30	0.253		<0.001
DHEA, nmol/L	5.51 $\pm$ 7.22	5.64 $\pm$ 7.31	6.97 $\pm$ 8.13	3.84 $\pm$ 4.97	0.087		3.51 $\pm$ 2.77	4.38 $\pm$ 4.72	3.35 $\pm$ 3.48	0.815		<0.001
P4, nmol/L	15.50 $\pm$ 14.60	15.64 $\pm$ 14.20	17.11 $\pm$ 14.52	13.16 $\pm$ 16.65	0.324		8.94 $\pm$ 5.30	9.09 $\pm$ 7.42	8.19 $\pm$ 6.83	0.644		<0.001
17OHP4, nmol/L	1.93 $\pm$ 0.91	1.91 $\pm$ 1.681	2.02 $\pm$ 13.23	1.87 $\pm$ 11.52	0.364		0.17 $\pm$ 0.04	0.22 $\pm$ 0.03	0.21 $\pm$ 0.01	0.067		<0.001 ^s
DOC, nmol/L	1.776 $\pm$ 1.887	1.873 $\pm$ 2.039	1.834 $\pm$ 1.566	1.362 $\pm$ 1.393	0.142		0.631 $\pm$ 0.391	0.856 $\pm$ 0.541	0.635 $\pm$ 0.339	0.968		<0.001
B, nmol/L	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	0.133 [#]		<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	0.550 [#]		0.044 ^s
11S, nmol/L	8.15 $\pm$ 9.56	8.94 $\pm$ 9.74	8.61 $\pm$ 10.50	5.07 $\pm$ 5.83 ^{b,c}	<0.001		7.79 $\pm$ 7.29	10.31 $\pm$ 7.29	6.35 $\pm$ 4.16	0.313		0.662
F, nmol/L	0.755 $\pm$ 0.710	0.743 $\pm$ 0.743	0.956 $\pm$ 0.689	0.608 $\pm$ 0.517	0.338		0.817 $\pm$ 0.559	1.002 $\pm$ 0.655	0.892 $\pm$ 0.592	0.613		0.029
A4, nmol/L	286.4 $\pm$ 105.8	296.3 $\pm$ 102.2	292.3 $\pm$ 118.7	241.1 $\pm$ 94.7 ^{b,f}	0.008		302.3 $\pm$ 93.7	351.5 $\pm$ 104.2	283.5 $\pm$ 104.8	0.469		0.007
T, nmol/L	2.34 $\pm$ 1.09	2.40 $\pm$ 1.09	2.48 $\pm$ 1.21	1.96 $\pm$ 0.85	0.369		1.06 $\pm$ 0.50	1.21 $\pm$ 0.53	1.27 $\pm$ 0.67	0.165		<0.001
DHT, nmol/L	0.768 $\pm$ 0.328	0.790 $\pm$ 0.326	0.730 $\pm$ 0.325	0.717 $\pm$ 0.342	0.154		0.552 $\pm$ 0.256	0.684 $\pm$ 0.368	0.524 $\pm$ 0.219	0.700		<0.001
E1, nmol/L	0.183 $\pm$ 0.128	0.193 $\pm$ 0.124	0.191 $\pm$ 0.155	0.151 $\pm$ 0.079 ^f	0.006		0.142 $\pm$ 0.063	0.139 $\pm$ 0.076	0.139 $\pm$ 0.154	0.597		<0.001
E2, nmol/L	0.212 $\pm$ 0.116	0.216 $\pm$ 0.118	0.201 $\pm$ 0.122	0.209 $\pm$ 0.099	0.319		0.069 $\pm$ 0.032	0.083 $\pm$ 0.038 ^f	0.098 $\pm$ 0.044 ^c	0.002		<0.001
	0.292 $\pm$ 0.277	0.323 $\pm$ 0.274	0.238 $\pm$ 0.300	0.251 $\pm$ 0.246	0.334		0.037 $\pm$ 0.022	0.036 $\pm$ 0.008	0.038 $\pm$ 0.023	0.640		<0.001

* vs NW, P < 0.001; ^b vs OW, P < 0.001; ^c vs NW, P < 0.010; ^d vs OW, P < 0.050; ^e vs OW, P < 0.010; ^f vs NW, P < 0.050; ^g Mann-Whitney test; ^h Reproductive vs menopausal by t-test. 17OHP5, 17-Hydroxyprogesterone; 17OHP4, 17-Hydroxyprogesterone; 11S, 11-deoxycorticosterone; A4, androstenedione; B, corticosterone; BMI, body mass index; DHEA, Dehydroepiandrosterone; DHT, dihydrotestosterone; DOC, 11-deoxycorticosterone; E1, estrone; E2, estradiol; F, cortisol; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment insulin resistance; P4, progesterone; T, Testosterone; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

Table 2 Impact of age, anthropometric and metabolic parameters on steroid circulating levels in women in reproductive and menopausal status. Data are shown as Cohen's effect size for multiple linear regression (f^2) and P value resulting from the stepwise multiple regression. The negative (-) or positive (+) nature of the relationship is reported.

Status	Age		BMI		WC		HOMA-IR		TC		HDL-C		TG	
	f^2	P	f^2	P	f^2	P	f^2	P	f^2	P	f^2	P	f^2	P
17OHP5														
Reproductive	0.12	<0.001 (-)		NS		NS		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
DHEA														
Reproductive	0.22	<0.001 (-)		NS		NS		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
P4														
Reproductive*		NS		NS		NS		NS		NS		NS		NS
17OHP4														
Reproductive*		NS	0.22	0.006 (-)		NS		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS	0.06	0.026 (+)
B														
Reproductive		NS		NS	0.20	<0.001 (-)		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
11S														
Reproductive		NS		NS	0.09	0.002 (-)		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS	0.07	0.011 (+)
F														
Reproductive		NS		NS	0.17	<0.001 (-)		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
A4														
Reproductive	0.28	<0.001 (-)		NS		NS		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
T														
Reproductive	0.22	<0.001 (-)		NS		NS		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
DHT														
Reproductive	0.11	0.002 (-)	0.09	0.004 (-)		NS		NS		NS	0.08	0.010 (-)		NS
E1														
Reproductive*		NS		NS		NS		NS		0.033 (-)		NS		NS
Menopausal		NS		NS	0.16	<0.001 (+)	0.08	0.012 (-)	0.10	NS		NS		NS
E2														
Reproductive*		NS		NS		NS		NS		NS		NS	0.15	0.011 (-)

*Data adjusted by the menstrual phase.

11S, 11-Deoxycortisol; 17OHP4, 17-Hydroxyprogesterone; 17OHP5, 17-Hydroxyprogesterone; A4, androstenedione; B, corticosterone; F, cortisol; DHT, dihydrotestosterone; E1, estrone; E2, estradiol; HDL-C, HDL cholesterol; HOMA-IR, homeostatic model assessment insulin resistance; HDL, high density lipoprotein; NS, not significant; P4, progesterone; TC, total cholesterol; T, testosterone; TG, triglycerides; WC, waist circumference.

Table 3 Reference intervals of serum androgens and pro-androgens according to age- and menopausal status. Data are reported in nmol/L. LRL and URL were calculated as the mean $\pm$ 1.96 $\times$ s.d. and mean $\pm$ 1.96 $\times$ s.d. of hormone distribution, respectively, according to the fractional polynomial regression by Royston and Wright.

Age (y) /status	17OHP5		DHEA		A4		T		DHT	
	LRL (90 CI)	URL (90 CI)	LRL (90 CI)	URL (90 CI)	LRL (90 CI)	URL (90 CI)	LRL (90 CI)	URL (90 CI)	LRL	URL (90 CI)
20	2.33 (1.65–3.21)	27.65 (17.63–51.44)	8.5 (6.2–11.0)	59.8 (44.8–92.8)	1.29 (1.06–1.50)	4.91 (4.38–5.69)	0.324 (0.234–0.392)	1.646 (1.418–1.894)	ND	ND
25	1.92 (1.57–2.30)	30.09 (22.51–44.45)	6.6 (5.5–7.7)	60.3 (49.2–81.5)	1.54 (1.36–1.71)	5.66 (5.09–6.48)	0.315 (0.257–0.357)	1.664 (1.514–1.894)	$\leq$ 0.135	0.535 (0.463–0.604)
30	1.68 (1.46–1.90)	29.96 (24.07–39.28)	5.5 (4.8–6.1)	57.1 (48.4–71.5)	1.37 (1.23–1.51)	5.28 (4.78–5.92)	0.308 (0.268–0.339)	1.664 (1.549–1.816)	$\leq$ 0.135	0.508 (0.453–0.575)
35	1.51 (1.35–1.67)	28.59 (23.88–35.50)	4.6 (4.1–5.1)	52.5 (45.5–62.8)	1.12 (1.01–1.22)	4.64 (4.27–5.10)	0.301 (0.271–0.327)	1.652 (1.546–1.790)	$\leq$ 0.135	0.472 (0.423–0.539)
40	1.38 (1.24–1.52)	26.70 (22.74–32.58)	4.0 (3.6–4.4)	47.5 (41.9–55.4)	0.88 (0.80–0.95)	4.03 (3.76–4.37)	0.294 (0.267–0.316)	1.630 (1.523–1.773)	$\leq$ 0.135	0.434 (0.391–0.496)
45	1.27 (1.13–1.41)	24.65 (21.11–30.00)	3.5 (3.1–3.9)	42.6 (37.9–48.9)	0.69 (0.62–0.75)	3.52 (3.31–3.79)	0.285 (0.259–0.307)	1.597 (1.485–1.747)	$\leq$ 0.135	0.396 (0.358–0.449)
50	1.18 (1.03–1.32)	22.59 (19.30–27.76)	3.1 (2.7–3.5)	38.0 (33.9–43.3)	0.54 (0.46–0.60)	3.12 (2.92–3.36)	0.275 (0.247–0.298)	1.556 (1.430–1.712)	$\leq$ 0.135	0.357 (0.325–0.406)
55	1.10 (0.94–1.25)	20.64 (17.41–25.59)	2.7 (2.3–3.1)	33.8 (30.1–38.7)	0.42 (0.33–0.50)	2.81 (2.59–3.05)	0.264 (0.232–0.290)	1.505 (1.357–1.679)	$\leq$ 0.135	0.317 (0.288–0.371)
60	1.03 (0.87–1.18)	18.81 (15.54–23.64)	2.4 (2.0–2.8)	30.0 (26.5–34.5)	0.33 (0.22–0.41)	2.58 (2.31–2.85)	0.251 (0.214–0.284)	1.446 (1.269–1.649)	$\leq$ 0.135	0.277 (0.245–0.342)
65	0.96 (0.80–1.12)	17.14 (13.87–21.86)	2.1 (1.8–2.6)	26.5 (23.2–31.1)	0.26 (0.15–0.34)	2.41 (2.08–2.74)	0.236 (0.191–0.277)	1.377 (1.168–1.614)	$\leq$ 0.135	0.234 (0.194–0.312)
70	0.90 (0.74–1.07)	15.61 (12.38–20.18)	1.9 (1.5–2.3)	23.5 (20.1–27.9)	0.20 (0.09–0.29)	2.29 (1.90–2.69)	0.221 (0.168–0.272)	1.301 (1.055–1.576)	$\leq$ 0.135	0.189 (0.135–0.281)
75	0.85 (0.68–1.02)	14.22 (11.05–18.71)	1.7 (1.3–2.1)	20.8 (17.5–25.2)	0.15 (0.05–0.25)	2.22 (1.74–2.71)	0.203 (0.144–0.266)	1.217 (0.938–1.544)	$\leq$ 0.135	0.141 (0.070–0.247)
MP	1.15 (0.99–1.33)	16.06 (13.15–19.69)	2.7 (2.3–3.2)	27.8 (24.0–32.1)	0.39 (0.32–0.46)	2.45 (2.25–2.66)	0.248 (0.220–0.276)	1.444 (1.280–1.629)	$\leq$ 0.135*	0.462*

* LRL and URL calculated as the 2.5th and 97.5th of hormone distribution, respectively.

LRL, lower reference limit; ND, not determined because of insufficient data points; URL, upper reference limit; 17OHP5, 17-hydroxyprogesterone; A4, androstenedione; T, testosterone; DHT, dihydrotestosterone; MP, menopausal

Figure 1

Distribution of steroid hormone serum levels by age. Continuous red lines: lower and upper reference limits; dashed green lines: 90% CIs.

Discussion

In the present study, to obtain RI as effective as possible when applied to the study of women health, we selected from the general population women who were drug- and disease-free and having no signs or symptoms of androgen excess. Nonetheless, our cohort included women with unmedicated excess weight, dyslipidaemia, impaired insulin sensitivity and hypertension, overall affecting a relevant portion of the general population.

Our results confirmed the dramatic decline in circulating estrogens, progestins, androgens and precursors with menopause (28). No age dependency was detected for estrogen and progestin levels in reproductive age, therefore, the 60–90% hormone reduction observed after menopause could totally be attributed to ovarian senescence. At variance, the circulating androgens and precursors peaked around age 25 years and started a declining trend long before the menopausal transition. A steep decrease from age 25 to 65 years was observed for A4, DHEA 17OHP5, and DHT, ranging 40 to 85%, while a moderate 20% decrease was found for T, overall in good agreement with previous estimates (29, 30, 31, 32, 33,

Table 4 Reference intervals of age-independent steroid circulating levels in the reference sub-cohorts, and comparison with limits in dysmetabolic sub-cohorts. *P* values refer to Z-test comparison of LRL ([§]) and URL ([#]) between dysmetabolic and reference subgroups. LRL and URL were calculated as the mean - 1.96 × s.d. and mean + 1.96 × s.d. of hormone distribution, respectively.

Steroid hormone/ Status	Reference sub-cohort				Dysmetabolic sub-cohort					
	Features	<i>n</i>	LRL (90% CI) (nmol/L)	URL (90% CI) (nmol/L)	Features	<i>n</i>	LL (90% CI) (nmol/L)	UL (90% CI) (nmol/L)	<i>P</i> value [§]	<i>P</i> value [#]
DOC* REP	All	186	<0.236 (<0.236)	0.404 (0.352–0.747)						
MP	All	127	<0.236 (<0.236)	0.308						
B										
REP	norWC	132	1.99 (1.65–2.40)	41.87 (34.31–51.14)	dysWC	52	1.39 (1.03–1.89)	31.16 (22.52–43.21)	0.102	0.204
MP	All	127	1.78 (1.40–2.25)	30.42 (26.28–35.09)						
11S										
REP	norWC	132	0.197 (0.168–0.231)	3.277 (2.681–4.020)	dysWC	52	0.212 (0.168–0.269)	2.891 (2.153–3.911)	0.667	0.568
MP	norTG	112	0.231 (0.186–0.284)	2.679 (2.334–3.065)	dysTG	10	0.574 (0.370–0.862)	2.666 (1.919–3.635)	0.003	0.981
F										
REP	norWC	132	148.2 (135.3–162.2)	566.1 (521.6–611.8)	dysWC	52	112.7 (94.8–133.5)	539.6 (465.1–624.9)	0.020	0.640
MP	norTG	112	158.7 (142.3–176.2)	548.9 (511.7–587.8)	dysTG	10	199.3 (137.4–276.9)	649.7 (510.9–811.0)	0.319	0.261
DHT										
REP	NW and norHDL	65	0.107 (0.093–0.123)	0.618 (0.513–0.749)	OW/OB or dysHDL	76	0.093 (0.081–0.106)	0.553 (0.461–0.666)	0.235	0.484
P4*					OW/OB only	40	0.087 (0.072–0.107)	0.553 (0.426–0.727)	0.178	0.578
MP	All	127	<0.16	0.28						
17OHP4										
MP	norTG	112	0.243 (0.204–0.287)	1.937 (1.711–2.186)	dysTG	10	0.301 (0.150–0.564)	3.001 (1.862–4.677)	0.613	0.141
E1										
MP	norWC and norHOMA	50	0.032 (0.027–0.038)	0.162 (0.137–0.192)	dysWC or dysHOMA	54	0.039 (0.033–0.045)	0.193 (0.164–0.227)	0.201	0.220
E2*					dysWC only	29	0.039 (0.031–0.050)	0.225 (0.177–0.286)	0.279	0.066
MP	All	127	<0.036 (<0.036)	0.088 (0.037–0.252)						

[§]Z-test comparison between dysmetabolic and reference subgroups (LRL vs LL); [#]Z-test comparison between dysmetabolic and reference subgroups (URL vs UL); *LRL and URL calculated as the 2.5th and 97.5th of hormone distribution, respectively.

11S, 11-deoxycortisol; 17OHP4, 17-hydroxyprogesterone; 90CI, 90% confidence interval; B, corticosterone; DOC, 11-deoxycorticosterone; dysHDL, HDL cholesterol level <1.29 mmol/L; dysHOMA, HOMA-IR ≥2.5; dysTG, TG level ≥1.69 mmol/L; dysWC, WC >88 cm; E1, estrone; E2, estradiol; F, cortisol; LL, lower limit; LRL, lower reference limit; MP, menopausal; norHDL, HDL cholesterol level ≥1.29 mmol/L; norHOMA, HOMA-IR <2.5; norTG, TG level <1.69 mmol/L; norWC, WC ≤88 cm; NW, normal weight; OW/OB; overweight/obese; P4, progesterone; REP, reproductive; TG, triglyceride; UL, upper limit; URL, upper reference limit; WC, waist circumference.

Table 5 Upper and lower reference limits of serum steroid levels varying with the menstrual phase. Data are reported in nmol/L. LRL and URL were calculated as the mean $\pm$ 1.96 $\times$ s.d. and mean $\pm$ 1.96 $\times$ s.d. of hormone distribution, respectively.

Steroid hormone	Reference cohort features	Early follicular (day 1–6)			Pre-ovulatory (day 9–13)			Mid-luteal (day 18–24)*		
		n	LRL (90% CI)	URL (90% CI)	n	LRL (90% CI)	URL (90% CI)	n	LRL (90% CI)	URL (90% CI)
P4*	All	31	<0.16	1.41	30	<0.16	7.66	22	13.09	82.14
17OHP4	NW	22	0.405 (0.301–0.538)	2.336 (1.788–3.042)	26	0.447 (0.334–0.608)	5.403 (3.536–8.501)	17	2.855 (2.028–3.693)	7.612 (6.742–8.488)
E1	norTC	20	0.069 (0.057–0.086)	0.367 (0.256–0.556)	25	0.091 (0.021–0.158)	0.516 (0.456–0.576)	17	0.131 (0.088–0.182)	0.523 (0.433–0.622)
E2	norTG	25	0.046 (0.032–0.065)	0.751 (0.464–1.254)	28	0.074 (0.010–0.171)	1.273 (1.048–1.513)	21	0.198 (0.132–0.274)	0.827 (0.701–0.961)

P4, progesterone; 17OHP4, 17-hydroxyprogesterone; E1, estrone; E2, estradiol.

*Women showing P4 levels <10 nmol/L were excluded.

*LRL and URL calculated as the 2.5th and 97.5th of hormone distribution, respectively. LRL, lower reference limit; NW, normal weight; norTG, triglyceride level <5.17 mmol/L; norTC, total cholesterol levels <5.17 mmol/L; URL, upper reference limit.

34, 35, 36). Given the relevance of peripheral androgen generation from adrenal precursors in females, this finding may be due to the combined effect of ovarian and adrenal senescence (30). Interestingly, recent studies showed that *zona reticularis*, but not *zona fasciculata*, undergoes a large involution with aging, thus explaining the impairment of pro-androgen secretion (33, 36).

In our cohort, corticosteroid levels were not influenced by age, however, in keeping with a recent study (33), DOC, 11S and F slightly increased after menopause. This is in contrast with studies reporting an age-dependent reduction of glucocorticoids (31, 32). The modulation of steroid metabolites we observed suggests an increased adrenal secretion with menopause rather than a peripheral reactivation of F (37), as supported by an elegant study demonstrating a slight increase in menopausal F secretion in response to ACTH (30).

When we tried to disentangle which specific metabolic component related to a particular steroid level, we found a different network of relationships before and after menopause. Notably, in our non-hyperandrogenic women, no associations of androgens and precursors with metabolic parameters were found, except for DHT, inversely correlating with BMI and HDL-cholesterol in reproductive age. Unfortunately, the limited sensitivity of our assay prevented a similar DHT evaluation in menopausal women. The link between androgen levels and excess weight in women still has not been clarified. Indeed, a variegated combination of unchanged or reduced levels of DHEA, A4, T and DHT was associated with increasing adiposity in non-PCOS women by studies using high-specificity MS-based assays (31, 33, 34, 36, 38, 39, 40, 41). Interestingly, low plasma DHT was associated with increasing dimension and lipogenesis function of omental adipocytes (38). Our findings, therefore, support the concept that, in obese women, low levels of the most active androgen associate with the lipid storage capacity of visceral depots.

Interestingly, increasing estrogen levels contributed to the healthy lipid profile of our young women, underlying the importance of a balanced orchestration among active sex steroids. Besides, after menopause, E1 inversely associated with insulin resistance and directly associated with waist circumference. While, on the one hand, this is consistent with adipose tissue being the predominant estrogen source after ovarian senescence (13, 28), on the other hand, it contrasts with the detrimental link between abdominal obesity and insulin resistance. The beneficial involvement of estrogens in energy metabolism and inflammatory response was widely described (28, 42),

and is evidenced by the vicious circle among abdominal adiposity, systemic low-grade inflammation, insulin resistance and increasing cardiovascular risk occurring with menopause (12). It is recognized that estrogen levels in menopause are proportional to body fat (13, 28); however, whether visceral or s.c. fat is the predominant source, and whether estrogens are associated with a favorable or unfavorable metabolic status in this life epoch, is still unclear. A study using LC-MS/MS found that visceral fat secreted E1 more than the s.c. fat in menopausal women, however, no correlations with the metabolic profile were performed (43). Another study found stronger associations of plasma estrogens with total rather than abdominal adiposity; moreover, direct associations between estrogens and insulin resistance were found, but these depended on the amount of fat (39). Given this scenario, our findings could be explained by the fact that, as we excluded medicated and diabetic subjects, our menopausal women are overall only mildly dysmetabolic. Unfortunately, in our population study we could not use techniques such as CT to distinguish between abdominal visceral and s.c. depots. Nonetheless, it is possible that fat amount reflected by waist circumference, be it s.c. or visceral, is prevalently metabolically healthy, so that the quantitative relationship between circulating E1, spilling-over from fat depots, and the beneficial result of its function in terms of insulin sensitivity, is still detectable. Unfortunately, due to the limited analytical sensitivity, we could not investigate the metabolic associations of E2 in menopausal age.

Waist circumference was the only independent factor negatively affecting glucocorticoid levels in reproductive age. Moreover, menstrual phase-adjusted 17OHP4 levels, possibly reflecting its role as adrenal glucocorticoid precursor, diminished with increasing BMI, as reported in a recent study (31). This may appear in contrast with the direct link expected between glucocorticoid tone and visceral obesity. Nonetheless, the dynamic of HPA axis in obesity is complex, and early morning F levels were previously found unchanged or inversely related with central adiposity, as in consequence of diminished ACTH-sensitivity of the adrenal, of flattening of HPA circadian rhythmicity, or of increased F clearance (44, 45). At variance, the positive association of glucocorticoids with triglycerides we found in menopausal women is in line with their role in the derangement of energy substrate utilization (46). Notably, we demonstrated that even mild metabolic dysfunctions, such as high waist circumference or triglycerides, can significantly alter the estimation of 11S and F limits, underlying the importance

of a proper metabolic characterization when generating glucocorticoid RI.

Menstrual fluctuation challenges the definition of robust RI for ovarian steroids. A limit of our study is that gonadotropins were not measured, and menstrual classification was performed according to the menstrual date, which may represent a source of bias. We could partially cope with this unpredictable bias in the mid-luteal phase, by excluding women showing P4 levels <10 nmol/L (27). However, we could not assess whether these women were actually misclassified or they had an occasional anovulatory cycle. Similarly, we cannot exclude that misclassification or anovulatory cycles were affecting women in early follicular and pre-ovulatory phases. Therefore, our menstrual phase RI are to be interpreted with cautions, and need to be refined in future studies including gonadotropin evaluation. Nonetheless, values we observed in early follicular phase are in reasonable agreement with previous reports (27, 31, 47). Androgen fluctuation throughout the menstrual cycle is still debated. Skiba *et al.* found slightly higher T and A4 in mid-cycle and luteal compared to follicular phase (34). Bui *et al.* observed minimal T fluctuation, with increasing values at mid-cycle in some women (48). We did not detect any androgen fluctuation, however, we found small but significant variations in DOC and 11S levels among menstrual phases. A proper exploration of this phenomenon would require repeated intra-subject evaluation across the menstrual cycle. Even though data are still inconclusive, we recommend to standardize the menstrual phase when studying the steroid dynamics in young women for clinical or research purposes.

Our steroid values are generally lower compared to other LC-MS/MS studies, except 17OHP5 and DHEA values which are higher, (27, 31, 32, 34, 35, 47, 48, 49, 50). Differences in analytical methods, sampling procedures, cohort selection and study design may account for variabilities in steroid levels and relationships with women's metabolic health. Time of sampling (50) as well as needle stress (51, 52) represents non-negligible sources of variability. In addition, though harmonization of LC-MS/MS measures seems an affordable goal, collective strategies are still awaited in this direction.

In conclusion, our study focusing on a carefully selected female cohort highlighted a different network of relationships between circulating steroid profile, obesity and metabolic status of women in reproductive and menopausal age. RI specific for age, reproductive and menstrual status were generated that will be useful for

the effective interpretation of the steroid involvement in women's health and disease.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-20-1147>.

Declaration of interest

The authors report no conflict of interest in this work. Alessandra Gambineri is on the editorial board of EJE. Alessandra Gambineri was not involved in the review or editorial process for this paper, on which he/she is listed as an author.

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Author contribution statement

M M 1 measured study samples, performed the statistical analysis and wrote the manuscript. A G, G D D, M B, V V and C P performed cohort recruitment and examination. A F and M M 2 measured study samples. U P designed the population study and wrote the manuscript. F F designed the study and wrote the manuscript.

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