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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Mezzullo, M., Gambineri, A., Di Dalmazi, G., Fazzini, A., Magagnoli, M., Baccini, M., et al. (2021). Steroid reference intervals in women: influence of menopause, age and metabolism. EUROPEAN JOURNAL OF ENDOCRINOLOGY, 184(3), 395-407 [10.1530/EJE-20-1147].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/858738> since: 2022-02-15

*Published:*

DOI: <http://doi.org/10.1530/EJE-20-1147>

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Marco Mezzullo, Alessandra Gambineri, Guido Di Dalmazi, Alessia Fazzini, Matteo Magagnoli, Margherita Baccini, Valentina Vicennati, Carla Pelusi, Uberto Pagotto, Flaminia Fanelli, Steroid reference intervals in women: influence of menopause, age and metabolism, *European Journal of Endocrinology*, Volume 184, Issue 3, Mar 2021, Pages 395–407

The final published version is available online at: <https://doi.org/10.1530/EJE-20-1147>

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

Marco Mezzullo, Alessandra Gambineri, Guido Di Dalmazi, Alessia Fazzini, Matteo Magagnoli, Margherita Baccini, Valentina Vicennati, Carla Pelusi, Uberto Pagotto and Flaminia Fanelli

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**Short title:** LC-MS/MS steroid reference interval in women

**Keywords:** liquid chromatography tandem mass spectrometry (LC-MS/MS), steroid profile, reference intervals, obesity, dysmetabolism, menopause, menstrual phase

**Word Count:** 3501

**Number of Tables and Figures:** 6

**Number of Supplemental files:** 2

## Abstract

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

**Design.** Cross-sectional.

**Methods.** 325 drug-free, healthy and eumenorrheic women were selected from the general population. Independent relationships of LC-MS/MS-determined steroid levels with age, body mass index (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 RI were estimated in menstrual phases and menopause. Age- and reproductive status-specific RI 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 RI were provided by LC-MS/MS for a broad steroid panel.

## 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 requires 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 ageing, 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 thirteen 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.

## **Materials 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 and 10:00 am. Waist circumference and body mass index (BMI) were recorded. Systolic (SBP) and diastolic (DBP) blood pressures were measured in supine position after 3 min rest. Inclusion criteria were: BMI  $\geq 18.5$  kg/m<sup>2</sup>, 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 (2000 g, 10 min, room temperature) and serum for LC-MS/MS was

stored at  $-80^{\circ}\text{C}$ . 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 (**Supplemental Table 1**) 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 ( $\geq 2.5$ , 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 ( $\geq 1.69 \text{ mmol/L}$ , dysTG;  $n=15$ ) 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×s.d.) of the transformed variables, respectively, then, values were back-transformed to the original unit [25]. Age-specific RI were estimated by modelling 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:  $eX = \exp \frac{(\log(0.01) \times (X - \min(X)))}{(\max(X) - \min(X))}$ . 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 were 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) (**Supplemental 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 analysed by MedCalc Software (v.18.2.1, Mariakerke, Belgium) except where specified.

## Results

### Anthropometric, metabolic and hormonal features of the cohort

**Table 1** reports the anthropometric, metabolic and hormonal features of our cohort. Compared to reproductive age, menopausal women showed worse BMI, waist circumference, SBP, DBP, glucose, total cholesterol, triglycerides (all  $P<0.001$ ) and HOMA-IR ( $P=0.013$ ), lower 17OHP5 (-31.2%), DHEA (-42.6%), P4 (-84.4%), 17OHP4 (-60.1%), A4 (-51.3%), T (-22.5%), DHT (-23.1%), E1 (-63.6%) and E2 (-87.4%) (all  $P<0.001$ ), but higher DOC (n.d.,  $P=0.044$ ), 11S (18.2%,  $P=0.029$ ) and F (11.3%,  $P=0.007$ ) (**Table 1**).

Worsening metabolic parameters at increasing BMI were observed both in reproductive age (min-max BMI: 18.5-42.9 kg/m<sup>2</sup>) and menopausal women (min-max BMI: 18.9-41.2 kg/m<sup>2</sup>). 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  $P<0.001$ ) (**Supplemental 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 NW and norHDL women in reproductive age and all menopausal women ( $n=65$  and  $n=127$ , respectively). Androgens and precursors peak around age 25 years and progressively decline. 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 (**Figure 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  $39.2\pm 10.3$  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; and 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**).

## 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; 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 ageing, 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 subcutaneous fat is the predominant source, and whether estrogens are associated with a favourable or unfavourable metabolic status in this life epoch, is still unclear. A study using LC-MS/MS found that visceral fat secreted E1 more than the subcutaneous 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 computed tomography to distinguish between abdominal visceral and subcutaneous depots. Nonetheless, it is possible that fat amount reflected by waist circumference, be it subcutaneous 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 dysfunction, such as high waist circumference or triglycerides, can significantly alter the estimation of IIS 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 [47]. 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; 48]. 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 [49]. 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; 48; 50; 51; 52]. 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 [52] as well as needle stress [53; 54] represent 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.

**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.

**Funding:** the study was supported by the European Union (REPROBESITY, FPVII-223713), by the Emilia-Romagna Region – University Program 2007-2009 (g.a. PRUa1a-2007-006), by the Emilia-Romagna Region, Alessandro Liberati Young Researcher Grants (g.a. PRUA 1-2012-004).

**Author contribution:** MM1 measured study samples, performed the statistical analysis and wrote the manuscript; AG, GDD, MB, VV and CP performed cohort recruitment and examination; AF and MM2

measured study samples; UP designed the population study and wrote the manuscript; FF designed the study and wrote the manuscript.

**Acknowledgements:** we thank Shimadzu for kindly providing the LC-MS/MS platform used to generate part of the data used in this study.

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## Figure Legend

**Figure 1.** Distribution of steroid hormone serum levels by age.

Continuous lines: lower and upper reference limits; dashed lines: 90% confidence intervals.

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

	Women in reproductive age					Women in menopausal age					P†
	All	Normal weight	Overweight	Obese	ANOVA trend, P	All	Normal weight	Overweight	Obese	ANOVA trend, P	
<i>n</i>	186	123	35	28		127	63	49	15		
Age, years	38.2±8.4	37.1±8.6	40.4±8.0	41.0±6.8	0.186	60.0±7.8	58.2±7.4	62.4±8.2	59.4±6.9	0.515	<0.001
Body mass index, kg/m <sup>2</sup>	21.8±5.3	20.4±1.7	25.8±1.4 <sup>a</sup>	33.8±3.7 <sup>a,b</sup>	<0.001	23.4±3.9	21.6±1.4	25.8±1.4 <sup>a</sup>	32.2±3.4 <sup>a,b</sup>	<0.001	<0.001
Waist circumference, cm	81.1±12.8	75.8±6.7	88.2±8.8 <sup>a</sup>	104.7±9.2 <sup>a,b</sup>	<0.001	86.8±9.8	81.5±7.1	90.0±5.6 <sup>a</sup>	104.7±8.2 <sup>a,b</sup>	<0.001	<0.001
Systolic blood pressure, mmHg	117.5±14.8	112.5±10.6	125.6±17.2 <sup>a</sup>	124.6±15.3 <sup>a</sup>	<0.001	130.7±17.6	129.1±15.1	129.7±17.7	140.7±22.2	0.027	<0.001
Diastolic blood pressure, mmHg	77.5±8.6	74.9±8.1	81.7±7.9 <sup>c</sup>	80.7±8.5 <sup>a</sup>	<0.001	82.8±8.3	81.7±7.0	81.7±7.8	88.8±11.2 <sup>c,d</sup>	0.003	<0.001
Glucose, mmol/L	4.53±0.73	4.42±0.67	4.45±0.75	5.11±0.74 <sup>d</sup>	<0.001	4.87±0.80	4.72±0.79	4.93±0.77	5.08±0.88	0.114	<0.001
Insulin, μU/mL	6.2±4.0	5.1±2.4	7.1±3.2 <sup>a</sup>	10.0±5.3 <sup>a,c</sup>	<0.001	7.1±4.1	5.9±2.5	7.7±4.3 <sup>c</sup>	9.5±5.3 <sup>a</sup>	<0.001	0.052
HOMA-IR	1.29±0.95	1.04±0.55	1.34±0.68 <sup>a</sup>	2.24±1.24 <sup>a,b</sup>	<0.001	1.55±1.12	1.27±0.65	1.67±1.19 <sup>f</sup>	2.14±1.50 <sup>e</sup>	<0.001	0.013
Total cholesterol, mmol/L	4.52±0.82	4.41±0.80	4.79±0.92	4.67±0.73	0.133	5.39±0.84	5.30±0.95	5.50±0.76	5.37±0.58	0.677	<0.001
HDL-cholesterol, mmol/L	1.50±0.37	1.57±0.35	1.51±0.31	1.31±0.44 <sup>a,d</sup>	<0.001	1.57±0.39	1.65±0.40	1.53±0.39	1.46±0.31	0.089	0.117
Triglycerides, mmol/L	0.70±0.34	0.64±0.22	0.80±0.38 <sup>a</sup>	0.86±0.53 <sup>a</sup>	<0.001	0.94±0.47	0.81±0.40	1.10±0.53	0.97±0.30	0.253	<0.001
17-Hydroxypregnenolone (17OHP5), nmol/L	5.51±7.22	5.64±7.31	6.97±8.13	3.84±4.97	0.087	3.79±3.77	3.51±2.77	4.38±4.72	3.35±3.48	0.815	<0.001
Dehydroepiandrosterone (DHEA), nmol/L	15.50±14.60	15.64±14.20	17.11±14.52	13.16±16.65	0.324	8.90±6.35	8.94±5.30	9.09±7.42	8.19±6.83	0.644	<0.001
Progesterone (P4), nmol/L	1.93±0.91	1.91±16.81	2.02±13.23	1.87±11.52	0.364	0.17±0.07	0.17±0.04	0.22±0.03	0.21±0.01	0.067	<0.001 <sup>§</sup>
17-Hydroxyprogesterone (17OHP4), nmol/L	1.776±1.887	1.873±2.039	1.834±1.566	1.362±1.393	0.142	0.710±0.466	0.631±0.391	0.856±0.541	0.635±0.339	0.968	<0.001
11-Deoxycorticosterone (DOC), nmol/L	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	0.133 <sup>#</sup>	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236)	0.550 <sup>#</sup>	0.044 <sup>§</sup>
Corticosterone (B), nmol/L	8.15±9.56	8.94±9.74	8.61±10.50	5.07±5.83 <sup>b,c</sup>	<0.001	8.49±7.14	7.79±7.29	10.31±7.29	6.35±4.16	0.313	0.662
11-Deoxycortisol (11S), nmol/L	0.755±0.710	0.743±0.743	0.956±0.689	0.608±0.517	0.338	0.892±0.605	0.817±0.559	1.002±0.655	0.892±0.592	0.613	0.029
Cortisol (F), nmol/L	286.4±105.8	296.3±102.2	292.3±118.7	241.1±94.7 <sup>b,f</sup>	0.008	318.9±101.9	302.3±93.7	351.5±104.2	283.5±104.8	0.469	0.007
Androstenedione (A4), nmol/L	2.34±1.09	2.40±1.09	2.48±1.21	1.96±0.85	0.369	1.14±0.54	1.06±0.50	1.21±0.53	1.27±0.67	0.165	<0.001
Testosterone (T), nmol/L	0.768±0.328	0.790±0.326	0.730±0.325	0.717±0.342	0.154	0.596±0.309	0.552±0.256	0.684±0.368	0.524±0.219	0.700	<0.001
Dihydrotestosterone (DHT), nmol/L	0.183±0.128	0.193±0.124	0.191±0.155	0.151±0.079 <sup>f</sup>	0.006	0.140±0.080	0.142±0.063	0.139±0.076	0.139±0.154	0.597	<0.001
Estrone (E1), nmol/L	0.212±0.116	0.216±0.118	0.201±0.122	0.209±0.099	0.319	0.077±0.037	0.069±0.032	0.083±0.038 <sup>f</sup>	0.098±0.044 <sup>c</sup>	0.002	<0.001
Estradiol (E2), nmol/L	0.292±0.277	0.323±0.274	0.238±0.300	0.251±0.246	0.334	0.037±0.022	0.037±0.028	0.036±0.008	0.038±0.023	0.640	<0.001

HOMA-IR: homeostatic model assessment insulin resistance; HDL: high density lipoprotein. <sup>a</sup> vs NW, P<0.001; <sup>b</sup> vs OW, P<0.001; <sup>c</sup> vs NW, P<0.010; <sup>d</sup> vs OW, P<0.050; <sup>e</sup> vs OW P<0.010; <sup>f</sup> vs NW, P<0.050; <sup>#</sup> Kruskal-Wallis test. <sup>§</sup> Mann-Whitney test; †Reproductive vs Menopausal by T=test

**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	0.05	0.038 (+)
A4														
Reproductive	0.28	<0.001 (-)		NS		NS		NS		NS		NS		NS
Menopausal		NS		NS		NS		NS		NS		NS		NS
TS														
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	0.10	0.033 (-)		NS		NS
Menopausal		NS		NS	0.16	<0.001 (+)	0.08	0.012 (-)		NS		NS		NS
E2														
Reproductive*		NS		NS		NS		NS		NS		NS	0.15	0.011 (-)

BMI: body mass index; HOMA-IR: homeostatic model assessment insulin resistance; HDL: high density lipoprotein; NS, not significant. \*data adjusted by the menstrual phase.

17OHP5, 17-Hydroxypregnenolone; DHEA, Dehydroepiandrosterone; P4, Progesterone; 17OHP4, 17-Hydroxyprogesterone; B, Corticosterone; 11S, 11-Deoxycortisol; F, Cortisol; A4, Androstenedione; TS, testosterone; DHT, Dihydrotestosterone; E1, Estrone; E2, Estradiol; WC, waist circumference; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglycerides

**Table 3.** Reference intervals of serum androgens and pro-androgens according to age- and menopausal status.

Age (y)/Status	17-Hydroxypregnenolone (17OHP5)		Dehydroepiandrosterone (DHEA)		Androstenedione (A4)		Testosterone (T)		Dihydrotestosterone (DHT)	
	LRL (90CI)	URL (90CI)	LRL (90CI)	URL (90CI)	LRL (90CI)	URL (90CI)	LRL (90CI)	URL (90CI)	LRL	URL (90CI)
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 (1.418-2.086)	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)	≤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)	≤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)	≤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)	≤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)	≤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)	≤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)	≤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)	≤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)	≤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)	≤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)	≤0.135	0.141 (0.070-0.247)
Menopausal	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 (0.222-0.276)	1.444 (1.280-1.629)	≤0.135*	0.462*

LRL: lower reference limit; 90CI: 90% confidence interval; URL: upper reference limit; ND: not determined because of insufficient data points. Data are reported in nmol/L. LRL and URL were calculated as the mean - 1.96xSD and mean + 1.96xSD of hormone distribution, respectively, according to the fractional polynomial regression by Royston and Wright. \* LRL and URL calculated as the 2.5<sup>th</sup> and 97.5<sup>th</sup> of hormone distribution, respectively.



**Table 4.** Reference intervals of age-independent steroid circulating levels in the reference sub-cohorts, and comparison with limits in dysmetabolic sub-cohorts.

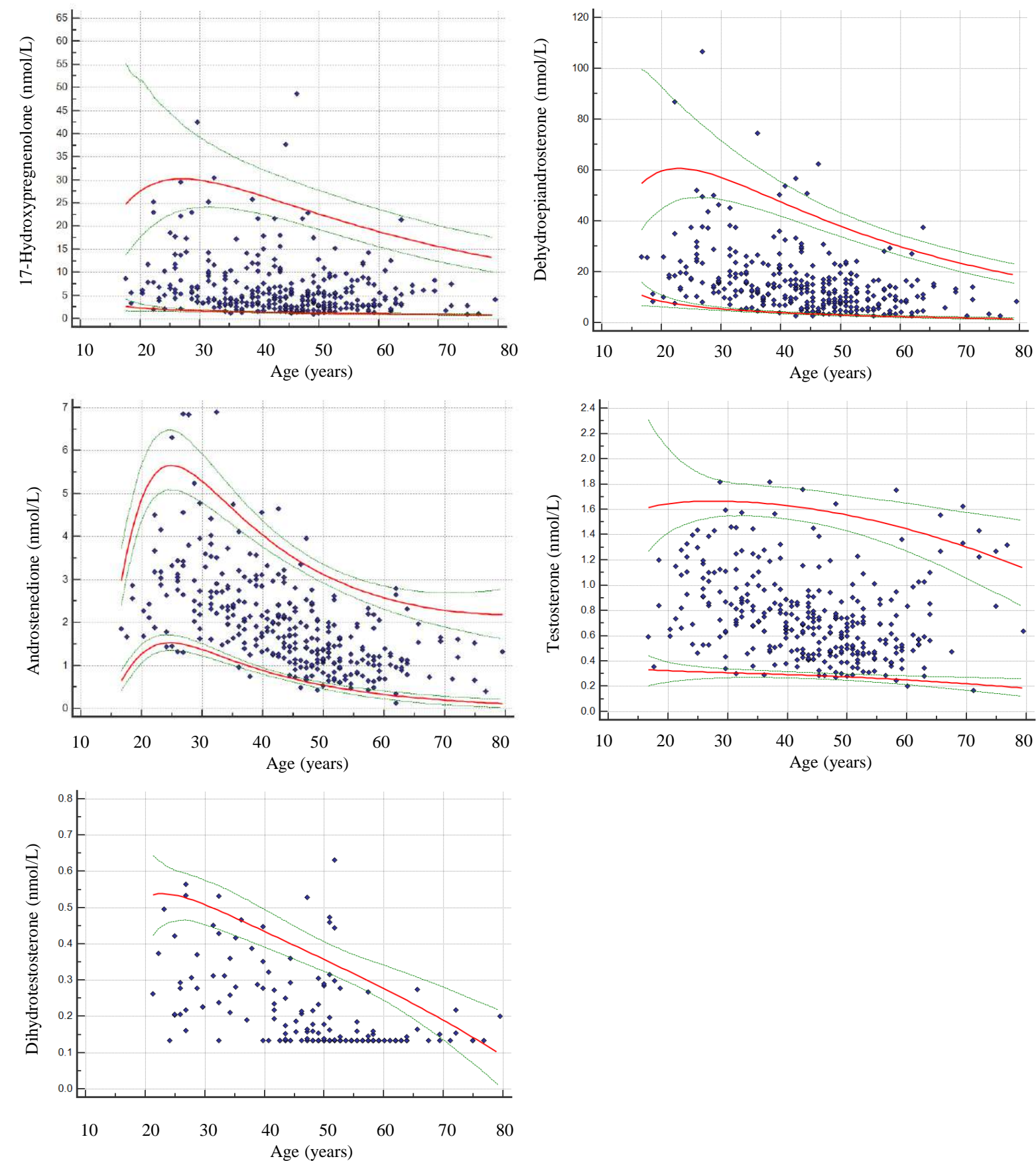
Steroid hormone	Status	Reference sub-cohort				Dysmetabolic sub-cohort					
		Features	n	LRL (90CI) (nmol/L)	URL (90CI) (nmol/L)	Features	n	LL (90CI) (nmol/L)	<i>P</i> value <sup>§</sup>	UL (90CI) (nmol/L)	<i>P</i> value <sup>#</sup>
11-Deoxycorticosterone (DOC)*	Reproductive	all	186	<0.236 (<0.236)	0.404 (0.352-0.747)						
	Menopausal	all	127	<0.236 (<0.236)	0.308						
Corticosterone (B)	Reproductive	norWC	132	1.99 (1.65-2.40)	41.87 (34.31-51.14)	dysWC	52	1.39 (1.03-1.89)	0.102	31.16 (22.52-43.21)	0.204
	Menopausal	all	127	1.78 (1.40-2.25)	30.42 (26.28-35.09)						
11-Deoxycortisol (11S)	Reproductive	norWC	132	0.197 (0.168-0.231)	3.277 (2.681-4.020)	dysWC	52	0.212 (0.168-0.269)	0.667	2.891 (2.153-3.911)	0.568
	Menopausal	norTG	112	0.231 (0.186-0.284)	2.679 (2.334-3.065)	dysTG	10	0.574 (0.370-0.862)	0.003	2.666 (1.919-3.635)	0.981
Cortisol (F)	Reproductive	norWC	132	148.2 (135.3-162.2)	566.1 (521.6-611.8)	dysWC	52	112.7 (94.8-133.5)	0.020	539.6 (465.1-624.9)	0.640
	Menopausal	norTG	112	158.7 (142.3-176.2)	548.9 (511.7-587.8)	dysTG	10	199.3 (137.4-276.9)	0.319	649.7 (510.9-811.0)	0.261
Dihydrotestosterone (DHT)	Reproductive	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)	0.235	0.553 (0.461-0.666)	0.484
						OW/OB only	40	0.087 (0.072-0.107)	0.178	0.553 (0.426-0.727)	0.578
Progesterone (P4)*	Menopausal	all	127	<0.16	0.28						
17-Hydroxyprogesterone (17OHP4)	Menopausal	norTG	112	0.243 (0.204-0.287)	1.937 (1.711-2.186)	dysTG	10	0.301 (0.150-0.564)	0.613	3.001 (1.862-4.677)	0.141
Estrone (E1)	Menopausal	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)	0.201	0.193 (0.164-0.227)	0.220
						dysWC only	29	0.039 (0.031-0.050)	0.279	0.225 (0.177-0.286)	0.066
Estradiol (E2)*	Menopausal	all	127	<0.036 (<0.036)	0.088 (0.037-0.252)						

LRL: lower reference limit; 90CI: 90% confidence interval; URL: upper reference limit; norWC: waist circumference  $\leq 88$  cm; dysWC: waist circumference  $> 88$ cm; norTG: triglyceride level  $< 1.69$  mmol/L; dysTG: triglyceride level  $\geq 1.69$  mmol/L; NW: normal weight; norHDL: high density lipoprotein cholesterol level  $\geq 1.29$  mmol/L; OW/OB: overweight/obese; dysHDL: high density lipoprotein cholesterol level  $< 1.29$  mmol/L; norHOMA: homeostatic model assessment insulin resistance  $< 2.5$ ; dysHOMA: homeostatic model assessment insulin resistance  $\geq 2.5$ . LRL and URL were calculated as the mean - 1.96xSD and mean + 1.96xSD of hormone distribution, respectively. \* LRL and URL calculated as the 2.5<sup>th</sup> and 97.5<sup>th</sup> of hormone distribution, respectively. *P* values refer to Z-test comparison of LRL (§) and URL (#) between dysmetabolic and reference subgroups.

**Table 5.** Upper and lower reference limits of serum steroid levels varying with the menstrual phase.

Steroid hormone	Reference cohort features	n	Early follicular (day 1-6)		n	Pre-ovulatory (day 9-13)		n	Mid-luteal (day 18-24) <sup>#</sup>	
			LRL (90CI)	URL (90CI)		LRL (90CI)	URL (90CI)		LRL (90CI)	URL (90CI)
Progesterone (P4)*	all	31	<0.16	1.41	30	<0.16	7.66	22	13.09	82.14
17-Hydroxyprogesterone (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)
Estrone (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)
Estradiol (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)

LRL: lower reference limit; 90CI: 90% confidence interval; URL: upper reference limit. Data are reported in nmol/L. NW: normal weight; norTC: total cholesterol levels <5.17 mmol/L; norTG: triglyceride level <5.17 mmol/L. LRL and URL were calculated as the mean - 1.96xSD and mean + 1.96xSD of hormone distribution, respectively. <sup>#</sup> Women showing P4 levels <10 nmol/L were excluded. \* LRL and URL calculated as the 2.5<sup>th</sup> and 97.5<sup>th</sup> of hormone distribution, respectively.

**Figure 1.** Distribution of steroid hormone serum levels by age.

Continuous lines: lower and upper reference limits; dashed lines: 90% confidence intervals.

**Supplemental Table 1.** Functional sensitivity limits by the LC-MS/MS assays used in the present study.

Steroid analyte	Abbreviation	Sensitivity limit (nmol/L)	Intra-assay CV%	Inter-assay CV%	Trueness %	Accuracy vs certified QC*	Assay	Ref.
17-Hydroxypregnenolone	17OHP5	0.117	3–4	7–9	89–114		2	21
Dehydroepiandrosterone	DHEA	2.71	7–8	8–10	95–102		1	18
Progesterone	P4	0.156	5–8	6–11		84–92	1	18
17-Hydroxyprogesterone	17OHP4	0.236	4–5	5–9		101–104	1	18
11-Deoxycorticosterone	DOC	0.236	5–6	6–9	100–104		1	18
Corticosterone	B	0.903	2–6	5–10	92–98		1	18
11-Deoxycortisol	11S	0.226	3–8	2–8	99–106		1	18
Cortisol	F	0.673	2–3	5–8		94–104	1	18
Androstenedione	A4	0.136	7–10	10–11	86–101		1	18
Testosterone	T	0.066	3–4	4–7		97–100	1	18
Dihydrotestosterone	DHT	0.134	4–6	3–9	81–112		2	21
Estrone	E1	0.018	2–3	3–9	83–111		2	21
Estradiol	E2	0.036	3–6	5–7	84–113	92–108	2	21

\* Quality control materials provided the Reference Institute for Bioanalytics.

The absence of interference was evaluated from prednisone, prednisolone, triamcinolone acetonide, methylprednisolone, dexamethasone, betamethasone, cortisone, 21-deoxycortisol, 20 $\alpha$ -dihydrocortisone, 20 $\beta$ -dihydrocortisone, epitestosterone, DHEA-sulfate, 16-hydroxyprogesterone, 11-hydroxyprogesterone, 17-hydroxypregnenolone and pregnenolone for assay 1, and from cortisol, DHEA, testosterone, epitestosterone, pregnenolone, progesterone, 17-hydroxyprogesterone and androstenedione for assay 2.

**Supplemental Table 2.** Anthropometric, metabolic and hormonal features of women sub-classified according to the menstrual phase.

	Early follicular (n=31)	Pre-ovulatory (n=30)	Mid-luteal (n=33)	ANOVA, P Value*
Age, years	39.8±8.7	39.2±8.2	38.7±8.3	0.845
Body mass index, kg/m <sup>2</sup>	22.8±4.2	22.2±2.4	23.7±6.1	0.217
Waist circumference, cm	80.7±10.4	78.0±8.9	80.5±13.4	0.516
Systolic blood pressure, mmHg	116.3±12.1	112.2±9.0	116.4±19.4	0.497
Diastolic blood pressure, mmHg	77.5±8.0	77.0±7.7	78.0±9.5	0.956
Glucose, mmol/L	4.48±0.83	4.36±0.74	4.57±0.51	0.601
Insulin, µU/mL	5.4±2.9	5.0±2.2	6.4±3.6	0.176
HOMA-IR	1.07±0.76	0.99±0.51	1.34±0.82	0.122
Total cholesterol, mmol/L	4.70±0.89	4.34±0.68	4.60±0.76	0.165
HDL-cholesterol, mmol/L	1.51±0.42	1.60±0.28	1.47±0.30	0.453
Triglycerides, mmol/L	0.70±0.38	0.60±0.26	0.69±0.33	0.150
17-Hydroxypregnenolone (17OHP5), nmol/L	6.51±9.30	4.89±9.15	6.16±5.52	0.302
Dehydroepiandrosterone (DHEA), nmol/L	18.2±12.2	13.7±12.6	16.0±10.1	0.178
Progesterone (P4), nmol/L	0.36±0.30	0.44±1.65	11.67±19.23 <sup>ab</sup>	<0.001
17-Hydroxyprogesterone (17OHP4), nmol/L	0.998±0.661	1.356±1.101	3.126±1.967 <sup>ab</sup>	<0.001
11-Deoxycorticosterone (DOC), nmol/L	<0.236 (<0.236)	<0.236 (<0.236)	<0.236 (<0.236) <sup>c</sup>	0.043 <sup>#</sup>
Corticosterone (B), nmol/L	8.99±9.89	6.19±9.65	8.92±11.59	0.135
11-Deoxycortisol (11S), nmol/L	0.821±0.736	0.591±0.555	0.878±0.774	0.048
Cortisol (F), nmol/L	300.3±101.7	253.6±107.9	292.3±115.6	0.167
Androstenedione (A4), nmol/L	2.25±1.45	2.22±0.73	2.44±0.95	0.585
Testosterone (T), nmol/L	0.691±0.330	0.755±0.279	0.741±0.334	0.659
Dihydrotestosterone (DHT), nmol/L	0.149±0.131	0.147±0.132	0.135±0.113	0.487
Estrone (E1), nmol/L	0.149±0.062	0.264±0.119 <sup>a</sup>	0.238±0.119 <sup>a</sup>	<0.001
Estradiol (E2), nmol/L	0.179±0.180	0.482±0.310 <sup>a</sup>	0.373±0.269 <sup>a</sup>	<0.001

Data are presented as the back-transformed means of the transformed variables ± SD, except 11-deoxycorticosterone which is shown as median (75° centile). Blood was withdrawn between 8:00–10:00 am after overnight fasting. HOMA-IR: homeostatic model assessment insulin resistance; HDL: high density lipoprotein. <sup>a</sup> vs follicular phase,  $P<0.001$ ; <sup>b</sup> vs pre-ovulatory phase,  $P<0.001$ ; <sup>c</sup> vs pre-ovulatory phase,  $P<0.050$ . # Kruskal-Wallis test.