Reliability of Serum Hormones in Premenopausal and Postmenopausal Women over a One-Year Period[1]

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Abstract

Serum hormones have been intensively investigated in association with several chronic diseases, but limited information exists on the reliability of a number of hormone determinations. The one-year reproducibility of dehydroepiandrosterone sulfate (DHEAS), total and free testosterone, total estradiol, insulin, C-peptide, and prolactin was studied in 60 premenopausal and 47 postmenopausal women recruited in Varese province, Italy, 1991–1992. The hormonal determinations were made in blood samples collected twice, one year apart, after 12-h fast, in the same month, day, and hour and for premenopausal women on the same day of the luteal phase of the menstrual cycle. Samples from the first drawing were stored at −80° C. Samples from both drawings were assayed simultaneously and in blind fashion. Total estradiol in postmenopause was not evaluated for limitation in the sensitivity of the laboratory method. The intraclass correlation coefficient in premenopausal women was 0.85 for DHEAS, 0.60 for total testosterone, 0.66 for free testosterone, 0.81 for insulin, 0.83 for C-peptide, 0.40 for prolactin, and 0.06 for total estradiol. In postmenopausal women, the coefficient was 0.90 for DHEAS, 0.88 for total testosterone, 0.71 for free testosterone, 0.67 for insulin, 0.73 for C-peptide, and 0.18 for prolactin. These data indicate that total estradiol measured during the luteal phase has a poor intraindividual reproducibility over time, and these findings may have important implications in studies of hormones in the etiology of chronic disease.

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Introduction

Numerous epidemiological investigators have analyzed the relationship between hormones, in particular serum sex steroids, and chronic disease such as breast and endometrial cancer (1, 2), cardiovascular disease (3), and osteoporosis (4) in women. The majority of these studies relied on single hormone measurements, and there is, however, very limited information about the intraindividual variability of these parameters. Intraindividual variability is a crucial factor in the design and investigation of epidemiological and clinical studies. Exposures characterized by large intraindividual variability require multiple measurements to reduce error in exposure determination. The present study was carried out to estimate the within-subject variability of a number of blood hormone determinations, over a one-year period, in premenopausal and postmenopausal women. The study design controlled for several sources of biological, hormonal (*i.e.*, the circadian and monthly rhythm), and laboratory variability. The hormones analyzed in the report include: DHEAS,[3] total and free testosterone, total estradiol, insulin, C-peptide, and prolactin.

Materials and Methods

Subjects. Participants for the present study were selected from members of a large prospective cohort study on hormones and diet in the etiology of breast cancer conducted in northern Italy (5). The cohort consists of 10,788 female residents of the Varese province, recruited between 1987–1991, and inclusion criteria were as follows: ages 35 to 69, no history of cancer, no bilateral ovariectomy and no use of hormonal drugs, and no pregnancy and breastfeeding in the 6 months prior to enrollment. From this cohort, 229 participants were subsequently recruited between November 1990 and April 1991. One hundred thirty-eight were in premenopause (60.3% of the participants) and 91 (39.7% of the participants) were in postmenopause at the time of recruitment. Premenopausal women were defined as women aged between 35 and 45 years and characterized by regular menstrual cycles (not shorter than 20 days and not longer than 39 days, with the last period occurring at least 23 days before the blood drawing). Postmenopausal women were defined as women aged 55 and 69 years and without periods for at least 12 months before the blood drawing. Participants were again contacted after 1 year from the initial visit for a second visit and blood drawing. Exclusion criteria were as follows: pregnancy or breastfeeding (three women), diagnosis of cancer (one premenopausal woman), and change in menopausal status between the first and the second visit (one woman). In addition, 53 participants (30 women in premenopause and 23 in postmenopause) were excluded from the reliability study because they were not able to return for the

[3] The abbreviation used is: DHEAS, dehydroepiandrosterone sulfate.

blood drawing at the same hour and the same day of the month (or on the same day of the luteal phase of the cycle in premenopause) in which the first blood drawing was performed. Additionally, some participants were not able to be included in the reliability study for the following reasons: 27 (23 premenopausal and 4 postmenopausal women) could not be recontacted, and 10 refused to participate in the second visit (3 premenopausal and 7 postmenopausal women). Furthermore, for 27 participants (17 women in premenopause and 10 in postmenopause) the complete set of the hormonal determinations could not be performed because of an insufficient amount of blood.

A total of 60 premenopausal and 47 postmenopausal women participated at both examinations and were included in the analysis. At the time of the second interview, the women repeated all of the study procedures according to the original study protocol. Alcohol consumption was ascertained as part of a semiquantitative food frequency questionnaire, and smoking status and number of cigarettes by questionnaire. Anthropometric measurements were taken by trained nurses according to a standardized protocol. Body weight was measured in kilograms, using a beam balance scale, with participants wearing light clothes and no shoes. Height was taken with a millimeter pole during a deep inhalation. Body mass index was computed as weight in kilograms divided by height in meters squared.

Blood Sampling. Both first and second blood drawings were performed 1-year apart on the same month, on the same day of the month, and at the same hour and minute of the day between 8:00 and 9:00 a.m. after approximately 12 h of fasting. In premenopausal women, the two blood drawings were taken on the same day of the luteal phase of the cycle, between the 20th and the 24th day, and in the same month. The blood samples were protected against light and kept at +4° C until they reached the laboratory (within 3 h). After processing and aliquoting, samples were stored at −80° C. Samples collected at the initial visit were stored at −80° C during the interval between the first and the second blood drawings, which ranged from 357 to 412 days. Samples collected during the second visit were stored at −80° C for a maximum of 117 days in premenopausal and 83 days in postmenopausal women before the hormonal determinations were performed. Samples from both visits were taken out simultaneously from the freezer and sent in the same parcel on dry ice to the laboratory for the hormonal determinations.

Laboratory Methods. For each participant, samples from the two blood drawings were assayed simultaneously in a blind fashion and by the same laboratory technician. DHEAS was measured by RIA using tritiated tracer, dextran-coated charcoal for free/bound separation, and highly specific antisera directly on diluted serum according to Buster and Abrahm (6). The antiserum (anti-DHEA-3-succinyl~BSA) had 0.4% and less than 0.1% cross-reactivity for androsterone and etiocholanolone sulfate, respectively. Total testosterone was assayed with a non-extraction, double-antibody RIA kit from Sorin Biomedica (Saluggia, Italy). Free testosterone was assayed by coated-tube RIA with a "coat-a-counts" kit from Diagnostic Products Corporation, Medical System (Genova, Italy). Total estradiol was measured with a RIA kit supplied by Ares Serono Diagnostics (Milan, Italy) with a secondary antibody supported on magnetizable particles as separating agent. Insulin and C-peptide were assayed with a non-extraction RIA kit from Ares Serono Diagnostics using a polyethylene glycol solution for the free/bound separation. Prolactin was measured with a "Maiaclone" kit supplied by Ares Serono Diagnostics based on a immunoradiometric assay technique with monoclonal antibodies and secondary antibody supported on magnetizable particles as separating agent. The intra- and interassay analytical variability, expressed as coefficient of variation (*CV%*) was measured during the study period by using a commercial lyophilized control serum at three concentration levels (Lyphochek; Bio-Rad, Milan, Italy). Quality control samples were added to unknown samples at the beginning, middle, and at the end of each single run to observe if drifts were present during the assay. No drift of the control values was observed, indicating the absence of a systematic error during the experiment measurements. The results are presented in Table 1.

As expected, in postmenopausal women, serum concentration of total estradiol reached the limit of sensitivity of the analytical method, and most of the women presented values of 55 pmol/liter (78.7% in the first blood samples and 93.6% in the second samples). Therefore, total estradiol was excluded from the reliability analyses in this group of women.

Statistical Methods. The hormonal values were logarithmically transformed for statistical analysis. Mean and standard deviation for all the variables considered were analyzed according to menopausal status. *t* test for unpaired data was applied to test the difference between premenopausal and postmenopausal women, and the intraclass correlation (*R*) was used to analyze the reliability of the hormone determinations (7). To indicate the limits of uncertainty concerning the degree of reliability, the lower limit of the 95% confidence interval of the intraclass correlation coefficients was computed, whereas the upper limit of the intraclass correlation coefficients was set at 1.0 (7). The minimum number of replicate determinations which would be necessary to correctly estimate the hormone level after setting a desired reliability level at 0.90 was computed using the Spearman-Brown formula (7). The degree of linear association between the first and the second measurement was analyzed by their product-moment Pearson's *r* correlation coefficient. To test the reliability of individual categorical classification, hormones were transformed to ordinal variables. The rank distribution of the second blood determination was compared with the rank distribution of the first blood sample using Kendall's tau (τ). The effects of changes between the first and the second visit in weight, alcohol consumption, and number of cigarettes smoked daily as potential determinants of changes in hormonal values between the two blood drawings were analyzed through multiple linear regression and found to be nonsignificant; therefore, they will not be reported. The software package used was the Statistical Package for Social Sciences (8).

Results

Table 2 summarizes the characteristics of premenopausal and postmenopausal women as determined at the first visit. Postmenopausal women were heavier and shorter than premenopausal women. Furthermore, premenopausal women had menarche and their first pregnancy at an older age than postmenopausal women. The differences in weight, alcohol consumption, and number of cigarettes between the first and the second visit were small and did not reach statistical significance.

Tables 3 and 4 show the mean (± SD) of serum hormones at the first and the second blood drawing, as well as the intraclass correlation coefficient, Pearson's coefficient, and Kendall's tau in premenopausal and postmenopausal women, respectively. Steroid hormones and prolactin had higher concentration in premenopausal than in postmenopausal women, with this difference reaching statistical significance at both determinations for estradiol and DHEAS. In both blood sam-

Table 1 Study on the analytical variability of hormone assays

Hormone	Mean concentration of control serum	Intra-assay variability		Inter-assay variability	
		N[a]	CV, (%)[b]	N[a]	CV, (%)[b]
DHEA-S	0.84 μmol	12	22.5	4	8.4
(RIA-tritiated)	4.82 μmol	12	7.9	4	4.2
In-house method	13.1 μmol	12	7.4	4	7.1
Total testosterone	2.88 nmol	12	8.5	4	5.3
(RIA-Sorin)	13.8 nmol	12	8.7	4	1.5
	55.9 nmol	12	6.0	4	12.8
Free testosterone	7.01 pmol	12	14.2	4	2.5
(RIA-DPC)	19.8 pmol	12	8.7	4	0.9
	86.4 pmol	12	4.7	4	7.3
Estradiol	245 pmol	16	13.7	4	5.0
(RIA-Ares Serono)	580 pmol	16	9.1	4	6.4
	118 pmol	16	7.7	4	7.8
Insulin	9.63 μIU/ml	20	7.3	4	9.9
(RIA-Ares Serono)	41.8 μIU/ml	20	5.8	4	4.9
	169 μIU/ml	20	6.4	4	5.0
C-Peptide	1.04 ng/ml	12	3.2	4	29.1
(RIA-Ares Serono)	2.17 ng/ml	12	4.6	4	35.2
	7.33 ng/ml	12	4.7	4	20.1
Prolactin (IRMA[c]-Serono)	11.8 ng/ml	20	6.2	4	5.8
	23.6 ng/ml	20	6.6	4	4.5
	72.6 ng/ml	20	4.7	4	2.8

[a] Number of determinations.
[b] Coefficient of variation.
[c] Immunoradiometric assay.

Table 2 Descriptive characteristics of the premenopausal and postmenopausal women at the first visit and differences in body weight, alcohol consumption, and number of cigarettes between the first and the second visit

	Premenopause (60 women) Mean ± SD	Postmenopause (47 women) Mean ± SD	Pre *versus* Post[a]
Age	40.1 (±3.1)	59.3 (±4.3)	
Age at menarche	12.4 (±1.3)	13.2 (±1.6)	NS
Age at first pregnancy	24.7 (±3.9)	25.6 (±4.1)	NS
Age at menopause		50.3 (±5.0)	
Number of children	1.8 (±0.09)	2.3 (±1.3)	NS
Weight (kg)	60.8 (±9.3)	66.2 (±10.3)	[b]
Height (m)	1.58 (±0.04)	1.56 (±0.08)	[c]
Body Mass Index (kg/m^2)	24.2 (±3.4)	27.00 (±3.8)	[b]
Difference[d] in weight (kg)	0.04 (±4.5)	0.2 (±1.9)	NS
Difference[d] in alcohol intake (gr)	2.86 (±4.4)	4.85 (±4.2)	NS
Difference[d] in number of cigarettes	4.00 (±4.65)[e]	1.00 (±0.00)[f]	NS

[a] *t* test for unpaired data. NS, not significant.
[b] $P < 0.000$.
[c] $P < 0.05$.
[d] Differences between the first and the second visit.
[e] Based on five smokers.
[f] Based on one smoker.

ples, the difference between premenopausal and postmenopausal women for total and free testosterone and prolactin reached statistical significance only in the first blood sample ($P < 0.05$). Insulin and C-peptide had higher levels in postmenopause than in premenopause (with the exception of C-peptide in the second blood sample), but the differences were small and not statistically significant.

For DHEAS the reliability (R), the index of association (r), and the rank correlation measure (τ) reached the highest values among all hormones in both premenopausal and postmenopause women. The Spearman-Brown formula indicates that a single DHEAS measurement would be sufficient to classify postmenopausal women, whereas two ($n = 1.8$) measurements would be required in premenopausal women. Total testosterone showed reliability values similar to DHEAS in postmenopausal women but somewhat lower reliability in premenopausal women. The Spearman-Brown formula indicated that the number of replicates needed to improve reliability of total testosterone are higher in premenopausal ($n = 6$) than in postmenopausal women ($n = 1.2$). The reliability and the measure of concordance for rank distributions in premenopausal women suggested a fairly high degree of misclassification of the two repeated determinations. Free testosterone showed a similar degree of concordance in premenopausal and postmenopausal women, with the highest correlation in the latter group. The number of replicates required to reach a reliability of 0.90 for this hormone were higher in premenopausal ($n = 4.6$) than in postmenopausal women ($n = 3.7$). In premenopausal women, total estradiol showed very poor reliability between the two determinations, and the Spearman-Brown formula estimated that 170 replicates would be required to improve its reliability to a 0.90 level. Insulin and C-peptide determinations showed similar reproducibility, with higher reliability in premenopausal than in postmenopausal women. In premenopausal women, 2.1 and 1.8 replicates would be needed for insulin and C-peptide, respectively, whereas in the postmenopausal group, 4.4 and 3.3 were the required number of repeated determinations. The magnitude of the intraclass correlation coefficient for prolactin was small in postmenopause ($r = 0.18$). In premenopause, a low to moderate reproducibility was observed ($r = 0.40$). The estimated number of replicates was 40.5 and 13.5 in postmenopause and in premenopause, respectively. In spite of this intra-individual variability in both premenopause and postmenopause, the degree of rank distributions showed to be in the range of values observed for the other hormones.

Table 3 Mean (±SD), intraclass correlation coefficient, correlation coefficient (Pearson's *r*), and rank correlation (Kendall's tau) between the first and the second blood sample (1 year apart) in premenopause women (60 subjects)

	No. of determinations	Mean (±SD)	Intraclass correlation coefficient I *versus* II sample[a]	Pearson's r[b]	Kendall's tau
Total estradiol (pmol/liter)	1	370.2 (159.0)			
	2	355.3 (163.5)	0.058 (0.00)	0.030	0.085
DHEAS (μmol/liter)	1	3.56 (1.3)			
	2	3.1 (1.2)	0.85 (0.72)	0.89[c]	0.69[d]
Total testosterone (nmol/liter)	1	1.7 (0.7)			
	2	1.5 (0.6)	0.60 (0.34)	0.72[d]	0.52[d]
Free testosterone (pmol/liter)	1	3.7 (1.8)			
	2	3.6 (1.7)	0.66 (0.43)	0.71[d]	0.52[d]
Insulin (μUI/ml)	1	8.3 (4.0)			
	2	8.9 (4.6)	0.81 (0.65)	0.81[d]	0.61[d]
C-peptide (ng/ml)	1	1.7 (0.5)			
	2	1.6 (0.6)	0.83 (0.70)	0.84[d]	0.64[d]
Prolactin (ng/ml)	1	15.6 (9.4)			
	2	13.6 (5.3)	0.40 (0.09)	0.69[d]	0.55[d]

[a] Number in parentheses, the lower 95% confidence limit of the intraclass correlation coefficient.
[b] Pearson's *r* adjusted for age.
[c] $P < 0.05$.
[d] $P < 0.000$.

Table 4 Mean (±SD), intraclass correlation coefficient, correlation coefficient (Pearson's *r*), and rank correlation (Kendall's tau) between the first and the second blood sample (1 year apart) in postmenopausal women (47 subjects)

	No. of determinations	Mean (±SD)	Intraclass correlation coefficient I *versus* II sample[a]	Pearson's r[b,c]	Kendall's tau[c]
DHEAS (μmol/liter)	1	2.2 (1.3)			
	2	2.0 (1.2)	0.90 (0.81)	0.90	0.78
Total testosterone (nmol/liter)	1	1.3 (0.6)			
	2	1.3 (0.7)	0.88 (0.77)	0.90	0.66
Free testosterone (pmol/liter)	1	3.1 (1.2)			
	2	3.2 (1.3)	0.71 (0.51)	0.76	0.61
Insulin (μUI/ml)	1	9.2 (3.5)			
	2	9.3 (3.3)	0.67 (0.44)	0.66	0.43
C-peptide (ng/ml)	1	1.8 (0.5)			
	2	1.3 (0.5)	0.73 (0.53)	0.72	0.53
Prolactin (ng/mlliter)	1	10.7 (6.6)			
	2	10.9 (11.9)	0.18 (0.04)	0.52	0.55

[a] Number in parentheses, the lower 95% confidence limit of the intraclass correlation coefficient.
[b] Pearson's *r* adjusted for age.
[c] $P < 0.000$.

Discussion

This study was conducted to investigate the reliability of several hormone determinations over a 1-year period in premenopausal and postmenopausal women. A number of possible sources of biological and laboratory variability potentially affecting hormonal determination were controlled for by the study design. Under these controlled conditions, DHEAS, total and free testosterone, insulin, and C-peptide were shown to be reliable in both premenopausal and postmenopausal women. Total estradiol showed very poor reliability in premenopausal women, whereas prolactin showed poorer reliability in postmenopausal women. To date, limited information is available on the reproducibility of hormone determinations.

Few studies have focused on estradiol and other hormone reliability indicators, either only in postmenopausal (9, 10) or in premenopausal and postmenopausal women (11). In the study by Toniolo *et al.* (11), samples from premenopausal women were collected, regardless of the time of the menstrual cycle; since total estradiol concentration varies greatly across the menstrual cycle, the reliability measure for this hormone could not be computed. However, the study evaluated also the reliability of free estradiol and found a small variability within the menstrual cycle and a consequent high reliability ($R = 0.72$ for premenopausal and $R = 0.77$ for postmenopausal women) for this hormonal free fraction. In a study focusing on the long-term stability of serum hormones at −80° C, total estradiol was shown to be stable at both high and low concentrations during the 3-year study period (12). Therefore, the lack of reliability for total estradiol in the present report cannot be due to the different lengths of storage between the first and the second

blood samples. Changes in body weight, daily alcohol intake, and number of cigarettes smoked each day between the first and the second visit were very small, and they cannot explain the poor reproducibility of total estradiol. A possible reason for this lack of agreement between the two determinations of estradiol might be the month-by-month variability of the menstrual cycle in terms of length and, consequently, hormonal fluctuation due to differences in length of follicular and luteal phases (13, 14). Furthermore, the lack of estradiol reliability does not seem related to the presence of women characterized by long menstrual cycles (31- to 39-day cycles) and, consequently, by possible anovulations. In fact, when from the sample we selected 34 women, characterized by regular menstrual cycles between 20 and 30 days, the reliability and the other two measures of concordance for total estradiol, prolactin, and free and total testosterone failed to show any improvement in comparison with the results shown in Table 3 (data not shown).

Our findings on androgens in postmenopausal women confirm previous reports on short (14 days) and long term (518 days) reliability and confirm that DHEAS determinations are characterized by the highest level of reliability (9, 10, 15). The lower reliability of total testosterone in premenopausal *versus* postmenopausal women could be due to slight fluctuations related with the menstrual cycle (16). Free testosterone showed good reliability in both premenopause and in postmenopause in our study.

Insulin and C-peptide showed good reliability (higher in premenopause *versus* postmenopause), confirming previous findings over a 14-day period in a group of men and women of ages 45–64 years. The previous report did not present data on reliability for men and women and for menopausal status separately. The modest prolactin reliability could be due to short-term fluctuations in prolactin serum concentration since prolactin is released in pulses of varying amplitude (17). Our results in premenopausal women are similar to results of Hankinson *et al.* (10) and Koenig *et al.* (18), but we found a worse reproducibility in postmenopausal women. This inconsistency might be due to the difference in the time of day in which the blood was collected in the three studies. Our samples were collected only during early morning, when serum prolactin levels are known to be lower in postmenopausal women (18) and, consequently, prolactin analytical variability might be larger than in serum samples collected later during the day.

To our knowledge, this is the first investigation in which several sources of biological and methodological variability have been controlled by both strict inclusion criteria and highly standardized blood drawing (*i.e.*, stability of menopausal status over the 1-year period of the study, exclusion of participants who were pregnant or breastfeeding at both visits or between the first and the second visit, exclusion of participants taking hormone therapy at both visits or between the first and the second visit, restriction of the blood drawing period between the 20th and the 24th day of the cycle, and same day of this interval in premenopausal women and same month of the year and day of the month in postmenopausal women, and same hour and minute at both visits in the whole sample; hormone determinations at both visits were performed in a single laboratory run), conditions that allowed us to investigate the hormone reliability in two homogeneous groups of premenopausal and postmenopausal women.

However, the study is limited by the undesirable selection of the sample by several components, which reduced the number of participants to about 50% both in the premenopausal and in the postmenopausal group. However, our data is, in general, consistent with data published in previous studies (9, 10, 15). In premenopausal women, blood was drawn during the luteal phase of the cycle. This might be an additional limitation of the study since, for instance, total estradiol may present better reliability in the follicular phase compared to the luteal phase.

It should be noted that the lack of reliability of our measurements taken 1 year apart could be due to temporal fluctuations in hormone levels. However, if long-term exposure to these hormones play an important role in the etiology of chronic disease (*i.e.*, breast cancer), the inability to characterize individuals with regard to the long-term exposure to the factors would severely limit our ability to conduct etiological studies, as emphasized by the large number of replicates needed to accurately estimate serum levels of these hormones.

Given the possibility that hormones may be important etiological factors for several chronic diseases, information concerning the reliability of their measurements is relevant for epidemiological studies focusing on this issue. Except for total estradiol and prolactin, the hormones investigated (*e.g.*, DHEAS, total and free testosterone, insulin, and C-peptide) appear to have fairly good reliability.

The poor reliability of estradiol in premenopausal women in the present study may have important implications in the interpretation of previous inconsistencies of studies focusing on the association between this hormone and diseases in premenopausal women.

For instance, the inconsistency of the association between total estradiol and premenopausal breast cancer compared to the more consistent findings of increased total estradiol in postmenopausal breast cancer patients (2) could not only be due to differences in the etiological role of estrogens in postmenopausal *versus* premenopausal breast cancer but could be due, at least in part, to the high intraindividual variability of total estradiol in the younger group of women. In the present study, we were not able to address specifically the issue of reliability of serum estradiol determinations in postmenopausal women because of the general limitation of the direct method assay that we used.

The intraindividual variability of total estradiol and its possible causes need to be further investigated to better evaluate the role of estrogen in the etiology of disease before menopause.

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