

# Effect of Delays in Processing Blood Samples on Measured Endogenous Plasma Sex Hormone Levels in Women

Michael E. Jones,<sup>1</sup> Elizabeth J. Folkler,<sup>2,4</sup> Deborah A. Doody,<sup>2,4</sup> Jhangir Iqbal,<sup>4</sup> Mitch Dowsett,<sup>2,4</sup> Alan Ashworth,<sup>3</sup> and Anthony J. Swerdlow<sup>1</sup>

<sup>1</sup>Section of Epidemiology, <sup>2</sup>Academic Department of Biochemistry, and <sup>3</sup>Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, University of London; <sup>4</sup>Biochemistry Department, Royal Marsden Hospital NHS Foundation Trust, London, United Kingdom

## Abstract

Time spent in transit may affect the concentration of various constituents of collected blood samples and, consequently, results of sex hormone assays. Whole blood was collected from 46 women, and one third was processed immediately, one third was stored at ambient conditions (22°C) for 1 day, and one third was stored for 2 days. Estradiol concentration increased by 7.1% [95% confidence interval (95% CI), 3.2–11.3%] after a delay in processing of 1 day and by 5.6% (95% CI, 0.2–11.4%) after a delay in processing of 2 days; the change was most apparent at lower than median concentrations. Progesterone concentrations showed no substantial change. Testosterone concentrations changed by 23.9% (95% CI, 17.8–30.3%) after a delay of 1 day but little thereafter. The

sex hormone-binding globulin concentration decreased by 6.6% (95% CI, 4.6–8.6%) and 10.9% (95% CI, 8.1–13.6%), follicle-stimulating hormone increased by 7.4% (95% CI, 4.2–10.7%) and 13.9% (95% CI, 8.7–19.3%), and luteinizing hormone increased by 4.9% (95% CI, 1.3–8.5%) and 6.7% (95% CI, 2.2–11.5%) after a delay in processing of 1 and 2 days. Increases in calculated values for biologically available levels of estradiol and testosterone were greater than the increases seen in measured total hormone concentrations. Similar changes are likely when samples are delayed in transit, and evidence of etiology may be obscured unless study designs or analyses take into account processing delays. (Cancer Epidemiol Biomarkers Prev 2007;16(6):1136–9)

## Introduction

Concentrations of endogenous sex hormones are related to risk of breast and endometrial cancers (1–3) and probably of several other cancers (4, 5). Quantifying these risks requires blood samples from many people, but there are practical difficulties in collecting and processing samples in large epidemiologic studies (6). For pragmatic reasons, whole blood samples may be collected at local centers or clinics and sent unfrozen by courier or routine post to central processing and storage laboratories. The time spent in transit at ambient temperatures may affect the integrity of a sample and, consequently, the result of subsequent assays and calculations for biologically available hormone levels, but there is exiguous information about this. We investigated the effect of delays in processing by measuring the change in concentration of five hormones and sex hormone-binding globulin (SHBG) in blood that had been held at ambient conditions for up to 2 days since venepuncture.

## Materials and Methods

Forty-six women who came to our phlebotomy clinic (Sutton, London, United Kingdom) from July 2004 to April 2005 to participate in the Breakthrough Generations Study, a cohort study of risk factors for breast cancer, agreed to provide blood samples. The women were volunteers from the general population who lived locally. A research nurse collected 9 mL whole blood into a tube containing K3-EDTA (Greiner

Bio-One Vacuette) and took the tube to the Biochemical Endocrinology laboratory at the Royal Marsden Hospital (Sutton, London, United Kingdom). Each 9 mL sample was split into three aliquots: one aliquot was centrifuged immediately and the plasma was frozen at –20°C, and the remaining two were stored at ambient temperature (22°C) for 24 and 48 h, respectively, in a temperature-controlled air-conditioned laboratory before separation and freezing. In July to August 2005, the frozen plasma samples were retrieved and sex hormone and binding globulin concentrations were measured in duplicate by RIA (i.e., there were six measurements made per woman, two at each time point over three time points). The samples were analyzed in batches, with samples for an individual woman being processed in the same batch.

Plasma estradiol was measured by RIA after organic extraction using a highly specific rabbit antiserum and “Third Generation Estradiol” <sup>125</sup>I reagent (Diagnostic Systems Laboratories, Inc.). The sensitivity of the assay is defined as 3 pmol/L by calculation from the 95% confidence limits of the zero standard (7). The coefficient of variation was 6.1% at 141 pmol/L ( $n = 22$ ). Progesterone was estimated by RIA (Diagnostic Systems Laboratories). The minimum detection limit is 0.32 nmol/L and the coefficient of variation was 5.9% at 2.5 nmol/L ( $n = 20$ ). Testosterone was measured using a solid-phase <sup>125</sup>I-RIA (Diagnostic Products Corp.). The analytic sensitivity of the assay is 0.14 nmol/L and the coefficient of variation was 3.4% at 2.4 nmol/L ( $n = 16$ ). SHBG was measured using a noncompetitive liquid-phase immunoradiometric assay (Orion Diagnostica). The detection limit is 1.3 nmol/L and the coefficient of variation was 2.5% at 53 mol/L ( $n = 20$ ). Follicle-stimulating hormone (FSH) was measured using a solid-phase two-site immunoradiometric assay (Diagnostic Systems Laboratories). The detection limit is 0.11 IU/L and the coefficient of variation was 4.9% at 13 IU/L ( $n = 18$ ). Luteinizing hormone (LH) was measured using a solid-phase two-site immunoradiometric assay. The detection limit is 0.5 IU/L and the coefficient of variation was 4.2% at 22 IU/L ( $n = 24$ ).

Received 1/10/07; revised 2/22/07; accepted 3/19/07.

**Grant support:** Breakthrough Breast Cancer and The Institute of Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Michael E. Jones, Section of Epidemiology, The Institute of Cancer Research, Sir Richard Doll Building, 15 Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom. Phone: 44-20-8722-4254; Fax: 44-20-8722-4019. E-mail: michael.jones@icr.ac.uk  
Copyright © 2007 American Association for Cancer Research.

doi:10.1158/1055-9965.EPI-07-0028

**Statistical Analysis.** Hormone and SHBG concentrations were analyzed after log transformation of the measurements. All results are presented on the original assay measurement scale. The change in concentration after a delay in processing of 1 and 2 days, relative to immediate processing, was estimated using a repeated measures random effects ANOVA model. The analysis made use of both duplicate measurements (i.e., the two replicates for each subject at each of the three time points) to estimate the measurement error in the assay itself. If duplicate measurements at any time point were not available, the single measurements still contributed to the analysis of the change in concentration with time. The average change per day over the 2-day period was estimated by fitting a linear trend that was constrained to pass through the individual woman-specific mean at baseline. The analysis was repeated only for those women who had average baseline measurements below, or above, the relevant median concentration. Data were analyzed using SAS/STAT PROC MIXED, version 9.1 (SAS Institute) for Windows (8).

## Results

The 46 women were on average 46.6 years old at the time of venepuncture; the youngest was 18 and the oldest was 74. Twenty-four women (52%) reported having menstruated in the last 2 months since venepuncture, 5 (11%) reported current use of oral contraceptives, and 5 (11%) reported use of hormone replacement therapy. Table 1 shows the mean hormone or SHBG concentration for each assay in the samples that were processed immediately (i.e., at baseline only). (Similar results for the samples processed at the two other time points are not presented because they show essentially the same pattern.) Not all measurements yielded valid assay results. For example, for LH, 43 women had duplicate measurements at baseline (Table 1), 1 woman had only a single valid measurement, and 2 had no valid measurements.

Table 2 shows the intra-assay coefficient of variation and mean percentage change in hormone and SHBG concentration after a delay in processing of 1 and 2 days compared with immediate processing. All 46 women had their aliquots processed immediately, 37 of these women had aliquots processed after a delay of 1 day and 39 women had aliquots processed after a delay of two days; 35 women had aliquots processed at all three time points.

There was evidence of a modest increase in estradiol concentration, of 7.1% after a delay in processing of 1 day and 5.6% after a delay in processing of 2 days (for completeness, the average percentage change per day is shown, although a daily trend may not accurately reflect the individual changes after 1 and 2 days if the response is nonlinear). Progesterone concentrations showed no substantial change over the 2-day period. Testosterone concentrations increased the most, by 23% after a delay in processing of either 1 or 2 days. SHBG concentrations decreased on average by 5.8% per day over the 2-day period (i.e., by -6.6% and -10.9%

after a delay in processing of 1 and 2 days). FSH concentrations increased on average by 7.0% per day, and LH concentrations increased by 3.8% per day over the 2-day period.

When the analysis was restricted to women who had concentrations below, or above, the median, the same pattern of results was seen, except the change in estradiol was greater at low concentrations (8.5% per day; 95% CI, 4.3-12.9%) than at high concentrations (-0.4% per day; 95% CI, -3.7% to 3.1%). Likewise, the change in estradiol was also greater among the women who had not menstruated in the last 2 months since venepuncture (7.8% per day; 95% CI, 2.9-12.8%) compared with those who had (0.9%; 95% CI, -2.3% to 4.2%).

## Discussion

The rationale for measuring endogenous plasma hormone levels in a clinical setting is different from that in epidemiologic studies. In a clinical setting, the usual purpose of an assay is to separate normal from abnormal levels and to assess degrees of abnormality, but in epidemiologic studies, moderate differences within the reference range are of etiologic interest. Delays in delivering blood specimens to the laboratory may introduce systematic changes in hormone concentrations and this could obscure associations in epidemiologic studies, in which it is not always practical to process blood samples immediately after venepuncture.

If blood samples from both cases and controls were subject to the same delay and all hormone concentrations were equally affected by the delay, then the delay should not affect the risk estimates in case-control or cohort studies. In practice, however, samples will not always be delayed by the same amount of time. In our cohort of more than 68,000 women from which the data in this article arise, for instance, delays vary between less than 1 day (samples taken at our clinic) and 3 or more days (but most samples are posted by the women in the study and arrive 1 or 2 days later).

Ellis et al. (9) and Evans et al. (10) examined the rate of change of FSH and LH in plasma from volunteers (age and sex were not reported) when blood samples were stored at 24°C to 30°C and reported no statistically significant changes over 24- or 120-h periods. The lack of statistical significance, however, may have been due to the small sample sizes based on only 10 and 6 subjects. In addition, Ellis et al. (9) and Evans et al. (10), writing from clinical perspectives, considered only changes of  $\pm 10\%$  or more as important and did not present numerical results for lesser effects; therefore, some of the modest changes we saw in FSH and LH may have been unreported. Livesey et al. (11) studied the changes in FSH and LH in plasma held at 4°C and 20°C for up to 8 days, from seven postmenopausal women, and concluded that these hormones were stable for up to 8 days; however, at 37°C, the concentrations dropped over time, with LH stable for only about 3 days and FSH stable for no longer than 1 day. Only Livesey et al.'s result for the decrease in LH reached statistical significance and it is at variance with the increase we observed in whole blood.

**Table 1. Mean hormone and SHBG concentrations and summary statistics on samples frozen immediately after venepuncture (i.e., at baseline) for 46 women**

Assay	No. women with two, one, or no replicate measurements (at baseline)			Hormone/SHBG concentration (at baseline)				
	Two	One	None	Mean	SD	Minimum	Median	Maximum
Estradiol (pmol/L)	44	0	2	253.6	324.8	5.6	85.0	1312.9
Progesterone (nmol/L)	44	2	0	11.4	21.2	0.8	2.0	94.8
Testosterone (nmol/L)	44	2	0	1.28	0.63	0.23	1.20	3.11
SHBG (nmol/L)	45	1	0	55.7	37.1	15.0	45.2	178.8
FSH (IU/L)	44	1	1	28.0	34.0	0.2	10.8	134.9
LH (IU/L)	43	1	2	23.4	20.4	1.3	16.1	87.7

**Table 2. Intra-assay coefficient of variation and mean percentage change within-person from baseline (immediate processing) in hormone concentration after postponement in processing of 1 and 2 d**

Assay	Intra-assay coefficient of variation (%)	Mean percentage change from baseline				Average percentage change per day	
		After 1 d	95% CI	After 2 d	95% CI	Trend	95% CI
Estradiol (pmol/L)	11.6	7.1	3.2-11.3	5.6	0.2-11.4	3.8	0.9-6.9
Progesterone (nmol/L)	10.4	3.2	-0.2 to 6.7	0.2	-3.1 to 3.5	0.9	-0.9 to 2.7
Testosterone (nmol/L)	15.8	23.9	17.8-30.3	23.1	15.5-31.3	14.3	10.0-18.9
SHBG (nmol/L)	2.2	-6.6	-8.6 to -4.6	-10.9	-13.6 to -8.1	-5.8	-7.3 to -4.4
FSH (IU/L)	9.1	7.4	4.2-10.7	13.9	8.7-19.3	7.0	4.4-9.7
LH (IU/L)	7.3	4.9	1.3-8.5	6.7	2.2-11.5	3.8	1.5-6.1

Wickings and Nieschlag (12) examined the effect of leaving whole blood for up to 3 days, and plasma for up to 24 h, at room temperature, and found no changes in testosterone concentrations. This report was based on samples from only two men and thus may have lacked power to detect modest changes.

Other studies have examined the effect of using frozen gel packs to keep whole blood chilled for at least part of the time in transit. Kristal et al. (13) found modest changes of <2% in testosterone and SHBG after 24-h refrigerated storage followed by 48-h storage with a -1°C gel pack (eventually increasing to 14°C at 48 h) in samples from 40 men. After 72 h of refrigeration and 72 h with a -1°C gel pack (eventually increasing to 21°C), testosterone levels had increased by 4.7% (95% CI, 0.3-9.5%) and SHBG had changed by -4.0% (95% CI, -6.5% to -1.2%). Hankinson et al. (14) evaluated among nine women the effect of 24- and 48-h delays in processing whole blood cooled with frozen gel packs (effectively 4°C for 20 h increasing to 9°C after 24 h and reaching 21°C after 41 h). Under these storage conditions, estradiol concentrations increased by 3.5% (95% CI, 1.7-8.7%) per day, testosterone increased by 9.5% (95% CI, 1.7-17.3%) per day, but SHBG showed only a modest increase of 1.3% per day (95% CI, -2.4% to 5.0%). We found a similar daily trend for estradiol but a large initial increase in testosterone after 1 day and little further increase after 2 days (i.e., the increase did not follow a linear trend). Our samples, however, were at ambient conditions for the entire period of delay in processing; this may explain the early increase we observed in testosterone levels and the greater decreases in SHBG when compared with samples that were chilled by frozen gel packs for at least a period of time.

Several studies have examined the effect of refrigeration of whole blood (i.e., storage at 4°C). Ellis et al. (9) reported no statistically significant effects of storage for up to 24 h for FSH and LH, and Evans et al. (10) reported no effects for up to 120 days when samples were kept refrigerated. Tsatsoulis et al. (15) examined changes in LH when plasma was held at 4°C for up to 24 h in four male subjects and found little variation over time. Key et al. (16) analyzed blood samples from 15 women and found increases in testosterone after 6 h of 1.0% (95% CI, -2.5% to 4.5%) in plasma samples and 5.2% (95% CI, 0.2-10.4%) in serum samples, and after 24 h slightly larger changes of 3.2% (95% CI, 1.4-7.9%) in plasma and 6.2% (95% CI, 0.2-12.5%) in serum.

It is difficult to compare studies directly because of the different temperature and storage conditions and analytic methods used to measure the hormone or SHBG concentrations. The studies that investigated changes at ambient temperatures (9-12), such as occur in regular mailing, with the exception of Livesey (11), did not detect significant changes in hormone concentrations but were much smaller in size than our study. Our results show that, after 1 or 2 days at ambient conditions, certain plasma hormone concentrations can change and thus similar changes are likely when samples are delayed in transit. We also found that, for estradiol, the percentage change per day was smaller for high concen-

trations than low ones and, likewise, for women who had menstruated in the last 2 months since venepuncture. Thus, estradiol measurements for premenopausal women may be less affected by delays in processing compared with measurements for postmenopausal women. The results of studies that simulated transport and storage with frozen gel packs or under refrigeration suggest that lower temperatures may slow the change. Refrigerating or chilling samples during transfer is not always feasible in large epidemiologic studies, and changes in hormone and SHBG levels are to be expected if samples are transported without being cooled for their entire journey.

The changes in hormone level may be due to effects on SHBG and other, lower affinity, binding agents. Although the changes in measured SHBG levels were modest, changes in the affinity of SHBG for steroids might be more marked; it is possible that weakening of the attraction between binding proteins and estradiol could allow a more complete organic extraction of the steroid hormone before analysis. Similarly, for testosterone, it is possible that a weaker binding to SHBG following a prolonged period at ambient temperatures may result in a change in the efficiency of the testosterone assay. Alternatively, enzyme activity in the blood samples during the period at ambient temperature may have resulted in the catabolism of some of the steroids present. For example, breakdown of androstenedione to testosterone may be a contributing factor to the increase in testosterone levels. This suggests that specific enzyme inhibitors or stabilizers could be added to blood collection tubes, but the approach would work only if the mechanism behind the changes was known. More general inhibitors or stabilizers (e.g., azide) frequently have effects on the analytic processes and would require substantial study for each of the analytes in an epidemiologic study before being accepted or rejected as suitable.

Theoretical models for calculating biologically available estradiol (17, 18) and testosterone (18, 19) provide considerable savings in assay costs and volume of sample required compared with direct measurement of biologically available hormone concentrations. The models have good validity, at least for postmenopausal women (18), but the consequences of processing delays on the validity of the calculations have not been considered previously. The models imply that an overestimation of total estradiol or testosterone will lead to an overestimation of bioavailable hormone and underestimation of SHBG would further compound this. For example, based on published values for variables in these models (18), the actual changes we observed in sex hormone and SHBG concentration, and a typical concentration of 45 nmol/L SHBG, we calculate that bioavailable estradiol and testosterone would be overestimated by about 10% and 30%, respectively. This holds equally for nonprotein-bound (free) and non-SHBG-bound hormone and varies little across a wide range of total hormone concentration. Thus, it is particularly important to consider taking account of delays in transit when calculating bioavailable hormone concentration.

It may be possible to adjust and correct individual measurements of hormone concentration if the shape of the "decay"



curve has been determined and measured sufficiently accurately, but this has not been done for sex hormones and would need a larger study than ours. Furthermore, our study was of samples held at an ambient temperature of 22°C, appropriate to the setting in northern Europe, but other studies would be needed to determine serum hormone changes in the much higher temperatures seen in summer months in southern Europe or parts of the United States. If, however, there is substantial heterogeneity between individual samples in the way in which hormone levels decay over time (e.g., due to different ambient temperatures), such an approach may be unrealistic and it may be necessary to use frozen chill packs and ensure overnight shipping, if possible and practical.

Alternatively, if the delay time was known, statistical analyses could take into account the changes in hormone or SHBG levels by using standard stratification or regression methods, or if many potential control samples were available (e.g., a case-control study nested within a cohort), the control selection procedure could ensure that matched case-control sets had the same processing delay time (i.e., by matching on delay time). This would be in addition to adjusting or matching on other factors, such as day of menstrual cycle, for premenopausal women. It might also be prudent to match on the weather temperature during transport (e.g., winter versus summer) because the comparison between our results and those of studies examining frozen storage suggests that temperature may affect the degree to which hormone levels change.

## Conclusion

In large epidemiologic studies that use standard postal or courier services to send blood to processing centers, it is inevitable that some delays will occur in receiving samples. Our results suggest that, of the hormones and SHBG we studied, average progesterone levels will be least affected by a delay in transit of 1 or 2 days, whereas testosterone levels will change the most. SHBG concentrations decrease, which exaggerates the bias in calculations for biologically available levels of estradiol and testosterone. Associations may be masked unless study design or analyses allow for delays in transit.

## Acknowledgments

We thank the women who participated in the study and our colleagues in the Breakthrough Generations Study Team, particularly the research nurses Alison Butlin, Alison Hart, and Margo Pelerin who collected the blood.

## References

1. Kaaks R, Berrino F, Key TJ, et al. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). *J Natl Cancer Inst* 2005;97:755–65.
2. Kaaks R, Lukanova A, Kurzer MS. Obesity, endogenous hormones, and endometrial cancer risk: a synthetic review. *Cancer Epidemiol Biomarkers Prev* 2002;11:1531–43.
3. The Endogenous Hormones and Breast Cancer Collaborative Group. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst* 2002;94:606–16.
4. Henderson BE, Ross RK, Pike MC, et al. Endogenous hormones as a major factor in human cancer. *Cancer Res* 1982;42:3232–9.
5. Key TJ. Hormones and cancer in humans. *Mutat Res* 1995;333:59–67.
6. Garcia-Closas M, Hankinson SE, Ho S, et al. Factors critical to the design and execution of epidemiologic studies and description of an innovative technology to follow the progression from normal to cancer tissue. *J Natl Cancer Inst Monogr* 2000;27:147–56.
7. Dowsett M, Goss PE, Powles TJ, et al. Use of the aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal breast cancer: optimization of therapeutic dose and route. *Cancer Res* 1987;47:1957–61.
8. SAS Institute, Inc. SAS 9.1.3 help and documentation. Cary (NC): SAS Institute, Inc.; 2002.
9. Ellis MJ, Livesey JH, Evans MJ. Hormone stability in human whole blood. *Clin Biochem* 2003;36:109–12.
10. Evans MJ, Livesey JH, Ellis MJ, et al. Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. *Clin Biochem* 2001;34:107–12.
11. Livesey JH, Hodgkinson SC, Roud HR, et al. Effect of time, temperature and freezing on the stability of immunoreactive LH, FSH, TSH, growth hormone, prolactin and insulin in plasma. *Clin Biochem* 1980;13:151–5.
12. Wickings EJ, Nieschlag E. Stability of testosterone and androstenedione in blood and plasma samples. *Clin Chim Acta* 1976;71:439–43.
13. Kristal AR, King IB, Albanes D, et al. Centralized blood processing for the selenium and vitamin E cancer prevention trial: effects of delayed processing on carotenoids, tocopherols, insulin-like growth factor-I, insulin-like growth factor binding protein 3, steroid hormones, and lymphocyte viability. *Cancer Epidemiol Biomarkers Prev* 2005;14:727–30.
14. Hankinson SE, London SJ, Chute CG, et al. Effect of transport conditions on the stability of biochemical markers in blood. *Clin Chem* 1989;35:2313–6.
15. Tsatsoulis A, Mavroudis K, Frost J, et al. The stability *in vitro* of bioactive and immunoreactive LH in human blood and plasma. *J Endocrinol* 1988;117:139–45.
16. Key TJ, Oakes S, Davey G, et al. Stability of vitamins A, C, and E, carotenoids, lipids, and testosterone in whole blood stored at 4 degrees C for 6 and 24 hours before separation of serum and plasma. *Cancer Epidemiol Biomarkers Prev* 1996;5:811–4.
17. Endogenous Hormones and Breast Cancer Collaborative Group. Free estradiol and breast cancer risk in postmenopausal women: comparison of measured and calculated values. *Cancer Epidemiol Biomarkers Prev* 2003;12:1457–61.
18. Rinaldi S, Geay A, Dechaud H, et al. Validity of free testosterone and free estradiol determinations in serum samples from postmenopausal women by theoretical calculations. *Cancer Epidemiol Biomarkers Prev* 2002;11:1065–71.
19. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 1999;84:3666–72.