

## Measuring Serum Melatonin in Epidemiologic Studies

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### Abstract

**Background:** Epidemiologic data on serum melatonin, a marker of circadian rhythms, and cancer are sparse due largely to the lack of reliable assays with high sensitivity to detect relatively low melatonin levels in serum collected during daylight, as commonly available in most epidemiologic studies.

**Methods:** To help expand epidemiologic research on melatonin, we assessed the reproducibility and refined a currently available melatonin RIA, and evaluated its application to epidemiologic investigations by characterizing melatonin levels in serum, urine, and/or plasma in 135 men from several ethnic groups.

**Results:** Reproducibility was high for the standard 1.0-mL serum [mean coefficient of variation (CV), 6.9%; intraclass correlation coefficient (ICC), 97.4%;  $n = 2$  serum pools in triplicate] and urine-based (mean CV, 3.5%; ICC, 99.9%) assays. Reproducibility for the 0.5-mL refined-serum assay was equally good (mean CV, 6.6%; ICC, 99.0%). There was a positive correlation between morning serum melatonin and 6-sulfatoxymelatonin in 24-hour urine ( $r = 0.46$ ;  $P = 0.008$ ;  $n = 49$  subjects). Melatonin levels in serum-plasma pairs had a high correlation ( $r = 0.97$ ;  $P < 1 \times 10^{-4}$ ;  $n = 20$  pairs). Morning serum melatonin levels were five times higher than those from the afternoon (before 9 a.m. mean, 11.0 pg/mL, versus after 11 a.m. mean, 2.0 pg/mL). Chinese men had lower melatonin levels (mean, 3.4 pg/mL), whereas Caucasian, African-American, and Ghanaian men had similar levels (mean, 6.7-8.6 pg/mL).

**Conclusions:** These results suggest that melatonin can be detected reliably in serum samples collected in epidemiologic studies in various racial groups.

**Impact:** With improved assays, it may be possible to investigate the role of melatonin and the emerging circadian rhythm hypothesis in cancer etiology in epidemiologic studies. *Cancer Epidemiol Biomarkers Prev*; 19(4); 932-7. ©2010 AACR.

### Introduction

Circadian rhythm disruption has recently been classified as a probable carcinogen to humans (1) and has been associated with an increased risk for cancers of the prostate (2-7), breast (8-11), and colon (12), and for non-Hodgkin lymphoma (13). Although the underlying mechanism explaining these observations is unknown, it has been suggested that melatonin, a hormone synthesized and secreted by the pineal gland in response to low-light conditions, may mediate the circadian rhythm-cancer relationship (1). Circulating melatonin exhibits a circadian rhythm with highest levels at night, moderate levels in the morning, and substantially lower levels during the afternoon (14). Light exposure can reduce the duration of melatonin secre-

tion and subsequent circulating melatonin levels (15). As such, melatonin may be a key biological intermediary of chronodisruption (16).

Traditionally, in sleep disorder studies, melatonin is measured in urine [as 6-sulfatoxymelatonin (aMT6s)] because overnight or morning first-void urinary aMT6s reflects melatonin levels during the night when melatonin levels are at their highest (17). However, in most large-scale prospective epidemiologic studies, overnight void urine is not routinely collected. In addition, most cohort studies collect blood samples during the day when melatonin levels are quite low. Therefore, reliable assays with high sensitivity are needed to quantify accurately the circulating levels of melatonin. For these reasons, few epidemiologic studies have measured serum melatonin levels and tested the hypothesis that altered melatonin levels are associated with increased cancer risk.

To extend the investigation of the circadian rhythm/melatonin hypothesis to epidemiologic studies, we conducted a methodologic study to measure melatonin levels in serum, plasma, and urine samples, and to assess the reproducibility of a currently available melatonin RIA and the feasibility of applying this assay to epidemiologic studies. Further, we measured serum levels in Caucasian, African-American, African, and Chinese men to assess ethnic differences in melatonin levels.

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## Materials and Methods

### Serum melatonin assays

To choose an acceptable assay for the study, we evaluated several commercial melatonin kits and concluded that the Buhlmann RIA kit (ALPCO) with a preceding extraction step is acceptable for measuring serum melatonin. This method was preferred over the direct assay because the extraction step can minimize interference from melatonin metabolites as well as remove lipids and other components that may be present in serum, which would result in overestimating melatonin levels. The Buhlmann RIA kit also uses the antibody raised by Kennaway and colleagues (18, 19), which has been validated by gas chromatography mass spectrometry (20). We used 60 samples to optimize the assay for both the standard (1.0 mL) and reduced (0.5 mL) serum volumes. Briefly, melatonin is purified from serum samples using reversed phase columns, dried, and reconstituted in assay buffer; melatonin standards for the standard curve were processed similarly. Samples were then incubated with  $\alpha$ -melatonin antibody and iodinated melatonin for 20 h, precipitated for the antibody-bound fraction using a solid-phase second antibody for 15 min, and centrifuged. The unbound fraction was aspirated and the antibody-bound fraction of iodinated melatonin was counted using a  $\gamma$  counter.

### Urinary aMT6s assays

Urinary aMT6s [expressed as ng/mg Creatinine (Cr)] were measured using the standard (1 mL) protocol by a competitive ELISA kit with a capture antibody technique (Buhlmann) as previously described (21). The assay was done on a microtiter plate coated with polyclonal antibodies specific for rabbit immunoglobulin. Urinary Cr was measured colorimetrically using a standard procedure.

### Study samples

For the study, only blood and urine samples collected from healthy men without a history of cancer were included. All samples were collected for our other epidemiologic studies and processed following standard procedures; serum samples were derived from blood that was processed within 4 h of collection and stored at  $-70^{\circ}\text{C}$ , and 24-h urine was collected, processed, and stored similarly.

**Serum and urine reproducibility assays.** Initially, reproducibility of the serum melatonin assay using the standard 1.0-mL volume was assessed with triplicate samples from two pooled serum samples; pooled serum samples were created by combining serum collected before 12 a.m. from multiple Chinese men. Subsequently, reproducibility of the refined (0.5 mL) assay was assessed using replicate morning serum samples from 14 Caucasian men (two to four replicates per sample). Reproducibility of urinary aMT6s assays using the standard

protocol was assessed with triplicate urine samples from two Chinese men.

**Serum-urine correlation.** For comparison of urinary aMT6s and serum melatonin levels, urine-serum paired samples collected from 49 Chinese men were used for this purpose, applying the standard protocols (1-mL sample volumes). All serum samples were derived from blood collected before noon. Urine samples were collected for the same participants during a 24-h period after blood collection.

**Serum-plasma correlation.** For comparison of melatonin levels in serum and plasma, serum-plasma paired samples from the same blood draw from 20 Caucasian men (13 morning blood draws and 6 afternoon blood draws) were measured using the refined (0.5 mL) assay.

**Descriptive studies.** To determine the serum melatonin profile by blood collection time, we measured serum melatonin levels in 43 Caucasian American men with blood drawn from one of four time blocks: (a) before 9:00 a.m. ( $n = 18$ ), (b) between 9:00 a.m. and 10:59 a.m. ( $n = 15$ ), (c) between 11:00 a.m. and 12:59 p.m. ( $n = 5$ ), and (d) 1:00 p.m. and later ( $n = 5$ ). To evaluate melatonin levels in various racial/ethnic groups, we measured morning serum melatonin in 50 Chinese, 35 Caucasian American, 11 African-American, and 30 Ghanaian men. For subjects with replicate measurements, the mean of the replicate measurement was used for analysis; all replicate samples were derived from the same blood draw.

### Statistical analysis

We used two measures of reproducibility to determine the precision of the melatonin assays: the coefficient of variation (CV) and the intraclass correlation coefficient (ICC). The CV, expressed as a percent, is calculated as 100 times the ratio of the SD to the mean:  $\text{CV} = 100 \times \sigma/\mu$ . Small CVs mean less dispersion from the mean and thus better reproducibility. The ICC, expressed as a percent, is calculated as 100 times the ratio of the variance associated with subjects ( $\sigma_{\alpha}^2$ ) to the sum of all variances [associated with subjects and other factors such as measurement error ( $\sigma_{\alpha}^2 + \sigma_{\epsilon}^2$ ):  $\text{ICC} = 100 \times \sigma_{\alpha}^2 / (\sigma_{\alpha}^2 + \sigma_{\epsilon}^2)$ ]. In our experience, the useful assays have CVs that are  $<20\%$  and ICCs of  $>80\%$ ; better assays have ICCs of  $>90\%$ . Pearson's correlation was used to measure the correlation between the paired serum and urine samples as well as between the paired serum and plasma samples.

## Results

Table 1 shows the means, SEMs, and medians of urine aMT6s, serum melatonin, and plasma melatonin levels in assays using either 1.0 or 0.5 mL of serum. The sensitivity of the melatonin RIA using both 1.0 and 0.5 mL serum volumes was 0.5 pg/mL. Urinary aMT6s levels among 49 men ranged from 0.49 to 12.36 ng/mg Cr, with a mean of 3.89 ng/mg Cr (SEM, 2.27 ng/mg Cr). In the same 49 men, melatonin concentrations measured by the assay using 1.0 mL of serum ranged from 0.50 to 10.44 pg/mL,

**Table 1.** Melatonin measurement in serum, urine, and plasma samples

	Assay using 1.0 mL of serum/urine*		Assay using 0.5 mL of serum/plasma†	
	Urine (ng/mg Cr)‡	Serum (pg/mL)	Plasma (pg/mL)	Serum (pg/mL)
<i>n</i>	49	49	20	20
Mean (SEM)	3.89 (0.32)	3.43 (0.34)	3.44 (0.66)	3.46 (0.66)
Minimum	0.49	0.50	1.10	1.25
Maximum	12.36	10.44	13.22	12.42
Median	3.72	2.61	2.41	2.11
<b>Replicate samples</b>				
<i>n</i>	2 subjects	2 pooled samples	—	14 subjects
Replicates/sample	3	3	—	2-4
CV range (mean CV)	2.8-4.1% (3.5%)	5.7-8.1% (6.9%)	—	1.7-14.2% (6.6%)
ICC	99.9%	97.4%	—	99.0%

\*Paired urine-serum samples from the same subject; all subjects were Chinese men.

†Paired plasma-serum samples from the same subject; all subjects were Caucasian American men.

‡Melatonin in urine is measured in the form of aMT6s and is adjusted for Cr.

with a mean of 3.43 pg/mL (SD, 2.41 pg/mL). Reproducibility for both urinary aMT6s and serum melatonin assays seems to be good, with mean CVs of 3.5% for the urine assays and 6.9% for serum assays. Reproducibility for serum melatonin assays using 0.5 mL of serum was similar to that using 1.0 mL of serum (mean CV, 6.6%) and ICCs were >97% for all assays. Serum and plasma levels were similar (plasma: mean, 3.44 pg/mL; range, 1.10-13.22 pg/mL; serum: mean, 3.46 pg/mL; range, 1.25-12.42 pg/mL).

Although direct comparisons between absolute concentrations of urinary aMT6s and serum melatonin are difficult, due to differences in units of measure (ng/mg Cr versus pg/mL, respectively), there was a positive and significant correlation between urine aMT6s and serum melatonin levels ( $r = 0.46$ ;  $P = 0.008$ ; Fig. 1A). There was also a strong correlation between plasma and serum melatonin levels ( $r = 0.97$ ;  $P < 1 \times 10^{-4}$ ; Fig. 1B).

Melatonin levels were detectable in serum throughout the day (Fig. 2A), although samples collected before 9 a.m. had levels that are five times higher than those collected after 11 a.m. (mean, 10.98 pg/mL; range, 1.61-26.67 pg/mL; and mean, 1.96 pg/mL; range, 1.25-4.08 pg/mL, respectively); these differences remained significant after adjusting for age (data not shown). In addition, serum melatonin was detectable in all four racial groups, with Chinese men (mean, 3.42 pg/mL) having lower serum levels, whereas Caucasian American (8.26 pg/mL), African-American (6.67 pg/mL), and Ghanaian (8.61) men had similar levels (Fig. 2B).

## Discussion

In this methodologic study, we showed that melatonin can be detected reliably in serum, plasma, and urine

samples collected in the morning from healthy men by RIAs with preceding extraction. In addition, there is good correlation between urine and serum levels as well as plasma and serum melatonin levels, suggesting that these biological specimens may be used for epidemiologic studies to quantify melatonin levels. We further showed that serum melatonin is detectable throughout the day, although at much lower levels in the afternoon, and in men of different ethnic backgrounds. These data suggest that it is possible to measure melatonin in biological samples, in particular in serum samples, to test the emerging hypothesis that circadian rhythm may play a role in cancer etiology.

In our study, the reproducibility of the serum assay was good but is considerably lower than that of the urine assay, due largely to the lower concentration of melatonin in serum. Although the absolute melatonin levels measured in urine and serum differ (ng/mg Cr versus pg/mL, respectively), the strong correlation between these two specimen types and between serum and plasma ( $r > 0.46$ ) suggests that when ranks or quartiles are used to categorize melatonin levels, measurements from each of these sample types are likely to distribute subjects into virtually identical classes, thereby yielding similar risk estimates for assessing the relationship between melatonin and cancer. Although we showed that the assay using 0.5 mL of serum has similar reproducibility as the 1.0-mL assay, for large-scale prospective studies, an even smaller amount is desirable.

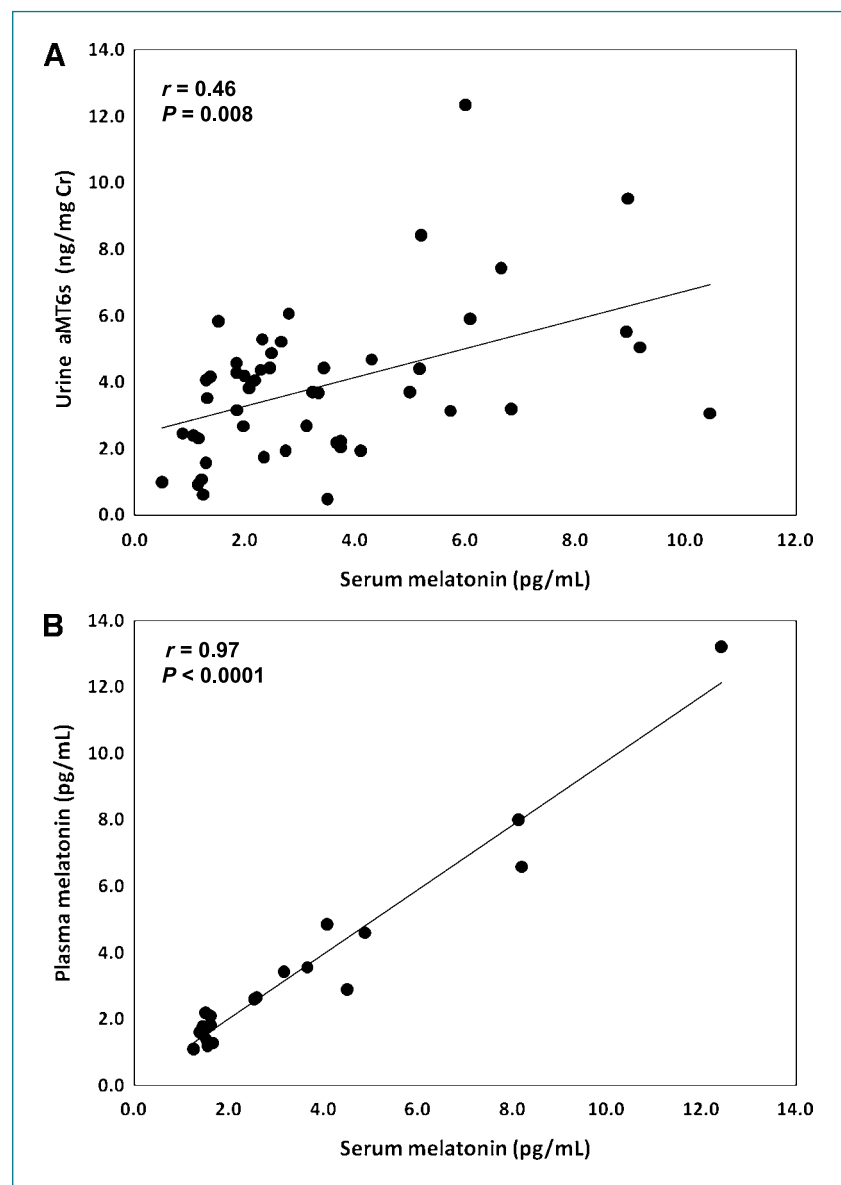
Similar to data from previous studies that measured melatonin in the same subjects throughout the day (22-26), in our study, higher melatonin levels were found in subjects with blood collected in the morning hours compared with those collected in the afternoon (up to a 5-fold difference). Although larger studies are needed to

confirm these findings and to characterize further the determinants of circulating melatonin (e.g., age, gender, body size, life-style factors, metabolic factors, and hormones, etc.), these data underscore the importance of using morning samples for the measurement of melatonin and the need to control for differences in time of blood collection in epidemiologic studies.

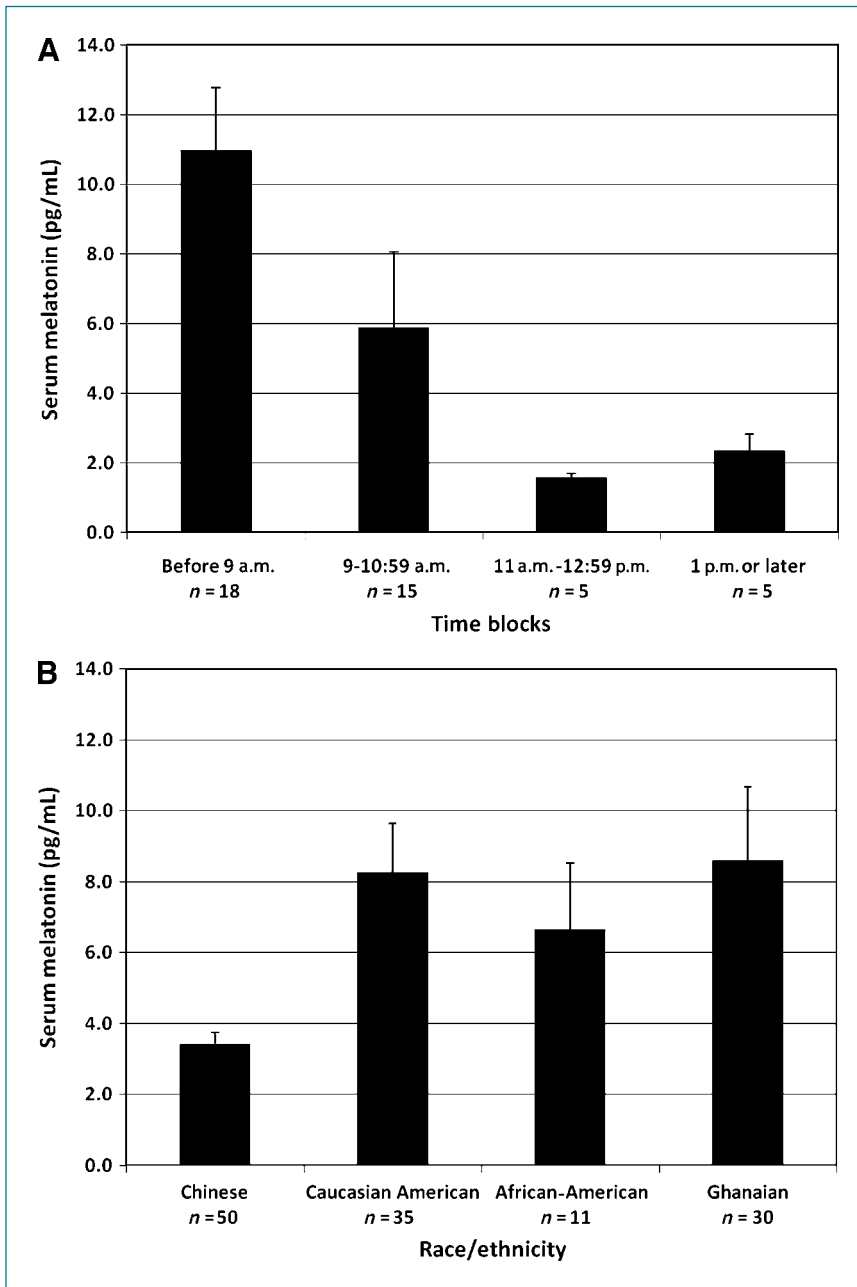
Emerging data suggest that circadian rhythm disruption may play a role in the etiology of several cancers. Recently, the IARC concluded that “shift-work that involves circadian disruption is probably carcinogenic to humans” (1). For example, data from observational studies suggest that increased risks for prostate cancer are seen for men with exposure to light at night, occupations that can disrupt their circadian rhythms, such work on a

rotating shift or as an airline pilot, and shorter sleep duration (2-7). Similar findings are also reported for breast cancer (8-11), colorectal cancer (12), and non-Hodgkin lymphoma (13). Genetic studies have shown that variants of circadian genes, which dictate the endogenous clock, may be associated with increased risk for prostate cancer (27, 28), breast cancer (29, 30), and non-Hodgkin lymphoma (31, 32). Together, these data suggest a potential role of circadian rhythms in several cancers, warranting further investigation.

It has been proposed that melatonin may be a key biological intermediary of chronodisruption (16). It is possible that cancer risk associated with chronodisruption may be due to low serum melatonin levels. Currently no epidemiologic study has addressed this hypothesis,



**Figure 1.** Correlations between urinary aMT6s and serum melatonin among 49 male subjects (A) and between plasma and serum melatonin among 20 male subjects (B).



**Figure 2.** Mean serum melatonin levels, by four time blocks in 35 Caucasian American men (A) and by race/ethnicity for measurements from serum collected before 12 p.m. (B). Columns, mean; bars, SEM. The refined 0.5-mL serum melatonin assay was used for all subjects except for Chinese subjects for whom melatonin was assayed using 1.0 mL of serum.

due largely to the lack of reliable assays with high sensitivity to detect relatively low melatonin levels in serum collected during daylight. Although future studies are needed to determine if our findings are generalizable to women, our data suggest that with improved assays, it may be possible to investigate the role of serum melatonin and the emerging circadian rhythm hypothesis in cancer etiology in epidemiologic studies.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

1. Straif K, Baan R, Grosse Y, et al. Carcinogenicity of shift-work, painting, and fire-fighting. *Lancet Oncol* 2007;8:1065–6.
2. Kubo T, Ozasa K, Mikami K, et al. Prospective cohort study of the risk of prostate cancer among rotating-shift workers: findings from the Japan Collaborative Cohort Study. *Am J Epidemiol* 2006;164:549–55.
3. Conlon M, Lightfoot N, Kreiger N. Rotating shift work and risk of prostate cancer. *Epidemiology* 2007;18:182–3.
4. Band PR, Le ND, Fang R, et al. Cohort study of Air Canada pilots: mortality, cancer incidence, and leukemia risk. *Am J Epidemiol* 1996;143:137–43.
5. Irvine D, Davies DM. British Airways flightdeck mortality study, 1950–1992. *Aviat Space Environ Med* 1999;70:548–55.
6. Pukkala E, Aspholm R, Auvinen A, et al. Cancer incidence among 10,211 airline pilots: a Nordic study. *Aviat Space Environ Med* 2003;74:699–706.
7. Kakizaki M, Inoue K, Kuriyama S, et al. Sleep duration and the risk of prostate cancer: the Ohsaki Cohort Study. *Br J Cancer* 2008;99:176–8.
8. Schernhammer ES, Laden F, Speizer FE, et al. Rotating night shifts and risk of breast cancer in women participating in the Nurses' Health Study. *J Natl Cancer Inst* 2001;93:1563–8.
9. Schernhammer ES, Kroenke CH, Laden F, Hankinson SE. Night work and risk of breast cancer. *Epidemiology* 2006;17:108–11.
10. Kakizaki M, Kuriyama S, Sone T, et al. Sleep duration and the risk of breast cancer: the Ohsaki Cohort Study. *Br J Cancer* 2008;99:1502–5.
11. Megdal SP, Kroenke CH, Laden F, Pukkala E, Schernhammer ES. Night work and breast cancer risk: a systematic review and meta-analysis. *Eur J Cancer* 2005;41:2023–32.
12. Schernhammer ES, Laden F, Speizer FE, et al. Night-shift work and risk of colorectal cancer in the Nurses' Health Study. *J Natl Cancer Inst* 2003;95:825–8.
13. Lahti TA, Partonen T, Kyrrönen P, Kauppinen T, Pukkala E. Night-time work predisposes to non-Hodgkin lymphoma. *Int J Cancer* 2008;123:2148–51.
14. Arendt J, Skene DJ. Melatonin as a chronobiotic. *Sleep Med Rev* 2005;9:25–39.
15. Cardinali DP, Pevet P. Basic aspects of melatonin action. *Sleep Med Rev* 1998;2:175–90.
16. Erren TC, Reiter RJ. Defining chronodisruption. *J Pineal Res* 2009;46:245–7.
17. Arendt J, Bojkowski C, Franey C, Wright J, Marks V. Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol. *J Clin Endocrinol Metab* 1985;60:1166–73.
18. Earl CR, D'Occhio MJ, Kennaway DJ, Seamark RF. Serum melatonin profiles and endocrine responses of ewes exposed to a pulse of light late in the dark phase. *Endocrinology* 1985;117:226–30.
19. Voultsios A, Kennaway DJ, Dawson D. Salivary melatonin as a circadian phase marker: validation and comparison to plasma melatonin. *J Biol Rhythms* 1997;12:457–66.
20. Lewy AJ, Markey SP. Analysis of melatonin in human plasma by gas chromatography negative chemical ionization mass spectrometry. *Science* 1978;201:741–3.
21. Wu AH, Wang R, Koh W-P, Stanczyk FZ, Lee H-P, Yu MC. Sleep duration, melatonin and breast cancer among Chinese women in Singapore. *Carcinogenesis* 2008;29:1244–8.
22. Thompson C, Franey C, Arendt J, Checkley SA. A comparison of melatonin secretion in depressed patients and normal subjects. *Br J Psychiatry* 1988;152:260–5.
23. Shochat T, Luboshitzky R, Lavie P. Nocturnal melatonin onset is phase locked to the primary sleep gate. *Am J Physiol* 1997;273:R364–70.
24. Benloucif S, Guico MJ, Reid KJ, Wolfe LF, L'Hermite-Baleriaux M, Zee PC. Stability of melatonin and temperature as circadian phase markers and their relation to sleep times in humans. *J Biol Rhythms* 2005;20:178–88.
25. Kumanov P, Tomova A, Isidori A, Nordio M. Altered melatonin secretion in hypogonadal men: clinical evidence. *Int J Androl* 2005;28:234–40.
26. Robeva R, Kirilov G, Tomova A, Kumanov P. Low testosterone levels and unimpaired melatonin secretion in young males with metabolic syndrome. *Andrologia* 2006;38:216–20.
27. Chu LW, Zhu Y, Yu K, et al. Variants in circadian genes and prostate cancer risk: a population-based study in China. *Prostate Cancer Prostatic Dis* 2007;11:342–8.
28. Chu LW, Li Q, Yu K, et al. Joint effects of circadian genes and serum androgens on prostate cancer risk in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Philadelphia (PA): Annual Meeting of the American Society of Human Genetics; 2008.
29. Zhu Y, Brown HN, Zhang Y, Stevens RG, Zheng T. Period3 structural variation: a circadian biomarker associated with breast cancer in young women. *Cancer Epidemiol Biomarkers Prev* 2005;14:268–70.
30. Zhu Y, Stevens R, Leaderer D, et al. Non-synonymous polymorphisms in the circadian gene NPAS2 and breast cancer risk. *Breast Cancer Res Treat* 2008;107:421–5.
31. Zhu Y, Leaderer D, Guss C, et al. Ala394Thr polymorphism in the clock gene NPAS2: a circadian modifier for the risk of non-Hodgkin's lymphoma. *Int J Cancer* 2007;120:432–5.
32. Hoffman AE, Zheng T, Stevens RG, et al. Clock-cancer connection in non-Hodgkin's lymphoma: a genetic association study and pathway analysis of the circadian gene cryptochrome 2. *Cancer Res* 2009;69:3605–13.