Clinical Endocrinology (2003) **58**, 710–717

Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men

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(Received 8 October 2002; returned for revision 28 October 2002; finally revised 9 December 2002; accepted 14 January 2003)

Summary

BACKGROUND Conflicting views are reported on the association between advancing age and gradually diminishing concentrations of serum total testosterone in men. The putative loss of diurnal rhythm in serum total testosterone in older men is reported to be in part due to low concentrations in the morning when compared to concentrations found in young men. We have measured total, free and bioavailable testosterone along with SHBG in samples taken every 30 min throughout a 24-h period in 10 young and eight middle-aged men.

RESULTS Both young and middle-aged men displayed a significant diurnal rhythm in all variables, with a minimum fall of 43% in total testosterone from peak to nadir in all subjects. Subjecting the data to a time series analysis by least squares estimation revealed no significant difference in mesor ($P = 0{\cdot}306$), amplitude ($P = 0{\cdot}061$) or acrophase ($P = 0{\cdot}972$) for total testosterone between the two groups. Comparing bioavailable testosterone in the two groups revealed no significant difference in mesor ($P = 0{\cdot}175$) or acrophase ($P = 0{\cdot}978$) but a significant difference ($P = 0{\cdot}031$) in amplitude. Both groups display a significant circadian rhythm (middle-aged group $P < 0{\cdot}001$; young group $P = 0{\cdot}014$). Free testosterone revealed a highly significant rhythm in both the young group ($P < 0{\cdot}001$) and the middle-aged group ($P = 0{\cdot}002$), with no significant difference between the groups in mesor ($P = 0{\cdot}094$) or acrophase ($P = 0{\cdot}698$). Although analysis of the SHBG data revealed a significant rhythm in the young group ($P = 0{\cdot}003$) and the older group ($P < 0{\cdot}001$), the acrophase occurred in the mid afternoon in both groups (15·12 h in the young and 15·40 h in the middle-aged). The older men had a significantly greater amplitude ($P = 0{\cdot}044$) but again no significant difference was seen in mesor ($P = 0{\cdot}083$) or acrophase ($P = 0{\cdot}477$) between the two groups. Acrophases for total, bioavailable and free testosterone occurred between 07·00 h and 07·30 h; for SHBG the acrophase occurred at 15·12 h in the young group and 15·40 h in the middle-aged group.

CONCLUSIONS The study suggests that the diurnal rhythm in these indices of androgen status is maintained in fit, healthy men into the 7th decade of life.

It is well recognized that circulating concentrations of testosterone in all forms (total, bioavailable and free) are characterized by a diurnal rhythm, with highest levels in the morning and a nadir in the late afternoon (Cooke *et al*., 1993 and references cited therein). This diurnal variation is reported (Marrama *et al*., 1982; Bremner *et al*., 1983; Deslypere & Vermeulen, 1984; Moroz & Verhratsky, 1985; Montanini *et al*., 1988; Plymate *et al*., 1989; Gray *et al*., 1991a) to be blunted in elderly men and is postulated (Bremner *et al*., 1983) to be, in part, due to lower morning concentrations in the elderly men.

Although a substantial body of opinion (Vermeulen *et al*., 1972; Bremner *et al*., 1983; Korenman *et al*., 1990; Gray *et al*., 1991b; Simon *et al*., 1992; Tennekoon & Karunanay, 1993; Morley *et al*., 1997; Harman *et al*., 2001) testifies to a gradual decrease in serum total, free and bioavailable testosterone with increasing age, this view is by no means universal, with several reports demonstrating concentrations of total testosterone in old men well into the reference range for young adults (Harman & Tsitouras, 1980; Sparrow *et al*., 1980; Nankin & Calkins, 1986; Schiavi *et al*., 1992; Mulligan *et al*., 1995; Ongphiphadhanakul *et al*., 1995; Janssen *et al*., 1998; van den Beld *et al*., 2000). In a group of 103 healthy men with a mean age of 67·7 years, Janssen *et al*. (1998) reported mean total testosterone concentrations of 20·4 nmol/l. In another more recent study of nearly 600 men between the ages of 51 and 85 years, no significant age-related

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decrease was found in total testosterone (Sculc *et al*., 2001). Similarly, no substantial change in total serum testosterone was found as a function of age when comparing 88 young (mean age 30·5 years), 97 middle-aged men (mean age 49·4 years) and 130 elderly (mean age 73·3 years; Khosla *et al*., 2001). We have also found a lack of significant correlation between total testosterone and age in an unpublished series of 130 subjects between 21 and 85 years of age.

In light of these findings and the reported relationship of the low morning total testosterone concentration with a blunted circadian rhythm in older men (Bremner *et al*., 1983), we have measured total, free and bioavailable testosterone along with SHBG, every 30 min over a 24-h period in a group of young men and compared the diurnal patterns with those measured in a group of elderly men.

Subjects and methods

Subjects

A group of 10 fit, healthy graduate students, aged 23–33 years, and a group of eight healthy middle-aged men, aged 55–64 years, who take regular moderate exercise had blood samples taken via an indwelling cannula every 30 min for 24 h. None of the subjects smoked or consumed more than 21 units of alcohol per week and none complained of, or were diagnosed as displaying any endocrinopathy. Sampling was started at 14·00 h and continued until 13·30 h the following day. Samples were allowed to clot and then centrifuged within 30 min of venesection. The serum was divided into 1-ml aliquots and frozen at –20 °C until analysed. The subjects were allowed to move around freely and eat and drink *ad libitum.* They lay down to sleep between 23·30 and 00·00 h and rose between 07·30 and 08·00 h. All samples from individual subjects were analysed sequentially, within-batch.

Methods

Total testosterone was measured on a Bayer Immuno 1® automated immunoanalyser (Bayer Diagnostics Corp, Tarrytown NY, USA) Within-batch and between-batch precision on this instrument is < 5% throughout the calibration range. Inclusion of regular QC samples throughout the batch eliminated the possibility of drift in the assay. Bioavailable testosterone was assayed as a percentage of the total testosterone using minor modifications of the method of Tremblay & Dube (1974). Briefly, this method employs ^{3}H-labelled testosterone as a tracer, where SHBG is precipitated from serum using saturated ammonium sulphate, and the radiolabelled testosterone measured in the supernatant as an estimate of the percentage non-SHBG-bound fraction. Between-batch precision in this assay was 5·2% at a concentration of 5 nmol/l. Free testosterone was calculated using the formula:

$$\%\text{Free testosterone} = 6 - 2{\cdot}38 \log_{10} [\text{SHBG}]$$ (Nanjee & Wheeler, 1985).

SHBG was measured using an IRMA supplied by Orion Diagnostics (Espoo, Finland). Both within- and between-batch precision was < 10% throughout the range of the assay.

The laboratory participates in the UK National External Quality Assessment Scheme (UKNEQAS) for total testosterone and SHBG. The total testosterone assay displays a 6-monthly running bias of ±3% to the all-laboratory (180 participating laboratories) trimmed mean. The SHBG assay has a 6-monthly running bias of between –6% and –11·5% to the all-laboratory (80 participating laboratories) trimmed mean.

The Liverpool Research Ethics Committee approved the study and all volunteers gave written informed consent prior to taking part in the study.

Statistical analyses

Individual and population mean cosinor analysis was performed as previously described (Ahmad *et al*., 2001) using Chronolab 3·0 (Universdade de Vigo, Vigo, Spain), a software package for analysing biological time-series analyses by least squares estimation (Mojon *et al*., 1992). Population mean cosinor analysis is based on the means of parameter estimates obtained from individuals in the study sample. The software therefore provides the circadian parameters as follows: (i) midline estimate statistic of rhythm (MESOR), defined as the rhythm-adjusted mean or the average value of the rhythmic function fitted to the data; (ii) amplitude, defined as half the extent of rhythmic change in a cycle approximated by the fitted cosine curve (the difference between maximum and MESOR of the fitted curve); (iii) acrophase, defined as the lag between a defined reference time (14·00 h of the first day in the study when the fitted period is 24 h) and the time of peak value of the crest time in the cosine curve fitted to the data. The circadian rhythm parameter, MESOR, is more reliable than the arithmetic mean obtained from single time-point measurements. A relatively small but statistically significant change in MESOR is not disregarded. Pulse analysis for each analyte profile in each subject was performed with the pulse-detecting algorithm, ULTRA (Van Cauter, 1988, 1990). The general principle of this algorithm is the elimination of all peaks of serum concentrations where either the increment (difference between peak value and preceding nadir) or the decrement (difference between peak value and the following nadir) that does not meet threshold criteria are eliminated from the data using an iterative process. This leaves a clean series in which all remaining peaks are assumed to represent significant pulses. The

Table 1 Percentage rhythm and significance of diurnal variation for total, bioavailable and free testosterone and for SHBG in young (Y) men and older (O) men

	% Rhythm		Significance		Goodness of fit	
	(Y)	(O)	(Y)	(O)	(Y)	(O)
Total testosterone	61·4	74	< 0·001	0·002	$P > 0·05$	$P > 0·05$
Bioavailable testosterone	51·1	48·8	< 0·001	0·014	$P > 0·05$	$P > 0·05$
Free testosterone	62·9	72·7	< 0·001	0·002	$P > 0·05$	$P > 0·05$
SHBG	27·8	42	< 0·001	0·003	$P > 0·05$	$P > 0·05$
Albumin		44·2		< 0·001		

Albumin was measured only in older men.

Table 2 Computed results of rhythmometric analysis of total, bioavailable and free testosterone and of SHBG in 10 young (Y) men and eight older (O) men

No. of men (Y or O)	Mesor ± SE	Percentage rhythm	Amplitude ± SE	Acrophase ± SE
Total T (nmol/l)				
10 (Y)	18·14 ± 1·77	61·4	4·47 ± 0·6	7·31 ± 21
Eight (O)	15·70 ± 1·33	74·0	2·87 ± 0·6	7·30 ± 39
Bioav.T (nmol/l)				
10 (Y)	4·25 ± 0·34	51·0	0·94 ± 0·1*	7·24 ± 60
Eight (O)	3·66 ± 0·19	48·8	0·5 ± 0·1	7·24 ± 23
Free T (pmol/l)				
10 (Y)	489·93 ± 22·87	62·9	124·01 ± 13·4*	7·19 ± 20
Eight (O)	393·47 ± 44·62	72·9	77·51 ± 13·5	7·05 ± 29
SHBG (nmol/l)				
10 (Y)	24·2 ± 1·37	27·8	0·99 ± 0·2*	15·39 ± 34
Eight (O)	28·8 ± 2·18	42·0	1·49 ± 0·1	15·11 ± 34
Albumin (g/l)				
Eight (O)	42·4 ± 1·65	44·2	1·74 ± 0·2	15·43 ± 30

*$P < 0·05$. Albumin was measured only in the older men.

threshold for pulse detection was set at a value 2 times the intra-assay CV. To test the difference between parameter estimates, Chronolab uses Bingham *et al*.'s (1982) test with a level of significance chosen as $P < 0·05$.

Cross-correlation analysis was performed to determine the relationship between the 24-h profile of SHBG and albumin using a previously described model (Markowitz *et al*., 1988). This analysis determines the correlation between two time series of equal length that have been paired, data point by data point, then one of the time series is shifted by one or more time points (lag time) and the correlation process repeated. This process can be repeated with the time series shifted backwards and forward, as many times as there are time points, minus 1. Calculating the mean value at each time point for all subjects derives each variable. Thus 49 means were determined for both albumin and SHBG.

Results

Individual and population mean cosinor analysis demonstrated a significant circadian rhythm for each of the four indices of androgen status in both young and elderly men (see Table 1). Table 2 summarizes the parameter estimates obtained by cosinor analysis: mesor, amplitude and acrophase for the four measured variables. Goodness of fit estimates for each individual subject were significant for each individual variable (Table 2). Figure 1(a–d) demonstrate the cosinor-derived circadian rhythms for these variables in both groups. Means of total, free and bioavailable testosterone were estimated for each young and each middle-aged subject as well as population means from grouped data, both approaches giving comparable estimates. Data analysis accorded equal weighting to each data point, revealing no strong relationship between each data point and its variance.

Total testosterone

Each subject in both the old and young groups showed a marked diurnal variation in serum total testosterone, with a minimum decrease (peak to nadir) of 43% of the peak value. In each group, 50% of the subjects reached a nadir of < 10 nmol/l, while all subjects in both groups had a peak level of > 10 nmol/l.

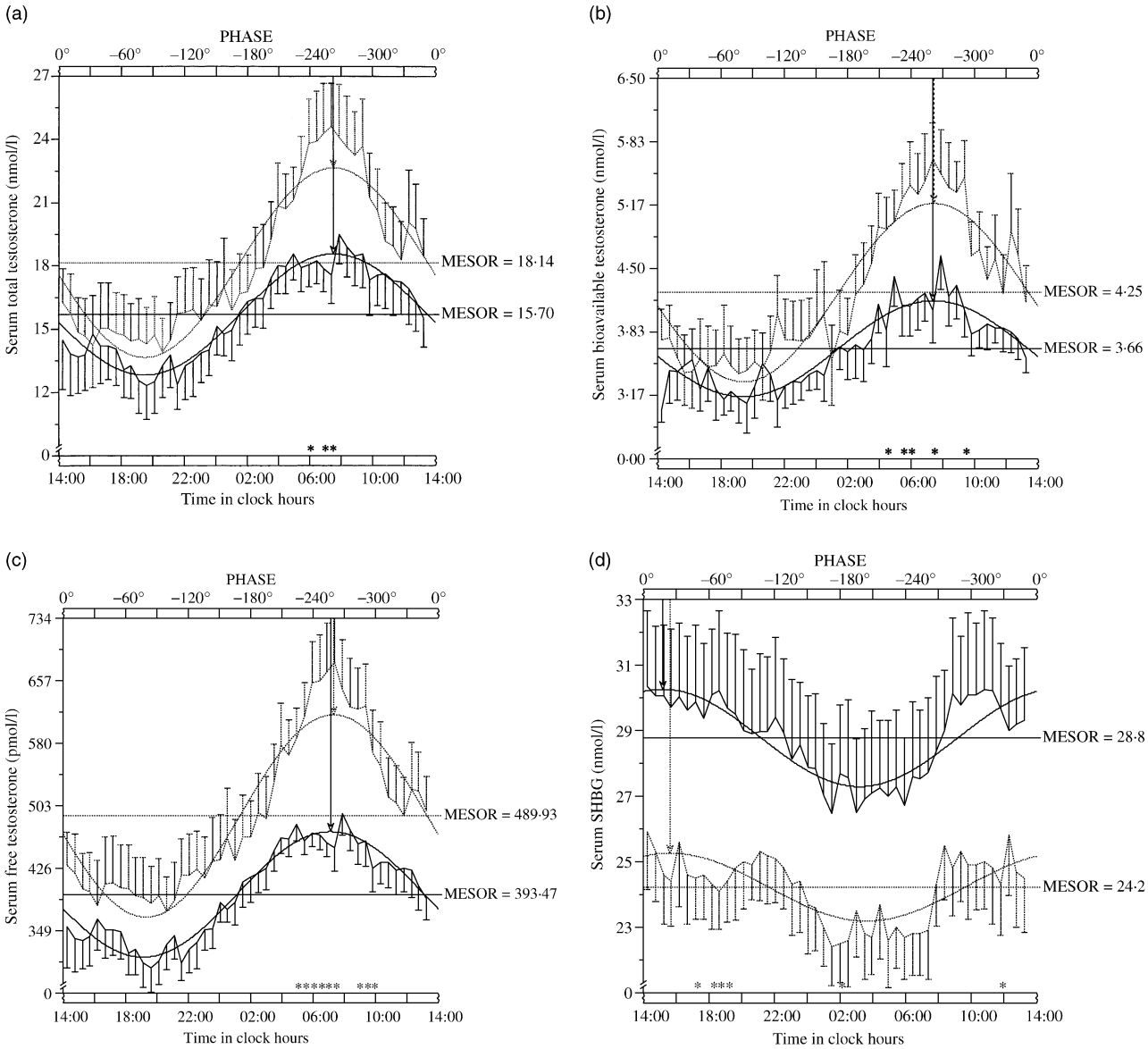

Fig. 1 Cosinor-derived circadian rhythmometry (CHRONOLAB) for serum (a), total testosterone (b), bioavailable testosterone (c), free testosterone (d) SHBG in young (dotted lines) and elderly (solid lines) men. The arrows (dotted = young: solid = elderly) indicate the time of acrophase. PHASE represents the time in degrees 360° = 24 h).

Population mean cosinor analysis showed a significant diurnal rhythm in the young group ($P < 0{\cdot}001$) and in the middle-aged group ($P = 0{\cdot}002$). No significant difference was seen in the mesor ($P = 0{\cdot}306$), amplitude ($P = 0{\cdot}061$), or acrophase ($P = 0{\cdot}972$) between the two groups (Fig. 1a). A significant difference between the young and older mean total testosterone was shown at three sampling times: 06·00, 07·00 and 07·30 h, when the young group showed a higher mean concentration at each of the three times. In both groups, the acrophase occurred at 07·30 h.

Bioavailable testosterone

Both groups display a significant circadian rhythm (see Table 1). No significant difference was demonstrated in mesor ($P = 0{\cdot}175$) or acrophase ($P = 0{\cdot}978$) but a significant difference ($P = 0{\cdot}031$) was shown in amplitude. The acrophase in both groups coincide at 07·24 h, similar to that for the total testosterone. Significant differences ($P < 0{\cdot}05$) between the young and middle-aged groups in the mean bioavailable testosterone were seen at 04·30, 05·30, 06·00, 07·30 and 09·30 h (Fig. 1b).

Free testosterone

A highly significant circadian rhythm was observed (Fig. 1c) in the young group ($P < 0{\cdot}001$) and in the older group ($P = 0{\cdot}002$). As with the bioavailable testosterone, there was no significant difference between the two groups in mesor ($P = 0{\cdot}094$) and acrophase ($P = 0{\cdot}698$) but a significant difference ($P = 0{\cdot}027$) was seen in amplitude. The acrophase was calculated as occurring at 07·18 h in the young group and 07·05 h in the older men. Significant differences ($P < 0{\cdot}05$) in the mean free testosterone concentration were seen at 05·00, 05·30, 06·00, 06·30, 07·00, 07·30, 09·00, 09·30, 10·00 and 18·00 h.

SHBG

Analysis of the SHBG data indicates a significant circadian rhythm (Fig. 1d) in both the young group ($P = 0{\cdot}003$) and in the older group ($P < 0{\cdot}001$). Again no significant difference was seen between mesor ($P = 0{\cdot}083$) and acrophase ($P = 0{\cdot}477$) in the two groups. A difference was demonstrated between the amplitudes in the two groups, the older men having a significantly greater ($P = 0{\cdot}044$) amplitude. The acrophase in the case of SHBG was seen in the afternoon (15·12 h in the young group; 15·40 h in the older men). Significant differences ($P < 0{\cdot}05$) in mean SHBG levels were noted at 12·00, 16·00, 17·00, 18·00, 18·30 and 19·00 h, the older men having the higher SHBG concentration.

Discussion

This is the first reported study where the diurnal rhythm in four indices of androgen status has been assessed simultaneously in young and middle-aged men. To our knowledge this is the first circadian rhythm study to employ an automated system of measuring serum total testosterone, allowing a large number of samples to be assayed concurrently with a high degree of precision (< 5%). No evidence of nonspecific interference was detected in this method, testified to by a lack of significant bias as recorded in UKNEQAS for male total testosterone. The work of Pardridge's group (Manni *et al*., 1985; Pardridge, 1986, 1988) and others (Cumming & Wall, 1985) led to the idea that bioavailable (i.e. free plus albumin-bound) testosterone, more than total testosterone, best reflects androgen status and this is now widely advocated (Garden *et al*., 1989; Plymate *et al*., 1989; Cooke *et al*., 1990; Korenman *et al*., 1990; Kaiser & Morley, 1994; Bhasin *et al*., 1998; Tremblay & Morales, 1998; Barrett-Connor *et al*., 1999; Morales *et al*., 2000; van den Beld *et al*., 2000; Khosla *et al*., 2001; Sculc *et al*., 2001). Although more technically demanding than measurement of total testosterone, many groups (Manni *et al*., 1985; Cooke *et al*., 1990, 1993; Sculc *et al*., 2001) have reported data based on the method of Tremblay & Dube (1974). The measurement has recently (Vermeulen *et al*., 1999) been advocated as a valid marker of bioactive androgen, correlating well with the 'gold standard' equilibrium dialysis method free testosterone assay. Similarly, the calculated free testosterone used here is also regarded (Vermeulen *et al*., 1999) as a more valid measure than the free androgen index (total testosterone × 100/SHBG). The method of calculation used in our study has previously been validated in both men and women (Nanjee & Wheeler, 1985), and reveals results similar to those produced using the Sodergard *et al*. (1982) formula. This is generally accepted as a measure of the fraction of total testosterone not bound to protein, and recognized as a valid means of calculating free testosterone concentration (English *et al*., 2001; Howell *et al*., 2001). The recent 2nd Annual Concensus Meeting of The Endocrine Society (Endocrine Society, 2001) has supported the use of these laboratory methods of biochemical assessment of male gonadal status.

This study has, as before (Bremner *et al*., 1983; Plymate *et al*., 1989; Cooke *et al*., 1993), been carried out on a small number of subjects but results in the young men confirm the well-documented circadian rhythm in total, bioavailable and free testosterone (Bremner *et al*., 1983; Plymate *et al*., 1989; Cooke *et al*., 1993). Results from the older group indicate that men in their 7th decade of life (six of the eight subjects were 62 or more years old) maintain these rhythms. Each of the middle-aged men displayed a marked diurnal rhythm in total serum testosterone, with a minimum decrease of 43% from peak to nadir; indeed, the middle-aged men show a mean percentage rhythm slightly greater than that in the young men.

Acrophases for all three measurements of serum testosterone (total, bioavailable and free), in young and middle-aged men, coincide between 07·00 and 07·30 h, similar to previous findings in young men (Clair *et al*., 1985). Other groups (Bodenheim *et al*., 1973; Rowe *et al*., 1974) who did not report an acrophase did, however, find peak total testosterone concentrations at a similar time. Although analysis of the data clearly indicates a diurnal rhythm in all variables in both groups, and every individual in both groups displayed these rhythms, the figures show that the absolute magnitudes of the mean peak to nadir difference for all variables are smaller in the older men. Nevertheless, the older men show a greater percentage rhythm in total and free testosterone and, despite the small sample size, a significant difference ($P < 0{\cdot}05$) in amplitude is displayed by bioavailable and free testosterone between the two groups. Cosinor analysis reveals a significant diurnal rhythm in SHBG in both groups with the acrophases found in the middle of the afternoon (15·39 h, young men; 15·11 h, middle-aged men). This is similar to some previous reports (Clair *et al*., 1985; Cooke *et al*., 1993) although others (Hamilton-Fairley *et al*., 1995) have, however, failed to find a significant diurnal rhythm in SHBG. Consideration of the half-life (4–5 days) of SHBG (Ruder *et al*., 1971; Namkung *et al*., 1988)

would tend to argue against a diurnal rhythm, reflecting increased or decreased synthesis or clearance.

Examination of the diurnal pattern of SHBG (Fig. 1d) might indicate that this reflects a shift of fluid and proteins out of the interstitial compartment into the plasma as the subjects rose in the morning and into the interstitial tissue as they lay down at night. This pattern is seen in some other plasma proteins such as albumin (Plymate *et al*., 1989; Cooke *et al*., 1993). We measured serum albumin only in the older group of men and results indicated that the diurnal pattern coincided with that of SHBG (Table 2). The strongest cross-correlation between SHBG and albumin ($r = 0{\cdot}57$) was observed at concurrent time points (0 lags), confirming coincidence of the diurnal patterns of SHBG and albumin. There is a clear difference in the timing of the acrophase for SHBG compared with that in the indices of androgen status (total, free and bioavailable testosterone). This may reflect the changes in synthesis and clearance of testosterone, which suggests that increased synthesis is the main influence on the increase in testosterone in the early morning. The fraction of total circulating testosterone that is bound to SHBG remains constant throughout the day so that the changes seen in total testosterone are mainly a reflection of increased free and bioavailable testosterone.

Our studies raise questions about the importance of the circadian rhythms for physiological function and end organ response. The absence or blunting of the circadian rhythm rather than the concentration measured at a single time-point during the day may better reflect the genesis of the pathophysiological effects observed in some elderly men (Turner & Wass, 1997). For example, some of these effects, such as decreased bone mass, purported to be due to gonad insufficiency, are not always associated with decreased serum testosterone concentrations. The circadian rhythms we have detected may be essential for the normal function/response of cells such as the osteoblast and osteoclast, regulating the circadian changes in bone formation and resorption well described in the literature (Eastall *et al*., 1992a, 1992b). In many areas of endocrinology the best responses to hormone therapy are seen when the normal or diurnal rhythms are emulated. This is particularly relevant for gonadotrophin-releasing hormone (pulsatile) and for cortisol (diurnal) replacement (Santoro *et al*., 1986; Thorner *et al*., 1998). Testosterone replacement is given either as bolus injections every 2–3 weeks, delivering an initial supraphysiological concentration throughout the day with a gradual decrease to hypogonadal levels prior to the next replacement dose, or as patches delivering a relatively constant concentration of hormone throughout a 24-h period. Treatment of hypogonadal men with transdermal testosterone by patch is reported to produce circadian variations in levels of total and bioavailable testosterone, these more physiological levels offering possible advantages in minimizing excessive stimulation of erythropoeisis, preventing or ameliorating gynaecomastia, while not oversuppressing gonadotrophins (Dobs *et al*., 1999).

In our study, the middle-aged men were younger than those previously reported in studies where the diurnal rhythm was blunted (55–64 years compared with a mean age of 70 years; Bremner *et al*., 1983; Plymate *et al*., 1989), and whilst we had fewer subjects than the original study (Bremner *et al*., 1983), we have sampled every half hour. The results of the studies might not therefore be considered directly comparable. Nevertheless, our numbers of subjects are similar to those used by others in studies examining patterns of 24-h testosterone secretion (Mulligan *et al*., 1995, 2001; Veldhuis *et al*., 2000). The age-range of our group of men does, however, represent the time in life when male patients are most likely to first present with erectile and/or gonadal dysfunction and loss of libido (Finkle *et al*., 1959; Bowers *et al*., 1963; Korenman *et al*., 1990; Bhasin *et al*., 1998; Korenman, 1998) or first begin to lose bone (Mazess *et al*., 1990; Wishart *et al*., 1995). The Massachusetts Male Ageing Study (Feldman *et al*., 1994) found a combined prevalence of minimal, moderate and complete impotence in 52% of males between 40 and 70 years.

In our study, middle-aged men had circadian rhythms mimicking those in the younger men. Our findings might be explained by the slightly younger ages of our men and by their lifestyle. All of our elderly men maintained a lifestyle not dissimilar to that of the younger men, took regular moderate exercise, were non-smokers, not overweight and generally enjoying good health. Reports (Kuoppasalami *et al*., 1976; Zmuda *et al*., 1996) have suggested that exercise can result in increased levels of circulating testosterone, while chronic illness can result in hypogonadism (Turner & Wass, 1997). If age was a major influence on the circadian rhythms, the comparability of lifestyle in the two groups of men would have helped us define the differences due to ageing.

This report would therefore support the contention that fit healthy men in the 7th decade of life can achieve levels of circulating testosterone, in any form, within the concentration range expected in young men at all times of the day with maintenance of circadian rhythm.

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