

## Free Triiodothyronine Has a Distinct Circadian Rhythm That Is Delayed but Parallels Thyrotropin Levels

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**Context:** TSH is known to have a circadian rhythm, but the relationship between this and any rhythm in  $T_4$  and  $T_3$  has not been clearly demonstrated.

**Objective:** With a view to optimizing thyroid hormone replacement therapy, we have used modern assays for free  $T_4$  (FT4) and free  $T_3$  (FT3) to investigate circadian rhythmicity.

**Setting:** The study was performed at a university hospital.

**Design and Subjects:** This was a cross-sectional study in 33 healthy individuals with 24-h blood sampling (TSH in 33 and FT4 and FT3 in 29 individuals) and cosinor analysis.

**Results:** Of the individuals, 100% showed a sinusoidal signal in TSH, for FT4 76%, and for FT3 86% ( $P < 0.05$ ). For FT4 and FT3, the amplitude was low. For TSH the acrophase occurred at a clock time of 0240 h, and for FT3 approximately 90 minutes later at 0404 h. The group cosinor model predicts that TSH hormone levels remain above the mesor between 2020 and 0820 h, and for FT3 from 2200–1000 h. Cross correlation of FT3 with TSH showed that the peak correlation occurred with a delay of 0.5–2.5 h. When time-adjusted profiles of TSH and FT3 were compared, there was a strong correlation between FT3 and TSH levels ( $\rho = 0.80$ ;  $P < 0.0001$ ). In contrast, cross correlation revealed no temporal relationship between FT4 and TSH.

**Conclusions:** FT3 shows a circadian rhythm with a periodicity that lags behind TSH, suggesting that the periodic rhythm of FT3 is due to the proportion of  $T_3$  derived from the thyroid. Optimizing thyroid hormone replacement may need to take these rhythms into account. (*J Clin Endocrinol Metab* 93: 2300–2306, 2008)

It is well established that TSH has a distinct circadian rhythm (1–3), and this circadian rhythm of TSH is maintained in patients treated with  $T_4$  and  $T_3$  (4). It has generally been observed that TSH levels reach a maximum between 0200 and 0400 h and a nadir between 1600 and 2000 h, but there is considerable interindividual variability according to studies reported. TSH is essential to maintain normal circulating levels of the thyroid hormones,  $T_4$  and  $T_3$ , and deficiency in TSH results in thyroid

hormone deficiency. However, although rhythms of  $T_4$  and  $T_3$  have been reported, the relationship between the circadian rhythm of TSH and circulating levels of  $T_4$  and  $T_3$  has not been clearly established. This probably relates to the physiology of thyroid hormones, the small sample sizes studied, and the variety of assays used for thyroid hormone measurements.

In humans 100% of circulating  $T_4$  is secreted by the thyroid gland, but only 20% of  $T_3$  is derived from this source. The re-

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Abbreviations: BMI, Body mass index; CV, coefficient of variation; FT3, free  $T_3$ ; FT4, free  $T_4$ .

maining 80% of  $T_3$  is generated by peripheral conversion of  $T_4$  to  $T_3$ . The kinetics of  $T_3$  metabolism differs from those of  $T_4$  because of the 10- to 15-fold lower affinity of  $T_3$  for thyroid binding globulin. Thus, for circulating  $T_3$  approximately 0.3% is in the free active form and for  $T_4$  only 0.02%. The overall production rate of  $T_3$  per day is approximately half that of  $T_4$  (50 vs. 110 nmol), and circulating levels of free  $T_3$  (FT3) are approximately 3- to 4-fold less than that for free  $T_4$  (FT4) (5 vs. 20 pmol/liter). The half-life of circulating  $T_4$  is estimated at 6.7 d and that for  $T_3$  of 0.75 d. In view of the very long circulating half-life of  $T_4$ , it is probably not surprising that no circadian rhythm has been described. Absolute circulating levels are determined by secretion as well as clearance, and because  $T_3$  has a shorter half-life, one might predict that if TSH stimulates a proportion of  $T_3$  release from the thyroid, then a circadian rhythm would be evident.

From the limited data available,  $T_4$  and  $T_3$  have been reported as exhibiting a morning peak and an evening or nighttime nadir (2, 3, 5). A similar circadian rhythm in urine  $T_4$  but not  $T_3$  was reported (6), and a synchronous diurnal rhythm of TSH and  $T_3$  from morning to midnight was demonstrated (3), although other investigators have found no rhythm in  $T_4$  and  $T_3$  (7–9). The failure of change in free thyroid hormones in response to the nighttime increase in TSH was found by one group to relate to the secretion of differentially glycosylated TSH with reduced bioactivity at night (9). In rats there is a circadian rhythm in tissue levels of  $T_3$  with an increase in the level at night that relates to an increase in tissue type II 5'-deiodinase activity (10). However, in man the reported nighttime increase in circulating FT3 was thought not to be due to peripheral deiodination but due to secretion from the thyroid (11). The increase in TSH after metoclopramide was related to changes in both thyroid hormones (TSH: $T_3$ ,  $r = 0.59$ ; TSH: $T_4$ ,  $r = 0.41$ ), suggesting that TSH can induce secretion of both  $T_4$  and  $T_3$  (12).

After the first publication that a combination replacement therapy of  $T_4$  and  $T_3$  may improve quality of life for hypothyroid patients (13), there has been considerable debate as to the actual benefits. Despite a large number of studies, there is no conclusive evidence that combination therapy with  $T_4$  and  $T_3$  improves efficacy of therapy or health-related quality of life (14). A major criticism of most studies is the lack of physiological combination therapies. It has been proposed that: “the ideal substitution therapy for hypothyroidism might be a combination of  $T_4$  and  $T_3$  in a carefully determined ratio and in a form in which  $T_3$  is slowly absorbed in a time-release form” (15). In response to this, a

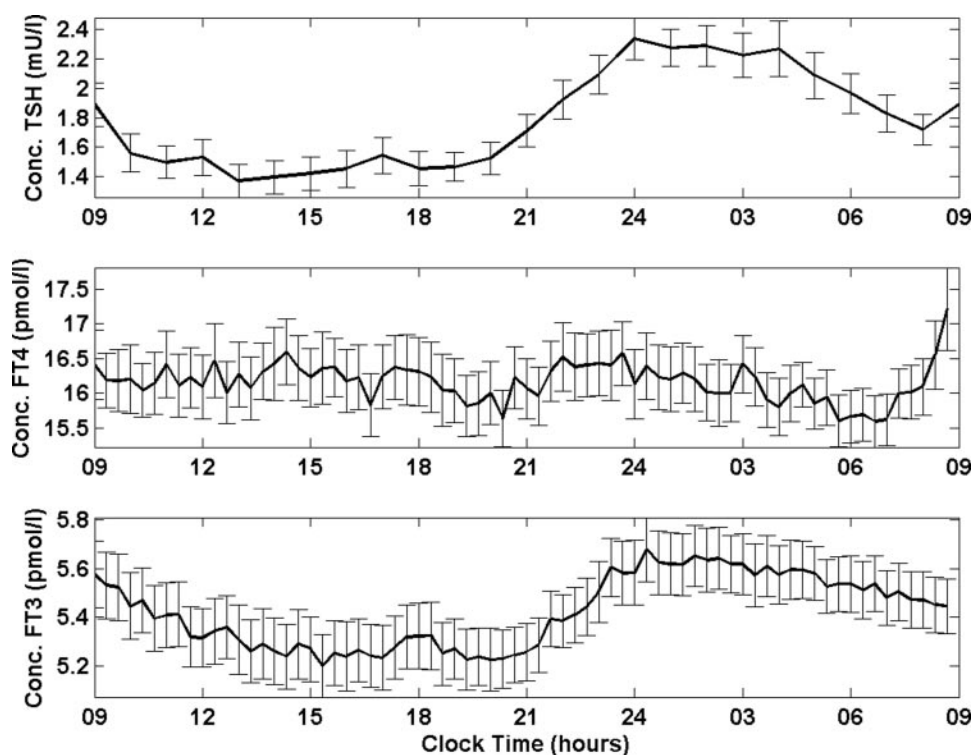


FIG. 1. Mean  $\pm$  SEM 24-h profiles for TSH (upper), FT4 (middle), and FT3 (lower). Conc, Concentration.

slow-release formulation of  $T_3$  has been developed, and initial proof of principle studies in humans suggests a good pharmacokinetic profile (16). In view of this debate and potential therapeutic developments, we felt it was important to establish the exact rhythms of FT4 and FT3 in the normal population. To do this we have studied FT4 and FT3 levels in a group of normal controls in which TSH levels had previously been measured and reported (17).

## Subjects and Methods

### Subjects

Samples were used from 33 healthy individuals who were studied as controls for previously published research on pituitary hormone levels in patients who have undergone cranial irradiation (17). The mean (range) age was 22.8 yr (17.3–56.5), body mass index (BMI) 22.9 kg/m<sup>2</sup> (16.3–28.9), and female to male ratio 9:24. The healthy subjects were taking no medication, and all had normal endocrine parameters that included normal stimulation tests for cortisol and GH (insulin tolerance test and GHRH + arginine), normal TRH test with 60-min TSH lower than 20-min TSH (no hypothalamic pattern), and no goiter clinically. Thyroid

TABLE 1. Percentage of individuals displaying significant rhythm for a range of significance levels

Significance level (P)	Percentage displaying significant rhythm		
	FT3	FT4	TSH
0.050	86	76	100
0.010	86	66	91
0.005	82	55	85
0.001	76	46	76

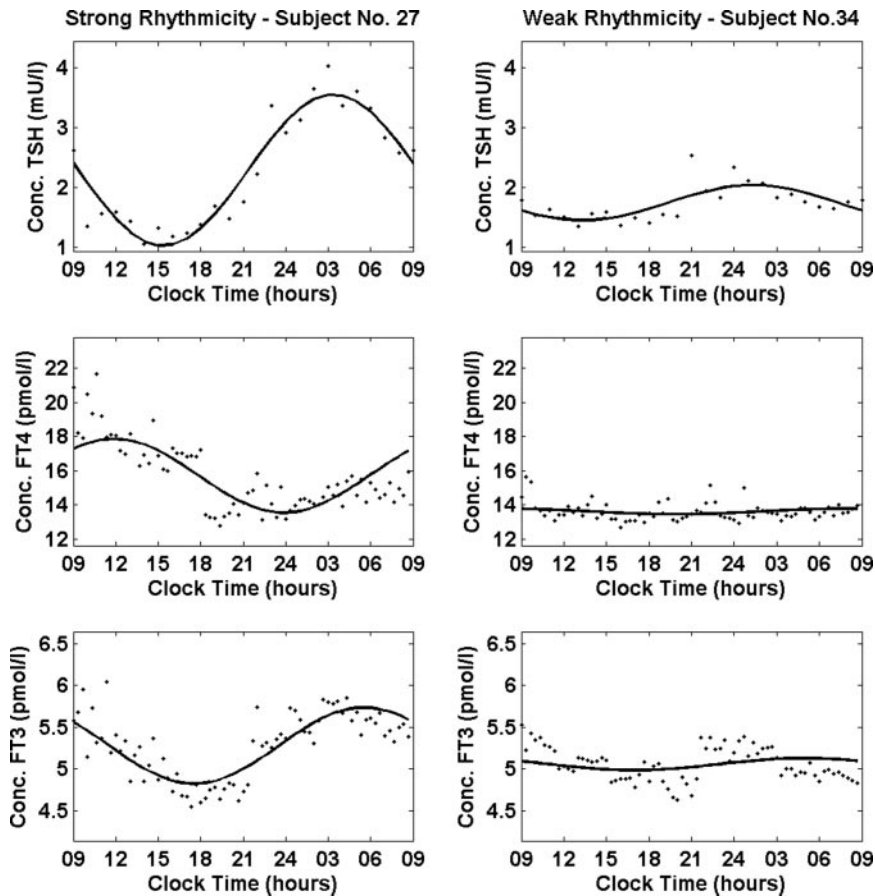


FIG. 2. Representative TSH (upper), FT4 (middle), and FT3 (lower) data from two individual subjects showing either strong or weak rhythmicity: Estimated cosinor (solid) and raw data (dots). Conc, Concentration.

antibodies were not checked, but subclinical hypothyroidism was excluded by a normal TSH. Women were profiled in the first half of the menstrual cycle, and there were no postmenopausal women. Sleep before the study was not controlled, but no shift workers were included in this cohort.

### Study procedures

The study was initially approved by the South Manchester local research ethics committee, and the additional analysis of FT4 and FT3 by the South Sheffield research ethics committee. Blood sampling at 20-min intervals was performed between 0900 and 0840 h the next morning. Three standard hospital meals were provided at 0830, 1230, and 1800 h, and physical activity was restricted to within the ward. Sera were separated and immediately frozen at  $-80^{\circ}\text{C}$ .

### Assays

TSH levels were measured hourly on samples from 33 subjects using a third-generation TSH assay (Heterogeneous Sandwich Magnetic Separation Assay) on the Immuno 1 System (Bayer, Pittsburgh, PA). The sensitivity of this method is 0.005 mU/liter, with a reported normal range of 0.35–3.5 mU/liter. The coefficient of variation (CV) at TSH levels of 0.028 mU/liter was 9.8% and at 0.5 mU/liter, 1.9%. TSH levels were only measured hourly in the initial analysis, and there was an insufficient sample to do 20-min sampling. FT4 and FT3 were measured on 20-min samples in 29 of the subjects (samples were not available on four subjects) using the Advia Centaur Chemiluminescence analyzer (Advia, Deerfield, IL). For FT4 the sensitivity is 1.3 pmol/liter, the normal range 10.3–21.9 pmol/liter, and the CV 6.6% at 6.1 pmol/liter and 3.0% at 13.9 pmol/liter. For FT3 the sensitivity

is 0.3 pmol/liter, the normal range 3.5–6.5 pmol/liter, and the CV, 4.0% at 2.9 pmol/liter and 2.9% at 6.6 pmol/liter.

### Statistical analysis

Linear interpolation was applied to accommodate the small number of missing values ( $<0.5\%$  overall, zero in TSH), all of which were distinct and verified by eye.

Single cosinor models of the form

$$z(t) = M + A\cos(2\pi t/T + \varphi) + e(t)$$

$$= M + \gamma\cos(2\pi t/T) + \beta\sin(2\pi t/T) + e(t)$$

were used to model the variation in measured hormone concentration as a function of time,  $t$  (hours).  $z(t)$  and  $e(t)$  represent the measured concentration and the error between the cosinor model and the measurement, respectively. The parameter  $M$  represents the mesor (value about which the variation occurs),  $A$ , the amplitude (distance from mesor to peak), and  $\varphi$  (radians), the acrophase (the time of occurrence of the peak equals  $\varphi T/2\pi$ ).  $T$  is the period, chosen here as 24. For a fixed value of  $T$  and known values of  $t$ , simple rearrangement of the model using trigonometric identities gives a linear model in the coefficients,  $M$ ,  $\gamma$ , and  $\beta$ , that can be fitted using conventional least-squares methods (18). Individual single cosinor models were fitted for each subject using least squares and the hypothesis that the data are better explained by the null hypothesis,  $H_0$ : a constant value (mesor) than,  $H_1$ : a single sinusoid with a 24-h period and mean value equal to the mesor, was tested via a likelihood ratio,  $F$ , test: reject  $H_0$  for large values of  $F$  ratio. For FT3, FT4, and  $F$  ratio,  $F_{2,69}(0.95) = 3.13$  was considered significant, whereas for TSH, it was  $F_{2,22}(0.95) = 3.44$ .

A group cosinor model was computed by averaging the coefficients from the individual fits, and the same null hypothesis was tested via the multivariate generalization of the likelihood ratio, the modified Wilks'  $\lambda$  statistic, which can be shown to be well approximated by the  $\chi^2$  distribution with appropriate degrees of freedom for the circumstances obtained here (relatively large sample, few coefficients). For FT3 and FT4, a  $\chi^2$  statistic,  $\chi^2_{58}(0.95) = 76.8$ , was deemed significant, whereas for TSH, it was  $\chi^2_{66}(0.95) = 86.0$ .

To look for similarities between pairs of hormone signals (TSH and FT4, TSH and FT3), the hormone profiles were shifted to maximize the correlation between the two signals by first identifying the peak value in the cross covariogram<sup>1</sup> and then realigning the two signals by the corresponding interval. To do this, the TSH profiles were first resampled on a 20-min interval via linear interpolation. Again, all records were inspected visually to ensure reasonable intersample behavior. Profiles with a time shift exceeding 12 h were omitted, and, therefore, data from only 24 are presented. A scatter plot of the remaining time-adjusted samples was then analyzed for correlation (Pearson coefficient).

To examine for relationships between age, height, weight, BMI, and markers of rhythmicity, correlations were calculated among these variables, and SD, CV, and cosinor amplitudes for FT3, FT4, and TSH.

<sup>1</sup> The cross covariogram is analogous to the Pearson correlation coefficient but for entire signals. It measures how similar two signals are to one another for all possible time shifts, thus, if two signals were identical but one lagged behind the other by 1 h, there would be a peak at  $\pm 1$  h.

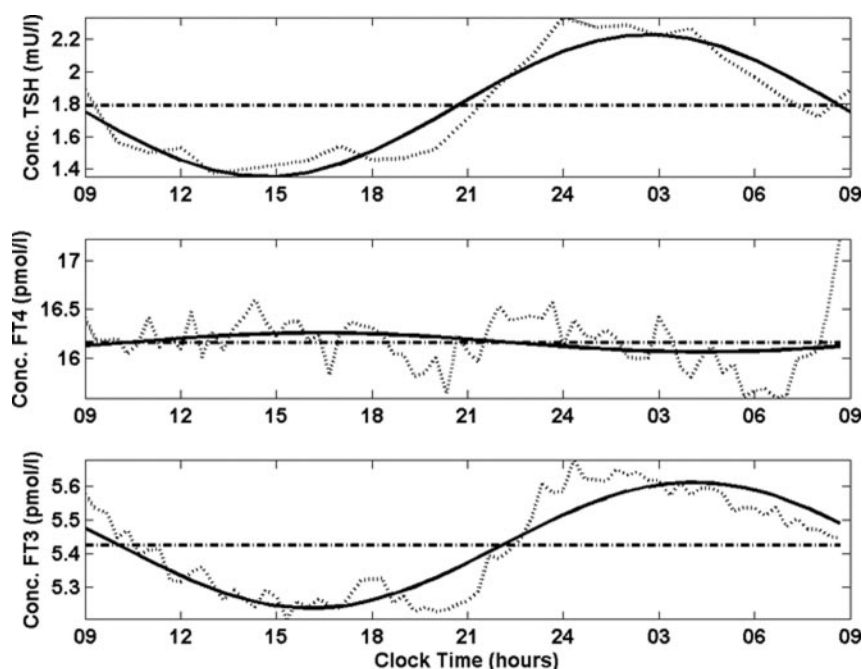


FIG. 3. Mean TSH (upper), FT4 (middle), and FT3 (lower) data (dotted) with group cosinor model superimposed (solid). Conc, Concentration.

## Results

### Cosinor analysis of individual hormone profiles

The mean profiles for TSH, FT4, and FT3 during the 24-h are shown in Fig. 1. It should be noted that in computing these averages, no account was taken of the fact that individuals peak at different times, and so a degree of smoothing should be expected. Nonetheless, visual inspection of the traces strongly supported the existence of a circadian rhythm for TSH and FT3, but not for FT4. Based on this observation, we tested the hormone data for a periodic signal using single cosinor analysis and an assumption of a 24-h period. Individual data from subjects displaying either strong or weak rhythmicity are shown in Fig. 2. Table 1 summarizes the percentage of subjects for which rejection of the null hypothesis, a straight line is better than a sinusoid, was demonstrated. It is evident that a very high proportion of subjects displayed rhythmicity in TSH and FT3: between 86 and 100% at  $P < 0.05$  and 76% at  $P < 0.001$ , whereas FT4 achieves only 76% at  $P < 0.05$  and 46% at  $P < 0.001$ .

### Group cosinor analysis

A single cosinor model for the group for each hormone was constructed by averaging the coefficients,  $M$ ,  $\gamma$ , and  $\beta$ , across the group (group amplitude and acrophase are then easily computed) (18). Rejection of  $H_0$  is indicated for all three hormones ( $P < 0.001$ ), suggesting that the group data are well supported

TABLE 2. Cosinor parameters for TSH, FT4, and FT3

	Mesor	Amplitude	Acrophase (h)
TSH	1.78 (1.64–1.93)	0.439 (0.174–0.703)	0240 (0222–0258)
FT4	16.2 (13.5–18.8)	0.097 (–5.81–6.01)	1620 (1521–1719)
FT3	5.42 (5.34–5.51)	0.187 (0.002–0.372)	0404 (0340–0428)

Mean with 95% confidence intervals shown in parentheses.

by the adoption of a single cosinor model. Figure 3 shows the mean of the raw data for each hormone with the group cosinor prediction superimposed. TSH and FT3 exhibited a close fit with their group means, whereas this was less evident for FT4. Table 2 summarizes the values of the cosinor parameters for each of the hormones. TSH exhibited the greatest amplitude, and the mean  $\pm$  SEM percent relative amplitude (= amplitude/mesor  $\times$  100) was  $36 \pm 2.6\%$  for TSH, for FT4 it was  $4.5 \pm 0.6\%$ , and for FT3,  $5.6 \pm 0.7\%$ . Thus, the mean peak to nadir change as a percentage of the mesor was 72% TSH, 9% for FT4, and for 11.2% for FT3. For TSH, acrophase occurred at a clock time of 0240 h, and for FT3, approximately 90 min later at 0404 h. The model also predicts that TSH hormone levels remained above the mesor between 2020 and 0820 h, FT3 from 2200–1000 h.

### Correlation of individual hormones

The cross covariograms and scatter plots for TSH and FT4 and FT3 are shown in Figs. 4 and 5, respectively. The cross covariograms for individual subjects suggest a close relationship between TSH and FT3 with 20 of 24 subjects showing peak correlation at between  $-0.5$  and  $-2.5$  h, suggesting that FT3 lags behind TSH by these amounts. There was a strong correlation between the time-adjusted FT3 and TSH levels ( $\rho = 0.80$ ;  $P < 0.0001$ ). In contrast, the cross covariogram showed no strong temporal relationship between FT4 and TSH with the peaks being spread quite uniformly. The computed group delay was zero. The scatter plot revealed a weak correlation between FT4 and TSH ( $\rho = 0.42$ ;  $P < 0.0001$ ). There was no significant evidence of relationships between strength of rhythmicity and age, weight, height, BMI, and gender.

## Discussion

We have confirmed that in humans there is a circadian rhythm of TSH with a peak level occurring at around 0240 h and levels remaining above the mesor from 2020–0820 h. We have also confirmed that FT3 shows a circadian rhythm (2, 3, 5), although with lower amplitude than TSH. We have now demonstrated that FT3 levels peak approximately 90 min after TSH levels at around 0404 h and remain above the median level from 2200–1000 h. Although FT4 showed significant rhythmicity, this was not evident from the raw data and did not correlate with either the TSH or FT3 rhythm.

We used single cosinor analysis as our primary tool for interrogating the data for a circadian rhythm. Our objective was to detect the presence of a circadian rhythm, should one exist, without regard to its detailed shape. A necessary condition for the existence of a periodic function of a given period is the presence of its fundamental. Therefore, the detection of a single cosinor

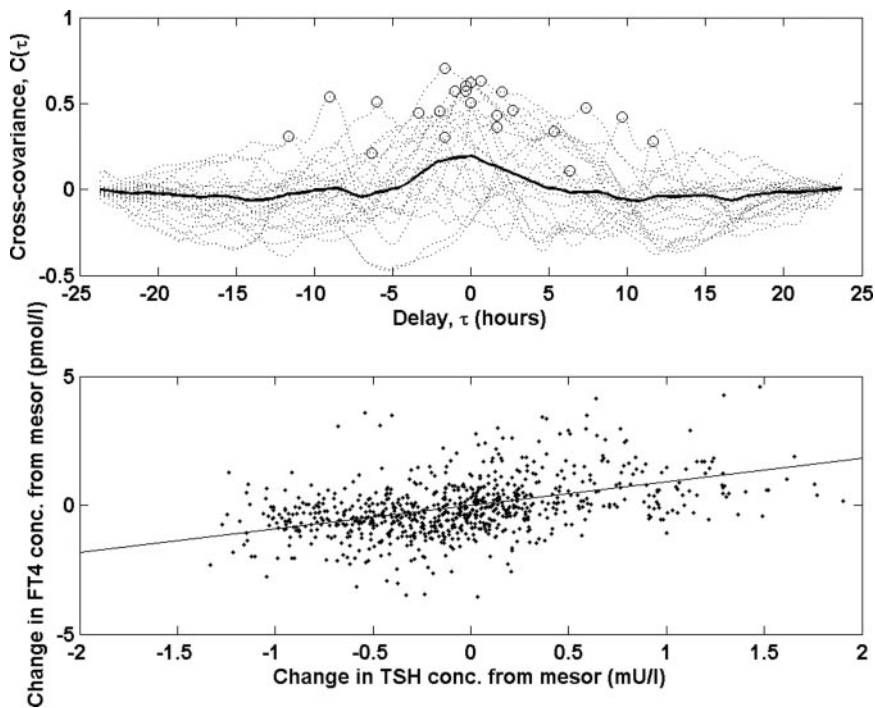


FIG. 4. Top Panel, Cross-covariance function [ $C(\tau)$ ] of individual profiles of FT4 and TSH. The graph shows the correlation between the two profiles on the y-axis for different periods of delay or advance on the x-axis. If both profiles were identical both in profile and timing, there would be a peak correlation of one at zero delay. The mean function is superimposed (solid line); a negative value of delay indicates that FT4 follows TSH. It is evident that there is negligible correlation between the FT4 and TSH profiles. Lower Panel, Scatter plot of time-shifted FT4 and TSH profiles ( $\rho = 0.42$ ;  $P < 0.0001$ ). conc, Concentration.

was adequate for testing our hypotheses. A limitation of this analysis was the availability of only a single 24-h profile from each subject. Although longer sampling periods would improve statistical accuracy, this restriction only precludes detection of rhythms of a longer period, outside the current hypothesis. In addition, it was assumed that the period of any rhythm would be 24 h instead of estimating it directly from the data. Exploratory studies with T ranging from 23.5–24.5 h made small quantitative differences (data not shown). TSH levels were measured hourly and FT4 and FT3 every 20 min because there was an insufficient sample to remeasure TSH. Previous studies using more frequent sampling have shown that TSH has an ultradian pulsatile secretion in addition to its diurnal rhythm (19, 20).

Despite the aforementioned limitations, it is evident from the data that the TSH rhythm is well described by a single sinusoid. For TSH, 100% of the profiles showed a significant sinusoidal component, and when comparing the group cosinor prediction with the mean levels of TSH, there was a close fit for a single sinusoid. The FT3 data showed a similar profile to TSH but with smaller amplitude and a time lag of approximately 90 min. For FT4 cosinor analysis showed that in 75.9% of subjects, a sinusoid was better than a straight line to describe the data, but the rhythm was not evident from inspection of the raw data, and the amplitude was low.

We observed that the peak in FT3 lagged behind that in TSH by approximately 90 min, and there was a strong correlation between the time-adjusted FT3 and TSH levels ( $\rho = 0.80$ ;  $P < 0.0001$ ). The strong temporal relationship between TSH and

FT3 levels and the positive correlation between time-adjusted FT3 and TSH levels suggest that the variation in FT3 levels is determined by TSH. This would be consistent with the observation that the increase in TSH and  $T_3$  levels correlates after treatment with the dopamine antagonist metoclopramide (12). Only 20% of  $T_3$  is derived from thyroid secretion and the remainder through peripheral conversion to  $T_3$ . Thus, it is possible that TSH either stimulates  $T_3$  release from the thyroid or increases conversion to  $T_3$  in the tissues. A previous study addressing this issue concluded that the change in FT3 was not due to peripheral conversion (11), and, therefore, it seems likely that the change in FT3 relates to TSH stimulation of thyroid hormone release. The failure to show a circadian rhythm of  $T_3$  in previous studies may relate to small sample size and the assay sensitivity at that time (7, 8).

There are many variables that determine the biological action of a hormone: rhythmicity, absolute level, affinity for receptor, and receptor occupancy required for maximal intracellular signaling. The observation that FT3 levels are dependent on an approximate 72% change in TSH

levels from nadir to peak confirms that at these serum concentrations, the variation in level of TSH has a biological action. The change in FT3 from nadir to peak was only 11.2% of the mean FT3 level. The low amplitude could be explained by the fact that only 20% of  $T_3$  is derived from thyroid gland secretion, that 97% of  $T_3$  is bound to thyroid binding globulin, and that the serum half-life of  $T_3$  is 0.75 d. The biological significance of this variation in FT3 is not known. For  $T_4$  and  $T_3$  sampled at the same time of day at monthly intervals, there is little variation in serum  $T_4$  and  $T_3$  within individual subjects compared with the variation within the population, and maybe a small change in thyroid hormone has physiological significance for the individual subject (21). Many hormones in addition to having a circadian rhythm may also show pulsatility. This is true for TSH, which has an ultradian rhythm with frequent small amplitude pulses (19), although the physiological significance of this is not established.

The clinical significance of any circadian rhythm in thyroid hormones has yet to be established. All the anterior pituitary hormones have a circadian rhythm with an increase in hormone levels overnight. ACTH and, thus, cortisol have a very distinct circadian rhythm with levels low before sleep and increasing from about 0200–0400 h (22). Although the role of the cortisol circadian rhythm in normal physiology has not been established, the loss of this rhythm does result in significant pathology. This is seen in congenital adrenal hyperplasia, in which recent attempts to replace the circadian rhythm have resulted in better biochemical disease control (23), and oral preparations of hydrocortisone that deliver circadian

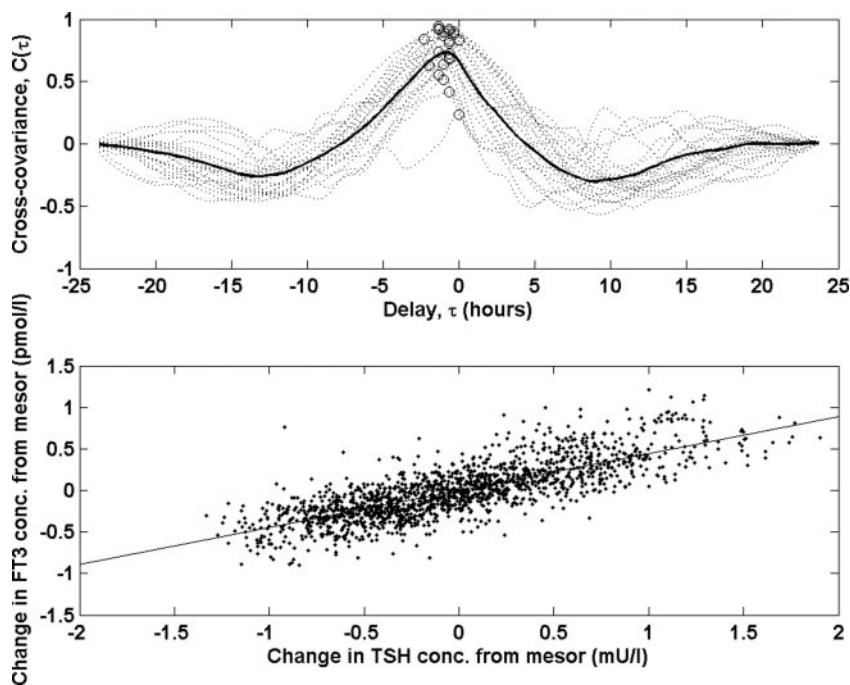


FIG. 5. *Top Panel*, Cross-covariance function [ $C(\tau)$ ] of individual profiles of FT3 and TSH. The graph shows the correlation between the two profiles on the y-axis for different periods of delay or advance on the x-axis. If both profiles were identical both in profile and timing, there would be a peak correlation of one at zero delay. The mean function is superimposed (*solid line*): a negative value of delay indicates that FT3 follows TSH. It is evident that there is considerable correlation between the FT3 and TSH profiles. *Lower Panel*, Scatter plot of time-shifted FT3 and TSH profiles ( $\rho = 0.80$ ;  $P < 0.0001$ ), conc, Concentration.

therapy have been developed (24). The TSH rhythm is distinct from ACTH, and TSH levels appear to increase earlier around the time of normal bedtime, and from our data, FT3 levels are above the median levels from 2200–1000 h. Current preparations of  $T_4$  and  $T_3$  result in immediate hormone release, with an increase in FT4 levels within the first 2-h administration. Most patients take their  $T_4$  dose in the morning, although a recent study examined biochemical control from taking  $T_4$  in the evening and found better control (25). Combination therapies of  $T_4$  and  $T_3$  have been tested, but none has reproduced the circadian rhythm of thyroid hormones, and there is no clear evidence that these treatments benefit patients (14). We have carefully examined and defined the normal circadian rhythm of thyroid hormones in anticipation of testing a more physiological replacement, but it remains to be proven that this would be of any benefit to patients.

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