Absence of Diurnal Variation of C-Reactive Protein Concentrations in Healthy Human Subjects

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Background: The concentration of C-reactive protein (CRP) in otherwise healthy subjects has been shown to predict future risk of myocardial infarction and stroke. CRP is synthesized by the liver in response to interleukin-6, the serum concentration of which is subject to diurnal variation.

Methods: To examine the existence of a time-of-day effect in baseline CRP values, we determined CRP concentrations in hourly blood samples drawn from healthy subjects (10 males, 3 females; age range, 21–35 years) during a baseline day in a controlled environment (8 h of nighttime sleep).

Results: Overall CRP concentrations were low, with only three subjects having CRP concentrations >2 mg/L. Comparison of raw data showed stability of CRP concentrations throughout the 24 h studied. When compared with cutoff values of CRP quintile derived from population-based studies, misclassification of greater than one quintile did not occur as a result of diurnal variation in any of the subjects studied. Nonparametric ANOVA comparing different time points showed no significant differences for both raw and \( z \)-transformed data. Analysis for rhythmic diurnal variation using a method fitting a cosine curve to the group data was negative.

Conclusions: Our data show that baseline CRP concentrations are not subject to time-of-day variation and thus help to explain why CRP concentrations are a better predictor of vascular risk than interleukin-6. Determination of CRP for cardiovascular risk prediction may be performed without concern for diurnal variation.

C-reactive protein (CRP)7 is the most prominent serum marker of the “acute-phase response”, the formation of various plasma proteins by the liver in response to an inflammatory stimulus in humans (1, 2). The role of CRP in the inflammatory process, although not well defined, may involve modulation through binding to inflammatory mediators such as platelet-activating factor (2) or complement-inhibitory factor H (3). Although CRP concentrations may increase many-fold in the acute-phase response, there is considerable evidence that CRP is present at low concentrations in asymptomatic individuals and may reflect baseline activity of circulating cytokines (4, 5). The determination of CRP concentrations in healthy individuals by a high-sensitivity assay (hs-CRP) has only recently become widely available (6, 7). Baseline hs-CRP concentrations have been shown to be one of the most powerful predictors of both long- and short-term cardiovascular risk in men and women (8–10), and they appear to add to the predictive value of lipid screening (9, 11).

CRP is synthesized and secreted by the liver in response to cytokines, predominantly interleukin-6 (IL-6) (IL-6, 12, 13). Studies of inflammatory markers in healthy sub-

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7 Nonstandard abbreviations: CRP, C-reactive protein; hs-CRP, high-sensitivity CRP; IL-6, interleukin-6; GCRC, General Clinical Research Center; and MESOR, midline estimating statistic of rhythm.
Projects have shown that the concentration of cytokines in plasma follows a diurnal variation (14, 15). The concentration of IL-6 has consistently been shown to be low in the morning and high just before bedtime when measured both in vivo (16, 17) or in stimulated blood samples ex vivo (18–20). This variation in the IL-6 concentration may reflect feedback inhibition of cytokine concentrations by endogenous cortisol, which is subject to circadian variation (21), although others have challenged this view (12, 22, 23).

The present study was designed to look for the presence of a diurnal variation in the concentrations of hs-CRP in healthy subjects using a newly available assay (7). Such variations may have important implications for the timing of blood sampling when measuring hs-CRP for the assessment of cardiovascular risk stratification.

**Materials and Methods**

Thirteen subjects (10 men and 3 women), 21–35 years of age, spent 16–18 days (24-h periods) in the University of Pennsylvania Sleep and Chronobiology Laboratory or the General Clinical Research Center (GCRC). Each subject gave signed informed consent to participate in the research protocol, which was approved by the institutional review board of the University of Pennsylvania, before undergoing screening tests. Participation in this study required sleep log evidence of a stable sleep-wake cycle as defined by habitual nocturnal sleep duration of 6.5–9.0 h, habitual morning awakening between 0600 and 0900, and no evidence of habitual napping (i.e., no more than once a week). Subjects were free of any sleep disorders as determined by history, actigraphy, and baseline polysomnography. Subjects had not undergone any periods of irregular sleep-wake schedules in the past 60 days, nor were they shift workers. All participants were screened for history of depression, mania, psychosis, epilepsy, and thyroid disorders; had no evidence of drug use by urinary toxicology screens; and had no indication of infection or disease by blood chemistry tests. Medical history and examinations revealed no chronic or debilitating medical conditions. Subjects were not permitted to smoke tobacco during the study, although two of the subjects reported being occasional tobacco smokers.

The data presented in this report were taken from baseline nights from two different experimental protocols where, after the baseline period, subjects remained in the GCRC for 14–16 days participating in studies on the effects of shortened sleep duration. The baseline sleep periods were either 8.2 or 8 h/night. Throughout the 24 h of these test days, wakefulness was monitored polygraphically to ensure that subjects did not sleep outside of the scheduled nocturnal sleep period. Nutritionally balanced meals were provided at appropriate times for breakfast, lunch, and dinner (caffeine and alcohol were prohibited).
Meals were prepared in the GCRC metabolic kitchen for the subjects and served at regular meal times. None of the subjects was permitted to have visitors during the portion of the protocol included in this report.

**SPECIMEN SAMPLING AND PROCESSING**

Blood was drawn via an indwelling forearm catheter at intervals of 15 or 30 min, depending on the protocol. Subjects remained in bed for the collection period and were permitted out of bed only for bathroom breaks.

Vacutainer Tubes with EDTA were used to collect the blood samples, and the samples were subsequently centrifuged immediately at 2600g for 7 min at 4 °C. After centrifugation, plasma was pipetted into polypropylene tubes and frozen to −70 °C for later assay. Hourly samples were used for the determination of CRP by an ultrasensitive latex-enhanced immunoassay (Dade Behring) (7). The day-to-day imprecision (CV) for the hs-CRP assay at concentrations of 0.6 and 12.6 mg/L was 8.8% and 4.3%, respectively.

**DATA ANALYSIS**

Missing values made up 5.8% of the total number of hs-CRP data points, or 1.5 missing values per subject. Each individual time series was displayed in a chronogram to inspect data for patterns, trends, and outliers. Data points below the detection limit of the hs-CRP assay (<0.2 mg/L) were converted to values of 0.1 mg/L (this was done for only one subject). Outliers, defined as points more than 3 SD from the subject’s mean score, were removed from analysis. Kruskal–Wallis one-way ANOVA of ranks was performed to detect significant differences for different time points in raw data and after transformation into normalized scores.

For the analysis of a rhythmic diurnal variation, individual as well as combined raw data and normalized scores were submitted to a computer program (Chronolab 3.0) (24), which fit a single cosine curve to the data using linear regression techniques. The analysis yielded a value for “percent rhythm”, explained by the fitted cosine curve, and a P value for the “goodness-of-fit” statistic. The midline estimating statistic of rhythm (MESOR) is the value midway between the highest and the lowest values of the fitted cosine curve. Amplitude is the measure of one-half of the extent of the rhythmic change estimated by the fitted cosine curve. Acrophase represents the crest-time of the fitted cosine curve relative to a defined reference time point (e.g., the beginning of the sampling period), expressed in degrees (360 degrees = 24 h).

**Fig. 2.** Rhythmograms of group averaged raw data (left) and the standardized z-scores (right) of hourly hs-CRP concentrations in 11 healthy subjects. Data are means ± SE. Tables at the bottom contain values for percent rhythm (PR), explained by the fitted cosine curve and P for goodness of fit, as well as MESOR, amplitude (AMP), and acrophase (ACR) of the fitted cosine curve. The dark bar indicates the time of the nocturnal sleep period.
**Results**

hs-CRP concentrations

Individual time series of baseline hs-CRP data are displayed in Fig. 1, and averaged data are shown in Fig. 2. Only three subjects had hs-CRP concentrations >2 mg/L, and only one subject had values >10 mg/L, probably reflecting the good general health of the study population. On the other extreme, four subjects had values consistently <0.5 mg/L, including one subject with all values below the detection limit of the assay (<0.2 mg/L).

The dashed lines in Fig. 1 represent cutoffs for the quintiles of baseline hs-CRP concentrations derived from population studies (25). Inspection of raw and combined data suggested that overall there is little variation of hs-CRP concentrations from the mean values over time, suggesting an absence of systematic variation in hs-CRP over the course of a single 24-h day. Nonparametric Kruskal–Wallis one-way ANOVA for detection of significant differences between individual time points confirmed this observation. Testing was negative for raw data and z-scores (H = 23.0, 23 degrees of freedom; P = 0.461).

Analysis for presence of a circadian rhythm in the data was performed by submitting raw data and standardized scores to a program fitting a cosine curve and testing for the likelihood that variation in the data could be explained by this curve. Two subjects for whom removal of outliers produced constant values throughout the 24-h period were excluded from this analysis. Results for the group-averaged data are displayed as rhythmograms in Fig. 2. Statistical testing for goodness of fit of the fitted cosine curve yielded nonsignificant P values for raw (P = 0.492) and standardized data (P = 0.897), failing to determine a significant circadian pattern of variation in these data. When the cosine-fitting function was applied to individual data series, the goodness-of-fit statistic was significant (P <0.05) in 7 of 11 subjects. However, the amplitudes of the detected variations were low (median amplitude, 0.2 mg/L), and acrophases of the detected variations were distributed evenly across the nycthemeron, with five acrophase peaks occurring during the day (0900–2100) and six at night (2100–0900).

**Discussion**

Visual inspection and statistical analysis of these data indicate that baseline hs-CRP concentrations in healthy subjects have remarkable stability throughout the course of the 24-h day. Thus, when comparing individual data in relation to hs-CRP concentrations representing quintiles of a larger study population (25) (dashed lines in Fig. 1), significant misclassification is unlikely to occur. These data demonstrate that hs-CRP testing in clinical situations can be performed without regard to time of day. These findings, therefore, extend prior studies that have demonstrated stable hs-CRP concentrations, not considering time-of-day effects, over a 5-year follow-up period (26).

CRP measured in healthy subjects has been validated as a powerful predictor of future risk of myocardial infarction or stroke (25). This effect appears to be present even among very young populations (27). The current data demonstrating a lack of circadian variation for hs-CRP also help to explain why hs-CRP concentrations appear to be a better predictor of vascular risk than IL-6, an inflammatory marker with a shorter half-life and a well-documented circadian variation (16, 17).

Prior studies evaluating diurnal variation of CRP concentrations have been limited to patients with active rheumatoid arthritis and markedly increased CRP concentrations. Results of these studies have been conflicting (28, 29). Until recently, technology has not permitted measurement of the very low concentrations of CRP seen in healthy individuals (29). To our knowledge this is the first study to investigate the presence of variability in hs-CRP concentrations in healthy subjects.

The absence of a diurnal variation in hs-CRP concentrations across the 24-h sleep-wake cycle may have several explanations. Studies of the appearance of CRP in plasma have shown that increased concentrations in response to exogenous IL-6 administration occur ~6–12 h after IL-6 challenge (30, 31). This is consistent with data on the increase of CRP after acute myocardial infarction (32). A single study has examined the half-life of the disappearance of allogeneic CRP in humans and found it to be ~15–19 h, independent of underlying disease or degree of CRP increase (33), whereas the measured decline of CRP concentrations after acute myocardial infarction in an earlier publication suggests an even longer plasma half-life (32). The combined effects of delayed production and prolonged half-life would thus dampen any variability in CRP concentrations attributable to variation in IL-6.

The fact that cosine fitting detected a significant variation in some subjects should not be construed as evidence for an underlying significant diurnal rhythm. The detected variations were evenly distributed in phase across the 24-h period, and the detected amplitudes were low. Thus, when data for the group were averaged, all systematic variability was lost (Fig. 2).

In conclusion, no diurnal variation of hs-CRP concentrations was detected in hourly samples of 13 healthy subjects under baseline conditions in the course of 24 h. Thus, determination of hs-CRP for cardiovascular risk prediction can be performed without concern for time of day.
References


