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Ibhar Al Mheid, Emory University
Frank Edward Corrigan III, Emory University
Farheen Shirazi, Emory University
Emir Veledar, Emory University
Qunna Li, Emory University
Wayne R. Alexander, Emory University
W Taylor, Emory University
Edmund K Waller, Emory University
Arshed Ali Quyyumi, Emory University

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Circadian Variation in Vascular Function and Regenerative Capacity in Healthy Humans

Ibhar Al Mheid, MD; Frank Corrigan, MD; Farheen Shirazi, MD; Emir Veledar, PhD; Qunna Li, MS; Wayne R. Alexander, MD, PhD; W. Robert Taylor, MD, PhD; Edmund K. Waller, MD, PhD; Arshed A. Quyyumi, MD

Background—Progenitor cells (PCs) are mobilized in response to vascular injury to effect regeneration and repair. Recruitment of PCs requires intact nitric oxide (NO) synthesis by endothelial cells, and their number and activity correlate with cardiovascular disease risk burden and future outcomes. Whereas cardiovascular vulnerability exhibits a robust circadian rhythm, the 24-hour variation of PCs and their inter-relation with vascular function remain unknown. We investigated the circadian variation of PCs and vascular function with the hypothesis that this will parallel the pattern observed for cardiovascular events (CVEs).

Methods and Results—In 15 healthy subjects (9 men, 37±16 years), circulating PCs and vascular function were measured at 8 AM, noon, 4 PM, 8 PM, midnight, 4 AM (only PCs counts), and 8 AM the following day. Circulating PCs were enumerated as mononuclear cells (MNCs; CD45med) that express CD34 as well as CD133, and their activity was assessed as the number of colonies formed by culturing MNCs. Vascular function was evaluated by measurement of endothelium-dependent, flow-mediated vasodilation (FMD) of the brachial artery and tonometry-derived indices of arterial stiffness. Higher CD34+ and CD34+/CD133+ cell counts were observed at 8 PM than any other time of the day (P-ANOVA=0.038 and <0.001; respectively) and were lowest at 8 AM. PC colony formation was highest at midnight (P-ANOVA=0.045) and lowest in the morning hours. FMD was highest at midnight and lowest at 8 AM and 8 PM, and systemic arterial stiffness was greatest at 8 AM and lowest at 4 PM and midnight (P-ANOVA=0.03 and 0.01; respectively).

Conclusion—A robust circadian variation in PC counts and vascular function occurs in healthy humans and both exhibit an unfavorable profile in the morning hours that parallels the preponderance of CVEs at these times. Whether these changes are precipitated by awakening and time-dependent physical activity or governed by the endogenous circadian clock needs to be further investigated. (J Am Heart Assoc. 2014;3:e000845 doi: 10.1161/JAHA.114.000845)

Key Words: arterial stiffness • circadian variation • endothelial function • progenitor cells

Vulnerability to adverse cardiovascular disease (CVD) events, such as myocardial ischemia, infarction, sudden cardiac death, and cerebrovascular accidents, are subject to an intrinsic circadian rhythm with greatest prevalence during the morning hours.¹⁻⁴ A similar circadian variability is observed in likely physiological precipitants, including heart rate and blood pressure (BP), vasoconstrictor tone, blood viscosity and fibrinolytic activity, platelet aggregability, epinephrine and norepinephrine levels, in addition to cortisol levels and plasma renin activity.⁵⁻¹²

Other precipitants of CVD events include impaired endothelial cell (EC) function and increased systemic arterial stiffness, which precede and contribute to the development of CVD and are long-term predictors of morbidity and mortality.¹³ Indeed, vascular dysfunction and reduced nitric oxide (NO) bioavailability promote a proinflammatory and -coagulatory milieu that also triggers acute vascular events.¹³,¹⁴ Whereas several studies confirm the presence of significant changes in vascular endothelial function by the time of day, which may differ between patient populations, others demonstrate no significant circadian or diurnal variation.¹⁵⁻²¹

Recently, bone-marrow (BM)-derived progenitor cells (PCs) have been identified and quantitated in the circulation. PCs are released into the bloodstream in response to vascular injury, homing to areas of ischemia, and actively participate in tissue repair and regeneration.²² In experimental models, mobilization of PCs is dependent on endothelial nitric oxide...
synthase (eNOS) activity and bioavailability of NO. In humans, recruitment of PCs into the circulation is modulated by the burden of CVD and its risk factors, and lower counts predict adverse outcomes. Significant circadian variation of circulating PCs in hospitalized patients and diurnal changes over 2 or 3 time points have been observed; however, their detailed 24-hour variability and relationship to changes in vascular function remain unknown.

The aim of our study was to investigate the circadian variation of circulating PC counts and their in vitro proangiogenic activity, measured using a colony formation assay, in relation to changes in endothelium-dependent and -independent measures of vascular function and arterial stiffness in healthy subjects. We hypothesized that PC counts and activity, in addition to endothelial function and arterial stiffness, have significant circadian variation that parallels the risk of CVD events.

Methods

Subjects

Fifteen healthy nonsmoking subjects were recruited after careful screening to include only normotensive (systolic arterial pressure < 140 or diastolic pressure < 90 mm Hg), normolipidemic (total cholesterol level < 200 and low-density lipoprotein < 120 mg/dL), normoglycemic (fasting glucose < 100 mg/dL), and nonobese subjects (body mass index ≤ 25) subjects. Other exclusion criteria included pregnancy, history of CVD or chronic illnesses, or intake of medications over 4 weeks preceding screening. This study was approved by the Emory University Institutional Review Board and subjects gave informed consent.

Subjects were instructed to adhere to a regular wake-sleep cycle (no later than 7 AM and 11 PM, respectively) for at least 2 weeks before the study. On the evening preceding testing, subjects were admitted to the Atlanta Clinical and Translational Science Institute (ACTSI) at Emory University Hospital, and an intravenous catheter was placed and maintained in the nondominant arm for the duration of the study. Lights were turned off at 11 PM, and testing started at 7:30 AM the following morning. Blood draws and vascular measurements were repeated at 4-hour intervals (except for vascular testing at 4 AM) and took approximately 1 hour to complete. Standardized, low-fat meals prepared by the ACTSI food services were provided to participants after study measurements at the 8:00 AM, 12:00 PM, and 6:00 PM time points, with a minimum of 4 hours separating consecutive meals. Subjects abstained from caffeinated beverages and were allowed to engage only in sedentary activities.

Circulating Progenitor Cell Counts and Proangiogenic Activity

Peripheral blood mononuclear cell subsets that are considered to be enriched for hematopoietic and endothelial progenitors were measured in CD45^med cells expressing CD34^+, CD133^+, and vascular endothelial growth factor receptor 2 (VEGFR2)^+ surface markers, either singly or in combination. The CD34^+ population represents hematopoietic progenitors, and the CD34^+/CD133^− subset are immature or early progenitors, because the CD133 epitope disappears as PCs mature. The VEGFR2^+ cell population exhibits an intact VEGFR2 receptor, indicating these cells’ capability to participate in angiogenesis and vasculogenesis. We measured circulating numbers of CD34^+, dual-positive CD34^+/CD133^− and CD34^+/VEGFR2^+, and triple-positive CD34^+/CD133^−/VEGFR2^+ cell populations.

Flow cytometry

Peripheral blood PCs were analyzed for the expression of surface antigens using direct flow cytometry (FCM; BD FACS Canto II Flow Cytometer; BD Biosciences, San Jose, CA). Three hundred microliters of venous blood (anticoagulant: EDTA) was incubated with fluorochrome-labeled monoclonal mouse antihuman antibodies, namely, FITC-CD34 (BD Biosciences), PE-VEGF2R (also known as kinase insert domain receptor; R&D Systems, Minneapolis, MN), and APC-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes. Red blood cells were removed by lysis in 1.5 mL of ammonium chloride lysing buffer, which was added to the sample and incubated for an additional 10 minutes. The lysis process was stopped by adding 1.5 mL of staining medium (PBS with 3% heat-inactivated serum and 0.1% sodium azide). Five million events were acquired from the cytometer with FlowJo software (Treestar, Inc., Ashland, OR) used for subsequent analysis of accumulated data. Absolute numbers of each cell subset per milliliter were determined by multiplying the counts with the number of monocytes per milliliter of blood.

Reproducibility testing

Twenty samples were repeatedly analyzed on 2 occasions by 2 technicians to assess for reproducibility. The percent repeatability coefficients (%) were calculated as SD of differences between pairs of measurements/mean of measurements × 100. The repeatability coefficients for the various cell types were: CD34^+, 7.4%; CD133^+, 7.0%; CD34^+/CD133^−, 4.4%; CD34^+/VEGFR2^+, 16.3%; and CD34^+/CD133^−/VEGFR2^+, 19.2%.
**Colony-forming assay**

To assess proangiogenic activity of circulating PCs, we performed a culture assay that quantifies colony formation units (CFUs) from mononuclear cells (MNCs), as detailed previously. This assay measures BM-derived precursor cells that mature into both proangiogenic hematopoietic- and endothelial-like cells, serving as a biomarker of a “combined” proangiogenic cell activity.

In brief, MNCs were isolated by density-gradient centrifugation from a 20-ml sample of blood using CPT (Becton Dickinson, Franklin Lakes, NJ) tubes, suspended in growth medium (DMEM supplemented with 20% FBS and 6.5% EC growth supplement) and plated on dishes coated with human fibronectin (Biocoat; Becton Dickinson). To eliminate mature circulating ECs, cells adherent after 24 hours were discarded and nonadherent cells were replated onto new fibronectin-coated six-well plates (1 million cells per well). Growth medium was changed every 2 days. Colonies, identified as multiple thin, flat cells emanating from central clusters of rounded cells, were counted 7 days after plating by a blinded observer and reported as the number of CFUs per 1 million cells.

Reproducibility was tested by measuring CFUs in 15 samples drawn 1 week apart from the same individuals. The overall correlation between the repeated assays was 0.84, and the mean number of colonies in each of the duplicates was 48±4 versus 50±3.

**Proliferation assay**

To determine whether the colonies were derived from mononuclear precursor cells by proliferation, 5-bromo-2'-deoxyuridine (BrdU; 1 μg/mL; Sigma-Aldrich, St. Louis, MO) was used to label cells for 24 hour on day 3 or 6 of growth. Subsequently, immunostaining with anti-BrdU (Dako, Carpinteria, CA) was used to detect proliferating cells within the colonies.

**Reverse-transcriptase polymerase chain reaction**

RNA was isolated from freshly isolated MNCs and from 7-day colonies using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from RNA samples, and reverse-transcriptase polymerase chain reaction (RT-PCR) was performed in an ABI 7900 (Applied Biosystems, Foster City, CA). RT-PCR was performed in an ABI 7900 (Applied Biosystems) instrument using pathway-focused gene expression PCR arrays from Super Array (human endothelial biology, angiogenesis). mRNA expression levels were determined using SYBR Green–based real-time PCR. Results were analyzed using the PCR Array data analysis Web portal to convert threshold cycles into fold changes.

**Immunocytochemistry**

We performed immunocytochemistry (ICC) for leukocyte and endothelial antigens. Seven colonies were fixed in 2% paraformaldehyde for 1 hour, washed in PBS, and blocked with 5% normal serum in 2% BSA in PBS for 30 minutes. Cells were incubated with the primary antibody (mouse anti-CD31, 1:300; eBioscience, San Diego, CA), mouse anti-CD45 (1:5000; eBioscience), mouse anti-CD3 (1:300; eBioscience), mouse anti-CD14 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti–vascular endothelial (VE) cadherin (1:50; Abcam, Cambridge, UK), rabbit polyclonal anti-eNOS (1:1000; Santa Cruz Biotechnology), and rabbit polyclonal anti–von Willbrand’s factor (vWF; 1:1000; Chemicon, Temecula, CA) in 2% BSA for 1 hour and then with biotinylated horse anti-mouse immunoglobulin G (IgG) for monoclonal primary antibodies and biotinylated goat anti-rabbit IgG for polyclonal antibodies (1:200; Vector Laboratories, Burlingame, CA) for 30 minutes. After another PBS wash, cells were stained with streptavidin-conjugated quantum dots (QDot 605; 1:100; Invitrogen, Carlsbad, CA) for 1 hour and counterstained with Hoechst nuclear stain. Images were acquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

To detect the surface expression of endothelial and hematopoietic lineage marker proteins, harvested colonies from 24-well plates were suspended in PBS after washing and incubated for 1 hour in the presence of the anti-CD14, -CD3, -CD45, -eNOS, -vWF, or -VE cadherin and then conjugated with biotinylated secondary antibodies. After washing with PBS, cells were stained with streptavidin-conjugated quantum dots (QDot 605; 1:100; Invitrogen), 1:100, and analyzed by FCM (FACSCalibur; Becton Dickinson).

ICC and FCM demonstrated cells expressing both leukocyte and endothelial antigens (80% of cells expressing CD45 and 20% of cells expressing eNOS and vWF), whereas reverse transcriptase demonstrated high levels of expression of proangiogenic factors as well as capillary-like tubes formed in culture.

**Assessment of Vascular Function**

After blood draw and an initial rest period of 10 minutes and with subjects in a supine position in a quiet, temperature-controlled room, BP was measured 3 times at 5-minute intervals by an automatic device (Omron, Kyoto, Japan) from the dominant arm and documented as the mean value of the final 2 measurements.

**Arterial stiffness**

We utilized the augmentation index (AIX) and carotid-femoral pulse wave velocity (PWV) for assessment of arterial wave...
Circadian Variation, PCs, Vascular Function  Mheid et al

**Statistical Considerations**

Study variables are described as the mean±SD for continuous variables, or as counts or proportions for categorical variables. To account for interindividual differences, mean values of study variables were calculated for each subject and used to determine percent deviation (Δ%) from the individual mean at every time point. A cubic (natural) spline function was used to smooth the interpolation line connecting different time points. Analyses were performed using SPSS (version 21.0; SPSS, Inc., Chicago, IL).

**Circadian Variation in Hemodynamic Measurements**

Arterial BP and heart rate measurements did not vary significantly by the time of day. Mean arterial pressure ranged between 87.4±7.1 and 90.4±11 mm Hg, and mean resting heart rate was between 58.6±11 and 61.6±10.5 bpm (Figure 1).

**Circadian Variation in PC Count**

There were significant circadian variations in circulating CD34+ (P<0.038) and dual-positive CD34+/CD133+ (P<0.001) counts by ANOVA, where counts were lowest at 8 AM and highest at 8 PM, with a difference of 551±175 and 412±85 cells/mL, respectively (Figure 1A,1B; Table 2). To account for interindividual differences, percent change from the individual mean was calculated for each subject. The percent change from the individual mean for CD34+ and CD34+/CD133+ PCs was lowest at 8 AM (−11±27% and −30±31%, respectively) and greatest at 8 PM (24±26%, and 44±40%, respectively; (Figure 2A,2B).

Similarly, within-subject differences were evaluated by repeated-measures ANOVA with Greenhouse-Geisser’s correction revealing significant differences in CD34+ and CD34+/CD133+ counts by the time of day (F=2.73 and 8.09; P=0.046 and <0.001, respectively).

**Results**

Baseline characteristics of study subjects are summarized in Table 1. The mean age of participants was 37 years, ranging from 21 to 67 years, 6 (40%) women, and 4 (27%) African-American subjects. Subjects were free of CVD risk factors.
Although a similar trend was observed in CD34+/VEGFR2+ and CD34+/CD133+/VEGFR2+ cell counts, these changes were not statistically different ($P$-ANOVA = 0.09 and 0.1, respectively).

### Circadian Variation in CFUs
There was a significant circadian variation in CFU counts across the 24-hour period ($P$-ANOVA=0.045; n=11), with the highest mean count (25.2±14.5) observed at midnight, compared to the nadir at 8 AM (13.2±14.5; Figure 2C). Within-subject differences were evaluated by repeated-measures ANOVA with Greenhouse-Geisser's correction revealing significant differences by the time of day ($F=3.96$; $P=0.014$).

The percent change from individual means was highest at midnight and lowest at 8 AM (54±36% and −36±38%, respectively; Table 2).

### Circadian Variation in Endothelial Function
There was a significant circadian variation in endothelial function ($P$-ANOVA=0.028), where brachial artery FMD peaked at midnight (5.9±2.2%). The percent change from the individual mean was greatest at midnight (30.4±34) and lowest at 8 AM and 8 PM (−9.9±31% and −11±28.8%, respectively; Figure 3A). Repeated-measures ANOVA with Greenhouse-Geisser’s correction revealed significant within-subject differences by the time of day ($F=3.38$; $P=0.047$).

### Circadian Variation in Arterial Wave Reflections
There was a significant circadian variation in AIX ($P$-ANOVA=0.014), with it being highest at 8 AM (22.2±8.4; Figure 3B). The percent change from the individual mean was highest at 8 AM (31.8±39.3%) and lowest at midnight (33.5±32.8%; Figure 3B). Differences evaluated by repeated-measures ANOVA with Greenhouse-Geisser’s correction confirmed significant within-subject differences by the time of day ($F=7.32$; $P=0.001$).

There was no significant circadian variation in PWV measurements, and the peak-to-nadir deviation did not exceed 30% in any of the subjects.

### Post-Hoc Analysis
Table 2 shows Tukey’s post-hoc HSD between the time point at which peak values were observed and other times of the day. There were significant within-subject differences in PC counts, endothelial FMD, and AIX between values observed at 8 AM and the time point at which the highest CD34+ and CD34+/CD133+ cell counts (8 PM) and at which peak FMD and lowest AIX (12 AM) were observed ($P<0.01$ for all; Figure 4).
Discussion

In this study of healthy subjects, we assessed the circadian variation of PC number and activity and their temporal relationship with the circadian variation in vascular function, measured as both endothelium-dependent vasodilation and arterial wave reflections/stiffness. We observed that the number of circulating PCs, measured as CD34+ and CD34+/CD133+ cell counts, and their proangiogenic activity, measured as CFUs in culture, were lowest in the early morning hours (8 AM). This coincided with the time when endothelial NO activity was lowest and arterial stiffness, measured as AIX, was greatest. We also found that PC counts were highest at 8 PM, and their proangiogenic activity was highest at midnight. In parallel, FMD was highest, and AIX was lowest, at midnight.

For the first time, to our knowledge, we have demonstrated a simultaneous and parallel 24-hour circadian variation in vascular function and PC numbers and activity. Our findings suggest that endogenous reparative or regenerative capacity varies by the time of day that may be related to, or reflected by, changes in vascular function. Whether these changes are the result of morning increases in physical activities after awakening (e.g., postural changes, mental activities, food intake, etc.) or are determined by an intrinsic circadian rhythm that is entrained over longer time periods (weeks or months) needs to be further investigated.

### Table 2. Tukey's Post-Hoc Honest Significant Differences (HSD) Between Peak and Values Observed at Other Times of the Day

<table>
<thead>
<tr>
<th>Time Point of Highest Value</th>
<th>Other Time Points</th>
<th>Mean Difference</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+, cells/mL</td>
<td>8 PM</td>
<td>8 AM</td>
<td>551.49*</td>
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<td>4 PM</td>
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<td>4 AM</td>
<td>506.53</td>
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<td></td>
<td></td>
<td>8 AM</td>
<td>522.3</td>
</tr>
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<td>CD34+CD133+, cells/mL</td>
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<td>412.28*</td>
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<td>12 AM</td>
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<tr>
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<td></td>
<td>4 AM</td>
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<tr>
<td></td>
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<td>FMD, %</td>
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<td>12.03</td>
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</table>

Δ % = percent deviation from the individual mean. AIX indicates augmentation index; CFU, colony formation units; FMD, flow-mediated vasodilation. *Denotes statistically significant difference between the time point of highest value and other individual time points, based on the 2-tailed P values (significance) for the studentized range statistic.

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Figure 2. Circadian variation in (A) CD34+ and (B) CD34+/CD133+ (C) colony forming unit (CFU) counts. Bottom box plots: middle band represents median, bottom and top of the box represent lower and upper quartiles, and whiskers represent highest and lowest values that are not outliers. Top: average percent deviation ($\Delta \%$) from the individual mean. $P$ values are for ANOVA, $N=15$ for A and B, $N=11$ for C.

Figure 3. Circadian variation in (A) brachial artery FMD and (B) AIX. FMD, brachial artery flow-mediated dilation (%). AIX, augmentation index. Bottom box plots: middle band represents median, bottom and top of the box represent lower and upper quartiles, and whiskers represent highest and lowest values that are not outliers. Top figures show average percent deviation ($\Delta \%$) from the individual mean. $P$ values are for ANOVA, $N=15$. DOI: 10.1161/JAHA.114.000845
We and others have previously shown circadian changes in vascular function that are similar to those observed in our subjects.\textsuperscript{5,15} In only 2 previous studies, diurnal variation over 3 time points in PC counts in selected subjects have been investigated. The circulating CD34\textsuperscript{+}/CD133\textsuperscript{+} cell count was higher at midnight than at noon or 6 PM in elderly inpatients,\textsuperscript{30} and higher PCs counts were observed at 10 PM, compared to 8 AM and 3 PM, in healthy men.\textsuperscript{31} These results are in line with our findings that detail the full 24-hour variation in PC counts in this study involving healthy men and women and further investigates circadian changes in their proangiogenic activity during culture.

Circulating PCs exhibited a single clear peak at 8 PM, and nadir values were observed in the morning hours. These pronounced differences (approximately 60\% for CD34\textsuperscript{+}/CD133\textsuperscript{+} cells) are not surprising in light of circadian changes of a similar magnitude in cytokines that mobilize BM progenitors, including granulocyte-colony stimulating factor and erythropoietin. The circadian pattern of these cytokines also parallels that of PCs, with marked evening increases, and is similarly observed in other peripheral blood components, such as the total circulating granulocyte or lymphocyte counts.\textsuperscript{36-40} PC mobilization is partly dependent on NO availability, which also exhibits significant circadian changes.\textsuperscript{41,42}

Several mechanisms may dictate the circadian pattern of PC egress from the BM, their circulating counts, and translocation into peripheral tissues,\textsuperscript{40,43} but the concomitant morning worsening of vascular function measurements and decrease in PC counts can be best explained by the surge of sympathetic nervous system activity observed in the morning hours. Indeed, sympathetic activity has been shown to blunt NO-mediated vasodilation and increase vascular tone, as well as regulate trafficking of PCs by adrenergic receptor activation.\textsuperscript{23}

Although some studies propose a counter-regulatory effect of enhanced endothelial function that may mitigate circadian increases in vascular tone,\textsuperscript{21,44} our findings demonstrate simultaneous worsening of brachial artery FMD and systemic arterial stiffness. Furthermore, AIX exhibited a lesser peak at 8 PM, alongside a significant decline in FMD at that same time point (Table 2). However,
the extent of improvements or worsening in arterial stiffness or wave reflections did not correlate with those in FMD at each time point. Nonetheless, both vascular function and PC counts exhibited an unfavorable morning profile with depressed PC counts and greater endothelial dysfunction, as well as increased arterial stiffness. This is also in agreement with recent findings of a possible lower hemostatic “set point” for vascular function parameters after a period of sleep.45

Limitations

Our study size may have been too small to detect circadian changes in rare cell populations (CD34+/VEGFR2+/CD133+ cells). Although both men and women across a wide age range were included, this study was designed to elucidate circadian changes in healthy individuals, thus limiting the generalizability of our findings. Similar to several other physiologic parameters, circadian variation in PC counts or vascular function may be blunted or accentuated in specific disease conditions, and this requires further investigation.

Whereas continuous wall motion tracking might have identified circadian variability in the postdeflation timing of the maximal dilatory responses, which may vary by age,46 we used the 1-minute time point to determine brachial artery flow-mediated dilation because of this technique’s high reproducibility and short duration.

This present study was not designed to detect BP or heart rate changes; however, we did not expect the absence of significant circadian changes. Although these measurements were performed before vascular testing, which required walking to the vascular lab and interrupted an otherwise sedentary day, subjects underwent an initial rest period of at least 10 minutes. Hemodynamic measurements were also performed after blood draws and, together with vascular testing, might have exerted a “white coat effect” on these measurements.

The study included 6 women (mean age of 28; range, 23 to 38 years), and all were premenopausal. Thus, differences that may occur after menopause cannot be addressed in this study population.

References

Circadian Variation, PCs, Vascular Function


