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Rapid Suppression of Bone Formation Marker in Response to Sleep Restriction and Circadian Disruption in Men

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WMK, PW, SWC, MM, and NV have nothing to disclose.

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Abstract

PURPOSE: A serum bone formation marker (procollagen type 1 N-terminal, P1NP) was lower after ~3 weeks of sleep restriction combined with circadian disruption. We now describe the time course of decline.

METHODS: The ~3 week protocol included two segments: "Baseline," ≥10 hours (h) sleep opportunity/day x 5 days; "Forced Desynchrony" (FD), recurring 28-h days (circadian disruption) with sleep restriction (~5.6 h sleep per 24 h). Fasted plasma P1NP was measured throughout the protocol in nine men (20–59 years old). We tested the hypothesis that PINP would steadily decline across the FD intervention because the magnitude of sleep loss and circadian misalignment accrued as the protocol progressed. A piecewise linear regression model was used to estimate the slope (β) as ΔP1NP per 24 h with a change point mid-protocol to estimate the initial vs. prolonged effects of FD exposure.

RESULTS: Plasma P1NP levels declined significantly within the first 10 days of FD ($\hat{\beta}$= −1.33 μg/L per 24-h, $p < 0.0001$) and remained lower than baseline with prolonged exposure out to 3 weeks ($\hat{\beta}$= −0.18 μg/L per 24-h, $p = 0.67$). As previously reported, levels of a bone resorption marker (C-telopeptide (CTX)) were unchanged.

CONCLUSION: Sleep restriction with concurrent circadian disruption induced a relatively rapid decline in P1NP (despite no change in CTX) and levels remained lower with ongoing exposure. These data suggest 1) even brief sleep restriction and circadian disruption can adversely affect bone metabolism, and 2) there is no P1NP recovery with ongoing exposure that, taken together, could lead to lower bone density over time.

MINI-ABSTRACT

We describe the time course of bone formation marker (P1NP) decline in men exposed to ~3 weeks of sleep restriction with concurrent circadian disruption. P1NP declined within 10 days and

remained lower with ongoing exposure. These data suggest even brief exposure to sleep and circadian disruptions may disrupt bone metabolism.

Keywords

Bone loss; sleep restriction; circadian disruption; bone formation; P1NP

1. INTRODUCTION

An adequate amount of sleep during the biological night is important for optimal health. Short sleep duration and circadian disruption (e.g., shiftwork, jet lag) are associated with insulin resistance and diabetes mellitus type 2 [1–3], obesity [4,3], cardiovascular disease [5] and impaired cognition [6]. Sleep and circadian disturbances are also associated with impaired bone health [7,8]. Postmenopausal women in the Nurses' Health Study who reported 20+ years of nightshift work had a 37% higher risk of wrist and hip fractures compared to those who never worked the nightshift [9]. Shiftwork has also been associated with lower bone mineral density (BMD) in postmenopausal nurses in Chile [10] and men and women in the Korean National Health and Nutrition Examination Survey [11], but not in middle-aged individuals in the National Health and Nutrition Examination Survey (NHANES) [12]. Data from animal studies also suggest that sleep and circadian disturbances may alter bone health [13–15]. Rats exposed to chronic sleep restriction had an early decrease in a bone formation marker despite initially stable levels of a bone resorption marker [14], decreased bone formation with no change in bone resorbing activity on bone histomorphometry [13], and lower bone mineral density [13] compared to ambulation controls.

We previously reported a significant decline in a bone formation marker (N-terminal propeptide of type 1 procollagen, P1NP) despite no change in a marker of bone resorption (C-terminal telopeptide of type 1 collagen, CTX) in ten healthy men after approximately three weeks of cumulative sleep restriction and concurrent circadian disruption, as can occur in people performing rotating shiftwork [16]. The P1NP decline was greater in young men (28%) who had higher levels of P1NP at baseline compared to older men (18%). This uncoupling of bone turnover, where markers of formation decline but resorption marker levels remain unchanged, parallel results from chronic sleep restriction studies in rats that were accompanied by declines in BMD [13,14], suggesting that sleep and circadian disturbance in humans could lead to bone loss, osteoporosis, and increased fracture risk if sustained over time. We sought to describe the time course and trajectory of the previously reported decline in serum P1NP observed in healthy men after ~3 weeks of sleep restriction combined with circadian disruption. [16].

2. METHODS

Study Design & Participants

Plasma samples from a previously performed clinical study [17] were used for this analysis. Participants were recruited through advertisements in the newspaper, on websites and via

flyers [17]. Healthy participants were eligible to participate if they passed physical and psychological exams performed by licensed physicians, had no sleep disordered breathing or other sleep disorder based on overnight polysomnography, and had no history of shift work or travel across more than two time zones for at least 3 months prior to enrollment[17]. Self-reported race/ethnicity were as follows: White/not Hispanic or Latino n = 6; White/Hispanic n = 2; Asian and White/not Hispanic or Latino n = 1.

The protocol (Figure 1) was performed in controlled laboratory conditions in the Intensive Physiological Monitoring Unit at the Center for Clinical Investigation at Brigham and Women's Hospital between 2007 and 2010. The protocol included a 3-week pre-admission phase during which participants were required to maintain a 10-hour per day sleep opportunity and a 5-day inpatient baseline segment where participants had at least 10 hours of sleep opportunity per day. The men then underwent a forced desynchrony (FD) protocol where they lived on a recurring 28-hour day with 6.5 hours in bed each 28-hours for approximately three weeks (the precise duration depended on each participant's circadian period length estimated from core body temperature recordings throughout the protocol, to enable measurements upon awakening at similar circadian phases at the beginning and end of the intervention). This protocol induced cumulative sleep restriction (5.6 hours of sleep opportunity per 24-hour period) and concurrent circadian disruption, akin to the stresses endured during rapidly rotating shiftwork. Nine of the original 10 healthy men who previously demonstrated a significant decline in serum P1NP from baseline to post FD intervention [16] had sufficient plasma samples for this analysis. As previously reported [16,17] all nine men were healthy (based on questionnaire and screening) and performed some habitual exercise prior to admission, including walking, endurance (e.g., jogging, swimming), and resistance exercise, but none was a competitive athlete. The study was performed in dim light (<0.02 lux during sleep opportunity; <15 lux at horizontal level during wake) and participants received a eucaloric, controlled nutrient diet (55% to 60% carbohydrate, 15% to 20% protein, and 15% to 30% fat) and ≥2.5 L of fluid per 24 hours. Timing of meals was controlled throughout the protocol relative to the midpoint of the time in bed interval.

All participants provided written informed consent [17]. All procedures were approved by the Partners Human Research Committee and were conducted in accordance with the Declaration of Helsinki. The current analysis used de-identified samples, was deemed nonhuman subjects research by The University of Colorado Institutional Review Board, and was approved by Brigham and Women's Hospital institutional review board.

Plasma Samples

Assays were performed at Oregon Health & Science University (OHSU) in the Oregon Clinical and Translational Research Institute (OCTRI) laboratory in September 2017. Samples were maintained at < −70°C until assayed. To determine the trajectory of P1NP decline, P1NP was measured in 7 to 14 fasted plasma samples from each participant throughout the protocol (represented as "F" in Figure 1). The forced desynchrony protocol evenly distributes sleep and wake across the circadian cycle [18]. Therefore, the fasted blood was collected at different times of the biological day/night but at least 12 hours after their

last meal. When these fasted samples were grouped across each week, the full range of circadian phases were captured.

Plasma P1NP was measured using the Orion Diagnostica assay that was used for previously reported serum assessments [16] (interassay coefficients of variation (CV) at low and high control were 3.9% and 8.2% respectively). Plasma samples were run in duplicate and averaged for the final result. The difference between duplicates was 8.9%. All plasma samples from each man were analyzed in the same assay to minimize inter-assay differences.

We previously reported a significant increase in *serum* sclerostin in the young men only [16], therefore, sclerostin was also measured in duplicate in the same *plasma* samples as P1NP using the ALPCO Biomedica assay. Inter-assay CV was 21% and the average difference in sclerostin levels between duplicate samples was 14%. These CVs were much larger than our prior analysis, possibly due to differences in the assay kit, sample type (serum vs. plasma), longer duration of sample storage, or sample volume. Thus, for quality control, sclerostin data were excluded due to unacceptably high variability in measurements and reproducibility concerns.

Actigraphically-Assessed Wrist Activity

As previously reported [16,17], actigraphically-assessed wrist activity (Actiwatch_L; Mini Mitter, Bend, OR) in arbitrary counts/minute was averaged for each condition (i.e., over all baseline days and over all FD days) when available to estimate each man's physical activity. Percent change in wrist activity from baseline through the FD intervention was calculated for each individual who had data from both conditions (data were missing for two participants at baseline due to technical device issues).

Statistical Analysis

A piecewise linear regression was set up for maximum likelihood estimates in a repeated measures model to estimate the group slope (β) as ΔPINP per 24 hours across the sleep/circadian (FD) intervention for all participants (n = 9) with a change point placed half way through the entire protocol. The slope (β) before and after this change point was assessed to estimate initial vs. prolonged effects of FD exposure. This model was also used to estimate the slopes in young and older men separately. We did not identify a significant diurnal variation in serum P1NP in these men [19], therefore no adjustment was made for circadian phase. The goal of this secondary analysis was to describe the trajectory, or time course, of P1NP decline across the forced desynchrony protocol for future hypothesis generation and study design considerations. Our initial study [16] had a predetermined sample size of 10 men. We had 80% power to detect a mean paired difference of 1.3 standard deviations in P1NP and CTX from baseline to post-intervention with a conservative α level of 0.0125 (adjusted for multiple comparisons)[17]. Previously obtained fasted morning cortisol levels drawn at baseline and at the end of forced desynchrony were used to calculate the change in cortisol across the protocol. Spearman correlations were used to determine if the changes in cortisol and P1NP (using the same previously obtained fasting morning levels) were

correlated. All analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC). All figures were generated using GraphPad Prism 7.02 (GraphPad software, La Jolla, CA).

3. RESULTS

The nine participants included six young (aged 20–27 years, mean 23.5 years) and three older (55–59 years old, mean 56.7 years) healthy men with an average BMI of 24.7 kg/m^2. As previously reported [16,17], all the participants were without pre-existing sleep disorders as verified by questionnaires and polysomnography.

The trajectory of P1NP decline for each man is depicted in Figure 2. On average, plasma P1NP declined 14.73 ± 13.51 μg/L (standard deviation, SD) from an average starting level of 50.04 ± 17.84 μg/L in these nine men across the protocol. In group statistical analysis, P1NP declined significantly ($\hat{\beta}$= −1.33 μg/L per 24-h, $p < 0.0001$) within the first 10 days of initial exposure to the FD protocol and remained lower than baseline with ongoing exposure (Figure 3). There was no evidence of a recovery in P1NP levels with prolonged exposure to sleep restriction and concurrent circadian disruption.

We explored whether the decline in P1NP differed in the young and older men. Plasma P1NP was higher at baseline in the young compared to older men ($mean_{young}$ = 54.31 ± 17.90 μg/L vs. $mean_{older}$ = 41.50 ± 17.55 μg/L). In the young men, P1NP declined −1.76 μg/L per 24 h with initial exposure ($p = 0.0005$) and continued to decline with ongoing exposure ($\hat{\beta}$= −1.04 μg/L per 24 h, $p = 0.002$). The decline in three older men was less steep and not statistically significant ($\hat{\beta}$= −0.49 μg/L per 24 h, $p = 0.47$ with initial exposure; $\hat{\beta}$= −0.23 μg/L per 24 h, $p = 0.52$ with ongoing exposure) but a larger study would be required to ascertain the clinical relevance of these differences.

As previously reported, serum CTX did not change significantly from baseline to post FD intervention [16]. Actigraphically-assessed wrist activity was consistently increased 46% ± 29% throughout the forced desynchrony intervention compared to baseline days (n = 7), indicating that, on average, the men's wrist activity counts increased as the protocol progressed, considered an effect of longer time awake. On average, there was a non-significant increase of 3% in fasted morning cortisol from baseline to the end of forced desynchrony in these nine men (Δ = 0.31 ± 2.82 mg/dL; range −3.51 to +4.84 mg/dL). The change in cortisol was not correlated with the change in P1NP (r = 0.28, p = 0.46).

4. DISCUSSION

Findings indicate that sleep restriction and concurrent circadian disruption, akin to rapidly rotating shiftwork, induce a significant decline in a plasma marker of bone formation (P1NP) in men within the first 10 days of exposure, despite as previously reported [16], no apparent change in a marker of bone resorption (CTX). The rapid decline in P1NP suggests that even intermittent, non-sustained exposure to sleep restriction and circadian disruption could have adverse skeletal effects. There was no evidence to suggest that the initial decline in P1NP reversed with ongoing exposure to sleep restriction and concurrent circadian disruption; indeed, P1NP levels remain lower or (for the young men) continued to decline

over 3 weeks of exposure. These changes in biochemical markers of bone turnover are similar to chronic sleep restriction studies performed in rats that also revealed decreased bone formation on histomorphometry and lower BMD over time [13,14]. If similar changes occur in women, these data may also explain the association between shiftwork and increased fracture risk previously reported in post-menopausal women [9]. Therefore, we hypothesize that even brief exposures to sleep restriction and circadian disruption can impair bone metabolism due to the relatively rapid uncoupling of bone turnover markers. Over time, either intermittent or chronic sleep and circadian disruptions could limit attainment of optimal peak bone mass if they occur early in life, or accelerate the age-related decline in bone mass later in life. Additional research is needed to confirm if the observed P1NP decline translates into changes in BMD and bone quality in humans exposed to sleep and circadian disruption. Moreover, the ability to recover from these detrimental changes in bone metabolism with either restoration of normal sleep/wake cycles or adaptation to prolonged exposure are important, unanswered questions.

We previously reported that P1NP levels declined more with cumulative sleep restriction and concurrent circadian disruption in the young compared to the older men [16]. Our current study extends those data to illustrate the time course of the previously observed P1NP decline, which was in fact steeper in the younger men than in the older men. The young men had an initial steep P1NP decline and continued to decline with ongoing exposure. The older men had lower baseline levels of P1NP and a more modest decline, however, the limited sample size (n = 3, one fewer than our prior analysis [16]) precludes strong conclusions in this subgroup. The steeper P1NP decline observed in some individuals may be related to participant age and/or baseline P1NP concentration. Future larger studies should investigate if and how the effects of sleep and circadian disruption are modified by age and baseline bone turnover marker concentrations and if sleep and circadian interventions in people with sleep and circadian disturbance can increase bone formation.

The mechanisms by which sleep restriction and circadian disruption alter bone formation more than resorption still need to be elucidated. The changes observed in P1NP may be due to differences in P1NP clearance or study conditions independent of the sleep/circadian interventions. However, the diurnal variation in bone turnover markers with a peak that occurs in the early morning hours [20,21,19] and differences observed in the skeletal phenotypes of clock gene knockout animal models [22–26] suggest there may be direct effects of sleep and circadian disruption on bone as well. In addition, it is unknown if or how P1NP clearance would be affected by the imposed sleep restriction and circadian disruption. Physical inactivity can increase sclerostin levels and decrease bone formation. However, it is unlikely that physical inactivity induced by the inpatient laboratory environment substantially contributed to these findings because actigraphically-assessed wrist activity was consistently increased through the intervention compared to baseline [17,16]. Furthermore, if these changes were due to decreases in mechanical loading (compared to pre-admission free-living conditions) or were mediated by other possible effects of the sleep restriction and circadian disruption (e.g., increased inflammation [27], decreased vitamin D), then an increase in bone resorption would have been expected [28,29] but this was not observed. Food intake can affect bone turnover marker levels. However, diet composition and timing were strictly controlled throughout the protocol and therefore, it is unlikely that

the observed changes in bone turnover markers were due to diet. Leptin, via the sympathetic nervous system, can inhibit bone formation and favor bone resorption [30–33]. As previously reported, the 24-hour profile of leptin actually decreased slightly (but significantly) during this sleep restriction and circadian disruption in a prior analysis of all 21 participants [17]. Furthermore, as previously reported [17], participants had a minimal loss of body mass (~1%) across this portion of the protocol and had worsened postprandial hyperglycemia [17]. The P1NP decline with sleep restriction and circadian disruption was likely unrelated to the negligible decrease in body mass. A prior analysis of all participants also reported a significant increase in fasted cortisol levels relative to baseline that remained stable from the first to the third week of the sleep and circadian disruption [17]. Similarly, the nine men in this analysis had a non-significant increase in fasted morning cortisol. The increase in serum cortisol may have contributed to the P1NP decline in some men. However, the change in P1NP was not correlated with the change in cortisol. Furthermore, given that the magnitude of change in cortisol from baseline to forced desynchrony in these nine men was small and less consistent than the decrease in P1NP, it is unlikely to be the sole mechanism responsible for the observed P1NP decline.

This study had several strengths, including a rigorously designed and demanding protocol performed in controlled laboratory conditions to simulate the stresses of rapidly rotating shiftwork. However, there were limitations. The data came from a relatively small sample of men only. Although the sample size was limited, results were relatively consistent across all participants with a statistically and clinically significant decline in P1NP in response to rapidly rotating shiftwork combined with sleep restriction. Although the P1NP assay used for this analysis suggests using serum, it has been used for plasma samples previously [21]. In addition, our internal analysis using recently collected blood found plasma and serum P1NP values had similar concentrations and variability. The original study was not specifically designed to address bone outcomes and did not include a direct measure of bone formation (e.g., bone biopsy). However, the study design was sufficient for investigating the bone biomarker response to concurrent sleep restriction and circadian disruption. Furthermore, previous studies of the effects of sleep restriction on bone turnover in rats showed similar patterns of change in bone biomarkers that were consistent with concurrent histomorphometry [13], and bone turnover markers correlate well with histomorphometry in humans [34]. Similar to other sleep/circadian intervention studies, there was no control group. Instead the focus was on within-subject changes from baseline sleep replete conditions. Therefore, it is possible that the observed changes were due to the study environment itself (e.g., food intake, lack of exercise) and not the sleep/circadian intervention. We feel this is less likely because meal composition and timing were identical throughout the protocol, participants were ambulatory and participants' actigraphically-assessed wrist activity increased from the beginning to the end of the protocol, likely because they spent more time awake and out of bed during the sleep restriction portion of the protocol. Nevertheless, future intervention studies to evaluate the skeletal effects of sleep and circadian interventions in humans should control for potential confounders (e.g., heparinized intravenous lines, physical activity, stress, light exposure, etc.,).

In conclusion, these data from healthy men indicate that plasma P1NP declines within the first 10 days of exposure to sleep restriction and concurrent circadian disruption and remains

lower than baseline with ongoing exposure despite no change in CTX. The rapid decline in P1NP with initial exposure to sleep restriction and circadian disruption and persistently lower P1NP with ongoing exposure is notable. This uncoupling of bone turnover markers may have an adverse clinical impact on bone health if sleep and circadian disruptions are brief (e.g., few days of shift work) or sustained (e.g., space missions, rotating shiftwork), especially if they occur during critical times of bone modeling and remodeling.

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Abbreviations:

BMD	Bone mineral density
P1NP	N-terminal propeptide of type 1 procollagen
CTX	C-terminal telopeptide of type 1 collagen
FD	Forced desynchrony
BTM	Bone turnover markers

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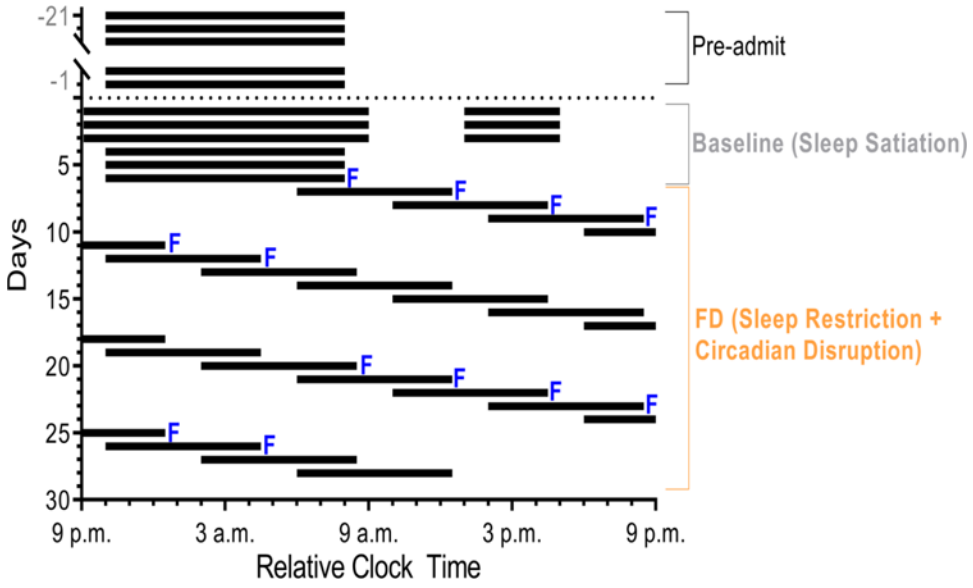

Figure 1. Study Protocol

(adapted from Swanson et al [16] and Buxton et al[17])

Horizontal black bars depict sleep opportunities. Blue “F” represent time points when fasted plasma was collected to measure P1NP for this analysis. All men had a 3-week pre-admission phase where they had 10 hours of sleep opportunity per day with consistent bed/wake times. Upon admission, men had at least 10 hours of sleep opportunity per day during baseline days. For the next ~3 weeks, men were exposed to a “forced desynchrony” (FD) protocol which is akin to the stresses endured during rapidly rotating shiftwork. The FD protocol required the men to live on a 28-hour day (instead of the typical 24-hour day) to induce circadian disruption and included the equivalent of 5.6 hours of sleep opportunity per 24 hours (6.5 hours per 28 hours). These study conditions induced circadian disruption and cumulative sleep restriction, akin to the stresses endured during rapidly rotating shiftwork.

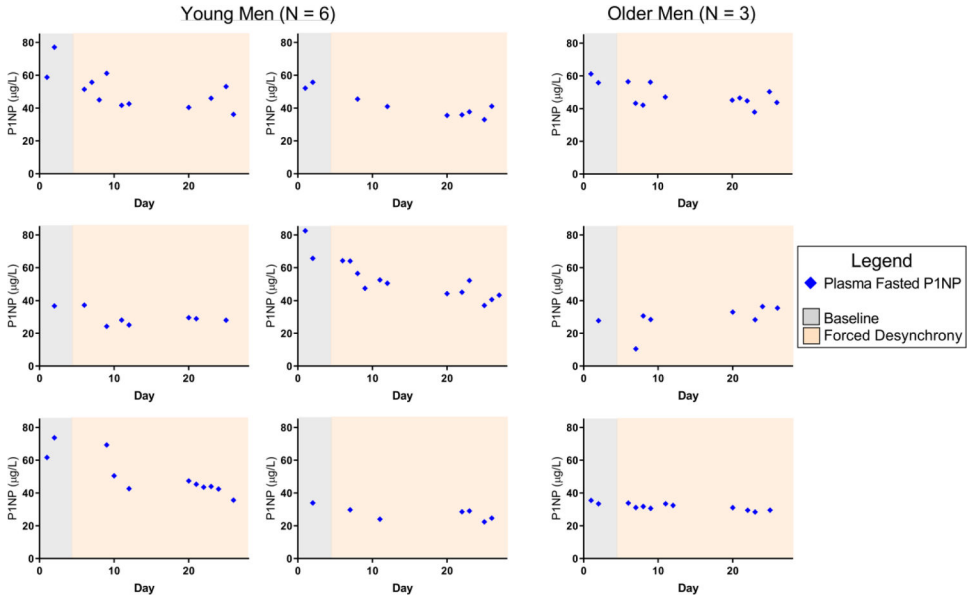

Figure 2. Individuals' Plasma P1NP Levels Across the Protocol

P1NP levels for the six young men in the first two columns and the three older men in the far right column. X-axis represents calendar days. Blue diamonds represent fasting plasma P1NP levels used for this analysis. Gray background indicates samples were obtained during the baseline segment of the protocol and orange background indicates samples were obtained during the forced desynchrony (FD) segment of the protocol. The decline in fasting P1NP from baseline appeared to occur within the first 10 days of the intervention and remained lower over the remaining weeks of sleep restriction with circadian disruption.

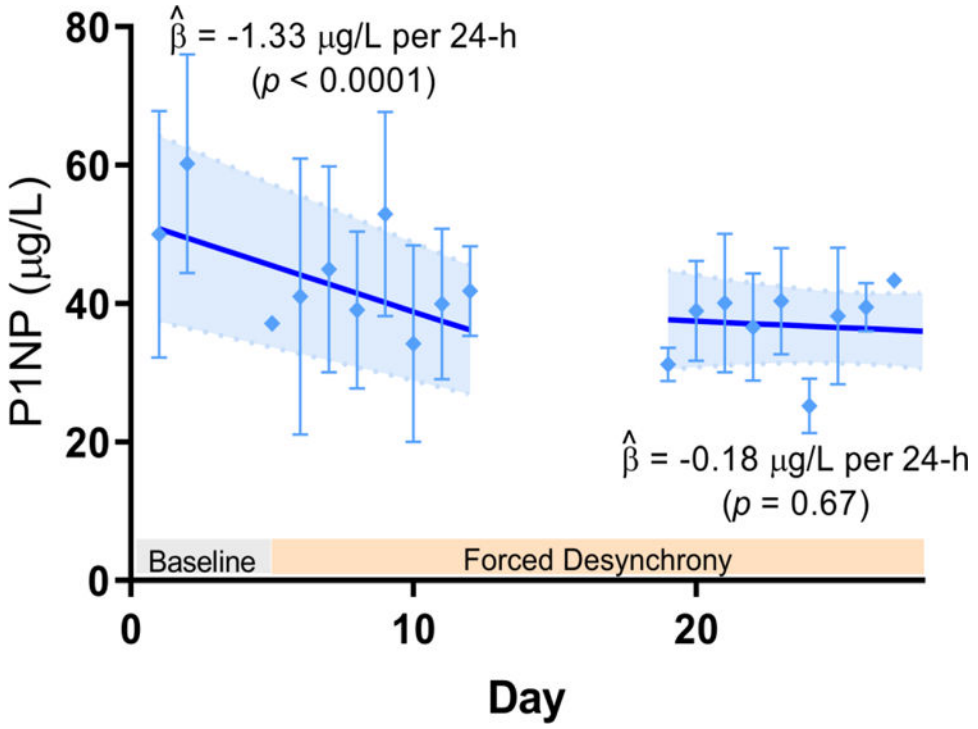

Figure 3. P1NP Decline Across the Forced Desynchrony Protocol

Fitted piecewise linear regression curve for all men (n = 9) with group averages showing the trajectory of P1NP decline across the study protocol. X-axis represents calendar days. Solid dark blue line indicates fitted curve, light blue dotted line and shading represent 95% confidence intervals. Blue diamonds with vertical standard deviation (SD) bars represent group averages for each available time point. The number of men contributing data to each time point varies, and time points with data from only one man have no SD bars. Horizontal shaded bars along the x-axis represent approximate phase of protocol (gray = baseline, orange = forced desynchrony). Gap in data points represents i.v. break for participants when no blood was drawn. P1NP levels declined significantly ($\hat{\beta}$= −1.33 µg/L per 24-h, $p <$ 0.0001) with initial exposure to the FD protocol and remained lower ($\hat{\beta}$= −0.18 µg/L per 24-h, $p = 0.67$) without evidence of recovery in P1NP levels with ongoing exposure. $p < 0.05$ indicates the slope of the line is significantly different from 0.