Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss

Rachel Leproult, PhD[1,2], Ulf Holmbäck, PhD[1,3] and Eve Van Cauter, PhD[1]

[1]Sleep, Metabolism and Health Center, Department of Medicine, University of Chicago, Chicago, IL, USA.

[2]Neuropsychology and Functional Neuroimaging Research Unit at the Center for Research in Cognition and Neurosciences and the ULB Neuroscience Institute, Université Libre de Bruxelles (ULB), Brussels, Belgium.

[3]Department of Public Health and Caring Sciences; Clinical Nutrition and Metabolism, Uppsala University, Uppsala, Sweden.

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Address for correspondence:
Rachel Leproult, PhD
Neuropsychology and Functional Neuroimaging Research Group (UR2NF) at the Center for Research in Cognition and Neurosciences (CRCN) and the ULB Neuroscience Institute (UNI)
Campus du Solbosch, CP191
Avenue F.D. Roosevelt 50
B-1050 Bruxelles
BELGIUM
Email: rleproult@gmail.com

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ABSTRACT

Shift workers, who are exposed to irregular sleep schedules resulting in sleep deprivation and misalignment of circadian rhythms, have an increased risk of diabetes relative to day workers. In healthy adults, sleep restriction without circadian misalignment promotes insulin resistance.

To determine whether the misalignment of circadian rhythms that typically occurs in shift work involves intrinsic adverse metabolic effects independently of sleep loss, twenty-six healthy adults were studied using a parallel group design. Both interventions involved 3 inpatient days with 10-h bedtimes followed by 8 inpatient days of sleep restriction to 5 hours, either with fixed nocturnal bedtimes (circadian alignment) or with bedtimes delayed by 8.5 hours on 4 of the 8 days (circadian misalignment). Daily total sleep time during the intervention was nearly identical in the aligned and misaligned conditions (4h48min[5min] vs. 4h45min[6min]). In both groups, insulin sensitivity significantly decreased after sleep restriction, without compensatory increase in insulin secretion, and inflammation increased. In male participants exposed to circadian misalignment, both the reduction in insulin sensitivity and the increase in inflammation doubled, compared to those who maintained regular nocturnal bedtimes.

Circadian misalignment as occurs in shift work may increase diabetes risk and inflammation, independently of sleep loss.

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Worldwide in industrialized countries, nearly 20% of working adults are shift workers (1-3). Prospective epidemiologic studies indicate that shift work is associated with an increased risk of type 2 diabetes and cardiovascular disease (4-9). Shift work is generally associated with chronic sleep loss, which adversely impacts glucose tolerance and cardiovascular function (10-13). Most shift workers also have irregular sleep schedules, resulting in circadian misalignment, a condition where the behavioral sleep-wake schedule and the feeding schedule are not aligned with endogenous circadian rhythms. Because the timing of sleep and food intake synchronizes a number of neural, endocrine and metabolic rhythms, while others remain locked to the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, circadian misalignment involves a lack of synchrony amongst endogenous 24-h rhythms. Two recent laboratory studies where healthy participants were exposed to circadian misalignment associated with reductions in total sleep time provided causative evidence for a deleterious impact on diabetes risk and cardiovascular function (14-15). Whether circadian misalignment has adverse cardio-metabolic effects that are distinct from those imparted by sleep loss is a fundamental and as yet unanswered question with important implications for the health of millions of shift workers.

We therefore designed an experimental study where the major determinants of diabetes risk (insulin sensitivity and beta cell function) as well as a predictor of cardiovascular risk (plasma levels of high sensitivity C-Reactive Protein (hsCRP), an inflammatory marker (16-19)) are compared in healthy adults under conditions of

circadian alignment versus circadian misalignment, keeping the amount of daily sleep identical.

RESEARCH DESIGN AND METHODS

Protocol and Participants

The protocol (Figure 1) was approved by the Institutional Review Board of the University of Chicago where all participants were studied after giving written informed consent.

We compared two 11-day interventions using a parallel group design. For logistic reasons primarily related to staffing, the two interventions could not be conducted simultaneously. Therefore, the participants were not formally randomized but assigned to the intervention that was implemented at the time of their recruitment, without being aware that there was an alternate intervention.

Participants from the local community responded to advertisements inviting healthy adults with normal body weight and ages 21 to 39 years to participate in a research study entitled "Extended work schedules and health: Role of sleep loss" and involving 2 weeks of hospitalization. Supplemental Figure 1 shows the flow diagram of subject recruitment and participation. Participants underwent a physical examination and laboratory tests to rule out endocrine, psychiatric and sleep disorders, medication use, smoking, excessive alcohol or caffeine consumption, shift work or travel across time zones during the past 2 months, self-reported habitual sleep less than 7.5 h or more than 8.5h.

During one week prior to the study, subjects were asked to comply with standardized schedules (23:00h-07:00h bedtimes). Compliance was verified with wrist

activity recordings (Actiwatch, MiniMitter Inc.). In women, the study was initiated during the early follicular phase of the menstrual cycle.

The interventions (Figure 1) involved three days with 10-hour bedtimes (22:00h-08:00h: B1-B3; baseline rested condition) followed by 8 days with 5-hour bedtimes (R4-R11), either with bedtimes always centered at 03:00h (00:30h-05:30h, circadian alignment) or with bedtimes delayed by 8.5 hours on 4 days (09:00h-14:00h on days R5-R6, R8-R9; circadian misalignment). Both interventions involved the same amount of bedtime restriction, representing 24 hours of lost sleep opportunity over 8 days and were followed by 3 nights of recovery sleep.

An intravenous glucose tolerance test (ivGTT) was performed after an overnight fast at 09:00h on B2 and R10. Frequent blood sampling via an intravenous catheter was performed during B3 and R11. Levels of hsCRP were measured at 4-h intervals. Saliva samples for melatonin assays were obtained every 30 minutes on R4 and R11, from 14:00h until bedtime.

Prior to the study, each participant met with a dietitian to determine food preferences and select three nutritionally balanced menus that were served on a rotating basis. On blood sampling days (B3 and R11), identical carbohydrate-rich meals were served at 14:00h, 19:00h and 09:00h and were completely ingested within 20 minutes. No other caloric intake was allowed on these 2 days. During the entire protocol, participants abstained from caffeinated beverages.

Sleep data

Polygraphic sleep recordings (Neurofax EEG-1100A, Nihon Kohden, Foothill Ranch, CA) were scored visually at 30-sec intervals in stages Wake, I, II, slow-wave sleep (SWS) and rapid-eye-movement (REM) according to standardized criteria (20). Total Sleep Time was defined as minutes of stages I+II+SWS+REM.

Intravenous glucose tolerance test (ivGTT)

Following three baseline samples, glucose (0.3 g/kg) was administered intravenously. Blood samples were taken at minutes 2, 3, 4, 5, 6, 8, 10, 12, 15, 19, 21, 22, 24, 26, 28, 30, 40, 50, 60, 70, 90, 100, 120, 140, 180, 210 and 240 after glucose injection. At minute 19, insulin (0.02 U/kg) was administered intravenously. Minimal model analysis (21) were performed using the software Minmod Millenium (22) and provided insulin sensitivity (SI), the acute insulin response to glucose (AIRg; a measure of beta-cell response), and the disposition index (DI=AIRg x SI), a marker of diabetes risk.

One woman experienced hypoglycemia during her baseline ivGTT. Her ivGTT data were not included in the analysis.

Assays

Glucose concentrations were assayed at bedside (Model 23A, Yellow Springs Instrument Company, Yellow Springs, OH, USA). Serum insulin and hsCRP concentrations were measured using high sensitivity chemiluminescence assays (Immulite, Diagnostic Products Corporation).

Melatonin was assayed in saliva and in serum by radioimmunoassay (Pharmasan Labs Inc, Osceola, WI, USA) with a limit of sensitivity of 3.5 pg/ml and an intra-assay coefficient of variation of 8 %.

Circadian phase

Circadian phase was determined via the "dim light melatonin onset" (DLMO) in saliva samples. Melatonin onset was defined as the first sample to exceed a threshold of 2 SD above the mean of the first 3 baseline samples (20:00h-21:00h) not followed by a return below this threshold. Light intensity was <50 lux at eye level. When the DLMO did not occur prior to bedtime, it was derived from serum levels for both study conditions. These estimations were made before the first (R4) and last (R11) short sleep periods.

High sensitivity CRP levels

Seven determinations of hsCRP at 4-h intervals, were obtained at baseline and at the end of sleep restriction. There were no consistent within-subject temporal variations and therefore we used the median of the 7 values as summary measure.

Statistical Analysis

Results are expressed as mean (SD) for normally distributed data or median (25th, 75th percentile) otherwise. Data were log-transformed where applicable.

To examine the impact of sleep restriction within each group, cardio-metabolic variables were submitted to repeated measures ANOVA.

Because of well-documented sex differences in the regulation of sleep (23-24), circadian rhythms (25) and glucose metabolism (26), sex was entered as a covariate in analyses comparing the two interventions. We examined the percent change from baseline to end of sleep restriction using a factorial ANOVA with intervention, sex, BMI and the interaction sex by intervention as factors for all cardio-metabolic variables.

All statistical calculations were performed using JMP software (SAS Institute Inc., Cary, NC).

RESULTS

Twenty-six participants completed the study, 13 in the circadian alignment group and 13 in the circadian misalignment group. The flow chart of enrollment is shown in Supplemental Figure 1 and Table 1 summarizes demographic data.

Sleep

At baseline, total sleep time, amounts of SWS and REM sleep were similar in the two groups (Table 1, see Figure 2 for daily total sleep time). Our experimental strategy (Figure 1) consisted of increasing sleep pressure using a first aligned night of bedtime restriction to achieve similar levels of total sleep time during the period of bedtime restriction, irrespective of the presence or absence of circadian disruption. This strategy was successful as the difference in total sleep time achieved over the 7 short sleep periods undisturbed by blood sampling averaged 22 min, i.e. approximately 3 min per bedtime period (Figure 2; Table 1).

Consistent with previous studies (27-28), SWS was better preserved than REM sleep when bedtimes were restricted. Amounts of SWS and REM sleep were similar in both groups both at baseline and during the one week intervention (Table 1).

Circadian phase (Figure 3)

Participants in the circadian alignment protocol experienced a non-significant delay of the DLMO of 30min (0, 60) whereas those exposed to circadian misalignment

had a delay of 3h08min (2h00min, 3h30min) (P=0.001). Of note, two men exposed to circadian misalignment did not shift circadian phase. The remaining 11 subjects shifted by 3h30min (2h23min, 3h38min).

Weight change and caloric intake

Participants were free to help themselves *ad libitum* during meals. In addition, they had unlimited access to various snack items. In the circadian alignment group, breakfast was served between 07:00h and 08:30h, lunch between 13:00h and 13:30h, and dinner between 18:30h and 19:30h. In the circadian misalignment group, on the days when bedtimes were scheduled during the day (R5-R6, R8-R9), a light meal (usually a sandwich) was served at 01:00h. A normal breakfast was served in the morning. Lunch was served at 15:00h and dinner was served between 18:30h and 19:30h. Thus, there were only minimal differences in the timings of breakfast, lunch and dinner between the two groups.

The two groups consumed excessive but almost identical amounts of calories, averaging 4061 (971) Kcal/24-h when bedtime periods were aligned and 4058 (888) Kcal/24-h when bedtime periods were misaligned. On average, daily caloric intake during sleep restriction included 62.8±11.3% carbohydrates, 33.0±3.9% fat and 10.4±2.1% protein in participants in the aligned condition, as compared to 57.2±5.2% carbohydrates, 34.6±3.8% fat and 10.8±1.8% protein in participants in the misaligned condition. None of the differences between the two conditions were significant (P>0.115). On average, over the 7 days of sleep restriction, the proportion of calories

consumed after 19:00h was 7% (4%) in the aligned condition versus 21% (8%) in the misaligned condition ($P<0.001$). Weight gain was significant ($P\leq0.002$) and similar in both groups (Table 1).

Cardio-metabolic variables

Twenty-four of the 25 participants experienced a decrease in SI following 7 days of sleep restriction. The findings were qualitatively similar in both groups in that a robust decrease in SI was not compensated for by a commensurate increase in beta cell responsiveness (as assessed by AIRg) and therefore the DI was decreased, consistent with an increased risk of diabetes (Table 2). Median hsCRP levels were higher after sleep restriction than at baseline in both groups but the difference was significant for the misaligned group only (Table 2). These findings were similar when analyses were adjusted for weight change.

To examine the effect of the two interventions, we compared the percent change in cardio-metabolic variables between the two groups (individual data in Figure 4). After controlling for BMI, the interaction sex by intervention for percent change in SI was significantly different (-34% (23%) in the aligned group (n=12) vs. -47% (20%) in the misaligned group (n=13), $P=0.026$). This interaction was not significant for percent change in insulin secretion (+28% (55%) vs. +18 (36%), $P=0.16$), percent change in DI (-17% (39%) vs. -39% (27%), $P=0.66$) or percent change in hsCRP (+50% (67%) vs. +119% (110%), $P=0.63$). Because of the small number of female participants, the remainder of the analysis included only men (individual data in Figure 4). Figure 5

illustrates the glucose and insulin temporal profiles during the ivGTT under rested condition and after sleep restriction for the aligned and misaligned groups and Figure 6 reports the changes in the summary measures derived from the ivGTT. Importantly, total sleep time for men only was not significantly different between the 2 groups during the sleep restriction period when baseline levels were controlled for (P=0.30). The relative decrease in SI in men was nearly twice as large in the misaligned as compared to the aligned group (-58%[13%] vs. -32%[25%]; P=0.011, Figure 6). There were no compensatory increases in AIRg in either intervention group (+24%[39%] vs. +21%[56%], P=0.66, Figures 5 and 6). Therefore, the reduction in DI, reflecting an increase in diabetes risk, tended to be greater following circadian misalignment than when the sleep-wake cycle remained aligned (-48%[24%] vs. -19%[43%], P=124, Figure 6). Increases in hsCRP following sleep restriction were higher in the misaligned than in the aligned groups (+146%[103%] vs. +64%[63%], P=0.049, Figure 6) in male participants. Interestingly, these hsCRP increases were correlated with the shift in circadian phase, as estimated via melatonin onset (r=0.487, P=0.040).

In women, differences in ivGTT and hsCRP variables between the aligned and misaligned intervention were non significant.

DISCUSSION

We designed the present study to determine whether circadian misalignment has adverse cardio-metabolic effects independently of sleep loss. Our experimental strategy was successful as the participants obtained nearly identical amounts of sleep irrespective of exposure to circadian alignment or misalignment. Sleep loss reduced insulin sensitivity and DI, but the reduction in insulin sensitivity was nearly twice as large when the week of sleep restriction included 4 days with bedtimes delayed by 8.5 hours than when the center of the sleep period remained fixed. Despite the greater decrease in insulin sensitivity in participants exposed to circadian misalignment, the beta cell response was similar to that observed in participants in whom synchrony between behavioral and endogenous rhythms was maintained. These findings demonstrate that circadian misalignment can have adverse effects on insulin action and insulin release that are distinct from those imparted by sleep loss alone. Similarly, the levels of hsCRP, a marker of systemic inflammation and a predictor of cardiovascular disease risk, were increased following sleep restriction, and to a greater extent in the participants who experienced circadian misalignment.

Our protocol involved restricting bedtimes to build sleep pressure and thereby achieving virtually identical amounts of sleep in both arms of the study. Thus, we controlled experimentally for total sleep time and caloric intake was also nearly identical in both arms of the protocol. We concluded that circadian misalignment has intrinsic adverse cardio-metabolic effects. A study design where bedtimes would not have been

restricted would have led to a greater amount of sleep loss in the circadian misalignment group, with a need to control statistically for total sleep time in the analysis, as performed in the only previous experimental study that attempted to demonstrate adverse cardio-metabolic effects of circadian misalignment (14). In this previous study, total sleep time varied according to the degree of misalignment and the conclusion that circadian misalignment has adverse cardio-metabolic consequences relied on the statistical significance of alignment versus misalignment, while controlling for sleep efficiency as a covariate in the statistical analysis. The present study provides instead a direct experimental demonstration.

The shift in circadian time could have influenced our estimations of the magnitude of the change in insulin sensitivity between the aligned and misaligned conditions. This issue was carefully considered when designing the protocol. Indeed, a phase delay, rather than a phase advance, was used to create circadian misalignment. In healthy non-obese individuals, insulin sensitivity is higher in the morning than 8-10 hours later, in the late afternoon or early evening (29). Our participants in the circadian misalignment condition experienced a delay of the melatonin onset of about 3 hours but the clock time of the ivGTT remained fixed at 9 am. Therefore, relative to internal circadian time, insulin sensitivity was assessed earlier –rather than later– in the biological day. In addition, there is evidence that peripheral clocks in metabolically relevant tissues shift at a slower rate than the central circadian pacemaker (30). Thus, it is likely that the delay in the diurnal variation of insulin sensitivity was smaller than 3 hours. If our estimations of morning insulin sensitivity were affected by this modest shift of peripheral

circadian time, it would therefore be in a direction that would result in a lower estimation of SI in the circadian misalignment than in the circadian alignment condition. Therefore, if affected at all by the shift of circadian time, the difference in the decrease of insulin sensitivity between the two conditions is underestimated, not overestimated.

We examined multiple putative mechanisms mediating the adverse metabolic impact of circadian misalignment. Previous studies in healthy young adults have shown that experimental reductions of sleep quality without change in sleep duration, either by near complete suppression of SWS (31) or by severe sleep fragmentation across all sleep stages (32), can result in decreases in insulin sensitivity that approximate the effect size of circadian misalignment observed in the present study. However, in the present study, the macrostructure of sleep, as assessed by the total amounts of SWS and REM sleep during the week of sleep restriction, was similar in both groups. Sleep restriction did not result in an increase in SWS in either group and REM sleep was markedly and similarly suppressed in both groups. Therefore, it seems unlikely that alterations of sleep quality played a major role in the adverse metabolic consequences of circadian misalignment.

Average daily caloric intake was excessive but similar in both study groups, as were the timings of breakfast, lunch and dinner. However, when participants in the circadian misalignment group were exposed to the 4 shifted nights, the overnight fast was interrupted by a small scheduled nighttime meal with continued access to snacks during the remainder of the night. Over the 7 days of sleep restriction, the proportion of daily caloric intake during the nighttime in the circadian misalignment group was three-fold higher than in the circadian alignment group. The night eating syndrome in humans (33-

34) and a shift of food intake from the active phase to the rest phase in laboratory rodents (35-36) have been associated with adverse metabolic consequences. Whether the disruption of dietary intake that occurred during shifted nights might have caused a further 20-30% decrease in insulin sensitivity as compared to a normal 12-h overnight fast is an important question with major public health implications that will need to be rigorously addressed. Importantly, weight gain was similar under both conditions and we verified that changes in body weight were not a significant predictor of changes in either insulin sensitivity or beta cell response.

The durations of exposure to light and dark, respectively, were identical in both groups, with similar levels of light intensity during periods of wakefulness and total darkness during periods of sleep. Exposure to light during the biological night during the 4 days with bedtime periods delayed by 8.5 hours resulted in a delay of the DLMO approximating 3 hours in all but two participants. The demographics, baseline DLMO and melatonin levels of the two participants who did not shift were similar to those of the remainder of the group. Further, these two individuals experienced qualitatively and quantitatively similar changes in insulin sensitivity as the other participants, suggesting that the timing of the melatonin rhythm was not a major determinant of the metabolic effects of circadian misalignment.

Consistent with previous studies of partial sleep deprivation in healthy young men (37-38), sleep restriction without circadian disruption resulted in a marked elevation of serum hsCRP levels in men. In those exposed to circadian misalignment, the relative increase was more than twice as large, revealing an adverse impact of circadian

disruption on this sensitive marker of cardiovascular risk. Inflammation could be involved in the mechanisms linking sleep loss and circadian disruption to alterations in glucose metabolism.

Novel concepts regarding organization of the mammalian circadian system and its interaction with metabolism have emerged over the past 10 years (39-43). The molecular mechanism generating circadian rhythmicity within pacemaker neurons of the SCN has been identified as a transcriptional-translational feedback loop of activators and repressors, including CLOCK and BMAL1 as positive elements and PER and CRY as negative elements. There is evidence for a direct metabolic input into the core clock mechanism. For example, REV-ERB and RORalpha, the products of two genes of the orphan nuclear hormone receptor family, repress or activate, respectively, the transcription of *BMAL1*, and contribute to the control of the amplitude and phase of the rhythms of clock gene expression. The same interacting circuitry of core clock and metabolic elements is present in many peripheral tissues, including muscle, liver, pancreas and fat. While the environmental light-dark cycle is the primary synchronizer of the central clock mechanism in the SCN, the timings of food intake and fasting have a direct impact on peripheral clocks. The central clock regulates behavioral rhythms, including the sleep-wake cycle and feeding schedule and also entrains peripheral clocks via neural and humoral mechanisms. In the present study, we created a misalignment between the central and peripheral oscillators by imposing an 8.5 h delay of the sleep-wake and dark-light cycles on 4 of the 7 days preceding metabolic testing. Assessment of the DLMO, widely considered as the most accurate marker of central circadian phase

(44), revealed that the central circadian signal had shifted by about 3 hours at the end of the study. Total sleep time and caloric intake were not affected by circadian misalignment. The timing of food intake was shifted with a higher proportion of caloric intake occurring during the biological night and a shorter fasting period. When sleep opportunities were delayed by 8.5 hours, peripheral organs were exposed to nutrients during the habitual period of overnight fast and thus received neuro-hormonal inputs out of phase with central circadian signals by an estimated 5 to 6 hours. This misalignment between metabolic cues and central circadian signals had adverse cardio-metabolic consequences that were not caused by reductions in sleep duration or quality or increases in total caloric intake.

Our study was performed under carefully controlled conditions and the results are unequivocal. The main limitation is the sample size. A significant sex by group interaction emerged from the statistical analysis, but the study was not powered to examine sex differences. Findings in men were robust, with a larger than expected effect size of misalignment relative to alignment.

Findings from this laboratory study provide evidence in support of an intrinsic adverse effect of circadian misalignment on glucose metabolism and cardiovascular risk. The increased risk of diabetes and cardiovascular disease documented in epidemiologic studies of shift work (4-9) is thus unlikely to be solely due to sleep loss and would not be fully mitigated by strategies designed to preserve sleep duration.

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Table 1: Demographics and sleep variables.

Data are expressed as mean (SD) when normally distributed and as median (25th, 75th percentile) when not normally distributed. Data are log-transformed when not normally distributed and *P*-levels are calculated using a t-test.

	Circadian alignment n=13	**Circadian misalignment n=13**	***P*-level**
Demographics			
Sex (F/M)	3/10	4/9	0.66
Age (years)	23 (21.5, 25.5)	22 (21.5, 24.5)	0.60
Weight at baseline (kg)	70.7 (11.0)	67.1 (11.3)	0.42
Baseline Body Mass Index (kg/m^2)	23.1 (2.4)	22.2 (2.5)	0.34
Weight gain during study (kg)	+ 1.4 (1.1)	+ 1.6 (1.5)	0.71
Baseline Sleep (mean of B1 and B2)			
Total sleep time	9h06 (8h47, 9h22)	9h05 (8h07, 9h14)	0.54
Slow-wave sleep (minutes)	70 (20)	64 (31)	0.56
REM sleep (minutes)	127 (24)	122 (27)	0.64
Sleep during bedtime restriction (mean R4-R10)			
Total sleep time	4h48 (5min)	4h45min (6min)	0.22
Slow-wave sleep (minutes)	85 (68, 94)	68 (63, 89)	0.60
REM sleep (minutes)	64 (14)	59 (12)	0.31

Table 2: Cardio-metabolic variables for both study groups.

Data are expressed as median (25th, 75th percentile) and are log-transformed to meet the assumptions of repeated measures ANOVA.

	Baseline	**Sleep restriction (after 7 nights of sleep restriction)**	***P*-level**
Circadian Alignment (n=12)			
SI ($(mU/L)^{-1}.min^{-1}$)	6.6 (4.2, 9.7)	4.0 (3.1, 5.5)	**< 0.001**
AIRg ($mU.L^{-1}.min$)	345 (267, 466)	456 (283, 555)	0.22
DI	1940 (1602, 3161)	1579 (1152, 2195)	0.075
hsCRP	0.048 (0.028, 0.161)	0.080 (0.042, 0.156)	0.061
Circadian Misalignment (n=13)			
SI ($(mU/L)^{-1}.min^{-1}$)	6.2 (5.8, 8.1)	2.9 (2.2, 4.7)	**< 0.001**
AIRg ($mU.L^{-1}.min$)	346 (276, 610)	385 (249, 720)	0.175
DI	2146 (1487, 3737)	1088 (690, 2378)	**< 0.001**
hsCRP (n=10)	0.031 (0.017, 0.047)	0.057 (0.028, 0.112)	**0.007**

SI = insulin sensitivity, AIRg = acute insulin response to glucose, DI = disposition index, hsCRP = high sensitivity C-reactive protein.

FIGURE LEGENDS

Figure 1. ***Schematic representation of the study design.***

The protocol followed a parallel group design with 2 experimental interventions: sleep restriction with circadian alignment (left) and sleep restriction with circadian misalignment (right). The black bars represent the periods allocated to sleep. In both groups, three baseline days of 10-hour bedtime (from 22:00h to 08:00h; B1, B2, B3) were followed by eight days of sleep restriction to 5-hour bedtimes (R4-R11). In the circadian alignment group, all short sleep periods were centered at 03:00h (bedtimes: 00:30h to 05:30h). In the circadian misalignment group, 4 of the 8 short sleep periods (R5, R6, R8 and R9) were delayed by 8.5 hours such that sleep occurred during the daytime (09:00h to 14:00h). In both groups, breakfast (B) was served between 07:30h and 08:30h, lunch (L) between 13:00h and 14:00h and dinner (D) between 19:00h and 19:30h. On shifted days in the misalignment group, lunch was served at 15:00h, one hour after wake up time and a sandwich (S) was presented at 01:00h. Snacks were available at all times. An intravenous glucose tolerance test (ivGTT) was performed at 09:00h on B2 and on R10. Two 24-h sessions of blood sampling at 15-30 min intervals were performed on B3 and R11 (dashed lines). Caloric intake during these sessions was limited to three identical carbohydrate-rich meals (HC). No snacks were allowed. Saliva sampling at 30-min intervals was performed from 16:00h to 00:30h on R4 and R11 to assess melatonin levels (grey bars).

Figure 2. ***Total sleep time.***

Minutes of total sleep time achieved on each day for both study groups. Represented values are mean (+SEM).

Figure 3. **Assessments of circadian phase**

Timing of dim light melatonin onset (DLMO) before the first (dark circles) and before the last (open circles) short sleep periods. For one subject (in the circadian misalignment group), circadian phase could not be determined.

Figure 4. ***Individual changes in cardio-metabolic variables.***

Values represent % change from baseline of SI (insulin sensitivity), AIRg (acute insulin response to glucose), DI (disposition index) and hsCRP (high sensitivity C-reactive protein).

Figure 5. **Temporal profiles of glucose and insulin levels during ivGTT.**

Mean (+SD) glucose and insulin levels during ivGTT performed under baseline (i.e. rested) condition and after 7 days of sleep restriction to 5 hours per day for the men in the circadian alignment (n=10) and the circadian misalignment (n=9) groups. Visual examination of these profiles suggests that the impact of sleep restriction on the decline of glucose concentrations is greater in the presence of circadian misalignment despite

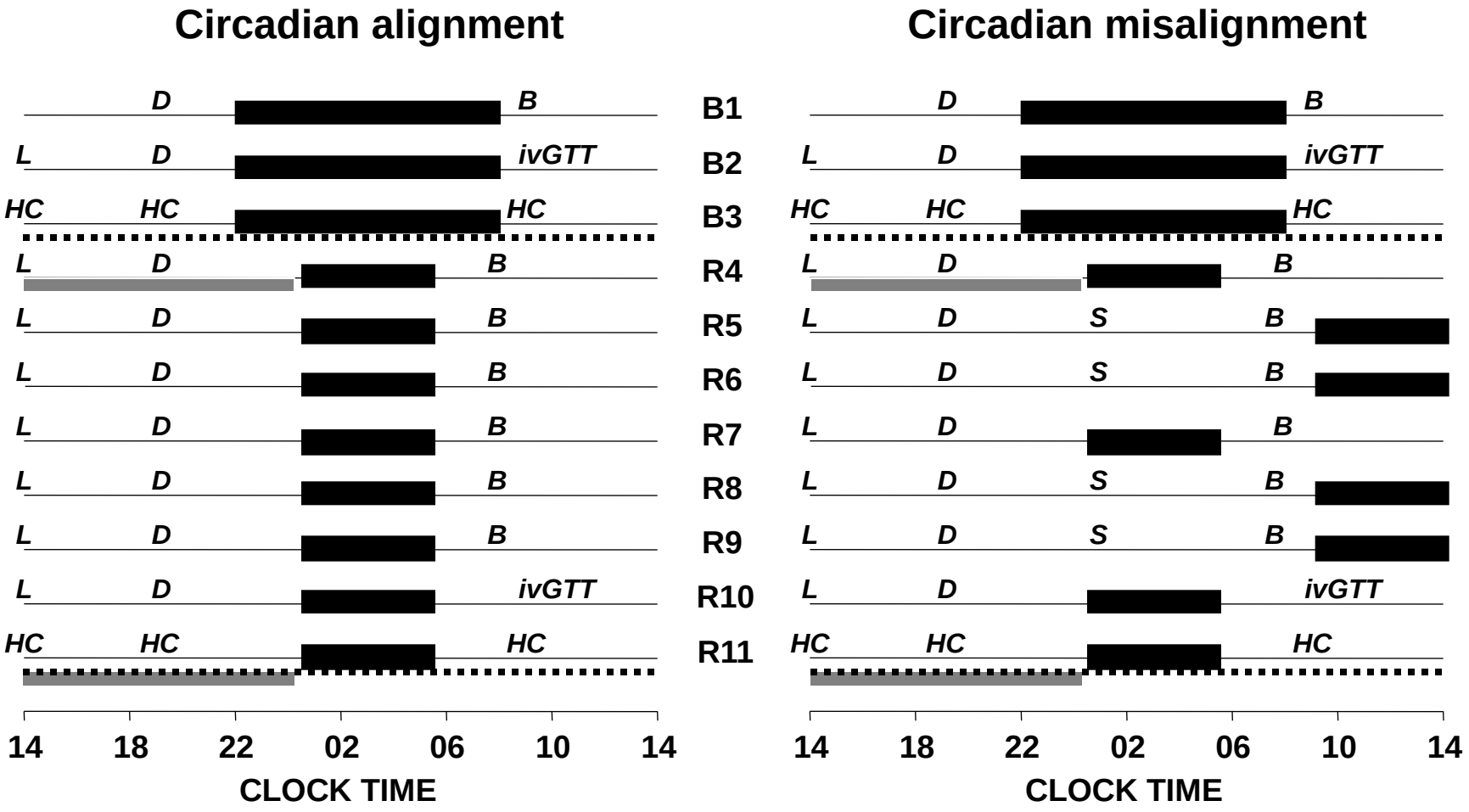
higher levels of insulin, consistent with a greater decrease in insulin sensitivity. Minimal model analysis of individual profiles confirmed this visual impression.

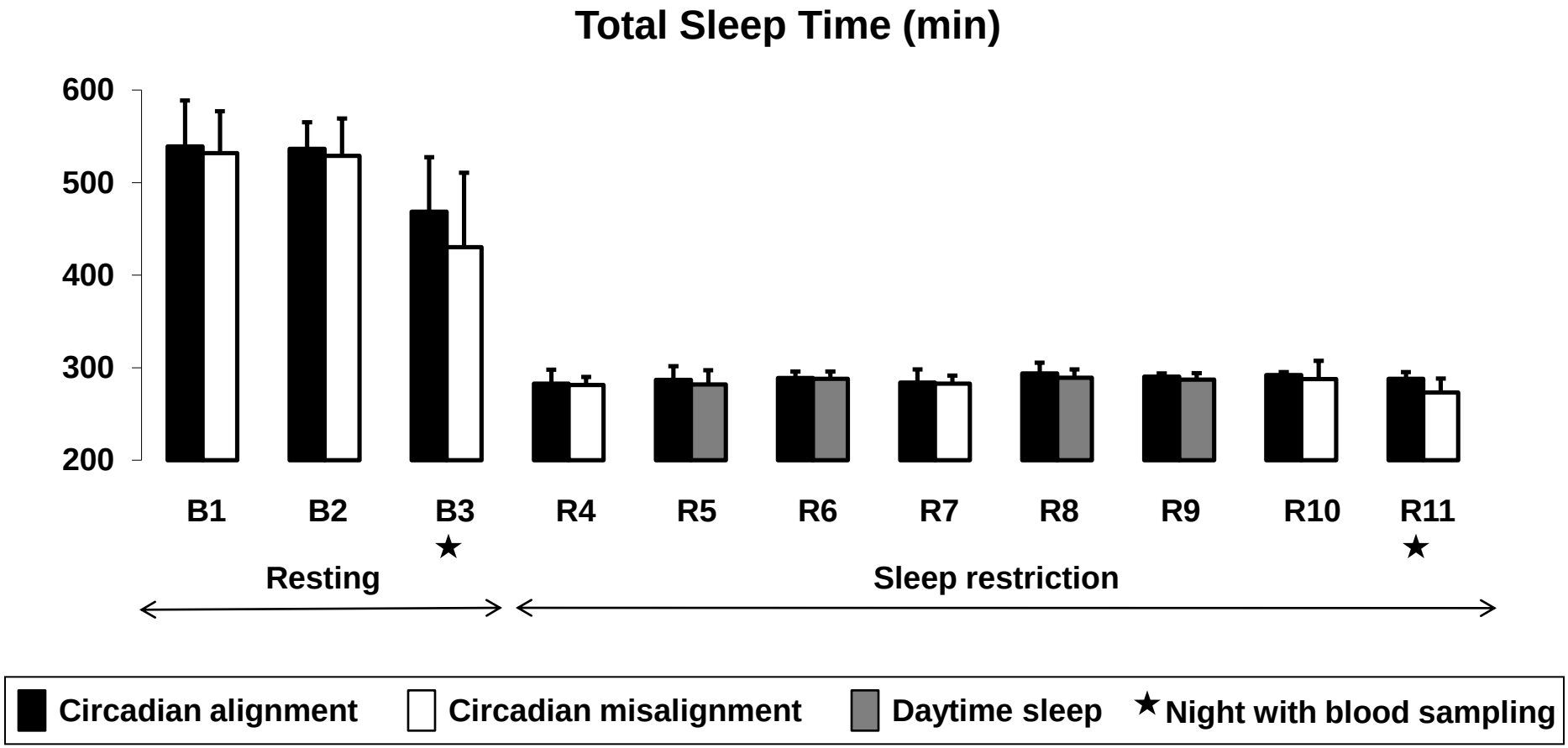
Figure 6. Changes in cardio-metabolic variables in male participants.

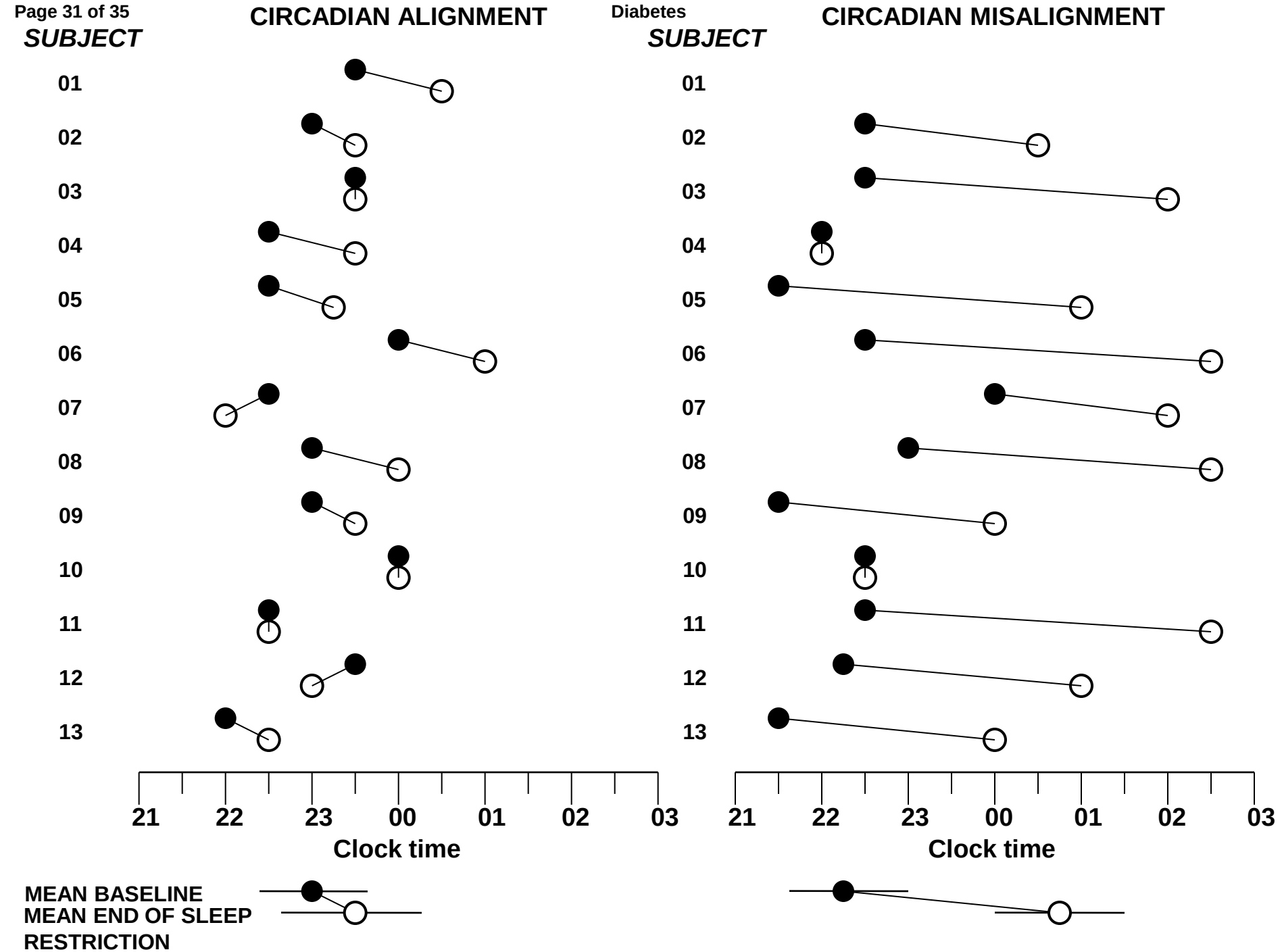
Mean (SD) changes in insulin sensitivity (SI), acute insulin response to glucose (AIRg), disposition index (DI) and high sensitivity C-reactive protein (hsCRP) from baseline to sleep restriction in both intervention groups.

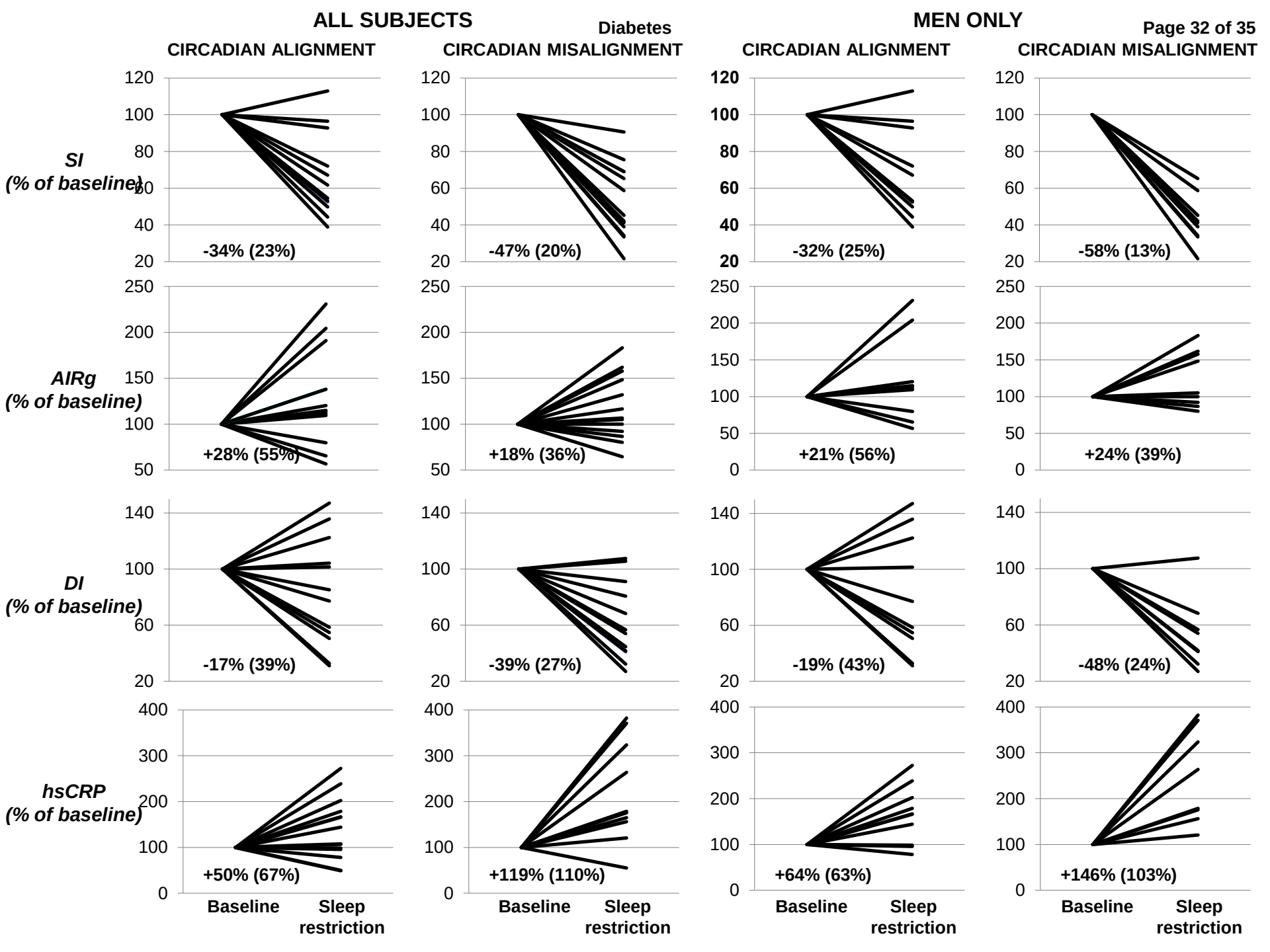
* $P < 0.05$.

Supplemental Figure 1. Flow diagram of recruitment and screening.


Circadian alignment
Circadian misalignment
B1
B2
B3
R4
R5
R6
R7
R8
R9
R10
R11
D
B
L
ivGTT
HC
S
14
18
22
02
06
10
14
CLOCK TIME
CLOCK TIME


Total Sleep Time (min)
600
500
400
300
200
B1
B2
B3
R4
R5
R6
R7
R8
R9
R10
R11
Resting
Sleep restriction
Circadian alignment
Circadian misalignment
Daytime sleep
Night with blood sampling

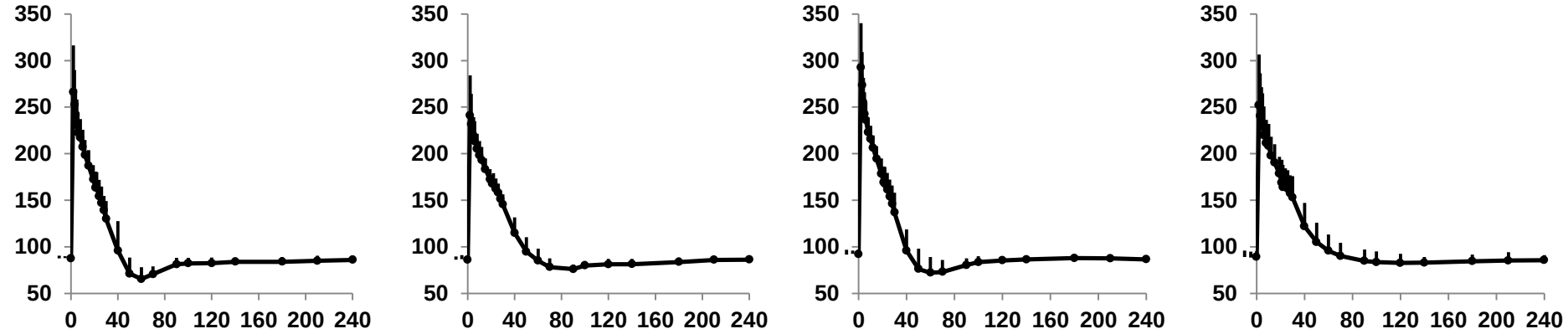

CIRCADIAN ALIGNMENT
SUBJECT
01
02
03
04
05
06
07
08
09
10
11
12
13
21
22
23
00
01
02
03
Clock time
CIRCADIAN MISALIGNMENT
SUBJECT
01
02
03
04
05
06
07
08
09
10
11
12
13
21
22
23
00
01
02
03
Clock time
MEAN BASELINE
MEAN END OF SLEEP
RESTRICTION


ALL SUBJECTS
MEN ONLY
CIRCADIAN ALIGNMENT
CIRCADIAN MISALIGNMENT
CIRCADIAN ALIGNMENT
CIRCADIAN MISALIGNMENT
SI (% of baseline)
AIRg (% of baseline)
DI (% of baseline)
hsCRP (% of baseline)
-34% (23%)
-47% (20%)
-32% (25%)
-58% (13%)
+28% (55%)
+18% (36%)
+21% (56%)
+24% (39%)
-17% (39%)
-39% (27%)
-19% (43%)
-48% (24%)
+50% (67%)
+119% (110%)
+64% (63%)
+146% (103%)
Baseline
Sleep restriction

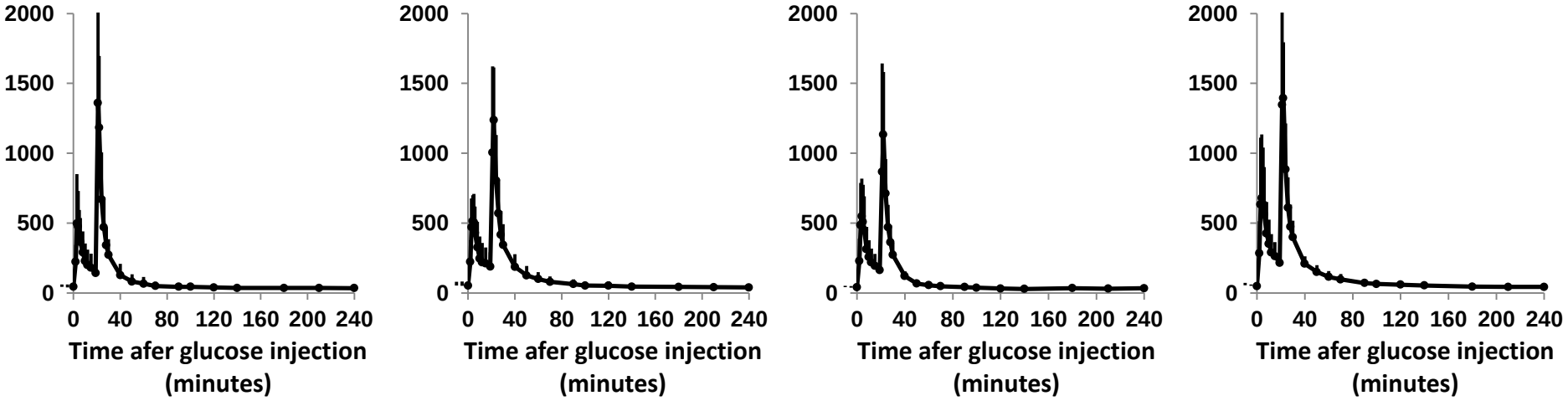
Circadian alignment *Circadian misalignment*

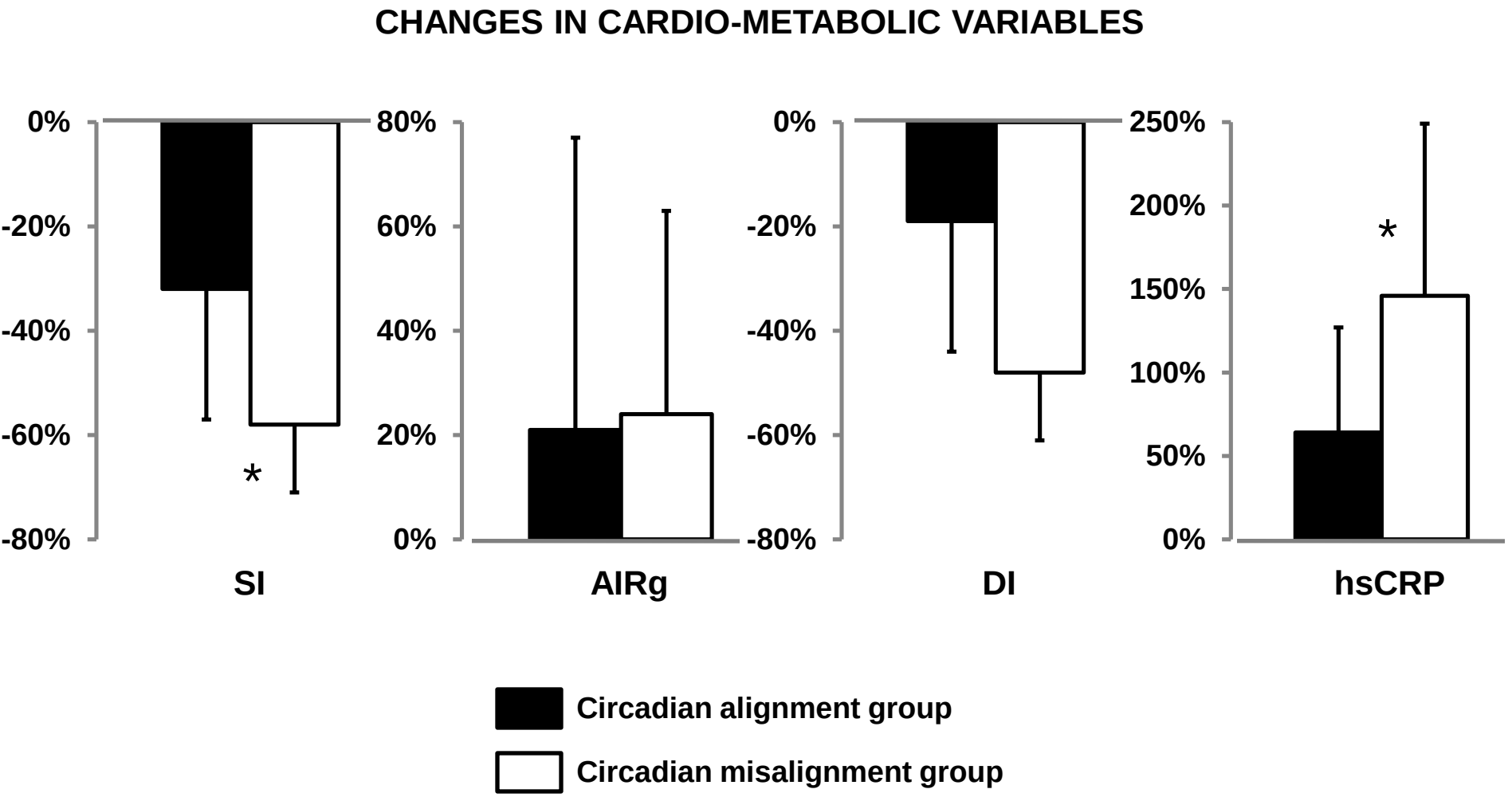
Rested condition Sleep restriction Rested condition Sleep restriction

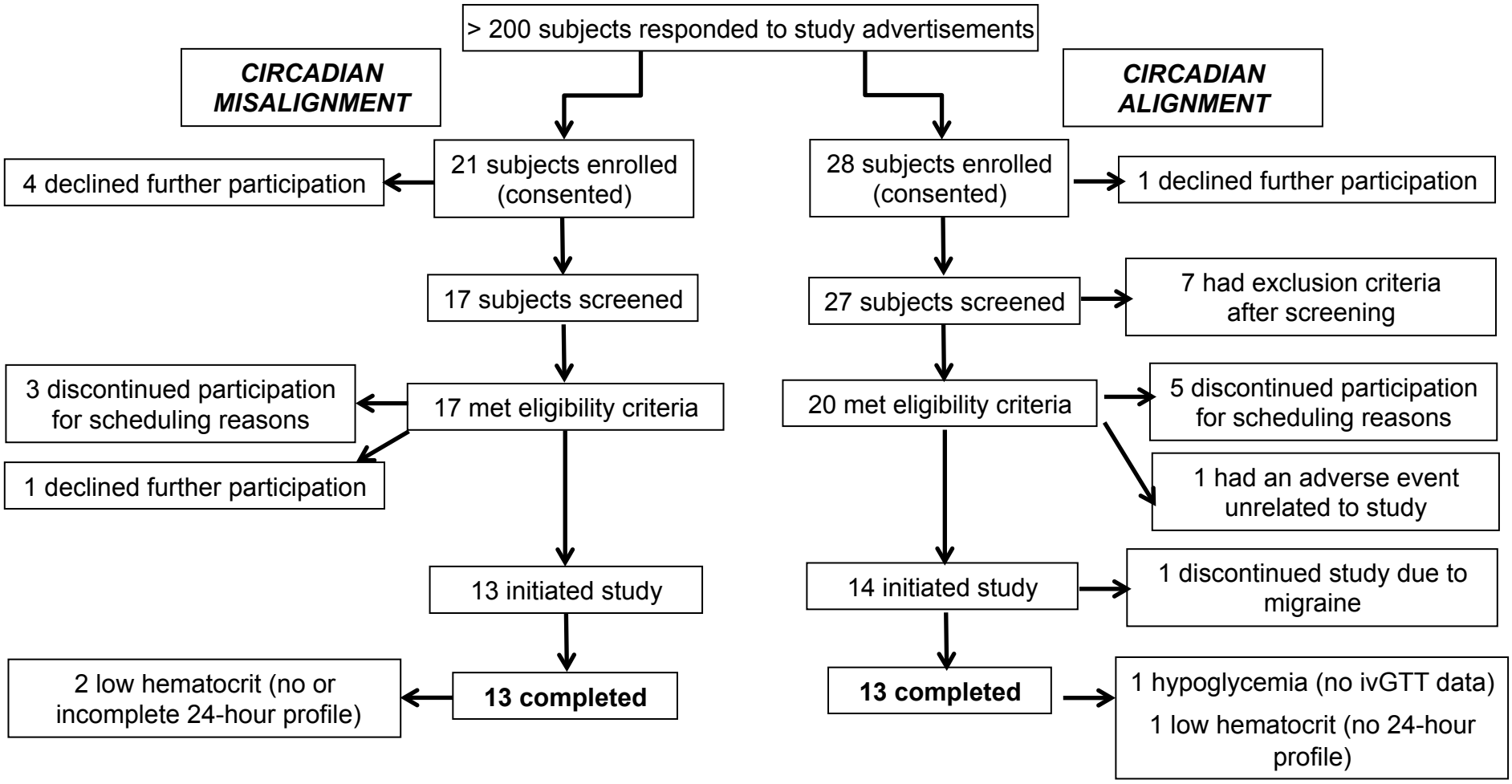
Glucose



Insulin




CHANGES IN CARDIO-METABOLIC VARIABLES
0%
-20%
-40%
-60%
-80%
*
SI
80%
60%
40%
20%
0%
AIRg
0%
-20%
-40%
-60%
-80%
DI
250%
200%
150%
100%
50%
0%
*
hsCRP
Circadian alignment group
Circadian misalignment group


> 200 subjects responded to study advertisements
CIRCADIAN MISALIGNMENT
CIRCADIAN ALIGNMENT
21 subjects enrolled (consented)
4 declined further participation
28 subjects enrolled (consented)
1 declined further participation
17 subjects screened
27 subjects screened
7 had exclusion criteria after screening
3 discontinued participation for scheduling reasons
17 met eligibility criteria
20 met eligibility criteria
5 discontinued participation for scheduling reasons
1 declined further participation
1 had an adverse event unrelated to study
13 initiated study
14 initiated study
1 discontinued study due to migraine
2 low hematocrit (no or incomplete 24-hour profile)
13 completed
13 completed
1 hypoglycemia (no ivGTT data)
1 low hematocrit (no 24-hour profile)