Project title: **Metabolic Effects of Sleep Extension in People with Obesity**

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A. SPECIFIC AIMS

Insufficient sleep causes metabolic dysfunction and is associated with an increased risk of developing obesity and cardiometabolic diseases, such as type 2 diabetes (T2D). The mechanism(s) responsible for the link between insufficient sleep and adverse metabolic outcomes is not known, but it has been proposed that disruption of circadian rhythms, oxidative stress, and/or inflammation are involved. The potential therapeutic effects of sleep countermeasures have not been evaluated in metabolically unhealthy people who are at increased risk of developing T2D. Therefore, the overall goals of this proposal are to assess sleep extension as a metabolic health countermeasure in people are metabolically unhealthy (MU) who habitually maintain chronic short sleep schedules.

Accordingly, we will conduct a randomized controlled trial to assess the effect of a 4-6 wk sleep extension intervention on key metabolic outcomes in MU people (BMI=25.0-50.0 kg/m^2, prediabetes [impaired fasting glucose, HOMA-IR ≥ 2.0, glucose intolerance or an elevated HbA1c]), who habitually sleep <7.0h/night and do not have clinically significant sleep disorders (e.g., sleep apnea, insomnia, narcolepsy). Participants will be studied at baseline during their habitually short sleeping pattern and then 4-6-wk after being randomized to i) continue their usual short sleep pattern (attention control) or ii) sleep extension, achieved by adding 1-2h of time in bed per night, objectively determined by using wrist actigraphy. Sleep will be assessed during the inpatient admissions by using polysomnography. Total energy intake will be adjusted as needed to ensure participants maintain a constant body weight.

The following specific aims will be evaluated:

Aim 1. Determine the effect of sleep extension on whole-body, multi-organ and cellular metabolic function in MU adults. We hypothesize that sleep extension implemented at home in people who chronically maintain short sleep schedules will: i) improve *in vivo* insulin sensitivity in the liver, adipose tissue and muscle; ii) have beneficial effects on 24h plasma insulin (concentration, secretion and clearance) glucose, and FFA profiles; iii) decrease intrahepatic triglyceride content; and iv) alter specific plasma metabolites (assessed every 6h for 24h) that are known to be associated with insulin resistance and the development of T2D.

In addition, untargeted metabolomics analyses will be performed to help identify novel metabolites associated with sleep extension-induced improvement in metabolic function that will serve as the basis for future investigation of these specific metabolites/bioactives.

Aim 2. Determine the effect of sleep extension on purported mechanisms responsible for metabolic dysfunction induced by obesity or sleep restriction: i) misalignment between the master clock of the suprachiasmatic nucleus (assessed by 24h plasma melatonin rhythm) and peripheral circadian clocks (assessed by serial measurements of peripheral blood mononuclear cell [PBMC] gene expression); ii) oxidative stress (urinary 8-iso-prostane F2α, plasma oxysterols and glutathione-to-oxidized glutathione ratio, and adipose tissue protein carbonylation); and iii) systemic and adipose tissue inflammation (proinflammatory cytokines and prostanoids). We hypothesize that sleep extension will: i) improve alignment between central and peripheral rhythms; ii) reduce oxidative stress; and iii) decrease plasma and adipose tissue markers of inflammation.

We will test these hypotheses by: i) a one-stage hyperinsulinemic-euglycemic clamp (HEC) procedure plus stable isotope tracer infusions; ii) 24h urine collection iii) hourly blood sampling for 24h; iv) muscle and adipose tissue biopsies; and v) magnetic resonance imaging.

This study represents a transdisciplinary collaboration between investigators at Washington University School of Medicine, the University of Colorado Boulder, and the University of California San Diego who have synergistic expertise in conducting complex metabolism and sleep studies in people, including sleep extension, required for this proposal. The results from this study will provide new information to assess whether sleep extension should be considered as a countermeasure to improve metabolic health in people at high risk for developing metabolic diseases.

B. SIGNIFICANCE, SCIENTIFIC PREMISE, AND INNOVATION

B1. Significance and Scientific Premise

The average daily sleep duration in American adults has decreased by nearly 2 hours in the last century from ~8.3h/night[1-3] to ~6.8h/night[4]. Sleep loss is often unavoidable in a busy modern society because of social activities, work-related deadlines, and work schedules of certain occupations, such as emergency responders, physicians, military personnel, and shift workers. There is a considerable body of evidence demonstrating that insufficient sleep has adverse effects on body weight and metabolic function. Data from epidemiological studies have found inadequate sleep is associated with an increased risk of obesity and type 2 diabetes (T2D)[5-7]. For example, a 6-yr longitudinal study of 1,455 people without diabetes found a three-fold greater risk of developing impaired fasting glucose in those sleeping less than 6h/night than those sleeping 8h/night[7]. A recent meta-analysis of prospective studies with a median follow-up of 7.5 years found the incidence of T2D was 37% greater in people sleeping ≤5h/night than those sleeping 7-8h/night[8]. Shorter sleep duration is also linked with increased energy intake, weight gain, and increased total body fat, abdominal fat and intrahepatic triglyceride content[9-16]. Every 1h/night reduction in sleep is associated with a 0.35 kg/m^2 increase in body mass index (BMI), and short sleepers (<5h/night) have a 55% greater risk of becoming obese than people who get adequate sleep (>5h/night)[6].

Although weight gain associated with inadequate sleep is likely an important contributor to its adverse effects on health, inadequate sleep *per se* can cause metabolic dysfunction independent of changes in body weight. Data from experimentally-induced sleep restriction studies conducted in people have demonstrated inadequate sleep alters glucose homeostasis, primarily by decreasing whole-body insulin sensitivity during hyperinsulinemic conditions[17-19], without an adaptive increase in insulin secretion when controlling for food intake[20,21]. These results demonstrate inadequate sleep impairs muscle insulin sensitivity, which is a key tissue for postprandial glucose disposal[22,23]. However, the effect of inadequate sleep on insulin action in other key metabolic organs, such as adipose tissue and liver, is not clear. The metabolic effects of sleep restriction on adipose tissue might be particularly important because we and others have found sleep loss induces an increase in plasma FFA concentration[21,24], which can affect insulin sensitivity in the liver (insulin-mediated suppression of glucose production) and skeletal muscle (insulin-stimulated increase in glucose uptake)[25,26]. However, we are not aware of any studies that have directly evaluated the effect of sleep restriction on *in vivo* adipose tissue insulin sensitivity during controlled energy intake. The effect of sleep restriction on liver metabolism is also unclear because of conflicting results from different studies that have found sleep restriction does[17] and does not[24] decrease hepatic insulin sensitivity.

The mechanisms responsible for the adverse effect of sleep restriction on glycemic control are not known, but several hypotheses have been proposed, including *circadian misalignment*[13,27], *increased oxidative stress*[28,29], and *altered pro-inflammatory and anti-inflammatory cytokines*[30,31]. Studies conducted in animal models have consistently demonstrated an interaction among sleep, circadian biology and metabolic physiology[32,33]. We and others have found that restricting sleep decreased whole-body insulin sensitivity[13] and resulted in high plasma melatonin levels for many hours after awakening (i.e., morning circadian misalignment)[13,27]. Moreover, the duration of high plasma melatonin correlated with the reduction in insulin sensitivity (see Preliminary Studies **C2.3**). These data suggest that sleep loss-induced circadian misalignment causes insulin resistance, which is a risk factor for subsequent T2D. An increase in markers of oxidative stress in blood and peripheral tissues are also associated with insulin resistance and the development of T2D in people[34-36]. We are aware of only one study that evaluated the effect of sleep restriction on oxidative stress in people, which found greater myeloperoxidase-modified oxidized low-density lipoprotein concentration after sleep restriction (5h/night for 5 nights) in healthy men[37]. Data obtained from rodent models have shown that sleep restriction causes oxidative stress in several regions of the brain, assessed as an increased ratio of oxidized-to-reduced glutathione, a decrease in reduced glutathione content, and diminished superoxide dismutase activity[38,39]. Chronic low-grade systemic and adipose tissue inflammation are associated with insulin resistance[40]. The effect of inadequate sleep on markers of inflammation is unclear, because of conflicting results among studies showing short sleep caused an increase, no change, or decrease in circulating markers of inflammation[41-44]. In rodents, sleep restriction increases adipose tissue macrophage infiltration and TNFα and IL-6 protein content[45]. The effect of sleep extension on diurnal rhythm, oxidative stress, or systemic and adipose tissue inflammation in people who are short sleepers is not known.

Data from several epidemiological studies show that people with obesity and metabolic dysfunction are short sleepers (sleep <7h/night)[46,47], supporting the notion that insufficient sleep can contribute to the metabolically unhealthy phenotype. Therefore, increasing sleep duration in this population could provide a

simple, low-cost therapeutic intervention. However, the potential beneficial effect of sleep extension on glucose homeostasis in habitual short sleepers who are metabolically unhealthy is not known. The data from one study conducted in healthy non-obese participants found 6 wk of sleep extension, achieved by increasing time-in-bed by 1h/night (resulting in an average increase of 44 min in sleep time/day), caused a decrease in fasting plasma insulin concentrations[48]. Another study, reported as an abstract only[49], found 3 weeks of sleep extension in people with impaired glucose tolerance or T2D produced a trend ($P = 0.06$) toward a decrease in glucose area under the curve (AUC) during a 3h oral glucose tolerance test. Moreover, the increase in sleep duration correlated with the decrease in glucose AUC ($r=0.81$, $P < 0.01$). Finally, another study found sleep extension to 10h/night for only 3 nights in healthy young men who regularly curtail their sleep to ~6h during the week resulted in an improvement in glucose tolerance during a 2h oral glucose tolerance test[50]. These data suggest chronic sleep loss is a potentially modifiable diabetes risk factor. However, we are not aware of any studies that have evaluated the effect of sleep extension on multi-organ system metabolic function in metabolically-abnormal, habitual short sleepers.

A non-targeted assessment of the metabolome (low molecular weight products of metabolism) could provide novel insights into the biological mechanisms involved in sleep-related alterations in metabolic function. Data from several studies that used a metabolomics approach have found that sleep restriction has a significant impact on the metabolomes in the blood, urine, and breath[51-56], which could be involved in the pathogenesis of the metabolic alterations induced by sleep restriction. However, we are not aware of any studies that have evaluated the effect of sleep extension on the plasma metabolome in people. The comprehensive mass spectrometry-based analysis of the systemic metabolome proposed in this application could provide insight into systemic metabolic alterations that influence whole body physiology and homeostasis, and identify novel metabolic targets for future studies of sleep related dysfunction.

Summary. Data from both epidemiological and sleep intervention studies have shown disrupted sleep has adverse effects on diabetes-related metabolism. However, the potential therapeutic benefits of sleep extension are not known. This proposal will help fill this important gap in our knowledge. The hypotheses being tested in this proposal are based on a sound foundation of data from our own studies, and studies conducted by other investigators in animal models and people. The assessment of sleep on metabolic function is difficult, because it requires careful manipulation of sleep time and a comprehensive cellular and *in vivo* assessment of multiple organ systems in controlled sleep/circadian protocols. We have assembled a transdisciplinary team of metabolic, sleep and circadian experts to conduct these complex studies in people.

B2. Innovation

Our proposal is innovative for several reasons: i) it involves a novel transdisciplinary collaboration among three research groups from different institutions, who have the combined expertise needed to conduct the complex studies proposed in this application (J. Broussard, K. Wright and B. Lucey [Co-Is] are experts in sleep interventions and sleep evaluation, S. Klein [PI] and G. Smith and J. Yoshino (Co-Is) are experts in evaluating *in vivo* and *ex vivo* metabolic function in people, and M. Jain [subcontract] is an expert in metabolomics/bioinformatics); ii) it combines a controlled sleep intervention with sophisticated, state-of-the-art, whole-body, multi-organ and cellular assessments of postabsorptive and 24h metabolic function in people, and iii) it provides a comprehensive assessment of potential therapeutic effects of sleep extension in people which has considerable physiological and potential clinical implications.

C. FEASIBILITY – INVESTIGATOR EXPERTISE AND PRELIMINARY DATA

C1. Transdisciplinary collaboration.

This application represents a new collaboration between the University of Colorado Boulder (UCB; Co-Is: J. Broussard and K. Wright) and Washington University School of Medicine (WUSM; PI: S. Klein; Co-Is: G. Smith, J. Yoshino and B. Lucey), in conjunction with specific additional support from M. Jain at the University of California San Diego (UCSD) (assessment of plasma metabolomics; see letter of support). The interaction between the PI (Klein) and Co-Is at UCB (Broussard, Wright) developed during a workshop, held by NIDDK in February, 2015, that focused on the "Impact of Sleep and Circadian Disruption on Energy Balance and Diabetes." This meeting led to a collaborative publication[57], frequent teleconference and face-to-face communications to design the current research proposal, and a construction of a dedicated room for sleep studies within the new Clinical Research Unit at WUSM. The involvement of M. Jain in this proposal developed from an ongoing collaboration between Drs. Klein and Jain that is evaluating the metabolome in blood samples from people with obesity undergoing different weight loss diets.

C2. Preliminary Studies.

The following preliminary data support the hypotheses being tested in this proposal, and demonstrate we have the experience needed to perform the precise sleep interventions and complex metabolic studies proposed in this application.

C2.1. Sleep loss impairs insulin sensitivity. We recently completed a 15 d inpatient protocol in 16 healthy volunteers that involved a 3 d baseline condition (9h sleep/night), followed by 5 d each of 5h and 9h of sleep/night in randomized order[13]. Insufficient sleep increased *ad libitum* energy intake and decreased whole-body insulin sensitivity, assessed by the Matsuda Index derived from an oral glucose tolerance test (Fig. 1A). Matsuda Index values returned to baseline when 9h sleep/night was instituted after the 5h sleep/night condition (Fig. 1B).

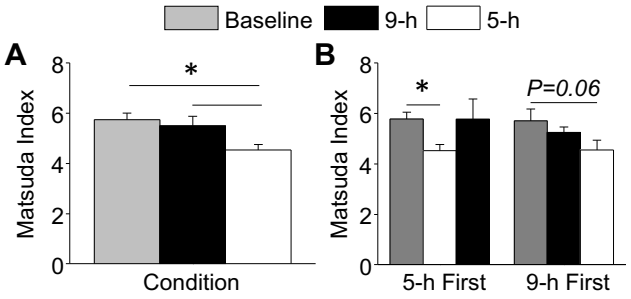


Fig. 1 Insulin sensitivity after insufficient sleep

C2.2. 24h blood sampling without disturbing participant sleep. We have implemented and routinely use a protocol for collecting frequent blood samples over 24h without awakening participants from sleep that is needed for this proposal. This protocol involves an indwelling intravenous catheter to a 12-ft long, small-lumen, extension tube passed through a wall portal for blood sampling[13,30,31,58-60].

C2.3. Sleep loss induces circadian misalignment. We have found that decreasing sleep to 5h/night leads to plasma melatonin levels being high for several hours after wake-time, indicating morning wakefulness during the biological night, or "morning circadian misalignment"[58]. Moreover, the duration of high plasma melatonin levels was negatively associated with insulin sensitivity, assessed by using the Matsuda Index, suggesting circadian misalignment contributed to decreased insulin action after insufficient sleep.

C2.4. Circadian misalignment increases plasma markers of inflammation. We have found that circadian misalignment, induced by extending day length by 36 min/d (to 24.6h) for 25 d, increased plasma C-reactive protein (CRP) concentrations (Fig. 2)[31]. In this proposal we will assess the effect of extended sleep on plasma and adipose tissue markers of inflammation.

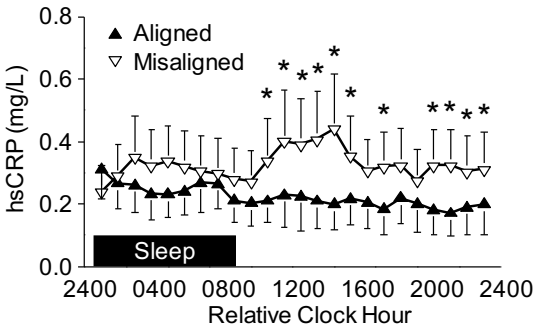


Fig. 2. Circadian misalignment and inflammation

C2.5. Effects of increased sleep opportunity on sleep duration, sleep architecture, and circadian timing. We conducted a study in 26 healthy, normal-weight, habitual short sleepers (<6.5h sleep/night), who were randomized to one of two sleep schedules for 2 wk: i) maintenance of habitual short sleep schedule (n=12); or ii) extend sleep opportunity by ≥2h/night (n=14). We used the same outpatient assessments (wrist actigraphy, participant call-in at bed-time and wake-time, and sleep diaries) and inpatient polysomonography (PSG)

proposed in the current study. The increase in sleep opportunity increased total sleep time by 2.4h/night (from ~5.8h/night to 8.2h/night) (Table 1). In addition, duration of N1, N2 and REM sleep significantly increased (data not shown) without altering sleep latency (time it takes to fall asleep) or sleep efficiency (sleep time divided by total time in bed). *These findings demonstrate our ability to increase sleep duration in people with short sleep schedules.*

Table 1. Effect of 2-week sleep extension intervention on PSG measured sleep

	Maintain habitual insufficient sleep		Extension from habitual insufficient to sufficient sleep	
	Before	After	Before	After
Time in bed (min)	336.1 ± 15.5	361.4 ± 8.7	348.8 ± 10.5	492.0 ± 10.4[a,b]
Total sleep Time (min)	322.2 ± 14.9	346.8 ± 9.5	329.3 ± 10.6	464.5 ± 10.0[a,b]
Sleep latency (min)	2.9 ± 1.8	2.1 ± 1.2	3.8 ± 2.0	7.6 ± 1.2
Sleep efficiency (%)	95.7 ± 0.8	96.0 ± 0.5	94.5 ± 1.2	94.5 ± 0.9

Values are means±SEM. [a]Value significantly different from baseline value; p<0.01;
[b]Value significantly different from effect of insufficient sleep condition, p<0.01.

C2.6. Use of high throughout liquid chromatography mass spectrometry (LC-MS) for untargeted, discovery metabolomics analysis. The reliable analysis of thousands of metabolites in biological samples, and an insightful interpretation of these complex datasets is not trivial. For this reason, we are collaborating with Dr. Mohit Jain at UCSD, who has a productive track record in using mass spectrometry-based approaches for targeted and untargeted discovery metabolomics[61-64], and in analyzing complex datasets[61-66]. *We will use these innovative LC-MS and computational approaches to assay the human metabolome in plasma to provide insights into the effects of sleep restriction/extension on metabolite determinants of metabolic function.*

C2.7. Use of peripheral blood mononuclear cells (PBMCs) to evaluate diurnal rhythm of peripheral clock genes expression. We will use circulating PBMCs to evaluate peripheral clock genes because of the burden of repeated tissue biopsies on study participants. We recently found PBMCs obtained every 3h for 24h from metabolically-healthy people who were overweight exhibit clear diurnal expression patterns of clock genes (Fig. 3, manuscript submitted). Diurnal expression patterns of clock genes in PBMCs are similar to those in skeletal muscle[67] and adipose tissue[68]. Data from previous studies that evaluated blood samples in people have shown that diurnal expression patterns of clock genes are affected by insufficient or mistimed sleep[69-72]. In addition, obesity is associated with the alterations in clock gene expression in human PBMCs[73]. Taken together, these findings suggest inter-relationship among metabolism, sleep homeostasis, and blood circadian clocks. Accordingly, we hypothesize that expression of PBMC clock genes will be affected by sleep extension in MU people who are short sleepers.

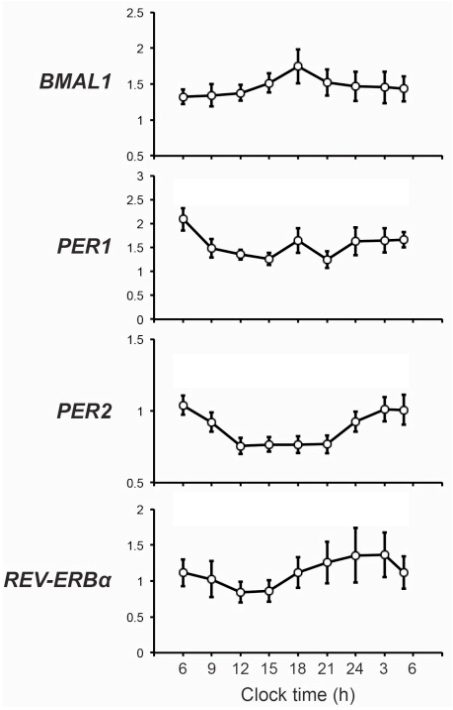


Fig. 3. PBMC clock gene expression

D. RESEARCH APPROACH

D1. Study Participants

Participants will consist of 50 MU men and women who habitually sleep ≤7.0h/night, and meet the following criteria: i) age: 21-65 years old; ii) BMI 25.0-50.0 kg/m^2; iii) prediabetes (fasting plasma glucose concentration between 100 and 125 mg/dl, or 2-h OGTT plasma glucose between 140 and 199 mg/dl, or HbA1c between 5.7 and 6.4%, or HOMA-IR ≥ 2.0); and iv) no evidence of clinically significant sleep disorders other than mild sleep apnea [apneas and/or hypopneas per hour of sleep (AHI) of 15 or fewer].

Screening visit 1. Written informed consent will be obtained from each subject before study participation. All participants will arrive in the Clinical Research Unit (CRU) in the morning after they have fasted for 10h-12h overnight at home, and will undergo a comprehensive screening, including a medical history, physical examination, standard blood tests, an oral glucose tolerance test and a urine toxicology screen. A clinical interview by a trained clinician will screen for psychiatric and sleep disorders and daytime

functioning. Screen for potential psychiatric and sleep problems will also include the following validated questionnaires: i) Beck Depression Inventory-II (BDI-II); ii) Beck Anxiety Inventory (BAI); iii) Pittsburgh Sleep Quality Index (PSQI); iv) Epworth Sleepiness Score (ESS); v) Insomnia Severity Index; vi) Berlin Questionnaire (indicates sleep apnea); and vii) Short form quality of life questionnaire. Prior to discharge, body composition including total body lean and fat mass will be determined by using dual energy X-ray absorptiometry (DXA).

Screening visit 2. After consuming a standard evening meal, a PSG recording[90] conducted during the night of admission (e.g. ~2300h until ~0500h, depending on the participant's actual habitual schedule) will be used as a clinical sleep disorders screen. If evidence of a clinically significant sleep disorder (e.g. obstructive sleep apnea, periodic limb movement disorder) is detected, the participant will be excluded from the study and discharged. PSG recordings will be obtained with Siesta digital sleep recorders (Compumedics). Sleep studies will be performed and scored by a polysomnographic technologist and reviewed by a board-certified sleep medicine physician according to American Academy of Sleep Medicine guidelines. Records will be scored for sleep staging[91] and we will perform quantitative EEG (e.g., delta power) analyses[92].

All potential participants will also be carefully screened to assess their ability to incorporate the sleep extension protocol into their daily lifestyle. This screen will include questions about sleep duration on workdays, free days and vacation to determine sleep need and whether participants will be able to extend sleep. In addition, we will extensively discuss any lifestyle modifications necessary to implement sleep extension, and examine evening and morning work, family and other obligations and commitments that make adherence to sleep extension very difficult or impossible.

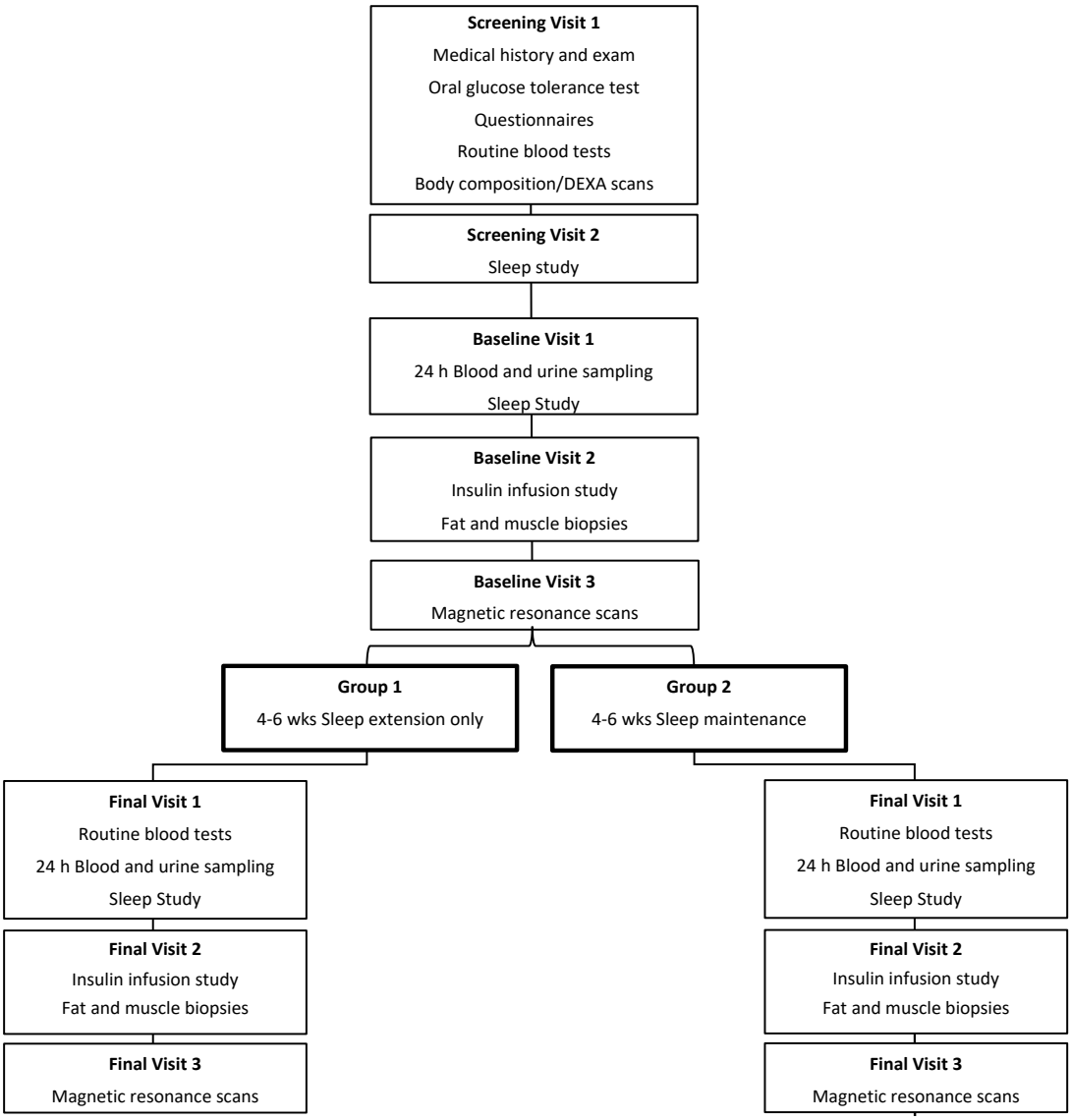
The following criteria will be exclusionary: i) history or evidence of abnormalities that affect sleep (e.g. moderate and severe obstructive sleep apnea [OSA] defined as AHI >15, periodic limb movements of sleep [PLMS], insomnia, narcolepsy, shift work in previous 6 months, or travel more than 1 time zone in 3 wk before the study); ii) significant organ system dysfunction/disease (e.g. diabetes, severe pulmonary, kidney or cardiovascular disease) and any evidence of active illness (e.g., fever); iii) history of seizure disorder; iv) history or current significant psychiatric disorder (e.g., prior or current diagnosis); v) pregnant/nursing; vi) use dietary supplements and/or medications known to affect sleep, circadian rhythms or metabolic function; vii) smoke >10 cigarettes/week or illegal drugs determined by medical history or urine screening; viii) consume excessive alcohol (>7 drinks/wk); ix) consume excessive amounts of caffeine daily (>500 mg; ~5 servings of coffee/espresso or ~8 12 oz caffeinated soft drinks per day) or consume less amounts of caffeine but believe withdrawal symptoms (e.g. headache) are likely if caffeine is stopped; x) participate in intense exercise (activity that causes heavy breathing and sweating, such as jogging) >70 min/wk or moderate exercise (e.g., brisk walking) for >150 min/wk; xi) unstable weight (>4% change during the last 2 months before entering the study); xii) have conditions that render subject unable to complete all testing procedures [e.g., metal implants that interfere with imaging procedures]; and xiii) unwilling/unable to provide informed consent.

The PI has an experienced participant recruitment core, a strong track record in recruiting subjects who meet specific requirements and excellent participant compliance and retention in studies as rigorous or more rigorous than the current proposal [e.g.,36,67,74-88].

D2. Experimental Design

D2.1. Study flow and overview. A randomized, controlled trial will be conducted in MU people to evaluate the effect of 4-6 wk of sleep extension (to account for the time needed to complete follow-up testing) on metabolic function, circadian biology, inflammation and oxidative stress (Fig. 4)**.** A 4-6 wk duration of sleep extension was chosen because a decrease in fasting plasma insulin concentration has been reported after 6 wk of sleep extension in healthy, non-obese subjects[48]. A randomized, cross-over study design (i.e. participants randomized to habitual short sleep or to extended sleep and then cross-over to the other condition) is not being done because of the concern that subjects would find that extending their sleep was beneficial and would not go back to their previous short sleep pattern. Indeed, in our previous sleep extension study (see preliminary data in section **C2.5)**, subjects who extended their sleep completed a 3-wk post-study sleep log which showed they continued to sleep more than 7h per night by their own choice.

Fig. 4. Study overview



D2.2. Baseline testing. Throughout baseline testing participants will be instructed to maintain their habitual sleep patterns. Participants will perform body composition analysis (magnetic resonance imaging will be used to determine intra-abdominal adipose tissue volume[77] and intrahepatic triglyceride content[89]) in the Center for Clinical Imaging Research (CCIR) after a 4 h fast. Participants who experience anxiety associated with the MR scans will have the option of receiving a single dose of Ativan or Xanax prior to the scans. In addition, participants will be admitted to the CRU on 2 occasions separated by 7-10 d. Before admission to the CRU for all inpatient visits, participants will maintain their habitual short sleep of ≤7.0h/night for 7 d, verified by actigraphy, sleep logs and bedtime and wake up time call-ins to a time stamped recorder to ensure subjects do not acutely or chronically sleep extend before the inpatient studies. Wrist actigraphy with concurrent light exposure (Actiwatch Spectrum Pro; Philips Respironics) will be used to assess sleep timing and total sleep time (TST) during the 7-10 d period prior to all inpatient testing visits, during the 4-6 wk sleep extension/habitual sleep intervention and throughout repeat testing. A daily sleep log will be maintained to assess subjective sleep onset latency, TST, sleep quality, number of awakenings, factors that might have disturbed sleep, and daily caffeine and alcohol intake. Bedtime and wake-up time call-ins to a time-stamped recorder will be used to help evaluate sleep and wake up times. Participants will be instructed to avoid alcohol for 7 d, avoid moderate or intense exercise for 3 d, and avoid caffeinated drinks for 3 d before admission. For 3 days before their in-patient visits, participants will consume packed-out meals produced by the metabolic kitchen, which will provide 50% of total energy as carbohydrate, 35% as fat and 15% as protein. Total energy

content will be estimated to meet individual daily energy requirements, calculated as 1.4 times resting energy expenditure (REE). Subjects will be informed that they should make every attempt to consume meals at specific times determined based on their habitual wake time (e.g. at ~0900h, 1500h, 2100h if wake time is 0800h [meals will be +1h, +7h and +13h after awakening]).

D2.2.1. Inpatient CRU Visit 1 (24h metabolic profile, and assessment of PSG, circadian misalignment, oxidative stress and systemic inflammation). Participants will be admitted at ~1800h and stay in the CRU for ~36h and will continue their usual ≤7.0h/night sleep schedule.

24h blood sampling and urine collection. Approximately 30 min after awaking on Day 2, after subjects fast for ~11.5h overnight, a catheter will be inserted into an antecubital vein. This catheter will be connected to a 12-ft long, small-lumen extension tubing that will be passed through a wall portal to allow blood sampling without disturbing the participant. Blood samples will be obtained hourly for 24h starting 1h after awaking (i.e., immediately prior to breakfast) to assess glucose, FFA, C-peptide, insulin, and melatonin concentrations [a total of 25 blood samples (3 mL each)]. Blood samples obtained every 3h will be used to assess: i) markers of inflammation (C-reactive protein, IL-6, TNFα, MCP-1, and selected prostanoids [prostaglandin [PG]E_2, PGD_2 $PGF_{2\alpha}$ and thromboxane A_2]); ii) markers of oxidative stress (plasma oxysterols and glutathione-to-oxidized glutathione ratio), and markers of insulin resistance (branched-chain amino acids, aromatic amino acids, glycine, and glutamine, and acetylcarnitine C2)[93]. An additional 10 ml of blood will be obtained every 3h to isolate PBMCs, which will be used to evaluate the expression of clock genes. PBMCs will be immediately isolated through a density gradient centrifugation by using the Histopaque-1077 (#10771; Sigma, St. Louis, MO). Subjects will be seated for at least 15 min before each blood sample because posture can impact melatonin levels[94]. Light exposure during 24h sampling will be <8 lux max (~1.9 lux angle of gaze), because bright light supresses melatonin, our primary marker of circadian phase[90,95]. At other times during the study subjects will be exposed to normal room lighting (e.g., 200 lux). Meals will be supervised to ensure all food is consumed within 30 min. Urine will be collected throughout the 24h blood sampling protocol to determine urinary 8-iso-prostane F2α content as a marker of oxidative stress.

Sleep study. Baseline PSG recordings will be obtained from ~2300h on Day 2 until ~0500h on Day 3. PSG recordings will be obtained with Siesta digital sleep recorders (Compumedics). Sleep studies will be performed and scored by a polysomnographic technologist and reviewed by a board-certified sleep medicine physician according to American Academy of Sleep Medicine guidelines. Records will be scored for sleep staging[91] and we will perform quantitative EEG (e.g., delta power) analyses[92].

Inpatient activity and diet. During free time, subjects will be permitted to ambulate (e.g., read, walk, watch movies, computer work) in their room. Additionally, participants will perform a standardized bout of exercise (5 min stepping at 60 steps/min) 2 h after each meal. Dietary energy content will be designed to meet individual total daily energy requirements, determined as 1.2 x REE. Meals will be provided 3 times/day, given every 6h beginning with breakfast 1h after awakening (e.g. if participant awakes at 0530h, breakfast, lunch and dinner will be given at 0630h, 1230h, and 1830h). The macronutrient content of the diet will be the same as the outpatient diet and will not contain any caffeine. Subjects will be instructed to finish meals completely within 30 min.

D2.2.2. Inpatient CRU Visit 2 (Hyperinsulinemic-euglycemic clamp (HEC) procedure in conjunction with stable isotopically labeled tracer infusion and muscle and adipose tissue biopsies). At ~1800h, 7-10 d after Inpatient CRU Visit 1, participants will be readmitted to the CRU. A standardized meal will be provided at 1900h. The macronutrient content of the diet will be the same as the outpatient diet and will not contain any caffeine. Subjects will be instructed to finish the meal completely within 30 min. At ~0700h in the morning after admission, after subjects have fasted for ~11.5 hours overnight, an intravenous catheter will be inserted into an antecubital vein to infuse stable isotope tracers, insulin and dextrose. A second catheter will be inserted into a hand vein for blood sampling, which will be heated using a thermostatically controlled box, to obtain arterialized venous samples. A primed-constant infusion of [6,6-$^2H_2$]glucose and a constant infusion of [U-^{13}C]palmitate will be started at ~0800h and maintained for 3.5 h to obtain basal glucose and fatty acid kinetics. At ~1130h, a one-stage HEC procedure will be initiated and continued for an additional 3.5 h. During the clamp insulin will be infused at a rate of 50 mU/m^2 body surface area [BSA]/min after initiation with a two-step priming dose: 200 mU/m^2 BSA/min for 5 min followed by 100 mU/m^2 BSA/min for 5 min. Euglycemia (~100 mg/dl) will be maintained by variable rate infusion of 20% dextrose enriched to 2.5% with [6,6-$^2H_2$]glucose. Adding glucose tracer to the dextrose infusion provides a more accurate measure of glucose kinetics by minimizing changes in

plasma glucose enrichment[96]. The infusion rates of [6,6-$^{2}H_2$]glucose and [U-^{13}C]palmitate will be stopped during the clamp because of the expected decreases in endogenous glucose production and palmitate release into the circulation. We have found this approach helps maintain steady tracer-to-tracee ratio (TTR) values throughout the HEC procedure. Blood samples will be obtained immediately before starting the tracer infusion and every 10 min during the final 20 min (4 samples) of the basal period and HEC procedure, to determine glucose and insulin concentrations and substrate kinetics. Additional blood samples will be obtained every 10 min during the HEC procedure to monitor blood glucose concentration. The insulin concentration achieved during the clamp reflects postprandial plasma insulin concentrations[97,98] and is ideal to evaluate insulin's effect on glucose disposal[99].

Abdominal subcutaneous adipose tissue sampling will be performed during the basal period of the HEC procedure. The periumbilical area on one side of the body will be cleaned and anesthetized with 1% lidocaine, then a small skin incision (~0.5 cm) will be made in the skin and a small liposuction cannula will be inserted into the incision to aspirate ~5 grams of subcutaneous adipose tissue under sterile conditions. The sample will be rinsed immediately in ice-cold saline, cleaned of blood, and submerged in liquid nitrogen and stored at -80 °C until further processing.

Thigh muscle sampling will be performed during the basal period of the HEC procedure. The site will be cleaned and anesthetized with 2% lidocaine, a small incision (~0.5 cm) will be made in the skin, with a muscle sample (~100 mg) obtained under sterile conditions by using conchotome forceps. The sample will be rinsed immediately in ice-cold saline, cleaned of blood and connective tissue, and submerged in liquid nitrogen and stored at -80 °C until further processing.

D2.3. Sleep extension intervention. After completing baseline testing, subjects will be randomized, stratified by sex, to either: 1) *sleep extension* for 4-6 wk (n=25) or 2) *continued habitual short sleep* for 4-6 wk followed by 4-6 wk *sleep extension* (n=25). Randomization will be accomplished using the online randomization module of REDCap, a secure, web-based application designed to support data capture for research studies[100]. Permuted blocks with varying block sizes will be used to ensure that there is no temporal bias in group assignment. When randomization occurs, the study coordinator will enter the REDCap system and will be asked to respond to a series of questions establishing the eligibility of the subject. The randomization assignment will only be revealed if all eligibility criteria are satisfied. We chose a 6-wk sleep extension intervention, because the only published study that showed a potential benefit of sleep extension on metabolic function used a 6-wk sleep extension protocol[48]. Sleep and circadian expert members of the research team will use the baseline assessments to develop an individualized targeted intervention for each subject randomized to the sleep extension group with the goal of increasing each subject's sleep schedule by ~2h/night to reach a minimum of 7h/night. We chose 7.0h/night as a cut-off for short sleepers, since it is the minimum recommendation from the American Academy of Sleep Medicine recommendation for our targeted age-range (i.e., 18-65 years old). Our preliminary data on sleep extension in this age-group demonstrate our ability to extend sleep by over 2h/night (Table 1, section **C2.5**).

The intervention will be designed to fit the lifestyle/school/work schedule of each subject (e.g. some subjects might add 1h to each end of the sleep opportunity whereas others may add 2h in the evening before usual bedtime). After setting the desired sleep extension timing with participants, they will be instructed to maintain a *consistent bedtime* (±30 min) and *wake time* (±15 min) 7 d/wk for 4-6 wk, with exceptions allowed for special occasions. We will also work with participants to arrange their bedrooms for optimal sleep hygiene, and instruct them to reduce exposure to light at night and increase light exposure in the morning to help achieve an earlier timed circadian clock that helps participant's go to bed at an earlier hour, thereby increasing the sleep opportunity. Maintaining a consistent wake time is the most important factor, because morning sunlight exposure is a strong cue for synchronizing the circadian clock[101-109], whereas increased flexibility with bedtime improves adherence. K. Wright (Co-I) has successfully implemented the proposed sleep extension strategy in adults who, by choice, maintain short insufficient sleep schedules (see preliminary data in section **C2.5**). He is also currently conducting similar sleep extension trials (NIH R01 HL131458 and R01 HL135598) to improve vascular function in patients with HIV infection and in people with high blood pressure.

To help ensure compliance with maintaining appropriate habitual or extended sleep patterns and body weight, participants will be seen weekly by the research team to review progress and problems, and obtain body weight. Participants' sleep and activity behavior will also be monitored by: i) wrist actigraphy (Actiwatch) worn for the duration of the study; ii) participant call-in at bed-time and wake-time to a time stamped recorder that will be reviewed daily; and iii) maintaining careful sleep logs. We will review call-ins daily, download the

Actiwatch weekly, and provide participants weekly feedback about maintaining a consistent schedule. When daily call-ins are reviewed, subjects will be sent a text message letting them know to keep on track or remind them to maintain their extended sleep schedule if they report deviations from the planned schedule (i.e. bedtime ±30 min from scheduled bed time and/or wake-time ±15 min away from scheduled wake-time). In our preliminary data extending sleep in this age group, 14 of 15 subjects were able to extend sleep. If participants report 3 consecutive unscheduled deviations of ±30 min for bedtime or ±15 min for waketime they will be asked to come to the laboratory and their sleep schedule will be verified by downloading the Actiwatch data. If participants are unable to adhere to the sleep extension protocol they will be withdrawn from the study. Lastly, a urinary toxicology screen will be performed at week 2 to ensure subjects continue to abstain from recreational drug use.

D2.4. Repeat testing. After subjects complete 4-6 wk of sleep extension or habitual sleep, all visits and procedures performed during baseline testing will be repeated. In addition, the DXA scan and questionnaires completed at screening will be repeated. Subjects will continue their assigned sleep duration until all follow-up tests are completed. Subjects randomized to the *continued habitual short sleep* group will have the opportunity to receive a personalized sleep treatment plan provided by our Sleep Medicine Center after the study is completed.

D2.5. Sample analyses and calculations

Blood samples. All blood sample analyses are routinely performed in our laboratories. Blood samples for inflammatory markers (C-reactive protein, IL-6, TNFα and MCP-1 concentrations) and estrogen and progesterone concentrations will be measured by using high sensitivity ELISA kits. Plasma C-peptide and insulin concentrations will be measured by using an Elecsys assay. Plasma melatonin will be analysed by Buhlmann RIA. Total, reduced and oxidized glutathione will be assessed in plasma by using a commercially available colorimetric assay (Cayman Chemicals, Ann Arbor, MI). Plasma FFA will be quantified by using gas chromatography[110]. Plasma glucose and palmitate TTRs will be determined by using gas chromatography-mass spectroscopy[86,110].

Metabolomics. State-of-the-art mass spectrometry techniques will be used to measure specific circulating metabolites that have been linked with metabolic dysfunction and/or sleep restriction, including markers of inflammation, oxidative stress, and insulin resistance (see section **D2.2.1).** These metabolites will be evaluated in blood samples obtained every 6h for 24h to provide an integrated assessment of plasma concentrations throughout the day. Additionally, we will measure a key marker of oxidative stress in a 24h urine collection (urinary 8-iso-prostane F2α)[62,63]. To capture these metabolites of interest, multiple complementary LC methods will be performed as previously described[62,63], each optimized to capture a chemical subset of the metabolome, and coupled to a high resolution Thermo Orbitrap QExactive mass spectrometer. This orthogonal chromatography will include hydrophilic interaction liquid chromatography (HILIC) using a Sequant Zic-pHILIC HPLC column to capture polar metabolites, reverse phase chromatography using a superficially porous Phenomenex Kinetex C18 UPLC column to capture lipids and peptides, and weak ion exchange chromatography using a Phenomenex Luna aminopropyl HPLC column to capture sugars, nucleotides, and phosphates. Metabolites of interest are definitively identified by using MS/MS analysis, with matching of LC measures and MS/MS fingerprint to commercial metabolite standards. In addition to targeted measures, simultaneous untargeted metabolomics analyses will be performed to comprehensively identify novel metabolites that may be associated with sleep related improvement in metabolic function. These metabolite markers will serve as the basis for future investigation and potentially enable discovery of novel metabolite biomarkers and bioactives involved in improved metabolic function induced by sleep extension in persons with MUO.

Molecular analyses. Total RNA will be isolated from PBMC, muscle and adipose tissue biopsy samples in Trizol reagent (Invitrogen, Carlsbad, CA) or RNeasy kit (Qiagen, Valencia, CA). Real time-PCR will be used to determine gene expression[36,67,87,88]. Expression of clock genes (e.g. *CLOCK, BMAL1, PER1, PER2, CRY1, CRY2, REV-ERBα, DBP*) in PBMC and markers of inflammation (e.g. *IL6, TNF, CCL2, CD68*) and oxidative stress (e.g. *NQO1, DHCR24, UCHL1*)[36] in muscle and adipose tissue will be determined. Adipose tissue protein carbonylation will be evaluated with an OxyBlot Protein Oxidation Detection Kit (Millipore)[111].

Circadian Melatonin Phase Assessment. Blood samples from the 24h blood sampling studies will be used to measure melatonin concentrations to determine the Dim Light Melatonin Onset (DLMO) and the Dim Light Melatonin Offset (DLMOff) (linear interpolation of 25% of the fitted peak to trough amplitude), as we have

done previously[108,112-114]. We will also calculate the timing of the midpoint between the DLMO and DLMOff. We considered other circadian phase markers (e.g. core body temperature and cortisol), but will use melatonin because it is the most precise and accurate marker of circadian phase in people[115].

Calculation of substrate and hormone kinetics. Endogenous glucose and palmitate Ra into the circulation and glucose Rd will be calculated as we have previously described[36,87]. The hepatic insulin sensitivity index will be calculated as the reciprocal of the product of endogenous glucose Ra and basal plasma insulin concentration[116]. The adipose tissue insulin resistance index will be calculated as the product of endogenous palmitate Ra and basal plasma insulin concentration[117]. Plasma substrate and hormone concentration areas-under-the-curve for 5 h after initiating meal consumption and for the entire 24h will be calculated by using the trapezoid method[118]. Insulin secretion will be estimated by C-peptide deconvolution during the 24h blood sampling study[119] and insulin clearance rates by dividing the ISR AUC by the insulin AUC over each meal period and over the full 24h and by compartmental modelling during the HEC procedure[120].

D3. Retention and Recidivism Plan. Several strategies will be used to enhance adherence with the study protocols and reduce drop-outs: i) a philosophy of partnership and collaboration will be encouraged between research site personnel and study participants; ii) reimbursement for time required for study visits will be provided; iii) participants will be seen weekly by the research coordinator and research nurse, sleep duration will be tracked weekly, and participants will be contacted frequently via text message and phone calls to encourage continued success with the sleep schedule or discuss deviations; and iv) participants who are not adhering to the study protocol will receive additional support by the study team with an intervention plan established after reviewing the participant's specific barriers for compliance.

D4. Statistical analyses

D4.1. Data analyses. Statistical evaluation of the study data will be conducted in collaboration with Dr. Kenneth Schechtman, a biostatistician in the Washington University Institute of Clinical and Translational Sciences (ICTS) Research Design & Biostatistics Group. We will use a modified intention-to-treat (mITT) analysis, in which all subjects that complete the study will be analyzed regardless of their adherence to the assigned sleep intervention (i.e., continued short sleeping pattern in the control group and successful sleep extension [adding ~2 h of time-in-bed per night ensuring at least 7h/night] in the sleep extension group). Analysis of covariance (ANCOVA) with the post-treatment value as the dependent variable will be used to determine whether there are between group differences in our metabolic outcomes assessed during the HEC procedure and 24-h blood sampling study with the pre-intervention value used as a covariate. Characteristics at baseline between groups will be compared by using t-tests or Wilcoxon's test for continuous variables and chi square tests for dichotomous variables; any significant differences between groups will be used to adjust subsequent statistical analyses when necessary. Violations of the assumption of homogeneity of variance and/or sphericity will be corrected, if necessary, via transformation of data to produce data that satisfy normality and equal variance assumptions[90,121-123]. As this protocol incorporates a 2-time point, pre-post study design there is no basis to input follow-up data when only baseline data are available. Because of this limitation, we will compare characteristics between completers and non-completers by using t-tests or Wilcoxon's test for continuous variables and chi square tests for dichotomous variables; these results will be reported in addition to the ANCOVA analysis. Metabolite analyses will be performed by using an ANOVA and Tukey post-hoc testing for statistical significance between conditions and across time. For discovery based untargeted metabolomics analysis, a conservative statistical threshold will be applied at the outset (Bonferroni corrected P value of $<10^{-5}$ to account for multiple hypothesis testing). To further minimize false discovery, we will also use principal component analysis as well as sparse partial least squares and LASSO regression with internal cross-validation. Metabolites identified using these methods will be prioritized for future investigation as potential mechanisms underlying sleep related metabolic improvements.

D4.2. Sample size considerations. Using G*Power 3.1.9.2[124] we estimate that 15 participants/group will be required to detect *realistic* and *clinically meaningful effects* of sleep extension (based on data from our own studies evaluating weight loss or weight gain[36,87,88] and to data from our own and other groups assessing the metabolic effect of sleep restriction[17-19,24]) with >90% power using two-sided tests at the $\alpha=0.05$ level of significance in all primary and secondary outcomes listed below.

D4.2.1 *Primary outcome*

Insulin-mediated glucose Rd. We have found the average insulin-mediated glucose Rd during high-dose insulin infusion to be 59.1 μmol/kg FFM/min in people with obesity with a day-to-day variability of 6.4 μmol/kg FFM/min (mean ± SD) [76]. Using this SD and assuming similar variance in the sleep restriction and extension groups, we estimate we will be able to detect a ~7.9 μmol/kg FFM/min (~13%) difference between groups. This difference is smaller than the ~20% reduction in insulin-mediated glucose Rd observed after acute (1 to 7 d) sleep restriction[17-19,24].

D4.2.2 *Secondary outcomes*

24-h glucose area-under-the curve (AUC). We have found average 24h glucose AUC is 2650 mg/dl/24h in people with obesity with a day-to-day variability of 80 ± 130 mg/dl/24h (mean ± SD). Using this SD and assuming similar variability in both groups, we estimate we will be able to detect a between group difference of ~137 mg/dl/24h (~5.2%). Five nights of sleep restriction increases average plasma glucose concentration by 11% over 10 h during the day[125]. Two nights of sleep restriction increases postprandial breakfast plasma glucose AUC by 7.4% after breakfast[126].

24-h insulin AUC. We have found average 24-h insulin AUC is 818 ± 333 μU/ml/24h in people with obesity with a day-to-day variability of 26 ± 85 μU/ml/24h (mean ± SD). Using this SD and assuming similar variability in both groups, we estimate we will be able to detect a between group difference of ~90 μU/ml/24h (~11%). Five nights of sleep restriction increases average plasma insulin concentration by 29% over 10 h[125]. Two nights of sleep restriction increases postprandial breakfast plasma insulin AUC by 52%[126].

Tissue gene expression and markers of inflammation and oxidative stress. It is difficult to make robust estimates of our study power to detect changes in molecular outcomes in adipose tissue and PBMCs, because of limited data evaluating these variables and the unknown effects of extending sleep. However, in our own experience, the population variance in PBMC and adipose tissue gene expression is ~30%[36,87,88]. In those studies, we used real-time PCR methods and detected transcriptional changes after diet-induced weight gain or weight loss[36,87,88]. Accordingly, we estimate that we have ≥80% power to detect ~35% difference in gene expression outcomes between groups, with a two-sided p-value of <0.05.

D5. Scientific Rigor.

We have made every effort to ensure we obtain robust results: i) the hypotheses being tested in this proposal are based on a sound foundation of preliminary data from our own studies and results from other studies; ii) we will use a randomized controlled study design, and well-established and validated techniques to obtain the study outcomes; iii) a carefully-defined population (metabolically unhealthy with strict exclusion criteria to remove factors known to influence outcomes) will be enrolled in the study; iv) we provide packed-out meals for 3 days before each inpatient study and provide standardized meals during admission to reduce variability; v) treatment groups will be balanced with regards to sex; and vi) the sample size has the statistical power needed to detect meaningful treatment effects.

D6. Timeline.

We plan to complete all inpatient studies within the first 4.5 years to leave time for final sample processing and data analyses in the last 6 months of the grant period (Table 2). This schedule will accommodate the time required to initiate the study, recruit and screen subjects, perform the experimental protocols before and after subjects complete the sleep extension intervention, process and analyze the study samples, collate the data, finalize the data analyses, and write manuscripts.

Table 2. Study timetable

	Subjects complete Admission 1 studies (baseline) (n)	Subjects complete Admission 2 studies (baseline) (n)	Subjects complete Admission 3 studies (post-intervention) (n)	Subjects complete Admission 4 studies (post-intervention) (n)
Year 1	9	9	5	5
Year 2	13	13	13	13
Year 3	13	13	13	13
Year 4	13	13	13	13
Year 5	2	2	6	6
Total	**50**	**50**	**50**	**50**

D7. Special considerations

D7.1 PI does not have experience in conducting sleep studies or sleep extension. Although the PI (S. Klein) has considerable experience in conducting the complex outcome measures needed for this study, he does not have experience in conducting sleep studies or sleep extension. Therefore, the inpatient sleep regulation and the outpatient sleep extension protocol will be assisted by supervision and input from experienced Co-Is at UCB (J. Broussard and K. Wright) and at WUSM (B. Lucey). K. Wright is Professor and Director of the Sleep and Chronobiology Laboratory at the University of Colorado Boulder and has 25 years of experience in sleep research, including oversight of multicenter research grants and in sleep extension proposed in this study. He will provide guidance on issues related to the inpatient studies and in implementing sleep extension. He will also attend the bi-weekly videoconference meetings and travel to WUSM once per year for in-person investigator meetings. J. Broussard is an early career investigator who has considerable expertise in conducting sleep studies in people. She will travel to WUSM for 2 weeks at the initiation of the study and then every 2 months in the first year to work with research staff and Dr. Lucey to ensure correct use of sleep and circadian data collection methods. During her initial 2-week visit, she will oversee initiation of the sleep extension protocol and the constant routine protocol used to assess circadian rhythms. After year 1, Dr. Broussard will visit WUSM 3 times per year to review study protocols and progress, as well as participate in data analyses. Finally, she will provide support for day-to-day operations by email or phone calls as needed and will participate in bi-weekly videoconferencing with the research group. B. Lucey is a physician-scientist who is a sleep neurologist and Interim Director of the Washington University Sleep Medicine Center at WUSM. He has considerable experience in evaluating sleep and sleep architecture, and participates in daily polysomnogram review at the Sleep Medicine Center. He is a member of our Nutrition Obesity Research Center (NORC), because of his current interest in the relationship between sleep and metabolic health. He will be responsible for: i) supervising the registered sleep technician in conducting the inpatient PSG studies; ii) reviewing the data from each study to ensure technical reliability and quality; iii) identifying participants who have moderate to severe sleep apnea or other exclusionary, clinically significant sleep disorders; iv) facilitating referral of such participants to our Sleep Medicine Center for further evaluation and therapy, if indicated, including providing a report to the Sleep Medicine Center physicians; and v) participating in the bi-weekly videoconference project meeting, which will include colleagues at UCB.

D7.2 Facilities for conducting sleep studies. The PI (S. Klein) is Director of the Clinical Research Unit, which now houses a newly-constructed, state-of-the-art sleep laboratory that coordinates services (certified polysomnographic technicians and equipment maintenance) with the Washington University Sleep Medicine Center. The CRU sleep laboratory was designed with direct and comprehensive input from the Co-Is of this proposal, B. Lucey, K. Wright and J. Broussard. The sleep laboratory is comprised of a 170 sq. ft. temperature-, sound- and light-controlled sleep study room which is equipped with a Compumedics Grael PSG/EEG system with video monitoring. A control room with a dedicated computer for PSG review and processing is located just outside the sleep study room. A sound-insulated, light-proof catheter portal passes from the control to the sleep study room which allows blood sampling without entering the room and infusion of tracers and hormones with the infusion pump located in the control room to eliminate infusion pump noise at the bedside. The sleep study room is connected via intercom to the control room and the CRU nurses station. A shared software license is available for off-site sleep scoring and two Alice PDx Portable sleep diagnostic systems (Philips Health) are available for off-site or home sleep monitoring.

D7.3 Compliance with sleep extension and attrition. We recognize the potential risk of attrition in this long duration sleep extension outpatient study. Therefore, we have developed a specific strategy to help ensure compliance with the sleep extension protocol that is described in section **D2.3.** Moreover, our Co-Is at UCB have shown that successful sleep extension is possible (see preliminary data in section **C2.5**) with good compliance and minimal drop-outs (only 1 of 15 subjects was not compliant with the sleep extension protocol and was removed from the study). In addition, the research coordinators who will be involved in the present proposal have demonstrated considerable success in retaining study participants in previous studies (>90%) that involved rigorous long-term lifestyle intervention (diet-induced weight loss and weight gain, and exercise training) with even more complex metabolic assessments [e.g., 36,87,88,127,128].

D7.4 Ambitious project that requires highly committed subjects, considerable organizational efforts, and interpretation of complex datasets. We have an experienced participant recruitment core with access to a large pool of potential participants, a strong track record in recruiting subjects who meet specific

requirements, excellent participant compliance and retention in studies as rigorous or more rigorous than the current proposal, extensive experience in performing the complex metabolic studies proposed in this application, and expertise in conducting all sample analyses and interpretation of data needed for this project [e.g.,21,31,36,67,74-88,90,92,94,108,110,129,130]. Dr. Kenneth Schechtman will serve as the biostatistician for this project and, will ensure appropriate statistical procedures and adjustments for potential confounding variables and drop-outs are used.

D7.5 Effect of study participant age on sleep architecture. The age of our study participants will range from 18-65 years old. We chose this range to increase our ability to identify people with MUO, which is much less common in younger age groups. This age range has implications for sleep, because there is a marked reduction in slow wave sleep (considered to be the most physically restorative sleep stage) and a reduction in sleep efficiency that begins in early adulthood[131,132]. Accordingly, people who are 18-65 years old are likely to have room for improvements in sleep. However, as noted in section **D1** and **D2.2**, participants who have insomnia or other clinically significant sleep disorders will be excluded and participants who are unable or unwilling to extend sleep will be removed from the study.

D7.6 Menstrual cycle in women. Women who are menstruating will be selected on the basis of a history of regular menstrual cycle ranging in length from 25-32 days with a maximum of 3 days variation month-to-month. We will use plasma progesterone and estrogen levels from the HEC procedure days and use menstrual phase as a co-variate in our analyses. To account for menstrual phase, we will initiate the pre- and post-intervention studies in the early follicular phase (confirmed by plasma progesterone and estrogen). Women with Late Luteal Phase Dysphoric Disorder will be excluded from participation because of alterations in sleep and circadian function associated with mood disorders. Postmenopausal women will also be included in this study to increase our ability to identify subjects with prediabetes or hyperinsulinemia. Since postmenopausal women could have lower sleep quality when not using hormone replacement therapy[133], participants who have clinically significant sleep disorders will be excluded.

D7.7 Clinical relevance. This study will determine whether sleep extension in people with MUO who are short-sleepers has therapeutic effects on a series of key metabolic and inflammatory outcomes that are associated with sleep restriction. Accordingly, this study will prove or disprove this specific question, but is not meant to establish whether sleep extension is possible in a real world setting or what specific approaches are needed to extend sleep in clinical practice.

E. PROTECTION OF HUMAN SUBJECTS

E1. Risks to the subjects

E1.1. Human Subjects Involvement and Characteristics. A total of 50 human subjects, aged 18-65 years old, will be studied. Subjects will be carefully screened with a medical history and physical examination, blood tests, an oral glucose tolerance test and resting electrocardiogram (see section **D1**. for details of inclusion/exclusion criteria). Mentally disabled persons, prisoners, pregnant and lactating women, and persons whose ability to grant voluntary informed consent is questionable will not be recruited.

E1.2. Sources of Materials. All specimens will be obtained solely for research purposes. Although these generally will be obtained specifically for the purposes of the study, use will be made, where appropriate, of existing records and data obtained as part of routine clinical care. Measures to be obtained include: 1) detailed medical history; 2) physical examination; 3) clinical blood tests, including measurement of complete metabolic panel, complete blood count, plasma lipids, and HbA1c; 4) resting 12 lead electrocardiogram; 5) PSG recordings, 6) DXA scan and MRI to assess body composition and fat distribution, 7) hourly blood samples for 24h to assess plasma melatonin, C-reactive protein, IL-6, TNF-α, MCP-1, glucose, FFA, and insulin concentrations; 8) urine to assess markers of inflammation and oxidative stress, 9) a HEC procedure to assess insulin sensitivity, 10) serial PBMC sampling to evaluate clock genes, and 11) muscle and adipose tissue biopsy specimens to evaluate markers of inflammation and oxidative stress. All data from each subject will be maintained confidentially and their names and identities will not be disclosed in any published document.

E1.3. Potential Risks. We anticipate no psychological, social or legal risks beyond those of participation in health-related research in general. The potential risks associated with participation in this study are small and are listed below. They will be explained to all subjects who desire to participate in this research project. In addition, subjects will be informed that there is a possibility of unforeseeable risk, although we consider this unlikely. The research coordinator, research nurse and/or the PI will ensure understanding of the consent and study procedures, as well as laboratory results. In addition, the research nurse and/or PI will explain the various procedures and answer any questions the subjects may have before initiating any procedures. Whenever concerns arise, subjects will be informed that they are free to withdraw from the study at any time with no bias or prejudice.

This research involves exposure to radiation from the dual-energy x-ray absorptiometry (DXA) for body composition measurements. The amount of radiation from these procedures, when averaged over the entire human body, is equivalent to a uniform whole-body dose of <1 mrem. This is equivalent to less than 3% of the amount of natural background radiation exposure all people in St. Louis receive each year.

Possible side effects of intravenous catheter insertion are discomfort, bruising, damage to the blood vessel, bleeding at the site of needle insertion, and infection at the site of catheter insertion. Some people experience dizziness or feel faint.

The intravenous infusion of any solution can cause infection if that solution is not sterile. The infusion of stable isotopically labelled tracers, per se, does not cause any additional risk because these tracers are infused at very small “tracer” amounts and already exist in our body and in the food we eat. In addition, only sterile and pyrogen-free stable isotopically labelled tracers are compounded, careful sterile technique are used in preparing the solutions, and all tracers are infused through a 0.22 micron filter during the study to decrease the risk of infection.

Fat biopsies can cause swelling, bruising, temporary numbness, or long-term (>1 year) loss of sensation and tingling at the biopsy sites. Some people experience dizziness or feel faint.

Blood sugar levels could change (become low or high) during the infusion of insulin and dextrose and may result in subjects feeling sweaty, shaky or nauseated. However, this risk is extremely small because blood sugar will be carefully monitored throughout the metabolic study.

There is a possibility that potentially adverse medical conditions (e.g., diabetes mellitus, OSA) will be identified as a result of the research testing. Unless it is a medical emergency, we will inform the participant of the finding so they can follow-up with their physician for further investigation/treatment. In addition, we will review the information with the subject’s physician if the subject gives us permission to do so.

E2. Adequacy of protection against risks

E2.1. Recruitment and Informed Consent. Subjects will be recruited by flyers posted in the WUSM Medical Center and in the community, through the Volunteers for Health program at WUSM, and if necessary via radio and TV ads. The objectives of the project, all experimental procedures, all of the requirements for participation, and any possible discomforts and risks and benefits of participation will be clearly explained in writing and orally, in lay terms, to the subject by the principal investigator, co-investigators, or research coordinator. After all questions have been answered, and the subjects have been informed orally and in writing that they are free to withdraw from the study at any time with no bias or prejudice, and the subject agrees to participate, written informed consent, which has been approved by the Institutional Review Board at WUSM will be obtained. The consenting procedure will be conducted in the CRU approximately 1 week to 1 month (but at least 48h) before initiating the research protocol described in section **D2**. Subjects will be recruited from the general public and will include persons of all races and ethnic groups. We have a strong track record in recruiting subjects who meet specific study requirements, and we do not anticipate any problems in recruiting a sufficient number of subjects for timely completion of these two study protocols.

E3. Protection Against Risks

E3.1. Confidentiality. All key personnel involved in the design or conduct of the research involving the human subjects will receive the required education on the protection of human research participants prior to the start of this project. All specimens will be obtained solely for research purposes. Study samples and data sheets will be coded with an identification number for each subject. All data will be treated confidentially and the subjects' names and identities will not be disclosed in any published reports. Clinical records will be maintained in locked file cabinets within a locked file room.

E3.2. Risks described in section E1.3. The potential risks of this study are small. Rigorous screening procedures will be used to screen out potential subjects with health problems and so to screen out subjects that might be at higher risk of adverse events. A pregnancy test will be conducted at screening and upon each admission to the CRU to ensure female subjects are not pregnant. If subjects become excessively sleepy and want to stop the study, they may do so and will be permitted to sleep before discharge. Subjects will be shown the sleep study rooms before the study, and study procedures will be described in detail to ensure there is no misunderstanding of the protocol. The sleep room is larger than a typical hospital room. Subjects will be informed about the continuous observation throughout the study. Subjects will be informed that they can stop the protocol at any time. All procedures will be performed by qualified and experienced personnel, and subjects will be carefully monitored during all procedures and during the intervention.

Intravenous tracer solutions will be prepared in a designated, sterile mixing room by an experienced technician, and only sterility- and pyrogenicity- tested stable isotopically labeled tracers will be compounded. In addition, the tracer solutions are infuse through a 0.22 micron filter during the study as an additional protection to ensure no bacteria (which are usually 1.0 micron) will be infused. Careful aseptic techniques will be used when inserting the catheters, and when obtaining blood and adipose tissue samples to decrease the risk of infection. The total amount of blood collected will be <625 ml. Blood sugar will be carefully monitored throughout the HEC procedures. If a participant develops a health problem or potential health problem the PI/study physician and, if necessary a medical consultant who is not part of the research team, will decide whether the participant should continue in the study, and/or what further steps regarding medical evaluation/treatment should be performed. The CRU and hospital facilities used for the studies are equipped with defibrillators and all appropriate emergency medications. Both subjects and their primary care physicians (if consent is provided by the participant) will be made aware of any abnormal findings while participating in the study. The participants will be given the phone numbers of the members of the research team (including an emergency phone number) and told to call one of us immediately if they develop any unusual signs or symptoms.

E4. Potential benefits of the proposed research to the subjects and others. Subjects participating in these studies will be reimbursed for their time and effort. Potential benefits to the subject, include information from the medical examination as well as the potential benefits of the sleep extension intervention. The benefits to society include a thorough evaluation of the effects of sleep extension on metabolic function in habitual short sleepers that may result in a low-cost intervention to improve metabolic function.

E5. Importance of the knowledge to be gained. The information gained from this work has important public health implications regarding the effect of reduced sleep duration on metabolic health.

F. DATA AND SAFETY MONITORING PLAN

Data from the study will be monitored on a continuous basis by the PI. All serious adverse events (SAEs), adverse events (AEs), and laboratory values will be reviewed by the PI on an ongoing basis. Dr. Klein will be responsible for annual reports to the WUSM IRB and for reporting any adverse events (AEs) to the IRB.

The IRB will be notified within 24 hours of any SAE occurring at that site via their online system for reporting SAEs and additional information will be forwarded to the IRB as it becomes available. The CRU will also be notified within 24 hours of any SAE by contacting the Research Subject Advocate and submitting to him/her the information provided to the IRB. In addition, all laboratory values outside of the normal range will be discussed with the study subject, and appropriate arrangements will be made for treatment, if necessary.

A Data and Safety Monitoring Board, chaired by David Carr M.D. (Professor of Medicine), and including Dominic Reeds M.D. (Associate Professor of Medicine) and David Alpers (Professor of Medicine) will meet every 12 months with the PI and Co-Is to review study data, discuss any safety issues, and ensure compliance with the protocol. Collectively, these individuals have decades of experience performing and monitoring the safety of complex metabolic studies, including those employing hyperinsulinemic-euglycemic clamp procedures and stable isotope tracer infusions, in people.

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