Document Title:	Statistical Analysis Plan
Protocol Title:	Menopausal Sleep Fragmentation: Impact on Body Fat Gain Biomarkers in Women
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Biostatistical Analysis

Analysis approach: Metabolic endpoints are: 1) fasting morning levels of leptin (primary), adipokines, ghrelin (secondary), and 2) the circadian leptin profile (exploratory), calculated as area under curve (AUC) to detect whether the overall profile is lower, as seen in sleep restriction studies,[53] and morning fasting AD levels. Behavioral endpoints are: 1) satiety (primary) and hunger (secondary) on a VAS scale, and 2) daily caloric intake during ad lib meals (exploratory). Analyses of each endpoint will be conducted in parallel as we seek to detect coherence among endpoints and focus on consistency across different outcomes in each hormonal and HF context.

Our experimental design, methodology, and analytic approach are robust and unbiased. While only women will be enrolled, analyses and interpretation of results will take into consideration age and other relevant biological variables. Key covariates will be measured and included as potential predictors in multivariate analyses, including specific *a priori* examination for age, race, BMI, SES, and stressful events to reduce unexplained variability in the outcomes.[141] Importantly, within-subject analyses using each woman as her own control and expected low attrition based on our prior experience with similar protocols will minimize potential bias.

To address **Aim 1** (*impact of menopause-related sleep fragmentation on metabolic and behavioral biomarkers of body fat gain*), the mean levels in each endpoint in response to experimentally fragmented sleep (**Hypothesis 1a & 1b**) will be examined using repeated-measures generalized linear mixed-model regression. Models will incorporate the 2 SB contrasts addressing **Hypothesis 1a and 1b** to simultaneously compare the impact of sleep fragmentation in the HF-free, estrogenized environment (**SB_{SF1} vs. SB_{UD1}**), and hypo-E2 environment with stable HF levels (**SB_{SF2} vs. SB_{UD2}**); exploratory analyses will adjust for HF, either categorized (e.g., any versus none) or number of HF. If there is an omnibus effect of experimental sleep fragmentation, we will conduct post-hoc analyses on each SB contrast to explore whether the impact of experimental sleep fragmentation is consistent across hormonal and HF contexts. To address **Hypothesis 1c**, mean levels in outcomes will be contrasted SP_{UD3} vs. SP_{UD1} to assess the impact of sleep disruption related to HF in the environment without experimental sleep fragmentation, comparing those developing HF (expected N=26) with those who do not develop HF; analyses will adjust for initial E2 level.

Aim 2 (*impact of HF on metabolic and behavioral biomarkers of body fat gain*) will be examined using a repeated-measures generalized linear mixed-model regression analysis to determine the effect of HF on mean levels of in metabolic and behavioral biomarkers. The regression will include SB contrasts that assess the impact of HF in the presence (**SB_{SF2} vs. SB_{SF1}**) and absence (**SB_{UD2} vs. SB_{UD1}**) of experimental sleep fragmentation. If an omnibus effect of HF is observed, post-hoc analyses will be conducted on each SB contrast to explore whether the impact of HF is consistent across sleep fragmentation contexts. Additional analyses will substitute objectively measured HF or night-time only HF.

To address **Aim 3** (*impact of estradiol withdrawal on metabolic and behavioral biomarkers of body fat gain*), the mean levels in each endpoint in response to estradiol withdrawal (**Hypothesis 3**) will be examined using repeated-measures generalized linear mixed-model regression. Models will incorporate the 2 estrogenized and hypo-E2 contrasts addressing simultaneously the impact of estradiol withdrawal in the unfragemented and fragemented sleep states. If there is an omnibus effect of estradiol withdrawal, we will conduct post-hoc analyses on each sleep state contrast to explore whether the impact of estradiol withdrawal is consistent across both sleep states. Exploratory analysis will include adjusting for HF – either categorical (yes/no or

none/infrequent/frequent) or continuous – and sleep HF-related disruption in the hypo-E2 state (second SB).

Exploratory Hypothesis #1 analyses will involve a repeated-measures generalized linear mixed-model regression similar to those proposed for the **Hypothesis 1a** analysis using the RQ as an indirect measure of resting EE as the dependent measure, and the same post-hoc paired contrasts and interaction terms for randomization order. **Exploratory Hypothesis #2** analyses will involve addition of interaction terms (between total body fat or visceral adiposity with sleep fragmentation, HF, or E2 withdrawal) to models described for **Aims 1–3**, to test for effect modification on each metabolic or behavioral endpoint. These analyses are by necessity exploratory in nature given the sample size of this complex experimental study. **Exploratory Hypothesis #3** will involve an omnibus repeated-measures regression model encompassing all 6 contrasts described in **Aims 1–3** with the PANAS Negative Affect scale score as the primary predictor and adjustment for fragmented versus undisturbed sleep given an expected association between sleep fragmentation and affect. Post-hoc analyses using paired t-tests on each SB contrast will be conducted to explore the potential variability of the impact of negative affect on each metabolic and behavioral biomarker across the sleep fragmentation, HF, and hormonal contexts. Psychological and cognitive factors driving eating behaviors on the DEBQ will also be explored.

Sample size: Forty subjects will complete all study procedures. Sample size calculations are based on pilot data correlations between the change in the number of EEG arousals and change in leptin levels from before to after receiving GnRHa. The observed Pearson and Spearman correlations are $r=-0.44$ and $r_s=-0.36$. The detectable correlation is $r=-0.43$ for a sample size of 40 and $r=-0.52$ for the subgroup analysis (n=26) in **Hypothesis 1c** ($\alpha=0.05$, 80% power). Because it takes 3 months (screening/menstrual tracking plus 2 months of active procedures) for each subject to complete the study and data analysis time is required after all procedures are completed, accrual is expected to be achieved in the first 39–42 months of the Award. The expected accrual rate of 1.1 subjects per month is therefore similar to that which [redacted]achieved in prior healthy volunteer studies with a similar number of in-lab nights. This rate is lower than the 1.7/month that Dr. Joffe achieved with healthy volunteers to leuprolide studies but with less intensive procedures. To ensure that accrual proceeds as planned, we will advertise widely using postcards we have used to enroll 2.4 women/month to menopause studies and which colleagues used to enroll 2.2 healthy volunteers/month to a study with fewer nights in the sleep lab.[142] Based on previous experience, we expect to consent up to 130 subjects in order to have 40 study completers, given that up to 10 women may not be eligible after completing screening procedures and an additional 8 women may decline to receive leuprolide after completing SB#1, and because some women will participate in pilot procedures only.