Statistical Analysis Plan (SAP)

Study Title	Heart rate and Breathing Effects on Attention and Memory (HeartBEAM)
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1. Objectives and hypotheses

   In our previous HRV-ER trial (NCT03458910), four weeks of daily slow-paced breathing designed to enhance parasympathetic activity reduced plasma Aβ levels in both younger and older adults. The current study aimed to extend those findings in adults aged 50 to 70 years using a 10-week intervention with three assessment time points. Secondary objectives included examining the effects of slow-paced breathing on brain structure (i.e., perivascular space and hippocampal volumes) and cognitive performance. We hypothesized that the 10-week slow-paced breathing intervention would reduce plasma Aβ levels and lead to improvements in brain structure and cognitive performance.

2. Sample Size & Power consideration

   For plasma Aβ, we estimated reliability using correlations between pre- and post-intervention time points in the HRV-ER trial. Effect sizes were derived from the pre-post difference in the HRV-ER older adult Osc+ (slow-paced breathing) group. We powered the study to detect small-to-medium effect sizes ($f = .2$) with a .7 correlation among repeated measures and aimed for 95% power at $\alpha = .05$. Based on these parameters, a total sample size of $N = 42$ (with three assessments) was required. Sixty-two participants (31 in each condition) ultimately completed the study.

3. Outcome Measures and Analyses

   We examined condition differences (slow-paced breathing vs. random-paced breathing) in intervention-related change in the following outcome measures. The average time between pre and post intervention assessment was 10 weeks.

   Primary outcome measures

   - **Change in plasma amyloid beta levels:** We computed an aggregate score based on plasma Aβ40 and Aβ42 levels (pg/mL), calculated as the average of their Z-scores. Higher values on this composite measure are associated with greater risk of Alzheimer's disease. This score was compared across three time points: Week 2 (pre-intervention), Week 7 (mid-intervention), and Week 12 (post-intervention). We conducted a time (Week 2, 7, 12) × condition ANOVA to test for a time × condition interaction in plasma Aβ levels, assessing group differences in change over time.
   - **Change in plasma Ab42/40 ratio**: The plasma Aβ42/40 ratio was calculated by dividing the plasma Aβ42 concentration (pg/mL) by the plasma Aβ40 concentration (pg/mL) at each time point. We conducted a time (Week 2, 7, 12) × condition ANOVA to test for an interaction effect, using plasma Aβ42/40 ratio scores as the dependent variable to assess group differences over time.

   Secondary outcome measures

   - **Change in brain perivascular space (pvs) volume:** PVS volume was defined as the percentage of PVS volume relative to white matter volume in the centrum semiovale, our main region of interest. To examine condition differences in changes over time, we conducted a time (Week 2, 7, 12) × condition ANOVA to test for an interaction effect.

- **Change in hippocampal volume:** We tested whether there were condition differences in changes in hippocampal volume. We performed a two-way mixed ANCOVA on hippocampal volume, with condition (slow-paced vs. random-paced) as the between-subjects factor and time point (Week 2, 7, 12) as the within-subjects factor, controlling for intracranial volume as a covariate.
- **Brain training performance on 12 Lumosity games during the breathing intervention (controlling for brain training performance pre intervention):** Increments in game play performance over the course of the intervention were captured on latent level, with all games contributing to the latent variable. To evaluate longitudinal changes in cognitive performance during the intervention, we fitted a linear mixed-effects model to participants' latent performance scores across the 10 time bins. We examined condition differences in performance changes over time.

Other pre-specified outcome measures

- Change in plasma pTau-181/tau ratio:
- Change in urine Ab42

Safety outcomes

Six non-serious adverse events were reported and subsequently resolved.

4. Populations and subgroups to be analyzed
   Population analyzed is composed by all randomization subjects who completed assessments and whose data quality was sufficient for data analysis.