COVER PAGE:

Non-invasive Auricular Fiber Vagus Nerve Stimulation (afVNS) for Autism Spectrum Disorder: A Study Protocol for an Open-Label Trial Investigating Clinical and Physiological Effects

Trial registration: This trial was registered in the clinical trials register http://www.clinicaltrials.gov (NCT06473623)

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Statistical Analysis Plan (SAP)

Study Title: Non-invasive Auricular Fiber Vagus Nerve Stimulation (afVNS) for Autism Spectrum Disorder (NCT06473623)

1. Analysis Principles & Data Handling:

- Analysis Sets: Primary analyses will follow the Intent-to-Treat (ITT) principle.
- Missing Data: For feasibility outcomes, missing post-intervention data is a failure. For clinical/ANS outcomes, complete-case analysis will be used initially. Sensitivity analyses using Last Observation Carried Forward (LOCF) will be performed if missingness exceeds 5%.
- Software & Significance: Analyses will use GraphPad Prism (v10+). A two-sided $\alpha=0.05$ defines significance. No formal adjustment for multiple comparisons will be made for exploratory analyses.
- Normality: Assessed via the Shapiro-Wilk test.

2. Analysis of Primary Feasibility & Usability Outcomes:

- Success will be determined by meeting all pre-defined criteria:
- Adherence Rate: Proportion of participants who complete >70% of scheduled afVNS sessions. Success Criterion: >70%.
- Assessment Completion: Proportion who complete the post-intervention assessment. Success Criterion: >70%.
- Safety/Tolerability: Incidence and severity of all Adverse Events (AEs). Success Criterion: No related Serious Adverse Events (SAEs).
- Device Usability: Mean score from a post-intervention usability questionnaire. Success Criterion: Mean score ≥ 70/100

3. Analysis of Intervention Adherence and Feasibility

3.1 Definitions and Data Adjustments:

- Device-Failure Session: A planned session where the participant intended to use the device, but a technical malfunction (e.g. software crash, connection error, hardware fault, rapid battery drain) prevented its initiation or completion. This requires verification by the study's technical support team.
- Participant-Adherent Session: A session where the device functioned as intended, but the participant chose to stop early, skip, or modify the session for reasons of tolerability, preference, or convenience (e.g. sensory discomfort, lack of time, forgetting).
- Device-Unavailable Period: Any consecutive calendar day(s) where the device was not in the participant's possession due to recall for repair, replacement, or

servicing. These days are excluded from the denominator for adherence calculations to assess tolerability fairly.

3.2 Analytic Metrics: Daily usage data from participant logs will be used to calculate the following metrics for each participant and for the cohort:

- Overall Usage Rate: (Total completed sessions/ 14 Planned sessions) x 100%. This measures adherence to the original 14 day protocol.
- Technical Failure Rate: (Total Device-Failure Sessions/ Total Planned Sessions) x 100%
- Device-Available Usage Rate: [Total completed session/ (14 planned sessions – Total Device Unavailable Days)] x 100%. This is the primary metric for participant tolerability, estimating adherence when the device was physically present and functional.
- Tolerability-Adjusted Usage Rate: [Total completed sessions/ (Total planned sessions – Total Device-Failure Sessions)] x 100%. This metric estimates adherence for sessions where a technical failure was not the primary barrier.

3.3 Analysis: All rates will be reported descriptively as median and Inter-Quartile Range (IQR)). The Overall Usage Rate and the Device-Available Usage Rate will be presented and compared descriptively to distinguish protocol feasibility from participant tolerability. Reasons for Device-Failure Sessions and Participant-Adherent Session non-completion will be coded, summarized by frequency, and reported separately to inform specific conclusions about technology readiness and protocol acceptability.

4. Analysis of Exploratory Clinical & Behavioral Outcomes

All clinical outcomes will be analyzed as paired, within-subject (pre- vs. post-intervention) comparisons.

Measure	**Analysis**	**Objective**
CGI-S, CGI-E, PRAS-ASD, ABC (subscales), PSQI/CASQ, COWAT	Paired t-test (parametric) or Wilcoxon signed-rank test (non-parametric).	To assess signal of change in core traits, anxiety, behavior, sleep, and verbal fluency.
CGI-I, Parent Target Symptoms	Descriptive statistics (frequency, proportion of responders). A "responder" is defined as a score of 2 ("much improved") or 3	To provide a global clinical impression of change and personalized symptom impact.

	("very much improved") on the CGI-I.	
Effect Size Calculation	Cohen's *d* (parametric) or rank-biserial correlation (non-parametric).	To quantify magnitude of change for future trial planning.

5. Analysis of Autonomic Nervous System (ANS) Outcomes

- Data Preparation: For each 30-minute recording, key variables will be calculated: Heart Rate (HR), Mean RR, NN50, pNN50, SDNN, RMSSD, HF Power, LF/HF Ratio, Poincaré Plot SD1, Poincaré Plot SD2.
- Between-Group Comparison: Welch's t-test or Mann-Whitney U test will compare baseline ANS activity between the ASD cohort and an age-matched neurotypical control group.
- Within-Subject Comparison: Paired *t*-test or Wilcoxon signed-rank test will analyze pre- vs. post-intervention changes within the ASD group.
- Exploratory Correlation: Spearman's correlation will examine associations between changes in key ANS variables (e.g., RMSSD, HF power) and changes in primary clinical scores (e.g., ABC Irritability, PRAS-ASD).

6. Sample Size Justification

- A sample of N=20 participants with ASD is targeted. This aligns with pilot study recommendations and provides:
- Feasibility: A 95% CI with ~±20% margin of error for binary outcomes.
- Exploratory Outcomes: 80% power ($\alpha=0.05$) to detect large effect sizes (Cohen's $d > 0.8$) in paired comparisons.