STUDY PROTOCOL

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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Objectives

We hypothesize afVNS represents an adoptable, non-pharmacological adjunct therapy for home healthcare. We anticipate this intervention will support improvements in core autistic traits and commonly co-occurring health challenges.

The objectives of this clinical trial are:

1. To test the feasibility and adoption of afVNS for autistic individuals in the home setting
2. To evaluate the short-term effects of afVNS on core autistic characteristics and associated conditions.
3. To assess the impact of afVNS on ANS biomarkers in autism, as well as on related domains including sleep quality, verbal fluency and anxiety.

These objectives correspond to specific aims regarding benefits and harms:

- Harms: Objective 1 includes systematic assessment of safety and tolerability, with comprehensive monitoring and reporting of adverse events.
- Benefits: Objectives 2 and 3 aim to collect preliminary data on potential clinical and physiological effects to inform future trial design.

Design and Methods

This is a single arm, open-label, exploratory study to be conducted at a single clinical site (Hong Kong). Following training and baseline assessments, afVNS feasibility and tolerance will be assessed over 14 days home healthcare application. The tolerability of afVNS application and completion of daily stimulation will be closely monitored, given the well-documented sensory sensitivities, particularly in the tactile domain, prevalent in the ASD population. A total of 20 ASD individuals will be assessed pre- and post- afVNS application to measure short-term effects on core autistic traits and commonly co-occurring conditions.

Population and Recruitment

The study population will consist of individuals diagnosed with ASD. Participants will be recruited through multi-faceted strategy to achieve the target sample size of 20. This includes active referrals from local clinical practices specializing in neurodevelopmental conditions, collaboration with non-governmental organizations (NGOs) serving the autistic community, and the use of approved study advertisements within these networks. Enrollment will be tracked weekly and outreach will be expanded to additional clinical partners if recruitment falls behind schedule. Written informed consent will be provided by all subjects or parents/ guardians prior to their participation. A qualified expert will assess the eligibility of each patient according to the inclusion and exclusion criteria as detailed below:

Inclusion criteria

- Written informed consent
- Age: between 7 and 22 years
- Participants and parent/ guardian have sufficient proficiency in English language
- Diagnosis of ASD as defined by Autism Diagnostic Observation Schedule 2nd edition (ADOS-2) or Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5)
- Full-Scale Intelligence Quotient (FSIQ) $\geq$70

Exclusion criteria

- Severe psychiatric disorders (e.g. bipolar, major depressive disorder)
- Severe neurological disorders (e.g. stroke, epilepsy)
- Presence of a known pathogenic genetic variant associated with neurodevelopmental disorders (e.g. Fragile X, Rett Syndrome, Tuberous Sclerosis)
- History of cardiovascular disease
- History of head trauma (surgery or tumor)

- Active medical implants (cochlear, VNS or pacemakers)
- Cerebral shunts
- Auricular skin disease that compromises placement of electrodes
- Pregnancy

Patient and Public Involvement (PPI)

This trial design was informed by clinical expert consultation. While formal PPI was not part of this initial protocol development, we have outlined a plan for involving the autistic community in the interpretation of results and future research.

Outcome measures

The primary outcome measure is feasibility measured from completion rate and neurostimulation tolerance of home healthcare application with successful utilization of daily afVNS. Participants or their caregivers will complete a daily usage log to record session completion, duration and any issues. This will be used to distinguish between sessions missed due to device-related technical problems and those missed due to participant choice or tolerability. This distinction is critical for interpreting the primarily feasibility endpoints and is detailed in the Statistical Analysis Plan (SAP).

The secondary measures enable assessment of afVNS clinical and physiological effects, which will be measured at pre- (‘baseline’) and post- (‘final day’) afVNS (Figure 1; Table 1). These measures include:

- Clinician Global Clinical Impression (CGI-I) for core social impairment and overall level of cognitive, adaptive and social functioning.
- Clinician Global Impression Severity (CGI-S) for assessment of social interactions, restricted or repetitive behaviors and overall autistic traits.

- Clinician Global Impression Efficacy (CGI-E) for assessment of therapeutic intervention on core autistic traits and associated functional domains.
- Parent-Rated Anxiety Scale for ASD (PRAS-ASD) for anxiety assessment consisting of 25 questions related to anxiety ranging from 0 (none) to 3 (severe).
- Parent-rated Aberrant Behavior Checklist (ABC) of 58 items on 1-4 Likert scale with five subscales including: irritability, hyperactivity, lethargy/ withdrawal, stereotypy and inappropriate speech.
- Parent defined target symptoms. One or two problems of greatest concern to parents at baseline rated on frequency, duration, intensity and functional impairment, assessed on a 9-point scale as 1 = normal, 2 = markedly improved, 3 = definitely improved, 4 = equivocally better, 5 = no change, 6 = equivocally worse, 7 = definitely worse, 8 = markedly worse; and 9 = disastrously worse. Ratings across the two target symptoms will be averaged.
- Pittsburgh Sleep Quality Index (PSQI) or Cleveland Adolescent Sleep Questionnaire (CASQ) for assessment of sleep quality.
- Controlled Oral Word Association Test (COWAT) for assessment of verbal fluency.
- Autonomic activity measured from wearable sensors with continuous data collection for 30 mins will be collected while the subject sits in a quiet room.

Study Protocol

Outcome measures will be assessed at baseline (Day 1) and final day (Day 15) (Figure 1). At baseline, research subjects or their caregivers will be trained in applying the neurostimulation device for use of the stimulators and controller unit. Research subjects or their caregivers will be required to demonstrate successful device operation and electrode placement before being approved to participate. Thereafter, the device will be utilized at home, and research subjects or caregivers will self-administer daily afVNS, ideally at a consistent time each day. Participants will complete daily usage records online, which will be reviewed weekly by the

study coordinator to monitor adherence. Research subjects with previously prescribed drug medications will continue their stable treatments without change during the clinical study. No new neuromodulation therapies are permitted for the duration of the trial.

Non-invasive VNS

afVNS will be performed by NX-08® device (supplied by Neuropix Technologies Ltd, Hong Kong). NX-08® device is a wireless neurostimulator with ear electrodes positioned at the cymba conchae of the auricle for afVNS. Stimulation intensity (mA) for treatment will be determined for each subject by tolerance measures at baseline (Day 1) visit, where patients will be assessed for their stimulation pain threshold by application of the device. Thereafter, afVNS will be applied for 1 hour per session, once daily, for 14 consecutive days.

Safety Monitoring and Assessment of Harms

Adverse events (AEs) will be defined as any untoward medical occurrence in a trial participant, which does not necessarily have a casual relationship with the intervention. Serious Adverse Events (SAEs) will be defined per standard ICH-GCP criteria. Harms will be assessed systematically through multiple channels: (1) participant/ caregiver reporting via a daily log and weekly check-in calls; (2) direct questioning by the study team during their final assessment, and (3) review of device usage data for signs of non-use that may indicate tolerability issues.

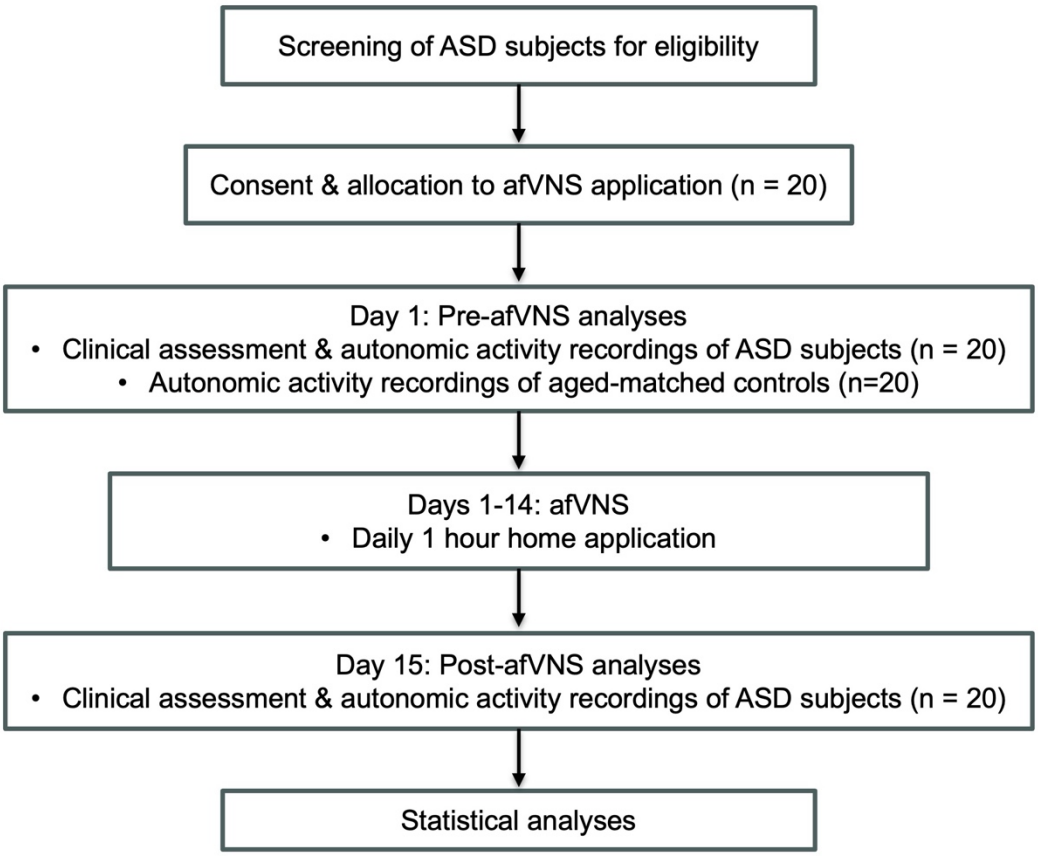

Figure 1. Study design for home application of auricular fiber Vagus Nerve Stimulation (afVNS) for Autism Spectrum Disorder (ASD) individuals. Recruitment of research subjects (n=20) will be followed by pre- (Day 1) and post- (Day 15) afVNS clinical assessments.

	Baseline Measures	Daily Measures	Endpoint Measures
Subject compliance and completion		Stimulation tolerance, successful deliverance, stimulation time and completion record; adverse effects; and picture of device placements.	Stimulation history and compliance rates.
Overall ASD – anxiety, social interaction	CGI-I, CGI-S, CGI-E, ABC		CGI-I, CGI-S, CGI-E, ABC
Specific ASD symptoms	Parent defined target symptoms		Parent defined target symptoms
Verbal Fluency	COWAT		COWAT
Anxiety	PRAS-ASD		PRAS-ASD
Sleep	PSQI or CASQ	Wearable Tracker	PSQI or CASQ
Autonomic Activity	Wearable Measure		Wearable Measure

Table 1. Study measures in Autism Spectrum Disorder (ASD) individuals for evaluation of clinical and physiological effects following auricular fiber Vagus Nerve Stimulation (afVNS). Clinician Global Clinical Impression (CGI-I); Clinician Global Impression Severity (CGI-S); Clinician Global Impression Efficacy (CGI-E); Parent-Rated Anxiety Scale for ASD (PRAS-ASD); Aberrant Behavior Checklist (ABC); Parent defined target symptoms; Pittsburgh Sleep Quality Index (PSQI); Cleveland Adolescent Sleep Questionnaire (CASQ); Controlled Oral Word Association Test (COWAT).

Recording Autonomic Nervous System (ANS) Activity

Autonomic activity of ASD individuals will be measured and characterized pre- ('baseline') and post- ('final day') afVNS. Each subject will wear a heart rate sensor for 30 min for continuous data collection while sitting in a quiet room (Figure 2). Real-time data will be acquired, and autonomic indices (Table 2) will be analyzed with HRV software (Kubios HRV Scientific). Autonomic activity of ASD individuals will be compared to aged-match controls.

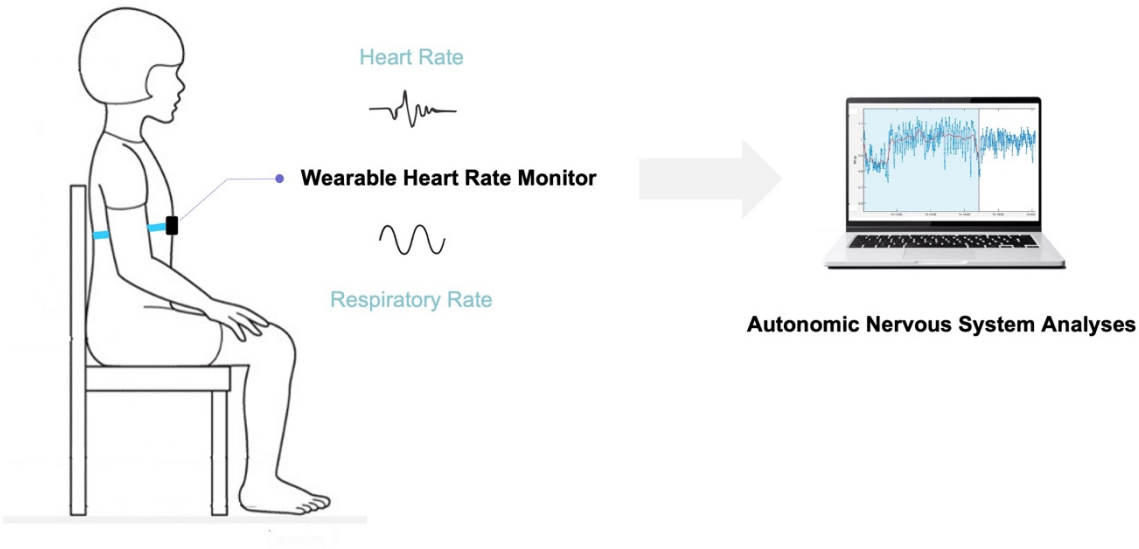

Figure 2. Research subjects will sit for 30 mins in a quiet room with a wearable heart rate sensor attached via a chest strap for measurement of the autonomic nervous system activity. Data will be analyzed for key autonomic indices.

ANS Indices	Name	Description
HR	Heart Rate	Heart beats per minute.
HRV	Heart Rate Variability	The variance in time between each heartbeat.
Mean R-R Intervals	Average number of intervals between adjacent R waves	The mean time interval between two consecutive heartbeats.
NN50	Number of pairs of successive NN (R-R) intervals that differ by more than 50 ms	The number of interval differences of successive R-R intervals greater than 50 ms.
pNN50	Percentage of adjacent NN (R-R intervals that differ by more than 50 ms	The proportion of NN-50 to the total of RR-intervals.
sdNN	Standard deviation of NN (R-R) intervals	Standard deviation of the R-R interval.
RMSSD	Root mean square of successive differences	Indicates high frequency influences of heart rate variability.
HF Power	High Frequency Power	Indicates the respiratory sinus arrhythmia (RSA) – the natural variation in heart rate that occurs with breathing.
LF/HF Ratio	Low Frequency to High Frequency Ratio	Indicates the index of sympathovagal balance.
Poincare Plot SD1	Poincare Plot Standard Deviation 1	The standard deviation of short-term heart rate variability; a direct non-linear equivalent of HF power.
Poincare Plot SD2	Poincare Plot Standard Deviation 2	The standard deviation of long-term heart rate variability; a direct non-linear equivalent of overall HRV.

Table 2. Summary and description of the main autonomic nervous system indices across time-, frequency- and non-linear domains.

Study Oversight

The trial sponsor serves as the coordinating centre responsible for overall trial management, regulatory compliance, and data analysis. The Principle Investigator holds responsibility for the scientific conduct at the study site. Given the pilot feasibility design, a formal steering committee or independent endpoint adjudication committee will not be constituted.

Oversight of data and safety will be managed by the Principal Investigator and the sponsor. A formal independent Data Monitoring Committee (DMC) is not required due to the short duration, single-arm design, and the low-risk, non-invasive nature of the afVNS intervention. No formal interim analyses for efficacy or futility are planned. The trial may be stopped prematurely on grounds of safety, such as occurrence of a serious adverse event deemed related to the device, or for clear futility in achieving feasibility endpoints.

Trial conduct will be monitored internally. The Principal Investigator and study coordinator will review recruitment progress, protocol adherence, and adverse events on a weekly basis. The sponsor will also perform periodic remote monitoring of the study database for data quality and completeness.

Participant Retention

To promote retention, participants will receive reminder calls/ messages before assessments. For participants who discontinue the intervention, outcome data will still be collected at the final timepoint where possible to minimize missing data.

Ethics, Consent, Study Organization and Registration

The clinical trial has been approved by the HKSTP Clinical Research Ethics Committee (CREC) IRB (No. 2025-031) and is registered in the clinical trials register http://www.clinicaltrials.gov (NCT06473623). The study complies with the code of Ethics of the World Medical Association Declaration of Helsinki. The investigator will explain the benefits

and risks of participation in the study to each research subject and will provide an informed consent form approved by the independent Ethics Committee. Only subjects or their parents/ guardians, who sign the form, will be included in the study. The research data will be published anonymously.

Data Collection and Management

Clinical and physiological data will be collected using standardized Case Report Forms (CRFs) by trained assessors according to the following schedule: (a) Screening Visit: Prior to the initiation of afVNS, participants will be screened for eligibility, and all relevant data will be documented; (b) Baseline and Follow-up Visits: All study application sessions and scheduled follow-up assessments will be documented upon completion; (c) Adverse Event Reporting: Any Adverse Events (AEs) will be documented promptly on occurrence. All forms will be dated and signed by the principal investigator or an authorized delegate. CRFs will be monitored for completeness and correctness. Only complete and correct data will be entered into a password-protected electronic study database by the designated data manager and stored on a secure server. Access to study data is restricted to authorized personnel. In all cases, it remains the responsibility of the investigator to check that CRFs are completely filled in. Original CRFs will be stored in a locked cabinet at the study site. All study related data (electronic as well as on paper) will be stored for 3 years after trial completion.

Confidentiality

All personal information will be kept strictly confidential. Participant data will be pseudonymized using a unique study ID at the point of data entry. Electronic records will be stored on password-protected, access-controlled secure servers with regular backups. Data collected is for the purposes of this study only; the consent form does not include provisions for the use of data or biological specimens in future ancillary studies.

Protocol Modifications

Any important modifications (e.g. change to eligibility criteria, outcomes, analyses) that may impact the study's conduct or participant safety will require prior approval from the Ethics Committee. Approved amendments will be communicated to all study site personnel and will be updated in the clinicaltrials.gov registry. Participants will be re-consented if the modification directly affects their participation.

Withdrawal from the study

In case of endangerment of personal safety, intolerable discomfort from the stimulation (as defined by a participant request to stop), lack of compliance, or withdrawal of informed consent, the research subject will be excluded from further participation.

The study involves a non-invasive neurostimulation device with a well-established safety profile. In the unlikely event of harm arising from participation in this trial, all participants are covered by insurance as required by local regulations. Standard clinical care will continue to be provided by the participant's treating physicians; no ancillary or post-trial care is provided by the study team beyond the trial period.

Sample Size Calculation & Statistical Analyses

The study protocol is designed with a primary objective of evaluating feasibility and usability of afVNS in individuals with ASD. A sample size of 20 participants has been determined to be appropriate for early-phase investigation. This number aligns with established methodological recommendations for pilot studies, which typically enroll 10-30 participants to inform the design of future definitive trials. The selected sample size provides sufficient precision for estimations on feasibility and tolerability, yielding 95% confidence intervals of approximately $\pm$20% for a binary outcome, and to detect large effect sizes (Cohen's $d>0.8$) in preliminary continuous outcomes, such as usability scores, with 80% power, $\alpha=0.05$. This 'fit-for-purpose' approach is consistent with FDA guidance for early-stage device development[60] and ensures

adherence to ethical principles by minimizing participant exposure, while addressing core questions of protocol viability.

Clinical outcome data, including severity of autistic traits, will be collected at baseline and following completion of afVNS intervention. All statistical analyses will be performed using Graphpad Prism. The normality of data distribution will be assessed using Shapiro-Wilk tests. Based on the data type and distribution, either parametric or non-parametric tests will be employed.

For the analysis of ANS activity, the mean value of each autonomic variable will be calculated from the 30 min data collection period for each subject. To compare autonomic activity between the ASD group and age-matched control group in a non-paired design, Welch's t-test (parametric data) or Mann-Whitney U test (Wilcoxon rank-sum test, non-parametric data) will be used.

For the primary within-subject (paired) analysis comparing pre- and post- afVNS application effects, pairwise statistical tests will be conducted. The paired t-test and Wilcoxon signed-rank test for parametric and non-parametric data will be used, respectively. These analyses will be applied to autonomic variables and clinical scores to evaluate the preliminary effects and within-subject change. Full details of statistical analyses are detailed in the SAP.