Medicine
Neuroscience

The effect of transcutaneous auricular vagus nerve stimulation on cardiovascular function in subarachnoid hemorrhage patients: a safety study

Reviewed Preprint
v2 • December 9, 2024
Revised by authors

Reviewed Preprint
v1 • August 22, 2024

Gansheng Tan, Anna L Huguenard, Kara M Donovan, Phillip Demarest, Xiaoxuan Liu, Ziwei Li, Markus Adamek, Kory Lavine, Ananth K Vellimana, Terrance T Kummer, Joshua W Osbun, Gregory J Zipfel, Peter Brunner, Eric C Leuthardt

Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA • Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA • Department of Neuroscience, Washington University in St. Louis, St. Louis, USA • Department of Neurology, Washington University in St. Louis, St. Louis, USA

eLife Assessment

The authors provide a **solid** set of data supporting the safety of transcutaneous auricular vagal nerve stimulation on cardiovascular parameters in the acute setting of critically ill patients presenting with subarachnoid hemorrhage. This **important** study also suggests a promising effect on autonomic balance.

https://doi.org/10.7554/eLife.100088.2.sa3

Abstract

Introduction

Subarachnoid hemorrhage (SAH) is characterized by intense central inflammation, leading to substantial post-hemorrhagic complications such as vasospasm and delayed cerebral ischemia. Given the anti-inflammatory effect of transcutaneous auricular vagus nerve stimulation (taVNS) and its ability to promote brain plasticity, taVNS has emerged as a promising therapeutic option for SAH patients. However, the effects of taVNS on cardiovascular dynamics in critically ill patients, like those with SAH, have not yet been investigated. Given the association between cardiac complications and elevated risk of poor clinical outcomes after SAH, it is essential to characterize the cardiovascular effects of taVNS to ensure this approach is safe in this fragile population. Therefore, we assessed the impact of both acute taVNS and repetitive taVNS on cardiovascular function in this study.

Methods

In this randomized clinical trial, 24 SAH patients were assigned to either a taVNS treatment or a Sham treatment group. During their stay in the intensive care unit, we monitored patient electrocardiogram (ECG) readings and vital signs. We compared long-term changes in heart rate, heart rate variability, QT interval, and blood pressure between the two groups. Additionally, we assessed the effects of acute taVNS by comparing cardiovascular metrics before, during, and after the intervention. We also explored acute cardiovascular biomarkers in patients exhibiting clinical improvement.

Results

We found that repetitive taVNS did not significantly alter heart rate, QT interval, blood pressure, or intracranial pressure. However, taVNS increased overall heart rate variability and parasympathetic activity compared to the sham treatment. The increase in parasympathetic activity was most pronounced from 2–4 days after initial treatment (Cohen's d = 0.50). Acutely, taVNS increased heart rate, blood pressure, and peripheral perfusion index without affecting the corrected QT interval, intracranial pressure, or heart rate variability. The acute post-treatment elevation in heart rate was more pronounced in patients who experienced a decrease of more than one point in their Modified Rankin Score at the time of discharge.

Conclusions

Our study found that taVNS treatment did not induce adverse cardiovascular effects, such as bradycardia or QT prolongation, supporting its development as a safe immunomodulatory treatment approach for SAH patients. The observed acute increase in heart rate after taVNS treatment may serve as a biomarker for SAH patients who could derive greater benefit from this treatment.

Trial registration

NCT04557618

Introduction

Subarachnoid hemorrhage (SAH) is a devastating subtype of stroke that represents a significant global health burden and causes permanent disability in approximately 30% of survivors.[1,6,45] Early brain injury can occur within the first 24 to 48 hours after ictus, which involves a cascade of elevated intracranial pressure and a subsequent drop of cerebral perfusion.[4] Systemic and local inflammation, cerebral edema, blood-brain barrier (BBB) disruption, sympathetic nervous system activation, autoregulatory failure, microthrombosis, spreading depolarizations (SDs), and inflammation have all been observed during this period.[11,12] These biological processes result in the inability of cerebral perfusion to match metabolic demands, leading to secondary brain injury and delayed cerebral ischemia that

typically occurs between 5 and 14 days after the SAH.[2,7,8,9] Delayed cerebral ischemia and deleterious inflammation are major predictors of poor outcomes and morbidity. The autonomic nervous system (ANS), comprising the sympathetic and the parasympathetic nervous system, plays a critical role in maintaining physiological homeostasis. SAH is believed to cause sympathetic predominance, which plays a key role in the development of cerebral vasospasm, renders patients more susceptible to non-neurological complications, and exacerbates deleterious inflammatory processes.

Numerous interventions have been explored to address the complex pathologies of subarachnoid hemorrhage (SAH) that contribute to secondary brain injury, aiming to improve patient outcomes.[14] Transcutaneous auricular vagus nerve stimulation (taVNS) is one of the most promising therapeutic options, as recent studies have demonstrated its efficacy in reducing inflammation, improving autonomic balance, and enhancing brain plasticity.[3,10,13,14] The auricular branch of the vagus nerve is a sensory nerve that innervates the external ear, including the cymba concha. Stimulating the auricular branch of the vagus nerve has been shown to activate the same brain regions as cervical vagus nerve stimulation[16]. Specifically, taVNS mediates cholinergic signaling and regulates proinflammatory responses via the inflammatory reflex (**Figure 1A**).[17,18] In this reflex, inflammatory mediators such as cytokines trigger afferent vagus nerve signaling. This afferent signal then prompts an efferent response from the vagus nerve that acts to reduce the production of pro-inflammatory cytokines.[19]

The vagus nerve also mediates cardiovascular function by regulating the autonomic system and metabolic homeostasis (**Figure 1A**).[20] Theoretically, taVNS increases parasympathetic activity, which can be measured as increased heart rate variability (HRV). While some animal studies have reported a potential risk of bradycardia and decreased blood pressure associated with vagus nerve stimulation, two reviews of human studies have considered the cardiovascular effects of taVNS generally safe, with adverse effects reported only in patients with pre-existing heart diseases. [21,22,23] However, its cardiovascular effect in SAH patients is largely unknown. Given that critically ill patients, such as those with SAH, are extremely vulnerable to cardiovascular complications, it is essential to thoroughly examine the cardiovascular implications of taVNS to ensure its safety in this fragile population. This is particularly notable as cardiovascular abnormalities following SAH, such as prolonged elevated heart rate and QT prolongation, are associated with an increased risk of poor outcomes[5,24,25]. However, our limited understanding of these effects constitutes a significant barrier, preventing the advancement of taVNS from a promising therapeutic approach to an established clinical treatment for SAH. To address this gap, we assessed the effects of acute and repetitive taVNS on cardiovascular function based on electrocardiogram (ECG) and other monitored vital signs from SAH patients in the intensive care unit (ICU). The current study is part of the NAVSaH trial (NCT04557618) and focuses on the trial's secondary outcomes, including heart rate, QT interval, HRV, and blood pressure.[32] This interim analysis aims to evaluate the cardiovascular safety of the taVNS protocol and to provide insights that will inform the application of taVNS in SAH patients. The primary outcomes of this trial, including change in the inflammatory cytokine TNF-α and rate of radiographic vasospasm, are available as a pre-print and currently under review.[26] Based on a meta-analysis, most aversive effects were seen in repeated sessions lasting 60 min or more; therefore, we hypothesized that repetitive taVNS increased HRV but did not cause bradycardia and QT prolongation.[23] To test this hypothesis, we compared changes in cardiovascular metrics at the phase of early brain injury (within 72 hours) and at the phase when delayed cerebral ischemia develops (after day 4) between patients receiving taVNS treatment and sham treatment. Root mean square of successive differences (RMSSD) and the standard deviation of normal RR intervals (SDNN) are two commonly used HRV metrics. RMSSD indicates parasympathetic activity, while lower SDNN is associated with increased cardiac risk.[27,28] Providing effective taVNS treatment modulates the autonomic system, we propose that heart rate or HRV following acute taVNS could inform which SAH patients may experience the most clinical benefit from taVNS treatment. To explore this possibility, we correlated the changes in heart rate

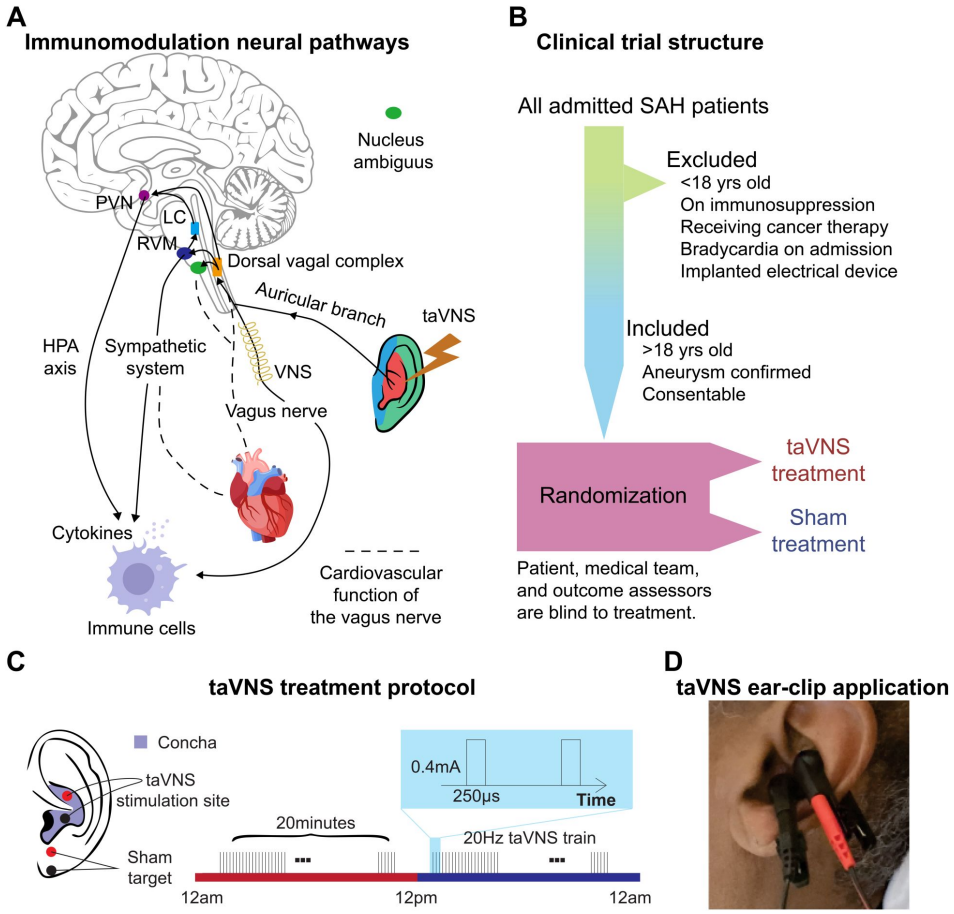


Figure 1.

Study rationale and clinical trial design.

A. Immunomodulation neural pathways associated with vagus nerve stimulation include cholinergic anti-inflammatory pathway, sympathetic nervous system, and hypothalamic-pituitary-adrenal (HPA) axis. Immunogenic stimuli activate vagal afferents terminating primarily in the dorsal vagal complex. Ascending projections from the dorsal vagal complex reach the paraventricular nucleus (PVN) and rostral ventromedial medulla (RVM), activating the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic system, respectively, to regulate the immune response. taVNS can affect cardiovascular function through the sympathetic system or efferent vagus nerve. **B**-**C**. Clinical trial structure and treatment protocol. Patients in the taVNS group received electrical stimulation (0.4 mA, 250 μs pulse width, 20 Hz) for 20 minutes twice daily. Sham group patients wore the ear clip on the earlobe for the same duration. **D.** Ear clip application for taVNS treatment.

and HRV following acute taVNS treatment and changes in the modified Rankin Score (mRS), which measures the degree of disability or dependence in the daily activities of people suffering from neurological disability.

Results

24 Participants were randomized to receive the taVNS (N = 11) or Sham (N = 13) treatment (**Table 1**, Supplementary Figure 8). Supplementary Table 3 shows the clinical characteristics of the two treatment groups. The participants, the medical team who dictated all management decisions for the patient's subarachnoid hemorrhage, and the outcomes assessors who assigned modified Rankin Scores (mRS) at admission and discharge were blinded to the treatment. The structure of this study is shown in **Figure 1B**. Following randomization, enrolled patients underwent 20 minutes of either taVNS or sham stimulation twice daily during their stay in the ICU. This treatment schedule was informed by findings from Addorisio et al., where a 5-minute taVNS protocol was administered twice daily to patients with rheumatoid arthritis for two days.[29] Their study found that circulating c-reactive protein (CRP) levels significantly reduced after 2 days of treatment but returned to baseline at the second clinical assessment by day 7. Given the high inflammatory state associated with SAH and our intention to maintain a steady reduction in inflammation, we decided to extend the treatment duration to 20 minutes per session. During treatment periods, a portable transcutaneous electrical nerve stimulation (TENS) device (TENS 7000 Digital TENS Unit, Compass Health Brands, OH, USA) was connected to the patient's left ear using two ear clips (**Figure 1C and D**). For taVNS treatments, these ear clips were placed along the concha of the ear, while for sham treatments, the clips were placed along the earlobe to avoid stimulation of the auricular vagus nerve from tactile pressure (**Figure *1C***). For the taVNS group, stimulation parameters were selected based on values reported in prior studies that sought to maximize vagus somatosensory evoked potentials while avoiding the perception of pain: 20 Hz frequency, 250 μs pulse width, and 0.4 mA intensity.[30] The stimulation parameters were designed to be imperceptible to the patients, and there were no reports of detection of taVNS, suggesting the success of the blinding. No electrical current was delivered during sham treatments. Please see [32] for a detailed protocol of this study.

Effects of repetitive taVNS on cardiac function

A study has shown that 15 minutes of taVNS reduced sympathetic activity in healthy individuals, with effects that persist during the recovery period.[33] This finding suggests that taVNS may exert long-term effects on cardiovascular function. Therefore, we investigated whether repetitive taVNS treatment affects heart rate and QT interval, key indicators of bradycardia or QT prolongation, using 24-hour ECG recording. We found no significant differences in heart rate between groups (Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value = 0.69, Cohen's d =-0.01, W-statistics = 4317, power = 0.93). Changes in heart rate from Day 1 were equivalent between groups (Two-tailed equivalence tests, p[*lower threshold*] = 0.006, test statistics[*lower threshold*] = 2.53; p[*lower threshold*] = 0.004, test statistics[*lower threshold*] = -2.72, N(VNS)=94, N(VNS)=95). We further confirmed that changes in heart rate were similar between treatment groups following SAH (**Figure 3A**, |Cohen's d| < 0.2 for Day 2-4, Day 5-8, Day 8-10, and Day 11-13). Moreover, changes in corrected QT interval from Day 1 were significantly higher in the Sham group compared to the taVNS group (**Figure 3B**, Mann– Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value < 0.001, Cohen's d = -0.57). Similarly, uncorrected QT intervals from Day 1 were higher in the Sham group (Supplementary Figure 10A, Cohen's d = -0.42). After the phase of early brain injury, the mean and median corrected QT interval were lower in the taVNS group with large effect sizes (**Figure 3B**, |Cohen's d| > 0.5). To ensure that repetitive taVNS did not lead to QT prolongation outside the stimulation period, we calculated the percentage of prolonged

Patient	Decade of life	Gender	Race	HH	mRS (admission)	mRS (discharge)	Vasospasm treated by blood pressure goal augmentation	Indwelling arterial lines	ICP monitoring
1	70s	M	White	3	5	5	Y	Y	Y
2	50s	M	Black/African American	3	4	3	N	Y	Y
3	50s	F	White	2	2	3	N	N	N
4	60s	M	White	2	3	4	Y	N	N
5	60s	F	White	2	2	3	Y	N	N
6	70s	F	White	2	3	2	N	Y	N
7	60s	M	White	4	5	4	N	Y	Y
8	60s	F	White	4	5	2	N	Y	Y
9	70s	F	White	2	3	0	Y	N	N
10	70s	F	White	2	3	3	Y	Y	N
11	40s	M	Black/African American	4	5	4	Y	Y	Y
12	40s	F	White	2	4	3	N	N	N
13	50s	F	Black/African American	2	2	1	Y	N	N
14	80s	F	White	1	3	3	Y	N	N
15	70s	F	Black/African American	4	5	3	N	N	Y
16	40s	F	Black/African American	2	2	3	N	N	N
17	60s	F	White	2	1	0	N	N	N
18	30s	F	White	3	4	2	N	N	Y
19	60s	F	White	3	4	2	N	N	Y
20	80s	F	White	2	2	3	N	Y	Y
21	50s	F	White	3	4	4	N	Y	Y
22	70s	F	White	1	2	4	N	N	N
23	40s	F	Black/African American	2	2	4	N	Y	N
24	60s	M	White	4	5	5	Y	Y	Y

Table 1

Patient demography

. HH: Hunt & Hess classification. mRS: modified Rankin Scale. Y: yes. N: no.

QT intervals. Prolonged QT intervals were defined as corrected QT interval >= 500 ms. We found that changes in prolonged QT intervals percentage from Day 1 were higher in the Sham group (**Figure 3F**, Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value < 0.001, Cohen's d = -0.72).

Subsequently, we investigated the effect of taVNS treatment on RMSSD and SDNN. We found that changes in SDNN using Day 1 as baseline were not significantly different between the treatment groups (T-test, N(taVNS) = 94, N(Sham) = 95, p = 0.479, Cohen's d =0.10, t statistics = 0.71, **Figure 2A**). Changes in RMSSD using Day 1 as baseline were significantly higher in the taVNS treatment group (T-test, N(taVNS) = 94, N(Sham) = 95, Bonferroni-corrected p = 0.025, Cohen's d =0.42, t statistics = 2.91, **Figure 2B**). We further studied the effects of taVNS in different phases following SAH. **Figure 2A-B** show the changes in SDNN and RMSSD in bins of three days for the two treatment groups. The taVNS treatment increased RMSSD over the course of the treatment, with a smaller effect size (Cohen's d = 0.29) observed between days 2-4, corresponding to the early brain injury phase, and large effect sizes at the later phases (Cohen's d = 0.41 for Days 5–7, Cohen's d = 0.54 for Days 8–10, Cohen's d = 0.66 for Days 11–13). We further tested if the RMSSD reduction rate was greater in the Sham treatment group with a linear regression model: RMSSD change ~ Day * Treatment. The results show that the RMSSD reduced slower in the taVNS treatment group when compared to the sham treatment, but this trend did not reach significance (coefficient of taVNS * Day interaction effect = 2.00, p = 0.21, Supplementary Figure 1).

RMSSD and SDNN are two of the most commonly used methods for quantifying heart rate variability. Bartlett's test indicated that there are significant correlations among these measures (p < 0.01, **Figure 3C**). To analyze the effect of taVNS treatment on the autonomic system, we used factor analysis to identify the underlying factors. We focused on the two factors with the greatest eigenvalue. **Figure 3D** shows the factor loading, that is, the variance explained by heart rate variability metrics on the two factors. The first factor correlates positively with metrics representing variability, including RMSSD, SDNN, pNNI_50, and total power, and therefore has been termed Overall Heart Rate Variability. The second factor correlated positively with RMSSD and normalized high-frequency power, representing parasympathetic activity, and negatively correlated with the cardiac sympathetic index. Hence, it is termed Parasympathetic Activity. Overall Heart Rate Variability change from Day 1 was significantly higher in the VNS group (**Figure 3E**, Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value = 0.04, Cohen's d =0.37). The effect size was trivial between days 2-4 and increased over the course of treatment. The parasympathetic activity was also significantly higher in the VNS treatment group, and we observed the largest effect size between days 2-4 (Cohen's d =0.50, **Figure 3F**).

We also investigated the potential association between clinical outcomes, as measured by changes in the mRS from admission to discharge, and heart rate variability metrics. We found that heart rate was lower in patients with improved mRS (i.e., <0) (Mann–Whitney U test, N(mRS < 0) = 122, N(mRS > 0)=98, p-value < 0.01, Cohen's d =- 0.54). Parasympathetic Activity, Overall Heart Rate Variability, and corrected QT interval did not differ significantly between patients with improved mRS and patients with worsened mRS (Supplementary Figure 4).

Effects of repetitive taVNS on vascular function

Elevated blood pressure is a common occurrence in SAH and is linked with a higher risk of re-rupture of cerebral aneurysms and vasospasm.[38,39] In this study, patients in both treatment groups received medical treatment determined by the medical team, including vasopressors and medication for blood pressure management. We investigated whether taVNS induced any additional blood pressure changes beyond those managed by the medical team. We found that the median and mean blood pressure change from the first hospitalized day were greater than 0 for both treatment groups (**Figure *4*B**). No significant differences were detected in changes in blood pressure and intracranial pressure (ICP) between the treatment groups (**Figure *4*B and C**). Equivalence testing confirmed that the ICP changes from the first hospitalization day were not significantly different between treatment groups, with a 2mmHg equivalence margin (two one-

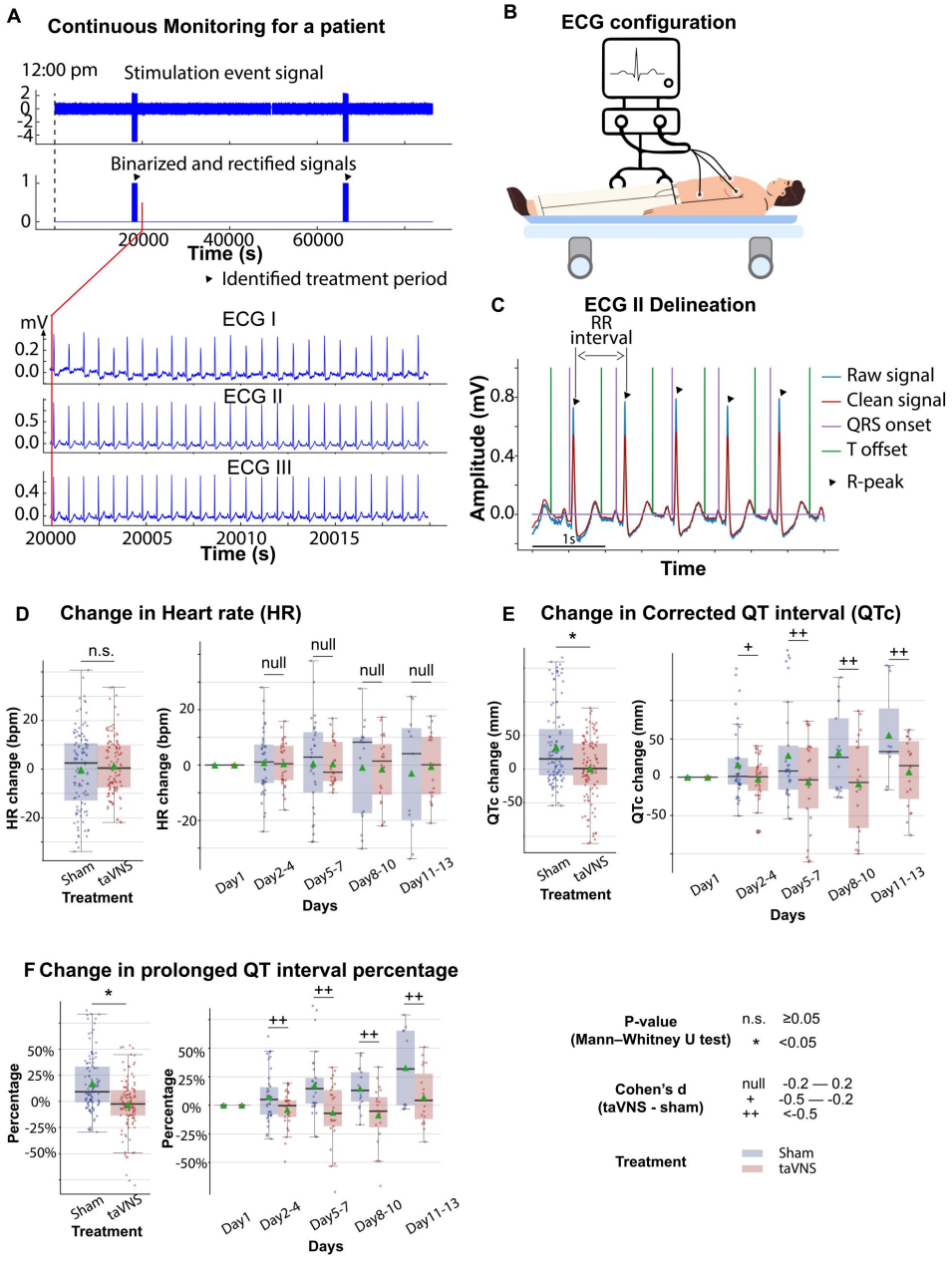


Figure 2.

The effects of taVNS on cardiac function.

A. Signals encoding treatment period and ECG signals in a representative patient. **B**. 3-lead ECG configuration in the intensive care unit. **C**. P wave, T wave, and QRS complex are delineated from clean ECG II signals. **D and E**. Heart rate and corrected QT interval changes from the first hospitalized day in the two treatment groups.

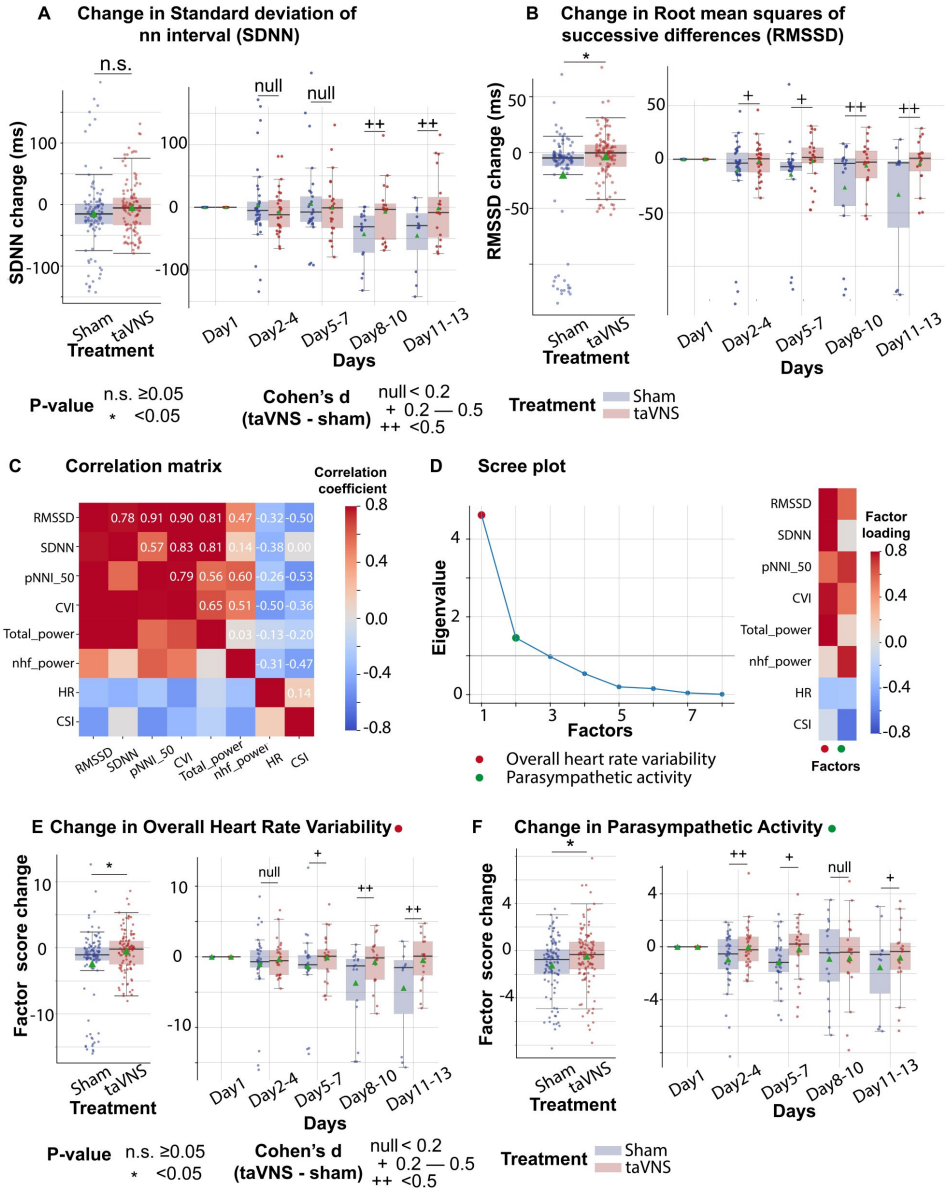


Figure 3.

The effects of taVNS on overall heart rate variability and parasympathetic activity.

A-B. Changes in standard deviation of NN interval (SDNN) changes and Root mean squares of successive differences over time for the two treatment groups. The color represents the treatment group. Green triangles represent the mean. **C**. Correlation between standard ECG features underlying autonomic activities. **D**. Factor analysis showed that there are two factors underlying the standard ECG features. The first factor is referred to as overall heart rate variability. The second factor is referred to as parasympathetic activity. **E-F**. The effect of taVNS on the two factors. pNNI_50: Percentage of Number of successive NN Intervals that differ by more than 50 ms. CVI: cardiac vagal index. Total power: total power below 0.4Hz of normal RR interval. nhf_power: relative power of the high-frequency band (0.15–0.4 Hz). CSI: cardiac sympathetic index.

sided t-tests, p[*lower threshold*] = 3.66 x 10^{-13}, t[*lower threshold*] = 8.07; p[*upper threshold*] = 3.33 x 10^{-10}, t[*upper threshold*] = -6.73). Equivalence testing also indicated that there were no significant different changes in blood pressure between treatment groups (two one-sided t-tests, p[*lower threshold*] = 0.07, t[*lower threshold*] = 1.51; p[*upper threshold*] = 0.002, t[upper threshold] = -3.00). We further verified that there were no significant changes in arterial line blood pressure obtained via continuous invasive monitoring between treatment groups (Supplementary Figure 5). We subsequently compared the Plethysmography Peripheral Perfusion Index (PPI) between the treatment groups as it is a proxy metric for cardiac stroke volume and vascular tone.[40,43] We found that PPI change was significantly lower (Mann–Whitney U test, N(taVNS) = 83, N(Sham)=95, Bonferroni corrected p < 0.01, Cohen's d =-0.49). In addition, respiration rate change was significantly higher (Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, Bonferroni corrected p = 0.02, Cohen's d =0.37) in the taVNS group, as compared to the Sham group (**Figure *4*D and E**). We hypothesized that the increase in respiratory rate was a compensatory mechanism to ensure similar oxygen delivery. We found a significant negative correlation between changes in PPI and changes in respiration rate only for the taVNS treatment group (Pearson correlation coefficient = -0.37, p < 0.001, t-test, Supplementary Figure 5D). The Pearson correlation coefficient for the sham treatment group is -0.08 (p = 0.36).

Acute effects of taVNS on cardiovascular function

Assessing the acute effect of taVNS on cardiovascular is crucial for its safe translation into clinical practice. We compared the acute change of heart rate, corrected QT interval, and heart rate variability between treatment groups, as abrupt changes in the pacing cycle may increase the risk of arrhythmias.[41] The change in heart rate from treatment onset is shown in **Figure *5*B**. We subsequently tested whether taVNS affects changes in heart rate between post-treatment and pre-treatment. We found that the changes in heart rate were not significantly different between treatment groups although heart rate increased in the taVNS group (Wilcoxon rank-sum test, N = 188, Bonferroni corrected p = 0.03, Cohen's d =0.11) but not in the Sham group (Wilcoxon signed ranked test, N = 199, Bonferroni corrected p = 0.72, Cohen's d =0.00) (**Figure *5*C**). However, the increase in heart rate after taVNS was within 0.5 standard deviations of daily heart rate. There were no significant differences in changes in corrected QT interval or heart rate variability, as measured by RMSSD, SDNN, and relative power of high-frequency band between treatment groups (**Figure 5D and E** and Supplementary Figure 6). Supplementary Figure 10B-C shows the acute changes in uncorrected QT interval. Supplementary Table 3 summarizes the absolute changes in cardiovascular metrics for the treatment groups. We further asked whether heart rate can serve as a biomarker that indicates which SAH patients would receive the greatest benefit from continuing taVNS treatment. We investigated the relationship between changes in heart rate from pre- to post-taVNS treatment and changes in mRS between admission and discharge using a linear mixed-effects model. In this model, the treatment group, mRS change, and their interaction were included as fixed effects, while subject was included as a random effect. Our analysis revealed that the slope between changes in heart rate and changes in mRS is significantly more negative for the taVNS treatment group (**Table 2**). This finding suggests that an increase in heart rate following acute taVNS treatment is associated with improved clinical outcomes (**Figure *5*F**). Post-hoc analysis showed that patients in the taVNS treatment group who had an improvement in mRS of -2 or greater compared to other patients had significantly greater increases in heart rate (Mann-Whitney U test, p=0.02, N(mRS change <-1) = 53, N(mRS change >= -1) = 135, Cohen's d=0.34, Supplementary Figure 6C). Conversely, HRV change, represented by RMSSD, was not significantly different based on mRS in SAH patients (Supplementary Figure 6D).

Subsequently, we compared changes in blood pressure, PPI, ICP, and respiration rate from pre- to post-treatment periods between treatment groups. We found that changes in PPI and blood pressure were significantly higher in the taVNS group, as compared to the Sham group (Mann-Whitney U test, blood pressure: p = 0.03, Cohen's d = 0.22, N = 180 for Sham and 159 for taVNS; PPI: p < 0.01, Cohen's d = 0.19, N = 227 for Sham and 186 for taVNS, Supplementary Figure 7). Only PPI

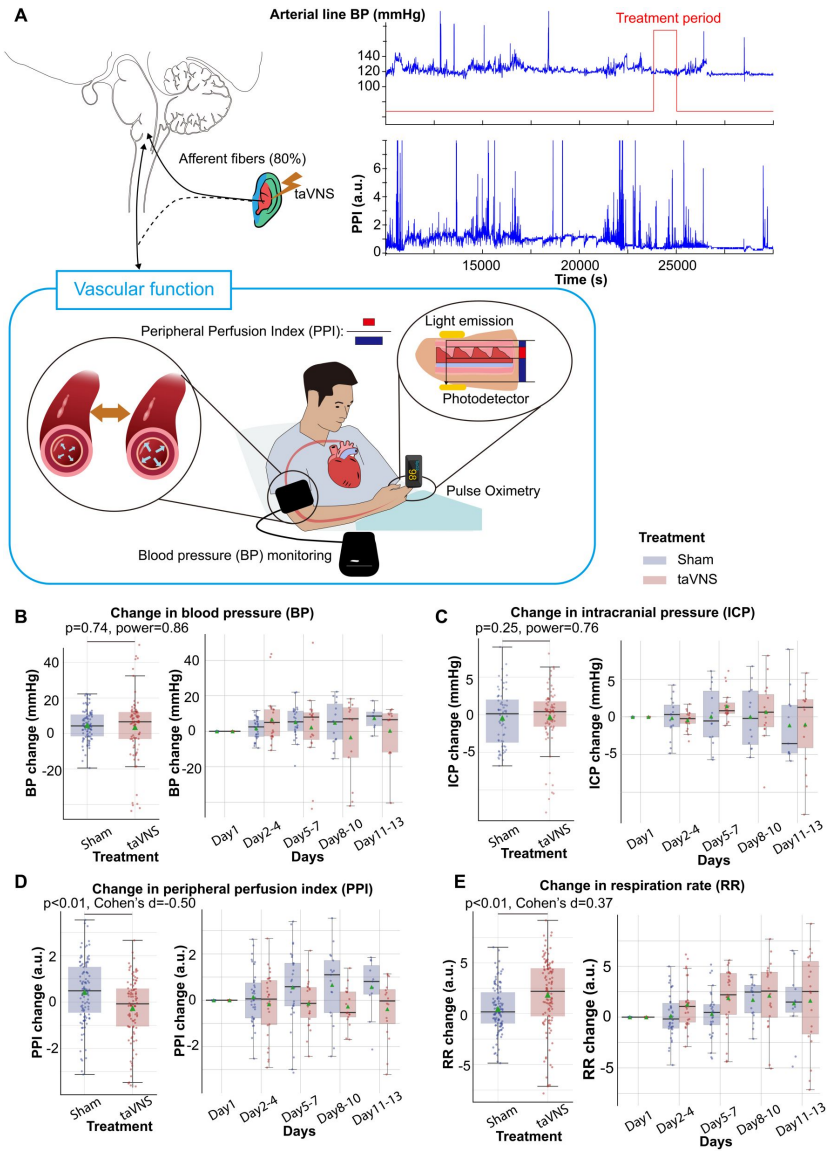


Figure 4.

Effects of repetitive taVNS on vascular function.

A. Representative vital signs and their physiology. Arterial line blood pressure (see supplementary Figure 5), intracranial pressure, and mean blood pressure measured regularly by nurses (BP) were recorded. Blood pressure is an index of vasodilation. PPI is the ratio between the pulsatile and the non-pulsatile blood flow, reflecting the cardiac output. **B-C.** Mean BP and ICP changes from the first hospitalization day did not differ significantly between the treatment groups. **D-E**. PPI change from the first hospitalized day was lower in the VNS treatment group, while RR change was higher.

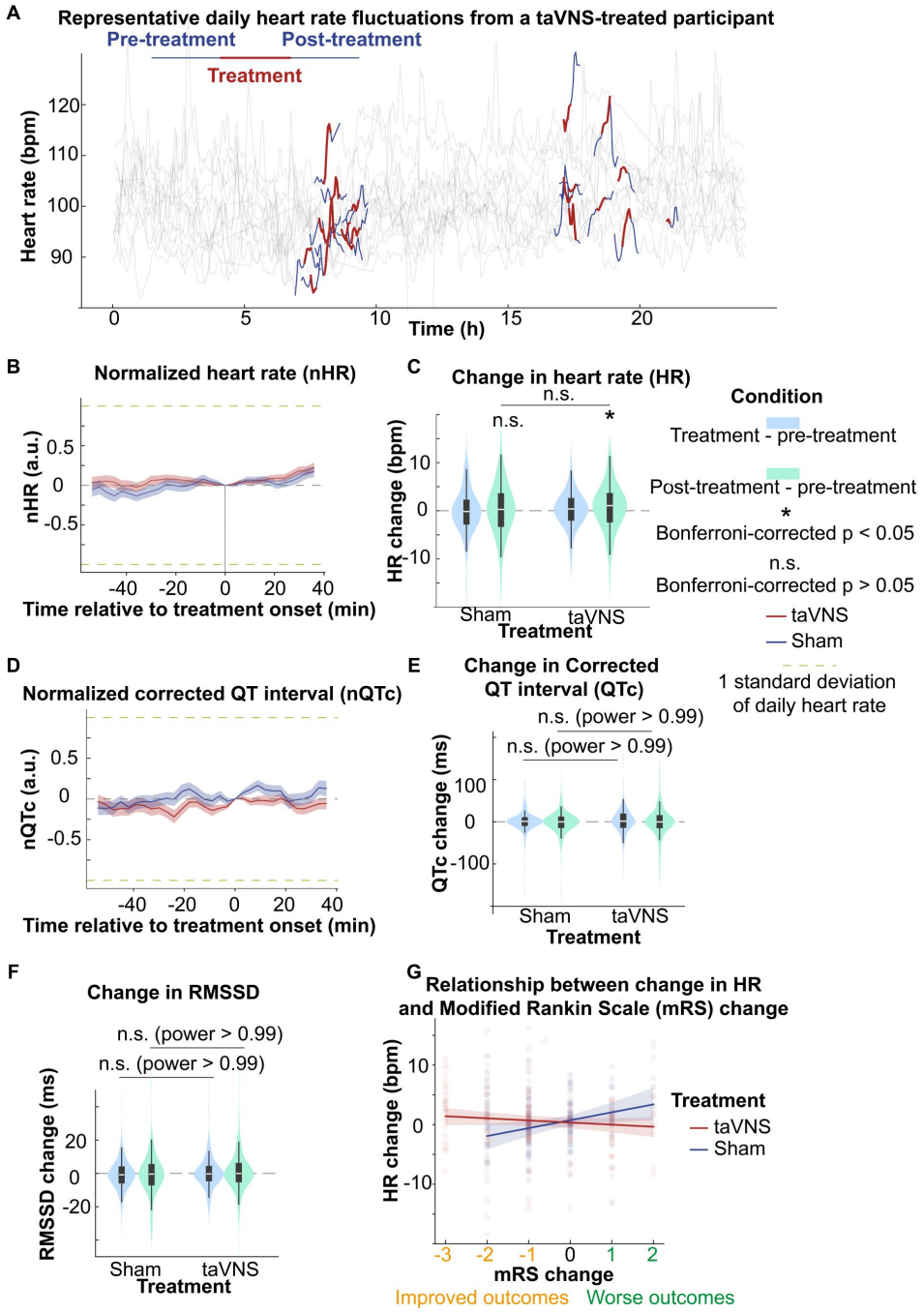


Figure 5.

The acute effects of taVNS on cardiac function.

A. Daily fluctuation of heart rate of a subject receiving VNS treatment. The treatment period, a 20-minute period before and after treatment, is highlighted. Note that a small proportion of ECG signals to derive heart rate was missing due to the expected cyclical restarting of the monitoring system. **B (D)**. Normalized heart rate (QTc) aligned at the treatment onset over time for the two treatment groups. The heart rate (QTc) is normalized based on the mean and standard error of heart rate for each day. **C**. The difference in HR between the treatment period, post-treatment period, and pre-treatment period for the two groups. Wilcoxon signed ranked test was used to test if the HR difference is statistically different from 0 in the VNS treatment group. Bonferroni-corrected p-value for HR difference between post-treatment and treatment period is 0.03 (N=188, Cohen's d = 0.1). Mann–Whitney U tests were used to compare cardiac function metric differences between the two treatment groups. **E-F**. The difference in QTc and RMSSD between the treatment period, post-treatment period, and pre-treatment period for the two groups **G**. The relationship between heart rate changes following acute taVNS and functional outcome.

Model	$HR_{subject,treatment}(mRS) = \beta_0 + \beta_{taVNS} \times 1_{treatment=taVNS} + (\beta_{mRS} + \beta_{taVNS,slope} \times 1_{treatment=taVNS}) \times mRS + u_{subject}$		
	Coefficient	P value	95% Confidence interval
β_0	0.73	0.211	-0.41 – 1.87
β_{taVNS}	-0.29	0.737	-1.95 – 1.38
β_{mRS}	1.47	**0.006**	0.43 – 2.51
$\beta_{taVNS,slope}$	-1.85	**0.005**	-3.12 - 0.57

Table 2

Relationship between HR changes following acute taVNS and clinical outcomes.

remained significantly different between treatment groups after Bonferroni correction. The acute changes in PPI and blood pressure remained within the daily standard deviation. No significant differences in post-treatment changes in ICP or respiration rate were observed between treatment groups.

Discussion

This study examined the effects of transcutaneous auricular vagus nerve stimulation (taVNS) on cardiovascular function in patients with subarachnoid hemorrhage (SAH). We investigated both the cumulative and acute impacts of taVNS. The findings in our study indicate that repetitive taVNS is not associated with previously suggested risks, such as bradycardia and QT prolongation. Furthermore, repetitive taVNS treatment increased overall heart rate variability and parasympathetic activity, which are indicators of a healthy cardiovascular system. When looking at the acute effects, taVNS only significantly increased the peripheral perfusion index but not heart rate, heart rate variability, corrected QT interval, blood pressure, or intracranial pressure. The findings are summarized in **Table 3**. Interestingly, we found that heart rate can serve as a biomarker for identifying SAH patients who are most likely to benefit from taVNS treatment. Collectively, this study substantiates the safety of treating SAH patients with taVNS and provides foundational data for future efforts to optimize and translate taVNS therapy toward clinical use.

taVNS and autonomic system

The autonomic nervous system (ANS), comprising the sympathetic and the parasympathetic nervous system, plays a critical role in maintaining physiological homeostasis. These two systems work synergistically to mediate interactions between the nervous and immune systems, which is thought to be the underlying mechanism for the immunomodulatory effect of taVNS. Our study is aligned with the finding that the autonomic balance is shifted toward sympathetic dominance following SAH (**Figure 3**, Supplementary Figure 2).[42,47] In addition, we found that dysregulation of sympathovagal balance toward sympathetic dominance could be restored by taVNS treatment.

A key metric that reflects this restored sympathovagal balance is heart rate variability (**Figure 3F**). Specifically, factor analysis based on heart rate variability metrics showed that the parasympathetic activity was significantly higher in the taVNS treatment group. This difference was most pronounced during the early phase, between Days 2 and 4 following SAH. In addition to analyzing the correlation between the parasympathetic activity factor and established HRV measures that reflect parasympathetic activity, such as RMSSD and pNNI_50 (**Figure 3C**), we also examined changes in a frequency-domain HRV measure—the relative power of the high-frequency band (0.15–0.4 Hz)—to validate the accuracy of the factor analysis. The relative power of the high-frequency band is widely used to indicate respiratory sinus arrhythmia, a process primarily driven by the parasympathetic nervous system. We found that both the change in parasympathetic activity factor and relative high-frequency power were higher in the taVNS group at the early phase (Day 2 – 4, Supplementary Figure 2). Conversely, we observed higher high-frequency power in the Sham group during the later phase. If the factor analysis successfully isolates the parasympathetic activity, there should be other factors than the parasympathetic activity affecting the relative power of the high-frequency band. One such factor is the respiration rate. The high-frequency range is between 0.15 to 0.4 Hz, corresponding to respiration's frequency range of approximately 9 to 24 breaths per minute. If the respiration rate increases and exceeds 24 breaths per minute, the respiratory-driven HRV might occur at a frequency higher than the typical high-frequency band. Given that the respiration rate was higher in the taVNS treatment group, a compensatory mechanism to ensure oxygen delivery (**Figure 4E**), we hypothesized that the observed lower high-frequency power in the taVNS treatment group compared to sham at later

Table 3.

Summary of effects of acute and repetitive taVNS on cardiovascular function in SAH patients

. Metrics for cardiovascular function include heart rate variability, heart rate, QT interval, blood pressure, intracranial pressure, peripheral perfusion index, and respiration rate.

		Positive findings	Null findings	Implications
Repetitive	Cardiac function	Increased HRV	QT interval Heart rate	Increased parasympathetic activity
	Vascular function	Reduced peripheral perfusion index Increased respiration rate	Blood pressure Intracranial pressure	Compensatory mechanisms to maintain autonomic balance
Acute	Cardiac function		Heart rate Heart rate variability QT interval	No to small acute effect
	Vascular function	Increased peripheral perfusion index	Intracranial pressure Respiration rate	

phases was a result of increased respiration rate. Indeed, we found the normalized high-frequency power was higher when RR was less than 25 bpm compared to when RR > 25 bpm (Cohen's d = 0.85, Supplementary Figure 3A). Moreover, an increase in RR in the taVNS treatment group was associated with a decrease in high-frequency power (Supplementary Figure 3B). These control analyses underscored the necessity of performing factor analysis to robustly measure parasympathetic activities and confirm that taVNS treatment mitigated the sympathetic overactivation during the early phase.

Additionally, taVNS led to a decreased QTc without a significant change in heart rate, mimicking the effects observed with propranolol administration, a beta-blocker that reduces sympathetic activity[53]. This finding suggests that repetitive taVNS reduces sympathetic overactivation and influences ventricular repolarization processes. Age affects sinus node function and is potentially associated with a higher risk of poor outcomes. To control for individual differences, including those due to age, our study compared the change in cardiovascular parameters from Day 1 within each subject across treatment groups. To further verify if age influences autonomic changes following SAH, we performed ANCOVA on autonomic function parameters with age included as a covariate. This analysis showed that age was negatively correlated with changes in heart rate, SDNN, and RMSSD from Day 1 but not with changes in QT intervals. After adjusting for age, we found that RMSSD and SDNN changes were significantly higher, while QTc changes were significantly lower in the taVNS treatment group (Supplementary Table 4). These results align with the conclusion that repetitive taVNS treatment increased HRV and was unlikely to cause bradycardia or QT prolongation. In addition, autonomic changes following SAH may be influenced by age. Specifically, lower RMSSD and SDNN in older individuals suggest a greater shift toward sympathetic predominance following SAH (Supplementary Table 4).

PPI is primarily influenced by cardiac output and vascular tone. Elevated PPI is associated with vasodilation and/or increased stroke volume. In the Sham group, increases in both PPI and blood pressure were observed when compared to Day 1 values (**Figure 3**). This effect may be due to higher stroke volume resulting from sympathetic activation following SAH. Alternatively, this could represent the heightened need for vasopressor interventions to improve cerebral perfusion due to more robust sympathetically driven cerebral vasospasm. The increase in PPI was less for the taVNS treatment group (**Figure *4***), suggesting a restored autonomic balance in the taVNS treatment group. However, the effects of taVNS on blood pressure require further investigation as more than half of the patients were on vasopressor and ionotropic drugs. Intuitively, sympathetic activation is associated with increases in both PPI and blood pressure. The blood pressure management might lead to similar blood pressure changes between the two treatment groups. Although repetitive taVNS increases heart rate variability days after initiation of the treatment, this effect is not seen acutely. Also, while repetitive taVNS was associated with a reduced PPI and no change in heart rate and blood pressure, there were small acute increases in PPI, heart rate, and blood pressure. All patients who were capable of verbal communication were asked if they felt any prickling or pain during all sessions. We confirmed that the current taVNS protocol is below the perception threshold for all trialed patients. Altogether, successful activation of the afferent vagal pathway by taVNS increased arousal, resulting in increased heart rate.[50,51] These speculative mechanisms warrant further validation through animal or pharmacological studies directly investigating the effects of taVNS on autonomic function and vascular tone.

Considerations for applying taVNS on SAH patients

Blood pressure management and cardiac function monitoring are crucial in patients following SAH.[38] This study shows that blood pressure, QT interval, and heart rate over days were not significantly different between taVNS and sham treatment groups. This suggests that adding taVNS in treatments for SAH patients is unlikely to cause adverse blood pressure alterations or cardiac complications. Our findings suggest that repetitive taVNS could enhance parasympathetic tone following SAH. This effect could lead to favorable clinical outcomes as lower HRV was found to be associated with neurocardiogenic injury.[47,48] Given the negative association between pro-

inflammatory markers and HRV, our finding that HRV was higher in the taVNS treatment group aligns with the findings of primary outcomes of this clinical trial, which showed that taVNS treatment reduced pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6.[26,52] The consistency between these findings strengthens the evidence supporting the anti-inflammatory effects of taVNS. In addition, the sympathetic predominance following SAH is implicated in an increased risk of delayed cerebral vasospasm, which is most commonly detected 5-7 days after SAH.[12] Given that taVNS treatment mitigated the sympathetic overactivation before the typical onset of cerebral vasospasm, it could potentially reduce the severity of this complication. Additionally, reduced PPI was associated with increased respiration rate only for the taVNS treatment group, suggesting that the autonomic system self-regulates to maintain cardiovascular homeostasis. Thus, it is important to consider autonomic system self-regulation when studying the therapeutic effects of taVNS.[44] Also, while acute cardiovascular changes were noted after taVNS, these changes were within normal daily variations in this study, making them unlikely to pose a risk to the patient. That said, the observed acute increases in PPI following taVNS necessitate caution when considering taVNS treatment for patients to whom peripheral vasodilatation is not desired.

As we pioneer the application of taVNS as an immunomodulation technique in SAH patients, we adopt parameters (20 Hz, 0.4 mA) reported in similar studies.[55] The current study provides a basis for future preclinical and clinical studies of taVNS in this patient population. To build on our findings, a systematic evaluation of the relationship between parameters such as frequency, intensity, and duration and taVNS's effects on the immune system and cardiovascular function is necessary to establish taVNS as an effective therapeutic option for SAH patients.[56]

Limitations and outlook

While this study supports the safety of taVNS treatment in SAH patients, we should be cautious when generalizing these findings to broader clinical populations. The current study did not explore the effects of taVNS on less commonly used cardiovascular metrics, such as QTc dispersion. Our study considers each day as an independent sample for the following considerations: 1. heart rate and HRV metrics exhibited great daily variations. Their value on one day was not predictive of the metrics on another day, which could be due to medications, interventions, or individualized SAH recovery process during the patient's stay in the ICU. 2. SAH patients in the ICU often experience daily changes in clinical status, including fluctuations in intracranial pressure, blood pressure, neurological status, and other vital signs. 3. Day-to-day cardiovascular function changes varied as the patient recovered or encountered setbacks. To conclusively establish that there is no significant cardiovascular effect of repetitive taVNS on any given day following SAH, we would need to perform statistical tests between treatment groups for each day. In this context, 64 subjects per treatment group are required to achieve 80% power, assuming a medium effect size (Cohen 's d = 0.5) and 0.05 type I error probability (two-sample t-test).

Mild cardiac abnormalities are common in SAH patients[5], complicating the precise calculation of cardiovascular metrics from ECG signals and the interpretation of the results. Systematic verification of methods for calculating cardiovascular metrics to ensure their applicability in SAH patients is crucial. We noticed a high variance of change in heart rate for days 5 – 7, 8 – 10, and 11 – 13 for both treatment groups (**Figure 2D**). This may be due to the small sample size in the later days, given that the mean duration of hospitalization for the 24 subjects included in this study was 11.3 days with a standard deviation of 6.4. Differences in medical history and clinical outcomes during hospitalization may also explain the variance of change in heart rate for the later days. For example. heart rate was lower in patients with improved mRS scores (Supplementary Figure 4B). Understanding the association between cardiovascular metrics and clinical assessments, such as vasospasm and inflammation, could help decide whether future taVNS trials should control for these factors when evaluating the effects of taVNS on cardiovascular function. Additional care

should be paid when interpreting the results of blood pressure, as hypertension was intentionally induced for some patients being treated for vasospasm. Patient medical histories are summarized in **Table 1**.

Kulkarni et al. showed that the response to low-level tragus stimulation (LLTS) varied among patients with atrial fibrillation.[54] Similarly, in our study, not all patients in the taVNS treatment group showed a reduction in mRS scores (improved degree of disability or dependence). This differential response may be inherent to taVNS and potentially influenced by factors such as anatomical variations in the distribution of the vagus nerve at the outer ear. These findings underscore the importance of using acute biomarkers to guide patient selection and optimize stimulation parameters. Furthermore, we found that increased heart rate was a potential acute biomarker for identifying SAH patients who are most likely to respond favorably to taVNS treatment. Translating this finding into clinical practice will require further research to elucidate the mechanisms by which an acute increase in heart rate may predict the outcomes of patients receiving taVNS, including its relationship with neurological evaluations, vasospasm, echocardiography, and inflammatory markers.

Conclusions

Utilizing taVNS as a neuromodulation technique in SAH patients is safe without inducing bradycardia or QT prolongation. Repetitive taVNS treatment increased parasympathetic activity. Acute taVNS elevated heart rate, which might be an acute biomarker to identify SAH patients who are likely to respond favorably to taVNS treatment.

Methods details

Study Participants

Participants in this study were recruited from adult patients who were admitted to the ICU at Barnes Jewish Hospital, St. Louis, MO, following an acute, spontaneous, aneurysmal SAH. Inclusion criteria were: **(1)** Patients with SAH confirmed by CT scan; **(2)** Age > 18; **(3)** Patients or their legally authorized representative are able to give consent. Exclusion criteria were: **(1)** Age < 18; **(2)** Use of immunosuppressive medications; **(3)** Receiving ongoing cancer therapy; **(4)** Implanted electrical device; **(5)** Sustained bradycardia on admission with a heart rate < 50 beats per minute for > 5 minutes; **(6)** Considered moribund/at risk of imminent death. Participants were randomized to receive either the taVNS (N = 11) or Sham (N = 13) treatment. Patients were enrolled prior to randomization by a member of the research team who went through the informed consent process with the patient or their legally authorized representative. Treatment group assignment was via a computer-generated randomization sequence, with the next number obscured until patient enrollment. Research team members who applied the ear clips and set stimulation parameters were not blinded to the treatment. The participants, the medical team who dictated all management decisions for the patient's subarachnoid hemorrhage, and the outcomes assessors who assigned modified Rankin Scores (mRS) at admission and discharge were blinded to the treatment. The structure of this study is shown in **Figure 1B**. This study was approved by the Washington University School of Medicine Review Board and was conducted in accordance with institutional and national ethics guidelines and the Declaration of Helsinki (Clinical trial number: NCT04557618).

taVNS protocol

Following randomization, enrolled patients underwent 20 minutes of either taVNS or sham stimulation twice daily during their stay in the ICU. During treatment periods, a portable transcutaneous electrical nerve stimulation (TENS) device (TENS 7000 Digital TENS Unit, Compass Health Brands, OH, USA) was connected to the patient's left ear using two ear clips (**Figure 1C and**

D). For taVNS treatments, these ear clips were placed along the concha of the ear, while for sham treatments, the clips were placed along the earlobe to avoid stimulation of the auricular vagus nerve from tactile pressure (**Figure 1** **Figure 1C**). For the taVNS group, stimulation parameters were selected based on values reported in prior studies that sought to maximize vagus somatosensory evoked potentials while avoiding the perception of pain: 20 Hz frequency, 250 μs pulse width, and 0.4 mA intensity[30]. The stimulation was not perceptible for the patients. No electrical current was delivered during sham treatments. For both groups, the TENS device was connected to the patient and a bedside recording computer. The computer recorded continuous ECG and vital signs, including blood pressure, temperature, respiration rate, peripheral perfusion index, intracranial pressure, and arterial blood pressure. The collection of intracranial pressure and arterial blood pressure data varied, being dependent on the treatment protocol assigned by the clinical team, and thus was not uniformly available for all patients throughout the study. Please see [32] for a detailed protocol of this study.

Data processing

A 3-lead system was used for electrocardiograms (ECG). ECG signals, sampled at 500 Hz, and other vital signs, such as blood pressure, sampled at 1 Hz, were recorded from the Intellivue patient monitor (Philips®, Netherlands) using vitalDB software.[34]

To calculate cardiac metrics, we first applied a 0.5 Hz fifth-order high-pass Butterworth filter and a 60 Hz powerline filter on ECG data to reduce artifacts. [35] We detected QRS complexes based on the steepness of the absolute gradient of the ECG signal using the Neurokit2 software package.[35] R-peaks were detected as local maxima in the QRS complexes. P waves, T waves, and QRS complexes were delineated based on the wavelet transform of the ECG signals proposed by Martinez J. P. et al. (**Figure 2A-C**).[36] This algorithm identifies the QRS complex by searching for modulus maxima, which are peaks in the wavelet transform coefficients that exceed specific thresholds. The onset of the QRS complex is determined as the beginning of the first modulus maximum before the modulus maximum pair created by the R wave. To identify the T wave, the algorithm searches for local maxima in the absolute wavelet transform in a search window defined relative to the QRS complex. Thresholding is used to identify the offset of the T wave. RR intervals were preprocessed to exclude outliers, defined as RR intervals greater than 2 s or less than 300 ms. RR intervals with > 20% relative difference to the previous interval were considered ectopic beats and excluded from analyses. After preprocessing, RR intervals were used to calculate heart rate, heart rate variability, and corrected QT (QTc) based on Bazett's formula: $QTc = \frac{QT}{\sqrt{RR}}$.[46] The corrected QT interval (QTc) estimates the QT interval at a standard heart rate of 60 bpm. Heart rate variability measures included the root mean square of successive difference of normal RR intervals (RMSSD), indicating parasympathetic activity, and the standard deviation of normal RR intervals (SDNN), a clinical measure of cardiac risk.[27,28] Heart rate variability calculations are detailed in Supplementary Materials.

To investigate the effect of repetitive taVNS on cardiovascular function, we compared heart rate variability, heart rate, corrected QT intervals, blood pressure, and intracranial pressure calculated over 24 hours between patients receiving taVNS and sham treatment. In addition, we compared the mean peripheral perfusion index and respiration rate over 24 hours between treatment groups to determine the effects of repetitive taVNS on the autonomic system. Data collection commenced on the first day of each patient's ICU admission. The average duration of continuous data recording was 11.1 days, with a standard deviation of 6.8 days. To analyze the effects of taVNS treatment more granularly, we segmented the changes in these metrics from the initial day at three-day intervals, facilitating comparison between the taVNS and sham treatment groups over the course of their ICU stay.

To study the effects of acute treatment over time, we focused on blood pressure, heart rate variability, heart rate, and corrected QT intervals 20 minutes before treatment (pre-treatment), during the 20-minute treatment (during-treatment), and 20 minutes after treatment (post-

treatment). The treatment event signals were rectified and binarized based on their half-maximum value to identify the treatment onset and offset (**Figure 2A**). We calculated metrics using 6-minute sliding windows over ECG data starting from treatment onset/offset and moving bi-directionally with a 3-minute step. To correct daily and between-subject variation, we applied the same sliding window strategy to calculate the mean and standard deviation of these cardiac metrics for each patient each day as a reference. Subsequently, heart rate variability, heart rate, and corrected QT interval around treatment onset/offset were normalized based on the reference. In addition, we calculated the difference in blood pressure, heart rate variability, and heart rate, and corrected QT intervals between during-treatment and pre-treatment, as well as the difference between post-treatment and pre-treatment for each patient and for each treatment. To study the effects of acute taVNS, we compared the two differences between the treatment groups.

Factor Analysis

We performed an exploratory factor analysis to identify the factors underlying autonomic system activity. Besides RMSSD and SDNN, variables derived from preprocessed RR intervals and used to perform factor analysis included the percentage of successive normal-to-normal (NN) Intervals that differ by more than 50 ms (pNNI_50), total power (below 0.4 Hz), normalized high-frequency power (0.15-0.4Hz), cardiac vagal index, and cardiac sympathetic index. The total power is thought to represent the overall heart rate variability, while normalized high-frequency power primarily reflects parasympathetic activity.[27] These variables were normalized using a z-score method based on individual daily means and standard deviations before factor analysis. Factor analysis was performed using the factor_analyzer Python package. The number of factors was set to 2 based on the Scree plot. The factor loadings were calculated using the Minimum Residual Method. After factor extraction, a Varimax rotation was applied for better interpretability so that each factor had high loadings for a smaller number of variables and low loadings for the remaining variables.

Statistical Analyses

To investigate the effect of taVNS at the phase of early brain injury and later phases, we grouped the change of heart rate variability, heart rate, and corrected QT interval from the first hospitalized day in bins of three days. The change in blood pressure, intracranial pressure, respiration rate, and peripheral perfusion index from the first hospitalization day were also compared between treatment groups. We used t-tests for comparisons between treatment groups when the data were normally distributed, as determined by the Shapiro-Wilk test. We employed Mann–Whitney U tests for non-normally distributed data. We used Wilcoxon signed-rank tests to compare the difference in heart rate between post-treatment and during-treatment against 0. To control the familywise error rate, we applied Bonferroni correction. Specifically, when investigating the cardiac effects of taVNS, we compared six metrics between treatment groups, including heart rate, corrected QT interval, RMSSD, SDNN, and two factors representing heart rate variability. Consequently, the p-values were corrected by a factor of six. In this study, we reported the statistical power achieved for tests that yielded non-significant results. The achieved power is calculated based on a two-sample t-test assuming a medium effect size (Cohen's d of 0.5) and a Type I error probability (a) of 0.05. We used two one-sided tests to confirm that taVNS did not induce long-term changes in heart rate, corrected QT interval, or blood pressure, with equivalency test margins set to 5 bpm for heart rate, 50 ms for QT interval, and 2 mmHg for blood pressure. A summary of statistical tests is provided in Supplementary Table 1.

Availability of data and materials

https://github.com/GanshengT/taVNS_SAH

Additional information

Funding

The American Association of Neurological Surgeons (ALH), The Aneurysm and AVM Foundation (ALH), The National Institutes of Health R01-EB026439, P41-EB018783, U24-NS109103, R21-NS128307 (ECL, PB), McDonnell Center for Systems Neuroscience (ECL, PB), and Fondazione Neurone (PB).

Authors' contributions

Gansheng Tan: Conceptualization, Methodology, Investigation, Formal analysis, Software, Writing – original draft, Writing – review & editing. Anna L. Huguenard: Conceptualization, Funding acquisition, Methodology, Data curation, Writing - original draft, Writing – review & editing. Kara M. Donovan: Writing – review & editing. Philip Demarest: Writing – review & editing. Xiaoxuan Liu: Methodology, Writing – review & editing. Ziwei Li: Methodology, Writing – review & editing. Markus Adamek: Methodology, Software. Kory Lavine: Writing – review & editing. Ananth K. Vellimana: Data curation, Writing – review & editing. Terrance T. Kummer: Data curation, Writing – review & editing. Joshua W. Osbun: Data curation, Writing – review & editing. Gregory J. Zipfel: Funding acquisition, Resources, Writing - review & editing. Peter Brunner: Funding acquisition, Resources, Supervision, Writing - review & editing. Eric C. Leuthardt: Conceptualization, Supervision, Funding acquisition, Writing – review & editing, Writing – original draft.

Declaration of competing interest

Eric Leuthardt has stock ownership in Neurolutions, Face to Face Biometrics, Caeli Vascular, Acera, Sora Neuroscience, Inner Cosmos, Kinetrix, NeuroDev, Inflexion Vascular, Aurenar, Cordance Medical, Silent Surgical, and Petal Surgical. He is a consultant for E15, Neurolutions, Inc., Petal Surgical. Washington University owns equity in Neurolutions.

Anna Huguenard has stock ownership in Aurenar.

Acknowledgements

The authors acknowledge physicians/nurses for helping administer treatment. The authors thank Dr. Paul Cassidy for his contributions to the scientific editing of this manuscript, supported by the Institute of Clinical and Translational Sciences grant UL1TR002345 from the National Center for Advancing Translational Sciences (NCATS).

References

1. D'Souza S (2015) **Aneurysmal Subarachnoid Hemorrhage** *Journal of Neurosurgical Anesthesiology* Ovid Technologies (Wolters Kluwer Health) :222–240

2. Provencio J. J (2012) **Inflammation in Subarachnoid Hemorrhage and Delayed Deterioration Associated with Vasospasm: A Review** *Acta Neurochirurgica Supplement* :233–238

3. Wu Z., et al. (2023) **Transcutaneous auricular vagus nerve stimulation reduces cytokine production in sepsis: An open double-blind, sham-controlled, pilot study** *Brain Stimulation* **16**:507–514

4. Schneider U. C., Xu R., Vajkoczy P (2018) **Inflammatory Events Following Subarachnoid Hemorrhage (SAH)** *Current Neuropharmacology* **16**:1385–1395

5. Norberg E., Odenstedt-Herges H., Rydenhag B., Oras J (2018) **Impact of Acute Cardiac Complications After Subarachnoid Hemorrhage on Long-Term Mortality and Cardiovascular Events** *Neurocritical Care* **29**:404–412

6. Weir B (2002) **Unruptured intracranial aneurysms: a review** *Journal of Neurosurgery* **96**:3–42

7. van Gijn J., Kerr R. S., Rinkel G. J. (2007) **Subarachnoid hemorrhage** *The Lancet* **369**:306–318

8. Tracey K. J. (2002) **The inflammatory reflex** *Nature* **420**:853–859

9. Lv S., et al. (2018) **Levels of Interleukin-1β, Interleukin-18, and Tumor Necrosis Factor-α in Cerebrospinal Fluid of Aneurysmal Subarachnoid Hemorrhage Patients May Be Predictors of Early Brain Injury and Clinical Prognosis** *World Neurosurgery* **111**:e362–e373

10. Bonaz B., et al. (2016) **Chronic vagus nerve stimulation in Crohn's disease: a 6-month follow- up pilot study** *Neurogastroenterology & Motility* **28**:948–953

11. Macdonald R. L., et al. (2012) **Randomized Trial of Clazosentan in Patients With Aneurysmal Subarachnoid Hemorrhage Undergoing Endovascular Coiling** *Stroke* **43**:1463–1469

12. Budohoski K., Czosnyka M., Kirkpatrick P., et al. (2013) **Clinical relevance of cerebral autoregulation following subarachnoid haemorrhage** *Nat Rev Neurol* **9**:152–163

13. Meyers E. C., et al. (2018) **Vagus Nerve Stimulation Enhances Stable Plasticity and Generalization of Stroke Recovery** *Stroke* **49**:710–717

14. Provencio J. J., Vora N (2005) **Subarachnoid Hemorrhage and Inflammation: Bench to Bedside and Back** *Seminars in Neurology* **25**:435–444

15. Huguenard A, Tan G, Johnson G, et al (2023) **O-055 Non-invasive auricular vagus nerve stimulation following spontaneous subarachnoid hemorrhage reduces rates of radiographic vasospasm and hospital-acquired infections** *Journal of NeuroInterventional Surgery* **15**:A43–A44

16. Frangos E., Ellrich J., Komisaruk B. R (2015) **Non-invasive Access to the Vagus Nerve Central Projections via Electrical Stimulation of the External Ear: fMRI Evidence in Humans** *Brain Stimulation* **8**:624–636

17. Sahn B., Pascuma K., Kohn N., Tracey K. J., Markowitz J. F (2023) **Transcutaneous auricular vagus nerve stimulation attenuates inflammatory bowel disease in children: a proof-of-concept clinical trial** *Bioelectronic Medicine* **9**

18. Pavlov V. A., Tracey K. J (2012) **The vagus nerve and the inflammatory reflex—linking immunity and metabolism** *Nature Reviews Endocrinology* **8**:743–754

19. Tynan A., Brines M., Chavan S. S (2021) **Control of inflammation using non-invasive neuromodulation: past, present and promise** *International Immunology* **34**:119–128

20. Keute M., Machetanz K., Berelidze L., Guggenberger R., Gharabaghi A (2021) **Neuro-cardiac coupling predicts transcutaneous auricular vagus nerve stimulation effects** *Brain Stimulation* **14**:209–216

21. Naggar I., et al. (2014) **Vagal control of cardiac electrical activity and wall motion during ventricular fibrillation in large animals** *Autonomic Neuroscience* **183**:12–22

22. Hua K., et al. (2023) **Cardiovascular effects of auricular stimulation -a systematic review and meta-analysis of randomized controlled clinical trials** *Frontiers in Neuroscience* **17**

23. Kim A. Y., et al. (2022) **Safety of transcutaneous auricular vagus nerve stimulation (taVNS): a systematic review and meta-analysis** *Scientific Reports* **12**

24. Schmidt J. M., et al. (2013) **Prolonged Elevated Heart Rate is a Risk Factor for Adverse Cardiac Events and Poor Outcome after Subarachnoid Hemorrhage** *Neurocritical Care* **20**:390–398

25. Zhang L., Qi S (2016) **Electrocardiographic Abnormalities Predict Adverse Clinical Outcomes in Patients with Subarachnoid Hemorrhage** *Journal of Stroke and Cerebrovascular Diseases* **25**:2653–2659

26. Huguenard A. L., et al. (2024) **Auricular Vagus Nerve Stimulation Mitigates Inflammation and Vasospasm in Subarachnoid Hemorrhage: A Randomized Trial** *medRxiv* https://doi.org/10.1101/2024.04.29.24306598

27. Kleiger R. E., Stein P. K., Bigger J. T. (2005) **Heart Rate Variability: Measurement and Clinical Utility** *Annals of Noninvasive Electrocardiology* **10**:88–101

28. Shaffer F., Ginsberg J. P (2017) **An Overview of Heart Rate Variability Metrics and Norms** *Frontiers in Public Health* **5**

29. Addorisio M. E., et al. (2019) **Investigational treatment of rheumatoid arthritis with a vibrotactile device applied to the external ear** *Bioelectronic Medicine* **5**

30. de Gurtubay I. G., Bermejo P., Lopez M., Larraya I., Librero J (2021) **Evaluation of different vagus nerve stimulation anatomical targets in the ear by vagus evoked potential responses** *Brain and Behavior* **11**

31. van der Bilt I. A. C., et al. (2009) **Impact of cardiac complications on outcome after aneurysmal subarachnoid hemorrhage** *Neurology* **72**:635–642

32. Huguenard A., et al. (2024) **Non-invasive Auricular Vagus nerve stimulation for Subarachnoid Hemorrhage (NAVSaH): Protocol for a prospective, triple-blinded, randomized controlled trial** *PLOS One* **19**

33. Clancy J. A., et al. (2014) **Non-invasive Vagus Nerve Stimulation in Healthy Humans Reduces Sympathetic Nerve Activity** *Brain Stimulation* **7**:871–877

34. Lee H.-C., Jung C.-W (2018) **Vital Recorder—a free research tool for automatic recording of high-resolution time-synchronised physiological data from multiple anaesthesia devices** *Scientific Reports* **8**

35. Schölzel C., Chen S. A (2021) **NeuroKit2: A Python toolbox for neurophysiological signal processing** *Behavior Research Methods* **53**:1689–1696 https://doi.org/10.3758/s13428-020-01516-y

36. Martinez J. P., Almeida R., Olmos S., Rocha A. P., Laguna P (2004) **A Wavelet-Based ECG Delineator: Evaluation on Standard Databases** *IEEE Transactions on Biomedical Engineering* **51**:570–581

37. Naredi S., et al. (2000) **Increased Sympathetic Nervous Activity in Patients With Nontraumatic Subarachnoid Hemorrhage** *Stroke* **31**:901–906

38. Minhas J. S., Moullaali T. J., Rinkel G. J. E., Anderson C. S (2022) **Blood Pressure Management After Intracerebral and Subarachnoid Hemorrhage: The Knowns and Known Unknowns** *Stroke* **53**:1065–1073

39. Hosmann A., et al. (2020) **Endogenous arterial blood pressure increase after aneurysmal subarachnoid hemorrhage** *Clinical Neurology and Neurosurgery* **190**

40. Elshal M. M., Hasanin A. M., Mostafa M., Gamal R. M (2021) **Plethysmographic Peripheral Perfusion Index: Could It Be a New Vital Sign?** *Frontiers in Medicine* **8**

41. Zaniboni M (2023) **The electrical restitution of the non-propagated cardiac ventricular action potential** *Pflügers Archiv - European Journal of Physiology* **476**:9–37

42. Chiu T.-F., Huang C.-C., Chen J.-H., Chen W.-L (2012) **Depressed sympathovagal balance predicts mortality in patients with subarachnoid hemorrhage** *The American Journal of Emergency Medicine* **30**:651–656

43. Coutrot M., et al. (2021) **Perfusion index: Physical principles, physiological meanings and clinical implications in anaesthesia and critical care** *Anaesthesia Critical Care & Pain Medicine* **40**

44. Bjerkne Wenneberg S., et al. (2020) **Heart rate variability monitoring for the detection of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage** *Acta Anaesthesiologica Scandinavica* **64**:945–952

45. Zhang A., et al. (2023) **Clinical Potential of Immunotherapies in Subarachnoid Hemorrhage Treatment: Mechanistic Dissection of Innate and Adaptive Immune Responses** *Aging and disease* **14**

46. Bazett H.C (1920) **An Analysis of the Time-Relations of Electrocardiograms** *Heart* **7**:353–370

47. Bai X., et al. (2023) **The Clinical Characteristics of Heart Rate Variability After Stroke** *The Neurologist* **29**:133–141

48. Megjhani M., et al. (2019) **Heart Rate Variability as a Biomarker of Neurocardiogenic Injury After Subarachnoid Hemorrhage** *Neurocritical Care* **32**:162–171

49. Sharon O., Fahoum F., Nir Y (2020) **Transcutaneous Vagus Nerve Stimulation in Humans Induces Pupil Dilation and Attenuates Alpha Oscillations** *The Journal of Neuroscience* **41**:320–330

50. Skora L., Marzecová A., Jocham G (2024) **Tonic and phasic transcutaneous auricular vagus nerve stimulation (taVNS) both evoke rapid and transient pupil dilation** *Brain Stimulation* **17**:233–244

51. Tan G., et al. (2024) **Does vibrotactile stimulation of the auricular vagus nerve enhance working memory? A behavioral and physiological investigation** *Brain Stimulation* **17**:460–468

52. Williams D. P., et al. (2019) **Heart rate variability and inflammation: A meta-analysis of human studies** *Brain, Behavior, and Immunity* **80**:219–226

53. Solti F., Szatmáry L., Vecsey T., Szabolcs Z (1989) **The effect of sympathetic and parasympathetic activity on QT duration. Clinical study in patients with normal and prolonged QT time** *Cor Vasa* **31**:9–15

54. Kulkarni K., et al. (2021) **Low-Level Tragus Stimulation Modulates Atrial Alternans and Fibrillation Burden in Patients With Paroxysmal Atrial Fibrillation** *Journal of the American Heart Association* **10**

55. Jelinek M., Lipkova J., Duris K (2024) **Vagus nerve stimulation as immunomodulatory therapy for stroke: A comprehensive review** *Experimental Neurology* **372**

56. Dusi V., Angelini F., Zile M. R., De Ferrari G. M (2022) **Neuromodulation devices for heart failure** *European Heart Journal Supplements* **24**:E12–E27

Author information

Gansheng Tan*
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA
ORCID iD: 0000-0001-8785-9499

*These two authors contributed equally to this work

Anna L Huguenard*
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA

*These two authors contributed equally to this work

Kara M Donovan
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA

Phillip Demarest
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA

Xiaoxuan Liu
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA

Ziwei Li
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA

Markus Adamek
Department of Neuroscience, Washington University in St. Louis, St. Louis, USA

Kory Lavine
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA

Ananth K Vellimana
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Neurology, Washington University in St. Louis, St. Louis, USA

Terrance T Kummer
Department of Neurology, Washington University in St. Louis, St. Louis, USA

Joshua W Osbun
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Neurology, Washington University in St. Louis, St. Louis, USA

Gregory J Zipfel
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA

Peter Brunner
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA
ORCID iD: 0000-0002-2588-2754

Eric C Leuthardt
Department of Neurosurgery, Washington University School of Medicine, St. Louis, USA, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, USA

For correspondence: leuthardte@wustl.edu

Editors

Reviewing Editor
Olujimi Ajijola
University of California, Los Angeles, Los Angeles, United States of America

Senior Editor
Olujimi Ajijola
University of California, Los Angeles, Los Angeles, United States of America

Reviewer #2 (Public review):

Summary:

This study investigated the effects of transcutaneous auricular vagus nerve stimulation (taVNS) on cardiovascular dynamics in subarachnoid hemorrhage (SAH) patients. The researchers conducted a randomized clinical trial with 24 SAH patients, comparing taVNS treatment to a Sham treatment group (20 minutes per day twice a day during the ICU stay). They monitored electrocardiogram (ECG) readings and vital signs to assess acute as well as middle -term changes in heart rate, heart rate variability, QT interval, and blood pressure between the two groups. The results showed that repetitive taVNS did not significantly alter heart rate, corrected QT interval, blood pressure, or intracranial pressure. However, it increased overall heart rate variability and parasympathetic activity after 5-10 days of treatment compared to the sham treatment. Acute taVNS led to an increase in heart rate, blood pressure, and peripheral perfusion index without affecting corrected QT interval, intracranial pressure, or heart rate variability. The acute post-treatment elevation in heart rate was more pronounced in patients who showed clinical improvement. In conclusion, the study found that taVNS treatment did not cause adverse cardiovascular effects, suggesting it as a safe immunomodulatory treatment for SAH patients. The mild acute increase in heart rate post-treatment could potentially serve as a biomarker for identifying SAH patients who may benefit more from taVNS therapy.

Strengths:

The paper is overall well written, and the topic is of great interest. The methods are solid and the presented data are convincing.

Comments on revisions:

The main previous weaknesses of the paper have now been fixed.

https://doi.org/10.7554/eLife.100088.2.sa2

Reviewer #3 (Public review):

Summary:

The authors characterized the cardiovascular effects of acute and repetitive taVNS as an index of safety and concluded that taVNS treatment does not induce adverse cardiovascular effects such as bradycardia or QT prolongation.

Strengths:

This study contributes important information about the clinical utility of taVNS as a safe immunomodulatory treatment approach for SAH patients.

Comments on revised version:

A number of limitations were identified previously: https://elifesciences.org/reviewed-preprints/100088/reviews#peer-review-2. These major concerns were largely addressed by the authors.

https://doi.org/10.7554/eLife.100088.2.sa1

Author response:

The following is the authors' response to the original reviews.

We are thankful to the reviewers and the editor for their detailed feedback, insightful suggestions, and thoughtful assessment of our work. The revised manuscript has taken into account all the comments of the three reviewers. We have also undertaken additional analyses and added materials in response to reviewer suggestions. In brief:

(1) We have conducted a more in-depth analysis of frequency domain HRV metrics to better depict the change of autonomic tone.

(2) We have revised the manuscript to provide justifications for the chosen taVNS protocol and to clearly articulate the objectives of the current study.

(3) In response to comments from reviewer #2, we have included two new tables that present the absolute changes in cardiovascular metrics, clinical characteristics for the two trial arms, and effects of taVNS adjusted for age.

Other significant amendments include:

(1) An expanded discussion linking our findings to the existing literature on the effects of taVNS on cardiovascular function, biomarkers for taVNS response, the safety of taVNS, and the dose-response relationship of taVNS.

(2) Revision to the Method section to provide details of QT interval calculation.

> ***Reviewer #1 (Public Review):***
>
> *The authors report the results of a randomized clinical trial of taVNS as a neuromodulation technique in SAH patients. They found that taVNS appears to be safe without inducing bradycardia or QT prolongation. taVNS also increased parasympathetic activity, as assessed by heart rate variability measures. Acute elevation in heart rate might be a biomarker to identify SAH patients who are likely to respond favorably to taVNS treatment. The latter is very important in light of the need for acute biomarkers of response to neuromodulation treatments.*
>
> *Comments:*
>
> *(1) Frequency domain heart rate variability measures should be analyzed and reported. Given the short duration of the ECG recording, the frequency domain may more accurately reflect autonomic tone.*

We sincerely appreciate this encouraging summary of our paper. We have analyzed and reported frequency-domain heart rate variability measures, including the relative power of the high-frequency band (0.15–0.4 Hz) and the relative power of the low-frequency band (0.04 – 0.15). We showed the distribution of the two frequency-domain HRV measures in supplementary Figure 2C-D. For 24-hour ECG recording, we found that the change in the

relative high-frequency power from Day 1 was not significantly different between the treatment groups. As both high-frequency band and low-frequency band power are relative to the total power, the comparison of the relative power of the low-frequency band between groups would be the opposite of the relative power of the high-frequency band. As both time-domain and frequency-domain HRV measures can reflect the autonomic tone, we performed factor analysis to identify the parasympathetic activity component (Figure 2D). Comparing the change in parasympathetic activity component and relative high-frequency power, we observed similarities and discrepancies. Specifically, both the change in parasympathetic activity component and the change in relative high-frequency power were higher in the taVNS group at the early phase (Day 2 - 4).

We also observed higher high-frequency power in the Sham group at the later phase. If the factor analysis successfully isolates the parasympathetic activity, there should be other factors than the parasympathetic activity affecting the relative power of the high-frequency band. One such factor is the respiration rate. The high-frequency range is between 0.15 to 0.4 Hz, corresponding to respiration's frequency range of approximately 9 to 24 breaths per minute. If the respiration rate increases and exceeds 24 breaths per minute, the respiratory-driven HRV might occur at a frequency higher than the typical high-frequency band. Given that the respiration rate was higher in the taVNS treatment group, a compensatory mechanism to ensure oxygen delivery (Figure 4E), we hypothesized that observed lower high-frequency power in the taVNS treatment group compared to sham at later phases is a result of increased respiration rate in the taVNS treatment group. Indeed, we found the normalized high-frequency power is higher when RR is less than 25 bpm compared to when RR > 25 bpm (Cohen’s d = 0.85, Supplementary Figure 3A). Moreover, an increase in RR in the taVNS treatment group is associated with a decrease in high-frequency power (Supplementary Figure 3B). These control analyses underscored the necessity of performing factor analysis to robustly measure parasympathetic activities and confirm that taVNS treatment mitigated the sympathetic overactivation during the early phase.

We have now discussed the results of frequency-domain HRV measures in the Discussion section: taVNS and autonomic system (p23): “A key metric that reflects this restored sympathovagal balance is the increase in heart rate variability (Figure 3F). Specifically, the factor analysis showed that the parasympathetic activity was significantly higher in the taVNS treatment group. This difference was most pronounced during the early phase, particularly between Days 2 and 4 following SAH. In addition to analyzing the correlation between the parasympathetic activity factor and established HRV measures that reflect parasympathetic activity such as RMSSD and pNNI_50 (Figure 3C), we also examined changes in a frequency-domain HRV measure—the relative power of the high-frequency band (0.15–0.4 Hz)—to validate the accuracy of the factor analysis. the relative power of the high-frequency band is widely used to indicate respiratory sinus arrhythmia, a process primarily driven by the parasympathetic nervous system (Supplementary Figure 2). We found that both the change in parasympathetic activity factor and relative high-frequency power were higher in the taVNS group at the early phase (Day 2 - 4). Conversely, we observed higher high-frequency power in the Sham group during the later phase. If the factor analysis successfully isolates the parasympathetic activity, there should be other factors than the parasympathetic activity affecting the relative power of the high-frequency band. One such factor is the respiration rate. The high-frequency range is between 0.15 to 0.4 Hz, corresponding to respiration's frequency range of approximately 9 to 24 breaths per minute. If the respiration rate increases and exceeds 24 breaths per minute, the respiratory-driven HRV might occur at a frequency higher than the typical high-frequency band. Given that the respiration rate was higher in the taVNS treatment group, a compensatory mechanism to ensure oxygen delivery (Figure 4E), we hypothesized that observed lower high-frequency power in the taVNS treatment group compared to sham at later phases is a result of increased respiration rate in the taVNS treatment group. Indeed, we found the normalized high-frequency power is higher when RR is less than 25 bpm compared to when RR > 25 bpm (Cohen’s d = 0.85,

Supplementary Figure 3A). Moreover, an increase in RR in the taVNS treatment group is associated with a decrease in high-frequency power (Supplementary Figure 3B). These control analyses underscored the necessity of performing factor analysis to robustly measure parasympathetic activities and confirm that taVNS treatment mitigated the sympathetic overactivation during the early phase."

We have also reported the changes in the relative power of the high-frequency band between the two treatment groups in Supplementary Figure 6. We did not find a significant change in relative high-frequency band power between the treatment groups (Treatment – pre-treatment difference: p = 0.74, Cohen's d = -0.08, N(Sham) = 199, N(taVNS) = 188, Mann-Whitney U test). We reported these results in the Results section: Acute effects of taVNS on cardiovascular function (p18): "There were no significant differences in changes in corrected QT interval or heart rate variability, as measured by RMSSD, SDNN, and relative power of high-frequency band between treatment groups (Figure 5D and E and Supplementary Figure 6)."

> *How was the "dose" chosen (20 minutes twice daily)?*

The choice of a 20-minute taVNS session twice daily was informed by findings from Addorisio et al. (2019), where the authors administered 5-minute taVNS twice daily to patients with rheumatoid arthritis for two days. They found that the circulating c-reactive protein (CRP) levels significantly reduced after two days of treatment but returned to baseline at the second clinical assessment by day 7. Given the high inflammatory state associated with subarachnoid hemorrhage (SAH) and our intention to maintain a steady reduction in inflammation, we extended the duration of taVNS to 20 minutes per session. We have clarified this stimulation schedule's rationale in the Results section (p5-6): "This treatment schedule was informed by findings from Addorisio et al., where a 5-minute taVNS protocol was administered twice daily to patients with rheumatoid arthritis for two days.29 Their study found that circulating c-reactive protein (CRP) levels significantly reduced after 2 days of treatment but returned to baseline at the second clinical assessment by day 7. Given the high inflammatory state associated with SAH and our intention to maintain a steady reduction in inflammation, we decided to extend the treatment duration to 20 minutes per session."

Addorisio, Meghan E., et al. "Investigational treatment of rheumatoid arthritis with a vibrotactile device applied to the external ear." Bioelectronic Medicine 5 (2019): 1-11.

> *The use of an acute biomarker of response is very important. A bimodal response to taVNS has been previously shown in patients with atrial fibrillation (Kulkarni et al. JAHA 2021).*

Thank you for this valuable insight and for bringing the study by Kulkarni et al. to our attention. Their study showed that the response to Low-Level Tragus Stimulation (LLTS) varied among patients with atrial fibrillation, which can be predicted by acute P-wave alternans (PWA) to some degree. We have discussed the implication of the bimodal response to taVNS in the Discussion section (p26-27): "Kulkarni et al. showed that the response to low-level tragus stimulation (LLTS) varied among patients with atrial fibrillation.49 Similarly, in our study, not all patients in the taVNS treatment group showed a reduction in mRS scores (improved degree of disability or dependence). This differential response may be inherent to taVNS and potentially influenced by factors such as anatomical variations in the distribution of the vagus nerve at the outer ear. These findings underscore the importance of using acute biomarkers to guide patient selection and optimize stimulation parameters. Furthermore, we found that increased heart rate was a potential acute biomarker for identifying SAH patients who are most likely to respond favorably to taVNS treatment. Translating this finding into clinical practice will require further research to elucidate the mechanisms by which an acute increase in heart rate may predict the outcomes of patients receiving taVNS, including its

relationship with neurological evaluations, vasospasm, echocardiography, and inflammatory markers."

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *This study investigated the effects of transcutaneous auricular vagus nerve stimulation (taVNS) on cardiovascular dynamics in subarachnoid hemorrhage (SAH) patients. The researchers conducted a randomized clinical trial with 24 SAH patients, comparing taVNS treatment to a Sham treatment group (20 minutes per day twice a day during the ICU stay). They monitored electrocardiogram (ECG) readings and vital signs to assess acute as well as middle-term changes in heart rate, heart rate variability, QT interval, and blood pressure between the two groups. The results showed that repetitive taVNS did not significantly alter heart rate, corrected QT interval, blood pressure, or intracranial pressure. However, it increased overall heart rate variability and parasympathetic activity after 5-10 days of treatment compared to the sham treatment. Acute taVNS led to an increase in heart rate, blood pressure, and peripheral perfusion index without affecting corrected QT interval, intracranial pressure, or heart rate variability. The acute post-treatment elevation in heart rate was more pronounced in patients who showed clinical improvement. In conclusion, the study found that taVNS treatment did not cause adverse cardiovascular effects, suggesting it is a safe immunomodulatory treatment for SAH patients. The mild acute increase in heart rate post-treatment could potentially serve as a biomarker for identifying SAH patients who may benefit more from taVNS therapy.*
>
> *Strengths:*
>
> *The paper is overall well written, and the topic is of great interest. The methods are solid and the presented data are convincing.*
>
> *Weaknesses:*
>
> *(1) It should be clearly pointed out that the current paper is part of the NAVSaH trial (NCT04557618) and presents one of the secondary outcomes of that study while the declared first outcomes (change in the inflammatory cytokine TNF-α in plasma and cerebrospinal fluid between day 1 and day 13, rate of radiographic vasospasm, and rate of requirement for long-term CSF diversion via a ventricular shunt) are available as a pre-print and currently under review (doi: 10.1101/2024.04.29.24306598.). The authors should better stress this point as well as the potential association of the primary with the secondary outcomes.*

Thank you for this valuable suggestion. The current study indeed focuses on the trial's secondary outcomes. The main objective is to evaluate the cardiovascular safety of the taVNS protocol and to provide insights that will inform the application of taVNS in SAH patients. Following your comments, we have clarified this in the Introduction section (p6): "The current study is part of the NAVSaH trial (NCT04557618) and focuses on the trial's secondary outcomes, including heart rate, QT interval, HRV, and blood pressure.32 This interim analysis aims to evaluate the cardiovascular safety of the taVNS protocol and to provide insights that will inform the application of taVNS in SAH patients. The primary outcomes of this trial, including change in the inflammatory cytokine TNF-α and rate of radiographic vasospasm, are available as a pre-print and currently under review.26"

The negative association between HRV and inflammatory cytokines has been reported in numerous studies such as (Williams et al., Brain, Behavior, and Immunity, 2019; Haensel et al., Psychoneuroendocrinology. 2008). There are some studies suggesting that increased sympathetic tone following SAH is associated with vasospasm (Bjerkne Wenneberg, S. et al.,

Acta Anaesthesiologica Scandinavica. 2020; Megjhani et al., Neurocrit Care. 2020). Based on the literature, we compared the effects of taVNS on primary and secondary outcomes. The findings from the two parallel analyses are consistent: taVNS treatment reduced pro-inflammatory cytokines and increased HRV. Furthermore, the analyses of the primary outcomes revealed a reduction in the presence of any radiographic vasospasm in the taVNS treatment group compared to the sham. We have now integrated these findings and discussed them in the Discussion section (p25-26): "Given the negative association between pro-inflammatory markers and HRV, our finding that HRV was higher in the taVNS treatment group aligns with the findings of primary outcomes of this clinical trial, which showed that taVNS treatment reduced pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6.26,52 The consistency between these findings strengthens the evidence supporting the anti-inflammatory effects of taVNS. In addition, the sympathetic predominance following SAH is implicated in an increased risk of delayed cerebral vasospasm, which is most commonly detected 5-7 days after SAH.12 Given that taVNS treatment mitigated the sympathetic overactivation before the typical onset of cerebral vasospasm, it could potentially reduce the severity of this complication."

> *(2) The references should be implemented particularly concerning other relevant papers (including reviews and meta-analysis) of taVNS safety, particularly from a cardiovascular standpoint, such as doi: 10.1038/s41598-022-25864-1 and doi: 10.3389/fnins.2023.1227858).*

Thank you for providing the relevant papers. We have provided these references in the Introduction section to provide a more comprehensive background of our study (p6): "While some animal studies have reported a potential risk of bradycardia and decreased blood pressure associated with vagus nerve stimulation, two reviews of human studies have considered the cardiovascular effects of taVNS generally safe, with adverse effects reported only in patients with pre-existing heart diseases. 21,22,23

> *(3) The dose-response issue that affects both VNS and taVNS applications in different settings should be mentioned (doi: 10.1093/eurheartjsupp/suac036.) as well as the need for more dose-finding preclinical as well as clinical studies in different settings (the best stimulation protocol is likely to be disease-specific).*
>
> *Overall, the present work has the important potential to further promote the usage of taVNS even on critically ill patients and might set the basis for future randomized studies in this setting*

Thank you for this valuable insight. Scientific understanding of the dose-response relationship and determining optimal parameters tailored to specific disease contexts has been recognized as an important part of taVNS research and, more generally, in the electrical neuromodulation field. Studies in this direction are often complex and time-intensive due to the multitude of possible parameter combinations. As such, most taVNS studies opted to use parameters that have been established in previous studies. For example, 20 Hz taVNS is extensively used as a therapeutic intervention in stroke (Matyas Jelinek ,2024, https://www.sciencedirect.com/science/article/pii/S0014488623003138). As we pioneer the application of taVNS as an immunomodulation technique in SAH patients, we also adopt parameters reported in similar studies, aiming to provide a basis for future preclinical and clinical studies of taVNS in this patient population. As you noted, the effects of taVNS are dose-dependent, necessitating systematic exploration of the parameter space, including frequency, intensity, and duration. Our findings of the acute biomarker (heart rate) hold the promise of close-loop taVNS. We have now emphasized the importance of investigating how parameters/dose affect taVNS's effects on immune function and cardiovascular function in SAH patients (p28): "As we pioneer the application of taVNS as an immunomodulation

technique in SAH patients, we adopt parameters (20 Hz, 0.4 mA) reported in similar studies.55 The current study provides a basis for future preclinical and clinical studies of taVNS in this patient population. To build on our findings, a systematic evaluation of the relationship between parameters such as frequency, intensity, and duration and taVNS's effects on the immune system and cardiovascular function is necessary to establish taVNS as an effective therapeutic option for SAH patients.56"

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *The paper is overall well written, and the topic is of great interest. The reviewer has some major comments:*
>
> *(1) It should be clearly pointed out that the current paper is part of the NAVSaH trial and presents one of the secondary outcomes of that study while the declared first outcomes (change in the inflammatory cytokine TNF-α in plasma and cerebrospinal fluid between day 1 and day 13, rate of radiographic vasospasm, and rate of the requirement for long-term CSF diversion via a ventricular shunt) are available as a pre-print and currently under review (doi: 10.1101/2024.04.29.24306598.).*

We have revised the manuscript following your comment. Please see comment Reviewer 2 Public Review and our response.

> *The authors should assess the relationship between the impact of taVNS on inflammatory markers in plasma and in cerebrospinal fluid and the autonomic responses. The association between inflammatory markers and noninvasive autonomic markers as well as sympathovagal balance should also be assessed. Specifically, the authors should try to assess whether the acute post-treatment elevation in heart rate was more pronounced in patients who experienced a more pronounced reduction in inflammatory biomarkers. Indeed, since all patients in the current study received the same dose of taVNS (20 Hz frequency, 250 μs pulse width, and 0.4 mA intensity), while in several cardiovascular studies (doi: 10.1016/j.jacep.2019.11.008, doi: 10.1007/s10286-023-00997-z) the intensity (amplitude) of taVNS was differentially set based on the subjective pain/sensory threshold, that might be a marker of acute afferent neuronal engagement.*

We agree that analyzing the change in cardiovascular metrics and changes in inflammatory markers is an important next step. In particular, testing whether the acute elevation in heart rate correlates with changes in inflammatory markers could further establish heart rate as a biomarker to guide patient selection and optimize stimulation parameters. (Please refer to comment 1.3 and our responses). However, in this paper, the primary objective is the cardiovascular safety of the current taVNS protocol in SAH patients. This association between inflammatory markers and autonomic responses extends beyond the scope of the current manuscript and would be more appropriately addressed in a separate publication.

Previous literature has shown a negative association between HRV and inflammatory markers in SAH patients (for example, Adam, J., 2023). It is reasonable to postulate that taVNS modulates the immune system and the autonomic system synergistically. We found that parasympathetic tone was higher in the taVNS treatment group, with the most notable differences observed between Days 2 and 4 following SAH (Figure 3F). In a separate study of the primary outcomes of this trial (Huguenard et al., 2024), serum levels of IL-6 (pro-inflammation cytokine) were also significantly lower in the taVNS treatment group on Day 4 (Figure 3A, in our preprint, https://doi.org/10.1101/2024.04.29.24306598).

We appreciate your input regarding the potential mechanism behind acute heart rate changes. In this trial, all patients who were able to engage in verbal communication were

asked if they felt any prickling or pain during all sessions. We confirmed that the current stimulation setting was sub-perception in all trialed patients, making it unlikely that the observed heart rate increase was due to pain or sensory perception. Our current hypothesis is that successful activation of the afferent vagal pathway by taVNS increased arousal, resulting in increased heart rate. We have revised the Discussion section based on your insight (p29): “All patients who were capable of verbal communication were asked if they felt any prickling or pain during all sessions. We confirmed that the current taVNS protocol is below the perception threshold for all trialed patients. Altogether, successful activation of the afferent vagal pathway by taVNS increased arousal, resulting in increased heart rate.50,51”

Huguenard, A. L. et al. Auricular Vagus Nerve Stimulation Mitigates Inflammation and Vasospasm in Subarachnoid Hemorrhage: A Randomized Trial. (2024) doi:10.1101/2024.04.29.24306598.

Adam, J., Rupprecht, S., Künstler, E. C. S. & Hoyer, D. Heart rate variability as a marker and predictor of inflammation, nosocomial infection, and sepsis – A systematic review. Autonomic Neuroscience vol. 249 103116 (2023).

> *A new table should be provided with the mean (or median) values of the two arms of the population (taVNS and sham) including baseline clinical characteristics, comorbidities (mean age, % of female, % with known hypertension, diabetes, etc), ongoing medications (% on beta-betablockers, etc), and pre, during and post-treatment absolute values (expressed as mean or median depending on the distribution) of the studied parameters (QT and QTc absolute values, heart rate, SDNN, etc) in order for the reader to have a better understanding of how SAH affects these parameters. Absolute changes in the abovementioned parameters should also be presented in the table. For instance, the reported absolute increase in heart rate, based on Figure 5, panel C, seems very modest, below 2 bpm. This is very important to underlying for several reasons, including the fact that the evaluation of the impact of treatment on heart rate variability as assessed in the time domain might be influenced by concomitant changes in heart rate due to the nonlinearity of neural modulation of sinus node cycle length. Indeed, time-domain indexes of HRV intrinsically increase when heart rate decreases in a nonlinear way, while frequency domain indexes (e.g. the low frequency/high frequency (LF/HF) ratio), appear to be devoid of intrinsic rate-dependency (doi: 10.1016/s0008-6363(01)00240-1).*

Thank you for your suggestion. We have added the new table to the manuscript. In this table, we include clinical characteristics, the median of absolute values of cardiovascular metrics from 24-hour ECG recording, and the median absolute changes in these metrics for both arms. We believe that absolute values of cardiovascular metrics from 24-hour ECG recording are more informative about how SAH affects these parameters than metrics for the pre-, during-, and post-treatment periods.

In Result (p7), we have added: “Supplementary Table 3 shows the clinical characteristics of the two treatment groups.” In Result, Acute effect of taVNS on cardiovascular function (p20), we have added: “Supplementary Table 3 summarizes the absolute changes in cardiovascular metrics for the treatment groups.”

Thank you for raising the concern about HRV and providing the reference. We have now reported frequency domain indexes in our results: relative power of high-frequency power, which is negatively correlated with the LF/HF ratio. The high-frequency power is used to capture sinus arrhythmia, reflecting the parasympathetic modulation of the heart. Although the frequency domain metrics might be less susceptible to the rate-dependency (doi: 10.1016/s0008-6363(01)00240-1), there are circumstances when the frequency domain metrics might not accurately reflect the autonomic tone (Please see Reviewer 1 Publice Review and our responses).

> *An attempt to correct the effect of taVNS on the evaluated autonomic parameters according to age should be provided, considering that there were no age limits and parasympathetic indexes, particularly at the sinus node level, are known to decrease with age, particularly for those older than 65 years.*

Thank you for the suggestion. We were aware of the influence of age on cardiac heart rate and heart rate variability. In our initial analysis, we compared the change in autonomic parameters from day 1 within each subject across the two treatment groups. This approach controls for individual differences, including those due to age. In addition to your comment, age is a risk factor for subarachnoid hemorrhage. Older individuals often face an increased risk of poor outcomes. To further verify if age influences autonomic changes following SAH, we performed ANCOVA on autonomic function parameters with age included as a covariate. This analysis showed that age was negatively correlated with changes in heart rate, SDNN, and RMSSD from Day 1, but not with changes in QT intervals. After adjusting for age, we found that RMSSD changes and SDNN changes were significantly higher in the taVNS treatment group, while QTc changes were significantly lower in this group. These results align with the main findings (Figures 2 and 3). In addition, autonomic changes following SAH may be influenced by age. Specifically, lower RMSSD and SDNN in older individuals suggest a greater shift toward sympathetic predominance following SAH. We have now reported these results in Supplementary Table 4 and discussed their implication in the Discussion section (p28): “To control for individual differences, including those due to age, our study compared the change in cardiovascular parameters from Day 1 within each subject across treatment groups. To further verify if age influences autonomic changes following SAH, we performed ANCOVA on autonomic function parameters with age included as a covariate. This analysis showed that age was negatively correlated with changes in heart rate, SDNN, and RMSSD from Day 1 but not with changes in QT intervals. After adjusting for age, we found that RMSSD changes and SDNN changes were significantly higher, while QTc changes were significantly lower in the taVNS treatment group (Supplementary Table 4). These results align with the conclusion that repetitive taVNS treatment increased HRV and was unlikely to cause bradycardia or QT prolongation. In addition, autonomic changes following SAH may be influenced by age. Specifically, lower RMSSD and SDNN in older individuals suggest a greater shift toward sympathetic predominance following SAH (Supplementary Table 4).”

> *The results of the current study should be discussed considering what was previously demonstrated concerning the cardiovascular effects of taVNS (doi: 10.3389/fnins.2023.1227858).*

We appreciate the suggestion to consider previous findings on the cardiovascular effects of taVNS. However, it is important to note that most studies investigating the cardiovascular effects of taVNS involve healthy individuals, whereas our study focuses on SAH patients who are critically ill. Given the influence of SAH on cardiovascular parameters, we should be cautious when generalizing our findings to the broader population. Previous studies involving stroke populations have reported cardiovascular parameters descriptively as part of their safety assessments (doi: 10.1155/2020/8841752). Our study is currently the only one systematically investigating the cardiovascular safety of taVNS in SAH patients. Furthermore, the review paper (doi: 10.3389/fnins.2023.1227858) includes a highly heterogeneous mix of studies, such as auricular acupressure, auricular acupuncture, and electrical stimulation applied to different parts of the ear. For the subset of studies involving electrical stimulation, there is considerable variation in the parameters used, with frequencies ranging from 0.5 Hz to 100 Hz, currents from 0.1 mA to 45 mA, and durations spanning from 20 minutes to 168 days. These variations make direct comparisons with our findings challenging.

> *It looks like QT measurements were performed automatically. It should be specified which method was used for the measurements (threshold, tangent, or superimposed method?).*

In our study, QT intervals were measured based on thresholding after wavelet transforming the ECG signals (Martínez, J. P., IEEE Transactions on Biomedical Engineering, 2004, doi: 10.1109/TBME.2003.821031). The local maxima of the wavelet transform correspond to significant changes in the ECG signal, such as the rapid upward or downward deflections associated with the QRS complex. The algorithm searches modulus maxima, that is, peaks of wavelet transform coefficients that exceed specific thresholds, to identify the QRS complex. R peaks are found as the zeros crossing between the positive-negative modulus maxima pair. After localizing the R peak, the Q onset is detected as the beginning of the first modulus maximum before the modulus maximum pair created by the R wave. To identify the T wave, the algorithm searches for local maxima in the absolute wavelet transform in a search window defined relative to the QRS complex. Thresholding is used to identify the offset of the T wave. Please refer to comments 3.4 and 3.5 and our responses for details. We have clarified the method for measuring QT in the Method section (p35): "This algorithm identifies the QRS complex by searching for modulus maxima, which are peaks in the wavelet transform coefficients that exceed specific thresholds. The onset of the QRS complex is determined as the beginning of the first modulus maximum before the modulus maximum pair created by the R wave. To identify the T wave, the algorithm searches for local maxima in the absolute wavelet transform in a search window defined relative to the QRS complex. Thresholding is used to identify the offset of the T wave."

> *QTc dispersion was not evaluated, and this should be listed as a limitation of the current study.*

We have added this limitation in the Discussion section: Limitations and outlook (p31): "The current study did not explore the effects of taVNS on less commonly used cardiovascular metrics, such as QTc dispersion."

> *It has been recently suggested (doi: 10.1016/j.brs.2018.12.510) that QTc, as a potential indirect marker of HRV, might be used as a biomarker for VNS response in the treatment of resistant depression. The author should try to assess whether in the current study baseline QTc before taVNS is associated with outcome and with taVNS response.*

Thank you for the suggestion. The conference abstract in the provided doi stated that QTc as an indirect marker of HRV before implantation was correlated with changes in the depression rating scale. The mechanism seems to be that QTc has information about the pathophysiology of the depression (10.1097/YCT.0000000000000684). The current study focused on the comparison between taVNS treatment and sham treatment. Our future study will further test if SAH patients' response to taVNS can be predicted by baseline QTc.

> *The dose-response issue that affects both VNS and taVNS in different settings should be mentioned (doi: 10.1093/eurheartjsupp/suac036.) as well as the need for more dose-finding preclinical as well as clinical studies in different settings (the best stimulation protocol is likely to be disease-specific).*

Please refer to our responses to comment 3.

> *Minor Comments*
>
> *Some typos or commas instead of affirmative points and vice versa.*

Thank you for pointing this out. We have carefully proofread the manuscript and made the necessary corrections to ensure proper punctuation and grammar throughout.

> *Table 1: why age is expressed as a range for each person?*

MedRxiv asks authors to remove all identifying information. Precise ages are direct identifiers, as opposed to age ranges. We have now revised the age column to 'decade of life' in the updated table. We believe this modification reduces confusion while adhering to MedRxiv's guidelines.

> *Although already reported in the study protocol (doi: 10.1101/2024.03.18.24304239), the heart rate limits for inclusion should be reported (sustained bradycardia on arrival with a heart rate < 50 beats per minute for > 5 minutes, implanted pacemaker or another electrical device).*

We have now added the specific inclusion and exclusion criteria in the Method details section (p33): "Inclusion criteria were: (1) Patients with SAH confirmed by CT scan; (2) Age > 18; (3) Patients or their legally authorized representative are able to give consent. Exclusion criteria were: (1) Age < 18; (2) Use of immunosuppressive medications; (3) Receiving ongoing cancer therapy; (4) Implanted electrical device; (5) Sustained bradycardia on admission with a heart rate < 50 beats per minute for > 5 minutes; (6) Considered moribund/at risk of imminent death."

> *Why did the authors choose a taVNS schedule of two times per day of 30 minutes each as compared for instance to one hour per day? Please comment on that also referring to other taVNS studies in the acute setting such as the one by Dasari T et al (doi: 10.1007/s10286-023-00997-z.) where taVNS was applied for 4 hours twice daily. For instance, Yum Kim et al (doi: 10.1038/s41598-022-25864-1) recently reported in a systematic review and meta-analysis of taVNS, safety, that repeated sessions and sessions lasting 60 min or more were shown to be more likely to lead to adverse events.*
>
> *The International Consensus-Based Review and Recommendations for Minimum Reporting Standards in Research on Transcutaneous Vagus Nerve Stimulation should be referred to and contextualized (doi: 10.3389/fnhum.2020.568051).*

Thank you for raising this question and providing relevant references. We have reviewed the proposed checklist for minimum reporting items in taVNS research (10.3389/fnhum.2020.568051) and have ensured that our manuscript complies with the recommended reporting items.

The current taVNS schedule was based on findings from Addorisio et al. (2019). We have revised the manuscript to clarify the rationale behind the current taVNS protocol. Please refer to our response to comment 1.2. The two studies mentioned in the comments were published after our trial was designed and initiated (https://clinicaltrials.gov/study/NCT04557618). Based on the meta-analysis by Yum Kim et al., the short duration of treatment sessions might explain the cardiovascular safety of the current taVNS protocol. We are also currently assessing the effects of our taVNS protocol on inflammatory markers.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *The authors aimed to characterize the cardiovascular effects of acute and repetitive taVNS as an index of safety. The authors concluded that taVNS treatment did not induce adverse cardiovascular effects, such as bradycardia or QT prolongation.*

> *Strengths:*
>
> *This study has the potential to contribute important information about the clinical utility of taVNS as a safe immunomodulatory treatment approach for SAH patients.*
>
> *Weaknesses:*
>
> *A number of limitations were identified:*
>
> *(1) A primary hypothesis should be clearly stated. Even though the authors state the design is a randomized clinical trial, several aspects of the study appear to be exploratory. The method of randomization was not stated. I am assuming it is a forced randomization given the small sample size and approximately equal numbers in each arm.*

Thank you for the suggestion. The current study is part of the NAVSaH trial (NCT04557618), aiming to define the effects of taVNS on inflammatory markers, vasospasm, hydrocephalus, and continuous physiology data. This study focuses on the effects of repetitive and acute taVNS on continuous physiology data to evaluate the cardiovascular safety of the current taVNS protocol. The primary hypothesis tested in our study is that repetitive taVNS increased HRV but did not cause bradycardia and QT prolongation. Following your comments, we have clarified this in the Introduction section (p6): “This interim analysis aims to evaluate the cardiovascular safety of the taVNS protocol and to provide insights that will inform the application of taVNS in SAH patients. The primary outcomes of this trial, including change in the inflammatory cytokine TNF-α and rate of radiographic vasospasm, are available as a pre-print and currently under review.26 Based on a meta-analysis, repeated sessions lasting 60 min or more are likely to lead to aversive effects; therefore, we hypothesized that repetitive taVNS increased HRV but did not cause bradycardia and QT prolongation.23”

> *(2) The authors "first investigated whether taVNS treatment induced bradycardia or QT prolongation, both potential adverse effects of vagus nerve stimulation. This analysis showed no significant differences in heart rate calculated from 24-hour ECG recording between groups." A justification should be provided for why a difference is expected from 20 minutes of taVNS over a period of 24 hours. Acute ECG changes are a concern for increasing arrhythmic risk, for example, due to cardiac electrical restitution properties.*

A human study (Clancy, L. A. et al., Brain Stimulation, 2017, https://doi.org/10.1016/j.brs.2014.07.031) has found that 15-min taVNS led to reduced sympathetic activity measured by low-frequency/high-frequency (LF/HF) ratio. The sympathetic activity remained lower than baseline levels during the recovery period, suggesting potential long-term effects of taVNS on cardiovascular function. In addition, the repetitive taVNS treatment in this clinical trial was intended to maintain a steady low-inflammatory state. Given the potential life-threatening implications of bradycardia and QT prolongation in these critically ill patients, we deemed it crucial to evaluate heart rate and QT interval both acutely and from 24-hour ECG monitoring. We have now provided the justification in the Result section (p11): “A study has shown that 15 minutes of taVNS reduced sympathetic activity in healthy individuals, with effects that persist during the recovery period.33 This finding suggests that taVNS may exert long-term effects on cardiovascular function. Therefore, we investigated whether repetitive taVNS treatment affects heart rate and QT interval, key indicators of bradycardia or QT prolongation, using 24-hour ECG recording.”

An additional value of analyzing 24-hour ECG recording is that we can detect bradycardia or QT prolongation that happen outside the period of the stimulation, which could caused by repetitive taVNS. To this end, we reanalyzed the data and calculated the percentage of prolonged QT intervals using 500ms criterion (Giudicessi, J. R., Noseworthy, P. A. & Ackerman,

M. J. The QT Interval. Circulation, 2019). When comparing the percentage of prolonged QT intervals between the treatment groups, we found that changes in prolonged QT intervals percentage from Day 1 were higher in the Sham group (Figure 3F, Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value < 0.001, Cohen's d = -0.72). We have now reported the results in the Result section (p11): "To ensure that repetitive taVNS did not lead to QT prolongation happening outside the period of stimulation, we calculated the percentage of prolonged QT intervals. Prolonged QT intervals were defined as corrected QT interval >= 500 ms. We found that changes in prolonged QT intervals percentage from Day 1 were higher in the Sham group (Figure 3F, Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value < 0.001, Cohen's d = -0.72).

The concern regarding acute ECG changes related to increased arrhythmic risk is valid. We have improved the reasoning behind analyzing acute ECG change, which now reads (p20): "Assessing the acute effect of taVNS on cardiovascular is crucial for its safe translation into clinical practice. We compared the acute change of heart rate, corrected QT interval, and heart rate variability between treatment groups, as abrupt changes in the pacing cycle may increase the risk of arrhythmias."

> *(3) More rigorous evaluation is necessary to support the conclusion that taVNS did not change heart rate, HRV, QTc, etc. For example, shifts in peak frequencies of the high-frequency vs. low-frequency power may be effective at distinguishing the effects of taVNS. Further, compensatory sympathetic responses due to taVNS should be explored by quantifying the changes in the trajectory of these metrics during and following taVNS.*

We appreciate your concerns regarding the potential effects on the autonomic system associated with taVNS treatment. We would like to clarify that the primary objective of our study was to evaluate the cardiovascular safety of the taVNS protocol in SAH, with a specific focus on detecting any acute changes in heart rate and QT interval. As you highlighted, such acute ECG changes are a concern for increasing arrhythmic risk. By directly studying the trend of heart rate, HRV, and QT over the acute treatment periods, we found no significant change in these metrics between treatment groups. In addition, these metrics remained within 0.5 standard deviations of their daily fluctuations during and following taVNS treatment (Figure 5 and Supplementary Figure 6). These findings support the conclusion that the current protocol is unlikely to cause cardiac complications.

In response to your suggestion to conduct a more rigorous analysis, particularly concerning peak frequencies within the high-frequency (HF) and low-frequency (LF) bands, we pursued this analysis to explore more nuanced effects of taVNS on the autonomic system. We compared the shifts in peak frequencies within these bands between the treatment groups and found no significant changes that would suggest a sympathetic or parasympathetic shift following acute taVNS.

In detail, we have made the following revisions following your comments:

(1) We have clarified the motivation behind studying the acute change of cardiac metrics following taVNS treatment – monitoring the cardiovascular safety of current taVNS protocol in SAH patients (p18): please refer to response to comment 3.2.

(2) We compared the peak frequencies of the high-frequency and low-frequency bands following taVNS. added the results in the supplementary materials:

We note that neurophysiology underlying peak frequencies has not been thoroughly studied in the literature compared to the LF-band power or HF-band power. Therefore, we report this result as an exploratory analysis.

(3) We have added the changes of QTc during and following taVNS in Figure 5 and showed that they were within 0.5 standard deviations of their daily fluctuations during and following taVNS treatment. We have now shown the changes of HRV during and following taVNS in Supplementary Figure 6 A-D. We added the change of high-frequency power following Reviewer #1's comment 1.1. Overall, our results suggest that repetitive taVNS increased parasympathetic activities, while there is no evidence that acute taVNS significantly affected heart rate or QT.

> *(4) The authors do not state how the QT was corrected and at what range of heart rates. Because all forms of corrections are approximations, the actual QT data should be reported along with the corrected QT.*

The corrected QT interval (QTc) estimates the QT interval at a standard heart rate of 60 bpm. In practice, we removed RR intervals outside of the 300 – 2000 ms range. Further, we removed ectopic beats, defined as RR intervals differing by more than 20% from the one proceeding. We used the Bazett formula to correct the QT intervals: $_{QTc} = \frac{QT}{\sqrt{RR}}$. We have now clarified how QT was corrected in the Method section – Data processing (p35-36): "R-peaks were detected as local maxima in the QRS complexes. P-waves, T-waves, and QRS waves were delineated based on the wavelet transform (Figure 2A-C).34 RR intervals were preprocessed to exclude outliers, defined as RR intervals greater than 2 s or less than 300 ms. RR intervals with > 20% relative difference to the previous interval were considered ectopic beats and excluded from analyses. After preprocessing, RR intervals were used to calculate heart rate, heart rate variability, and corrected QT (QTc) based on Bazett's formula: $_{QTc} = \frac{QT}{\sqrt{RR}}$.44 The corrected QT interval (QTc) estimates the QT interval at a standard heart rate of 60 bpm."

We have reported the actual QT data in the Result section (p10 and p 19):" Moreover, changes in corrected QT interval from Day 1 were significantly higher in the Sham group compared to the taVNS group (Figure 3B, Mann–Whitney U test, N(taVNS) = 94, N(Sham)=95, p-value < 0.001, Cohen's d = -0.57). Similarly, uncorrected QT intervals from Day 1 were higher in the Sham group (Supplementary Figure 10A, Cohen's d = -0.42)."

"Supplementary Figure 10B-C shows the acute changes in uncorrected QT interval."

> *(5) The QT extraction method needs to be more robust. For example, in Figure 2C, the baseline voltage of the ECG is shifting while the threshold appears to be fixed. If indeed the threshold is not dynamic and does not account for baseline fluctuations (e.g., due to impedance changes from respiration), then the measures of the QT intervals were likely inaccurate.*

A robust method to estimate the QT interval is essential in our study. To this end, we used the state-of-the-art method to calculate QT intervals. We first applied a 0.5 Hz fifth-order high-pass Butterworth filter and a 60 Hz powerline filter on the ECG recording. The high-pass filtering is used to correct potential baseline fluctuations. Subsequently, a wavelet-based algorithm was used to delineate the QRS complex and T wave (Martínez, J. P., IEEE Transactions on Biomedical Engineering, 2004). In short, this algorithm identifies QRS based on modulus maxima of the wavelet transform of ECG signals. After localizing the R peak, the Q onset is detected as the beginning of the first modulus maximum before the modulus maximum pair created by the R wave. The detection is performed on wavelet transform at a small scale rather than on the original signal, minimizing the effect of baseline shift (see III Detection methods, (5), Cuiwei Li et al., IEEE TBME, 1995, Detection of ECG Characteristic

Points Using Wavelet Transforms). T wave is detected similarly based on wavelet transform. Please refer to our response to comment 2.9.

Martínez, J. P., Almeida, R., Olmos, S., Rocha, A. P., & Laguna, P. (2004). A wavelet-based ECG delineator: evaluation on standard databases. IEEE Transactions on Biomedical Engineering, 51(4), 570-581.

In Figure 2C, the purple and green lines take the value of 1 at the QRS onset or the T wave offset; otherwise, 0, which might appear to be a threshold. We have now used verticle lines to denote the detected QRS onsets and T wave offsets. Please see below for a comparison of the annotation:

We have clarified the details of extracting QT intervals from ECG recordings in the Method section (p31): "To calculate cardiac metrics, we first applied a 0.5 Hz fifth-order high-pass Butterworth filter and a 60 Hz powerline filter on ECG data to reduce artifacts. 35 We detected QRS complexes based on the steepness of the absolute gradient of the ECG signal using the Neurokit2 software package.35 R-peaks were detected as local maxima in the QRS complexes. P waves, T waves, and QRS complexes were delineated based on the wavelet transform of the ECG signals proposed by Martinez J. P. et al. (Figure 2A-C).36 This algorithm identifies the QRS complex by searching for modulus maxima, which are peaks in the wavelet transform coefficients that exceed specific thresholds. The onset of the QRS complex is determined as the beginning of the first modulus maximum before the modulus maximum pair created by the R wave. To identify the T wave, the algorithm searches for local maxima in the absolute wavelet transform in a search window defined relative to the QRS complex. Thresholding is used to identify the offset of the T wave."

We have modified Figure 2C for better clarity:

> *More statistical rigor is needed. For example, in Figure 2D, the change in heart rate for days 5-7, 8-10, and 11-13 is clearly a bimodal distribution and as such, should not be analyzed as a single distribution. Similarly, Figure 2E also shows a bimodal distribution. Without the QT data, it is unclear whether this is due to the application of the heart rate correction method.*

Thank you for raising this concern. Several factors could contribute to the observed distribution of changes in heart rate for days 5-7, 8-10, and 11-13, as shown in Figure 2D. One such factor is the smaller sample size in the later days. The mean duration of hospitalization for the 24 subjects included in this study was 11.29 days, with a standard deviation of 6.43, respectively. Other factors include variations in medical history, SAH pathology, and clinical outcomes during hospitalization. Further analysis revealed that heart rate was lower in patients with improved mRS scores (Supplementary Figure 4B), suggesting that clinical outcomes might impact changes in heart rate. Understanding the association between cardiovascular metrics and clinical assessments, such as vasospasm and inflammation, could help decide whether future taVNS trials should control for these factors when evaluating the effects of taVNS on cardiovascular function. We are currently continuing to recruit SAH patients in this clinical trial, and we plan to perform such analyses in future studies.

In the manuscript, we reported the effect size between the treatment groups for days 5-7, 8-10, and 11-13. This should be interpreted in conjunction with the characteristics of the distribution. To provide a rigorous interpretation of our results, we have now discussed these considerations in the discussion section (p28): "We noticed a high variance of change in heart rate for days 5 – 7, 8 – 10, and 11 – 13 for both treatment groups (Figure 2D). This may be due to the small sample size in the later days, given that the mean duration of hospitalization for the 24 subjects included in this study was 11.3 days with a standard deviation of 6.4. Differences in medical history and clinical outcomes during hospitalization may also explain the variance of change in heart rate for the later days. For example. heart rate was lower in

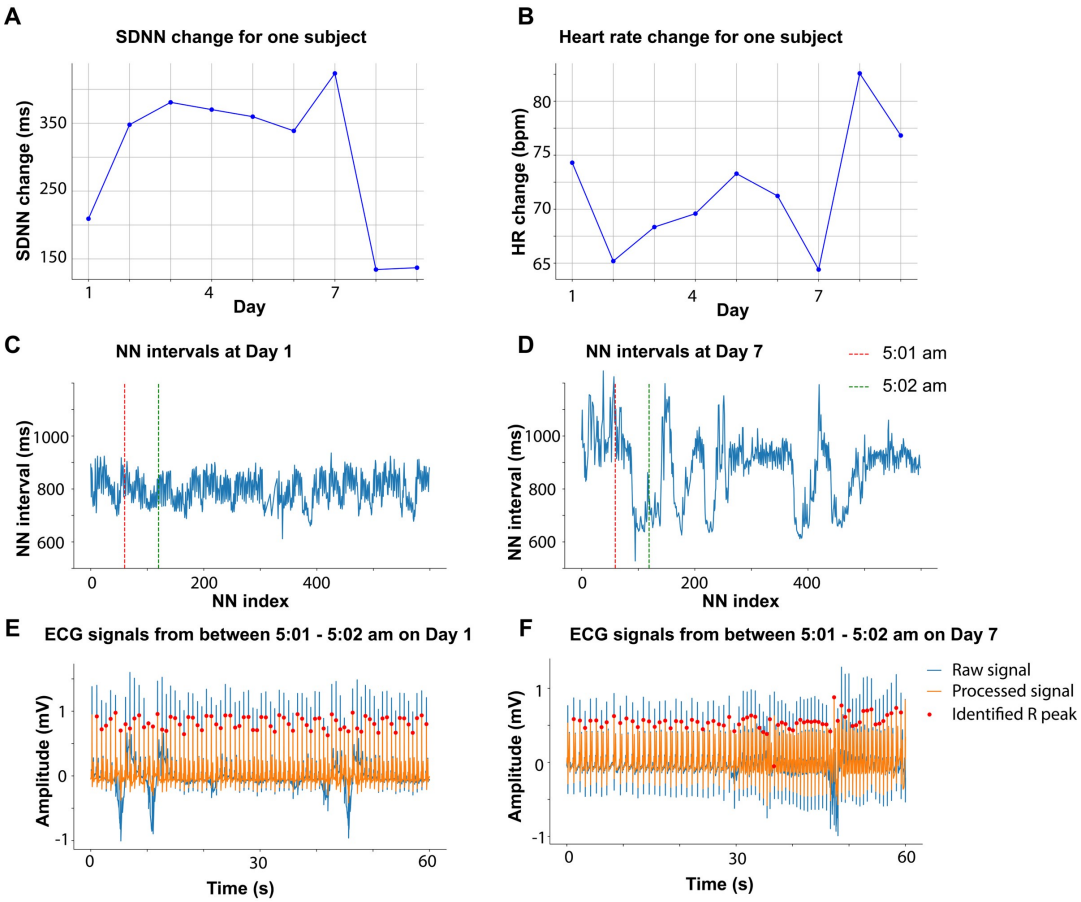
patients with improved mRS scores (Supplementary Figure 4B). Understanding the association between cardiovascular metrics and clinical assessments, such as vasospasm and inflammation, could help decide whether future taVNS trials should control for these factors when evaluating the effects of taVNS on cardiovascular function."

To test our hypothesis that repetitive taVNS does not induce significant heart rate change, we performed a two-tailed equivalence test of heart rate change between the two treatment groups, including data from days 2-13 (Figure 2D, left panel). To verify the validity of this approach, we calculated the Bimodality Coefficient (BC) and performed the Dip Test for unimodality for the distribution of heart rate change for the two treatment groups. The Bimodality Coefficient (BC) is a measure that combines skewness and kurtosis to assess whether a distribution is bimodal or unimodal. A BC value greater than 0.555 typically indicates a bimodal distribution, whereas a BC value less than or equal to 0.555 suggests an unimodal distribution. The Dip Test is a statistical test that assesses the unimodality of a distribution. A non-significant p-value (p-value ≥ 0.05) indicates that the distribution is likely unimodal. This analysis suggests that the distributions of heart rate changes in both treatment groups (days 2 - 13) are unimodal (BC = 0.457 and p = 0.374 for the taVNS treatment group; BC = 0.421 and p = 0.656 for the sham treatment group). This finding provides justification for our statistical approaches.

> *Figure 3A shows a number of outliers. A SDNN range of 200 msec should raise concern for a non-sinus rhythm such as arrhythmia or artifact, instead of sinus arrhythmia. Moreover, Figure 3B shows that the Sham RMSSD data distribution is substantially skewed by the presence of at least 3 outliers, resulting in lower RMSSD values compared to taVNS. What types of artifact or arrhythmia discrimination did the authors employ to ensure the reported analysis is on sinus rhythm? The overall results seem to be driven by outliers.*

Mild cardiac abnormalities are common in SAH patients. Therefore, change in cardiovascular metrics was expected to differ from healthy individuals, which makes studying the cardiovascular effect on taVNS extremely important in this context. Following your comment, we investigated whether the large SDNN change was due to arrhythmia or artifacts. Except for a single instance where one subject exhibited an SDNN change of 200 ms on a particular day, all other SDNN changes were less than 150 msec. We identified the subject and day associated with the largest SDNN change, which is Day 7. As shown in Author response image 1A and B, SDNN of this subject increased on day 7 while the heart rate (HR) of this subject decreased. Changes in HRV were inversely related to HR changes, suggesting shifts in sympathetic and parasympathetic tone. We checked the ECG recording and the extracted NN intervals (processed RR intervals) on that day. The NN intervals are more variate on day 7 compared to day 1 (Author response image 1C and D). To determine whether the significant variance observed between 5:01 am and 5:02 am was due to arrhythmia or artifacts, we closely examined the corresponding ECG signals (Author response image 1E and F). Based on our analysis, the elevated SDNN is unlikely to be attributed to artifacts.

Author response image 1.



Similarly, we identified the subjects and days corresponding to the most prominent RMSSD decrease in the sham treatment group. We verified the ECG quality for this subject and the accuracy of RR interval identification, and that there was no significant cardiovascular event during the subject's stay in the ICU. Based on the inclusion and exclusion criteria defined in our protocol (Huguenard A et al.m PLOS ONE, 2024), we did not exclude these data from our analysis.

Huguenard A, Tan G, Johnson G, Adamek M, Coxon A, et al. (2024) Non-invasive Auricular Vagus nerve stimulation for Subarachnoid Hemorrhage (NAVSaH): Protocol for a prospective, triple-blinded, randomized controlled trial. PLOS ONE 19(8): e0301154. https://doi.org/10.1371/journal.pone.0301154

To ensure accurate inferences about sympathetic and parasympathetic tone from these cardiovascular metrics, we have rigorously refined our methodologies, including correcting RR intervals outliers, correcting ectopic peaks, using state-of-art algorithms to identify QRS complex, P wave, and T wave (please refer to response to comment 3.5), and performing factor analysis. In addition, no significant cardiac complications have been reported by the attending physicians for the subjects included in this study. Nonetheless, it is important to note that ECG patterns in patients with SAH differ from those in healthy individuals, potentially impacting the accuracy of R peak identification. For example, one identified R peak (out of 73) was Q peak (F in the above figure). The pathology associated with SAH complicates the precise calculation of cardiovascular metrics and the interpretation of the results. We are committed to continually improving our methodologies for assessing

autonomic function in SAH patients. We have now discussed these limitations in the Discussion section (p31-32): “Mild cardiac abnormalities are common in SAH patients5, complicating the precise calculation of cardiovascular metrics from ECG signals and the interpretation of the results. Systematic verification of methods for calculating cardiovascular metrics to ensure their applicability in SAH patients is crucial.”

> *The above concern will also affect the power analysis, which was reported by authors to have been performed based on the t-test assuming the medium effect size, but the details of sample size calculations were not reported, e.g., X% power, t-test assumed Bonferroni correction in the power analysis, etc.*

Thank you for raising this concern. The current study is part of the NAVSaH trial (NCT04557618), focusing on the trial’s secondary outcomes (Please refer to comment 2.1 and our responses). The main objective of this interim analysis is to evaluate the cardiovascular safety of the current taVNS protocol. Goal enrollment for the pilot NAVSaH trial is 50 patients, based on power calculations to detect significant differences in inflammatory cytokines, radiographic vasospasm, and chronic hydrocephalus. The detailed power analysis is described in the protocol (Huguenard A et al.m PLOS ONE, 2024):

“Under a 2-by-2 repeated measures design consisting of two groups of patients, each measured at two time points, our goal is to compare the change across time in the taVNS group to the change across time in the Sham group. Based upon previous work from Koopman et al. [67], we assume our study will observe 1.1 standardized inflammatory cytokines mean change difference between the two groups. Using a two-sided, two-sample t-test, assuming both time points have equal variance and there is a weak correlation (i.e., 0.15) between measurement pairs, a sample size of 25 in each group achieves at least 80% power to detect a standardized difference of 1.1 in mean changes, with a significance level (alpha) of 0.05 [68].

Based on our preliminary data, we assume this study will observe 25% and 55% severe vasospasm in the taVNS and Sham groups, respectively. Under a design with 2 repeated measurements (i.e., 2 raters), assuming a compound symmetry covariance structure with a Rho of 0.2, at a significance level (alpha) of 0.05, a sample size of 25 in each group achieves at least 80% power when the null proportion is 0.55, and the alternative proportion is 0.25 [69–71].

As previously described, LV et al. [8] studied the relationship between cytokine levels and clinical endpoints in SAH, including hydrocephalus. From their outcomes, we predict a needed enrollment of approximately 50 to detect these endpoints. From our own preliminary data, with an incidence of chronic hydrocephalus 0% in treated patients and 28.6% in control (despite grade of hemorrhage), alpha = 0.05 and power = 0.80, the projected sample size to capture that change is approximately 44 patients.”

In this study, we used power analysis to report the achieved power of insignificant findings. For example, a Mann-Whitney U test on heart rate change between the treatment groups revealed no significant differences. We then used power analysis to calculate the achieved power. We have added the details of power analysis in the Method section (p34): “We calculated the achieved power of tests on heart rate change between the treatment groups assuming a medium effect size (Cohen’s d of 0.5) and a Type I error probability (a) of 0.05. Given that the Mann-Whitney U test is a non-parametric counterpart to the t-test and that the asymptotic relative efficiency of the U test relative to the t-test is 0.95 with normal distributions, we estimated the achieved power based on the power of a two-sample t-test, which is 0.93. We have clarified this in the introduction section and in the method section (p6 and p38):

"The current study is part of the NAVSaH trial (NCT04557618) and focuses on the trial's secondary outcomes, including heart rate, QT interval, HRV, and blood pressure.30 This interim analysis aims to evaluate the cardiovascular safety of the taVNS protocol and to provide insights that will inform the application of taVNS in SAH patients. The primary outcomes of this trial, including change in the inflammatory cytokine TNF-α and rate of radiographic vasospasm, are available as a pre-print and currently under review.24"

"In this study, we reported the statistical power achieved for tests that yielded non-significant results. The achieved power is calculated based on a two-sample t-test assuming a medium effect size (Cohen's d of 0.5) and a Type I error probability (a) of 0.05."

> *If the study was designed to show a cardiovascular effect, I am surprised that N=10 per group was considered to be sufficiently powered given the extensive reports in the literature on how HRV measures (except when pathologically low) vary within individuals. Moreover, HRV measures are especially susceptible to noise, artifacts, and outliers.*
>
> *If the study was designed to show a lack of cardiovascular effect (as the conclusions and introduction seem to suggest), then a several-fold larger sample size is warranted.*

The primary goal of this study is to assess the cardiovascular safety of the current taVNS protocol in SAH patients (please refer to comments 2.1 and 3.8 and our responses). More specifically, we want to assess whether the current taVNS protocol is associated with bradycardia or QT prolongation. The data in this study included ECG signals and vital signals from 24 subjects recruited between 2021 and 2024. The total number of days in the ICU is 271 days, which corresponds to 542 taVNS/sham treatment sessions. These data allow us to detect significant cardiovascular effects of acute taVNS with high power. For example, the comparison of heart rate from pre- to post-treatment sessions between treatment groups had power > 99% (N1 = 188, N2 = 199, assuming 0.05 type I error probability, medium effect size two sample t-test).

To safely conclude that there is no significant cardiovascular effect of repetitive taVNS on any given day following SAH, we would need to perform statistical tests between treatment groups on Day 1, Day 2, and Day N. In this context, 64 subjects per treatment group are required to achieve 80% power assuming medium effect size and 0.05 type I error probability (two-sample t-test). We have acknowledged this limitation in the Discussion section. Thank you for raising this concern!

The results reported in this study treat each day as an independent sample for several reasons. First, heart rate and HRV metrics exhibited great daily variations (Figure in comment 3.7, for example). Their value on one day was not predictive of the metrics on another day, which could be due to medications, interventions, or individualized SAH recovery process during the patient's stay in the ICU. Second, SAH patients in the ICU often experience rapid/daily changes in clinical status, including fluctuations in intracranial pressure, blood pressure, neurological status, and other vital signs. Also, the recovery process from SAH is highly individualized, with different patients exhibiting distinct trajectories of recovery or complications. Day-to-day cardiovascular function changes varied as the patient recovered or encountered setbacks. Moreover, we verified ECG signal quality, corrected outliers and artifacts in ECG processing, and employed a state-of-the-art QRS delineation method (Please refer to comment 3.5). All these ensure the accuracy of our reported results.

The revised Discussion section now reads (31): " Our study considers each day as an independent sample for the following considerations: 1. heart rate and HRV metrics exhibited great daily variations. Their value on one day was not predictive of the metrics on another day, which could be due to medications, interventions, or individualized SAH recovery process during the patient's stay in the ICU. 2. SAH patients in the ICU often experience daily

changes in clinical status, including fluctuations in intracranial pressure, blood pressure, neurological status, and other vital signs. 3. Day-to-day cardiovascular function changes varied as the patient recovered or encountered setbacks. To conclusively establish that there is no significant cardiovascular effect of repetitive taVNS on any given day following SAH, we would need to perform statistical tests between treatment groups for each day. In this context, 64 subjects per treatment group are required to achieve 80% power assuming medium effect size and 0.05 type I error probability (two-sample t-test)."

https://doi.org/10.7554/eLife.100088.2.sa0