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ORIGINAL RESEARCH

Low-Level Tragus Stimulation Modulates Atrial Alternans and Fibrillation Burden in Patients With Paroxysmal Atrial Fibrillation

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BACKGROUND: Low-level tragus stimulation (LLTS) has been shown to significantly reduce atrial fibrillation (AF) burden in patients with paroxysmal AF. P-wave alternans (PWA) is believed to be generated by the same substrate responsible for AF. Hence, PWA may serve as a marker in guiding LLTS therapy. We investigated the utility of PWA in guiding LLTS therapy in patients with AF.

METHODS AND RESULTS: Twenty-eight patients with AF were randomized to either active LLTS or sham (earlobe stimulation). LLTS was delivered through a transcutaneous electrical nerve stimulation device (pulse width 200 μs, frequency 20 Hz, amplitude 10–50 mA), for 1 hour daily over a 6-month period. AF burden over 2-week periods was assessed by noninvasive continuous ECG monitoring at baseline, 3 months, and 6 months. A 5-minute control ECG for PWA analysis was recorded during all 3 follow-up visits. Following the control ECG, an additional 5-minute ECG was recorded during active LLTS in all patients. At baseline, acute LLTS led to a significant rise in PWA burden. However, active patients receiving chronic LLTS demonstrated a significant reduction in both PWA and AF burden after 6 months ($P<0.05$). Active patients who demonstrated an increase in PWA burden with acute LLTS showed a significant drop in AF burden after 6 months of chronic LLTS.

CONCLUSIONS: Chronic, intermittent LLTS resulted in lower PWA and AF burden than did sham control stimulation. Our results support the use of PWA as a potential marker for guiding LLTS treatment of paroxysmal AF.

Key Words: atrial fibrillation ■ P-wave alternans ■ spectral method ■ vagal stimulation

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice, accounting for approximately one third of hospitalizations for cardiac rhythm disturbances.[1] AF has been associated with significant morbidity and decline of quality of life.[2]

Recent evidence suggests that the autonomic nervous system plays a central role in the pathogenesis of AF, especially in the early stages,[3] and several studies[4,5] have shown that autonomic modulation with vagus nerve stimulation (VNS) can suppress AF in experimental models.[4,5] More recently, in a proof-of-concept study in humans, we showed that in patients with drug-refractory AF undergoing AF ablation, noninvasive low-level transcutaneous VNS (LLTS) for just 1 hour significantly shortened AF duration and decreased inflammatory cytokines.[6] A recent clinical trial TREAT-AF (Transcutaneous Electrical Vagus Nerve Stimulation to Suppress Atrial Fibrillation) demonstrated that LLTS for 1 hour daily can significantly reduce AF burden in ambulatory patients with paroxysmal AF over a 6-month period.[7] Yet, the response to LLTS has been variable among individual patients, highlighting the need to optimize patient selection, in order to maximize the efficacy of this novel therapeutic strategy.

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CLINICAL PERSPECTIVE

What Is New?

- Chronic low-level transcutaneous electrical stimulation of the tragus (LLTS) of the ear significantly lowers both P-wave alternans (PWA) and atrial fibrillation (AF) burden in patients with paroxysmal AF.
- While acute LLTS causes an increase in PWA, chronic LLTS leads to a decrease in PWA, and an acute increase in PWA at baseline predicts lower AF burden at follow-up.

What Are the Clinical Implications?

- Chronic LLTS may be used as an alternative treatment option in selected patients with AF, and PWA may serve as a potential biomarker for identifying patients who are most likely to benefit from LLTS, guiding treatment of paroxysmal AF.

Nonstandard Abbreviations and Acronyms

AA	atrial alternans
HRV	heart rate variability
LLTS	low-level transcutaneous VNS/low-level tragus stimulation
PWA	P-wave alternans
VNS	vagus nerve stimulation

Given the evidence supporting the role of atrial alternans (AA) promoting an arrhythmogenic atrial substrate[8–12] or being a precursor to AF,[10,11,13–17] we hypothesized that P-wave alternans (PWA), a subtle beat-to-beat variation in the morphology of the atrial electrocardiographic waveform, can be used as a biomarker to assess the effect of LLTS on AF burden and thus guide patient selection.

METHODS

The data used in the analysis will be available to any investigator, upon request.

Human Study

This is an ancillary study of the recently published TREAT AF randomized clinical trial[7]; the last 28 patients were included in this ancillary study, after modification of the original protocol. In brief, patients with paroxysmal AF, who had at least 2 documented episodes of AF within 3 months of randomization, were eligible for inclusion in the study. Exclusion criteria included left ventricular ejection fraction <40%, significant valvular disease, recent (<6 months) stroke or myocardial infarction, severe heart failure (New York Heart Association class III or IV), recurrent vasovagal syncopal episodes, unilateral or bilateral vagotomy, and pregnancy or nursing. In addition, we excluded patients with sick sinus syndrome, second- or third-degree atrioventricular block, bifascicular block and prolonged first-degree atrioventricular block (PR >300 ms), in the absence of a pacemaker. The study was approved by the Institutional Review Board of the University of Oklahoma Health Sciences Center and patients provided informed consent before enrollment in the study. Enrollment in this study started after amendment of the original protocol. Thus, a total of 28 patients were enrolled in this study. A schematic of the study protocol is presented in Figure 1.

Low-Level Tragus Stimulation

Patients were randomized to either active or sham LLTS groups. LLTS was delivered by the patients themselves at home after individual training, through a transcutaneous electrical nerve stimulation device (Parasym device, Parasym Health, Inc, London, UK) for 1 hour daily over a 6-month period. Active LLTS was accomplished by attaching an ear clip to the tragus, which is innervated by the auricular branch of the vagus nerve.[18] Sham stimulation was delivered to the earlobe, which is devoid of vagal innervation.[18] The device was set at a pulse width of 200 μs and a pulse frequency of 20 Hz. The stimulation strength was gradually increased until the patient experienced mild discomfort, then decreased by 1 mA below that threshold.

Patient Follow-up

All patients were followed for 6 months. AF burden, defined as the percent of time spent in AF over a 2-week period, was assessed by noninvasive continuous ECG monitoring (Ziopatch; iRythm Technologies, Inc, San Francisco, CA) at baseline, at 3 months, and at 6 months. A 5-minute ECG was recorded as control for heart rate variability (HRV) and PWA analysis at each visit (baseline, 3 months and 6 months), using a PC-based ECG machine (SE 1515; Edan USA, Inc) with a sampling rate of 1000 Hz. Immediately after the control ECG, an additional 5-minute ECG was performed during active LLTS in all patients (active and sham), irrespective of randomization group (Figure 1), to gauge the acute effects of LLTS at each time point. Hence, while active patients effectively received both chronic and acute LLTS, sham patients received only acute LLTS.

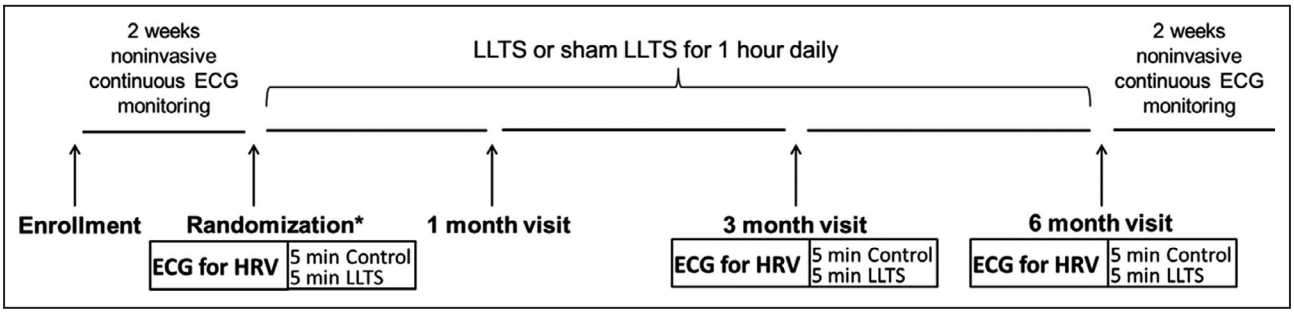

Figure 1. Schematic depicting the experimental protocol.
Patients with paroxysmal AF were randomized into sham (no chronic LLTS) and active (chronic LLTS) groups. Ten minutes of ECG was recorded at baseline, 3-month, and 6-month follow-up visits, wherein 5 minutes were recorded during control (no acute LLTS), and 5 minutes during acute LLTS. Two weeks of noninvasive ECG monitoring both before and after the 6-month study duration was performed to calculate AF burden before and after LLTS treatment. AF indicates atrial fibrillation; HRV, heart rate variability; and LLTS, low-level tragus stimulation. * denotes sham or active group.

AA Estimation

Extensive prior work by our laboratory has established the ability of microvolt T-wave alternans to predict short-[19–22] and long-term[23–25] susceptibility to ventricular tachyarrhythmias and sudden cardiac death. We have customized an algorithm currently used for estimating ventricular T-wave alternans so that it can be applied to estimating AA. Further details of the algorithm are presented in Data S1.

We estimated the level of PWA reflecting atrial depolarization (details presented in Data S1), during control (no stimulation) and LLTS (acute tragus stimulation), for sham and active groups at baseline, 3 months, and 6 months.

AA Burden

Estimates of alternans voltage and K_{score} were generated based on the spectral method as previously described.[19–24,26,27] Briefly, for each estimate, a matrix of 128 beats was used in which a window that reflected the atrial depolarization for each beat was created. Then, the power spectrum was estimated for each time-aligned sequence of sample points (Figure 2), within the selected atrial waveform (ie, P-wave reflecting atrial depolarization).[26] Subsequently, the power spectra for all sample points within the waveform were averaged and the statistical estimates of alternans (ie, alternans voltage, noise, and K_{score}), were obtained.[26] The alternans voltage is a direct measure of the presence of alternans, while the K_{score} is a measure of the statistical significance of the alternans voltage (Figure 2). Once estimates of alternans voltage and K_{score} were generated (for more details, please see Data S1), we calculated PWA burden for each patient as follows:

$$\text{PWA burden} = \text{positive PWA sequences/total sequences} \times 100\%,$$

where a positive PWA sequence was defined as any 128-beat sequence with K_{score} >3, alternans voltage >0.5 μV, and goodbeat % >80. In addition, ECG recordings with at least 50 good sequences across all leads were used for analysis.

Effect of Vagal Stimulation on HRV

We further sought to evaluate the effect of VNS on HRV. Clinically, high HRV has been associated with healthy cardiac tissue[28] while low HRV has been correlated with increased risk of lethal ventricular arrhythmias and sudden death.[29] Hence, evaluation of HRV has become an important method for assessing cardiac autonomic regulation. Here we calculated both linear time domain and nonlinear measures of HRV to gauge the effect of LLTS on autonomic regulation. Detailed methods documenting the calculation of individual HRV parameters are presented in Data S1.

Statistical Analysis

For each subject, sequences across all 12 leads with the number of good beats exceeding 80% and no respiration interference were combined to calculate the mean value, presented here. ECG recordings that did not meet the good beat threshold and did not have at least 50 good sequences were excluded from the analysis.

Continuous data are presented as mean±SD or median and interquartile range, as applicable. Categorical data are presented as percentages. Comparisons of continuous data were performed using a mixed linear model, with adjustment for the respective baseline values. Significant group-by-time interactions were followed by time-stratified analyses. For all pairwise testing, we adjusted for multiple comparisons using Tukey's method. The association of PWA burden with AF burden was examined using a polynomial, as well as logistic regression model, after adjustment for baseline values. Logarithmic transformation was performed as

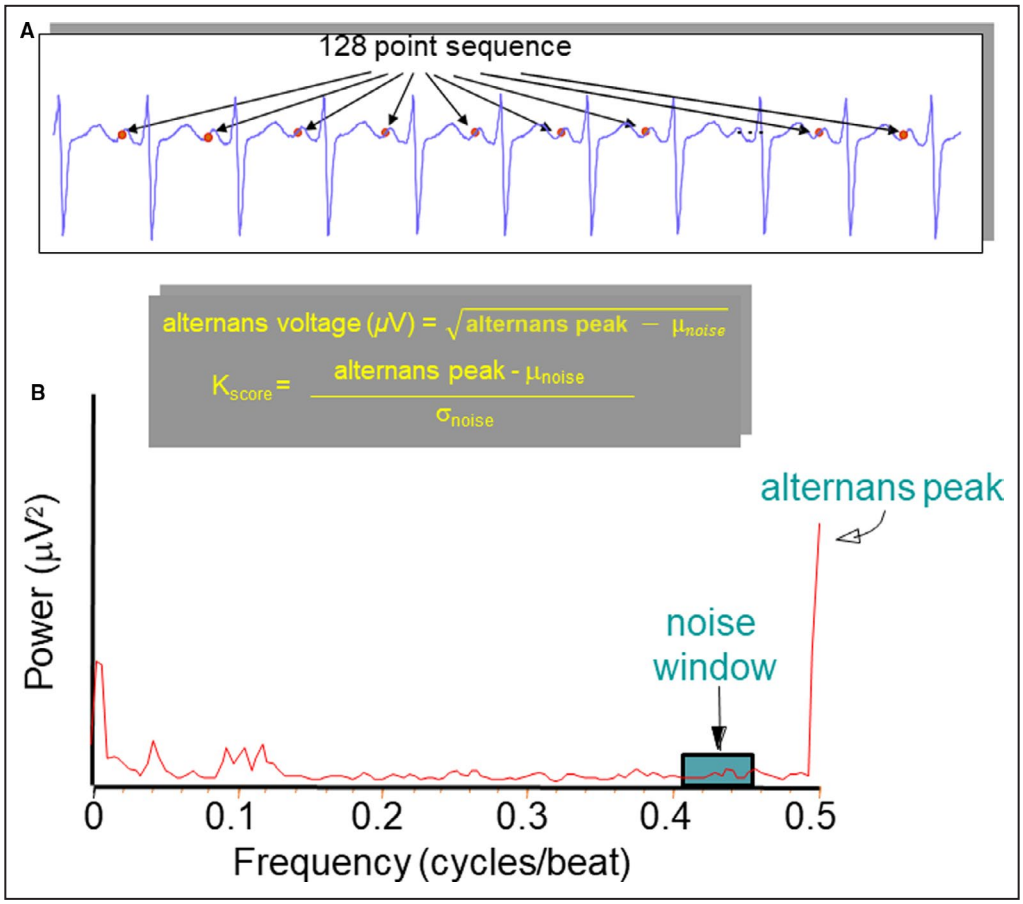

Figure 2. Spectral method for estimation of P-wave alternans.
A, One hundred twenty-eight time-aligned (with respect to the R-wave peak) P-wave points that are used in the power spectrum estimation. **B**, Representative example of power spectrum of beat-to-beat fluctuations in P-wave morphology. The alternans voltage is the square root of the amplitude of the power spectrum at the alternans frequency (alternans peak) minus the mean background noise level (μ_{noise}). The alternans ratio, K_{score}, is the amplitude of the spectrum at the alternans frequency (alternans peak) minus the mean background noise level (μ_{noise}), divided by the SD of the noise (σ_{noise}) in the reference noise band.

appropriate, to satisfy the model assumptions of normality and homoscedasticity. Categorical data were compared using Fisher's exact test. Values of $P<0.05$ were considered statistically significant. Statistical analysis was performed using SAS software version 9.4 (SAS Institute, Cary, NC) and MATLAB (MathWorks Inc, Natick, MA).

In figures, data are presented as median (horizontal solid line), 75% to 25% percentiles (box), and 90% to 10% percentiles (error bars).

RESULTS

Patient Population

The baseline clinical characteristics of the patients enrolled in this study are summarized in Table 1. Of the 28 patients enrolled at baseline, n=12 were randomized to sham and n=16 were randomized to the active LLTS group. There were no differences in clinical characteristics between the sham and active LLTS groups. Four patients (2 in each group) withdrew consent after 3 months of being in the study, while 1 patient in the sham group died of myocardial infarction. The rest of the patients completed the entire study. The stimulation intensity was similar in the 2 groups (sham 18.9±9.3 mA versus LLTS: 16.2±8.5 mA; $P=0.42$). No device-related adverse events were reported.

Effect of Chronic LLTS on Heart Rate, HRV, P-Wave Duration, QT, QTc, PR, and Tpeak-Tend

We first evaluated the effect of LLTS on heart rate for both sham and active patients. No significant changes were observed with chronic LLTS on either group, indicating that the selected stimulation parameters did not cause a change in resting heart rate (Figures S1 and S2). Chronic LLTS did not significantly alter any ECG

Table 1. Baseline Characteristics of the Patient Population

	Sham (n=12)	Active (n=16)	*P* Value
Age, y	60.3±12.5	66.3±7.6	0.17
Female sex, %	6 (50)	8 (50)	1.0
Body mass index, kg/m^2	31.7±5.4	30.6±8.2	0.71
Diabetes mellitus, %	3 (25)	3 (19)	0.28
Hypertension, %	9 (75)	12 (75)	1.0
Coronary artery disease, %	4 (33)	5 (31)	1.0
Heart failure, %	3 (25)	3 (19)	0.59
Obstructive sleep apnea, %	5 (42)	5 (31)	0.58
Antiarrhythmic drugs, %	7 (58)	5 (31)	0.14
Years in atrial fibrillation	6.0±5.3	4.1±3.3	0.37
$CHADS_2$-VA_2Sc score	2.6±1.7	3.0±1.7	0.61
Left ventricular ejection fraction, %	57.7±7.7	62.4±6.8	0.18
Left atrial diameter, cm	4.8±1.0	4.6±0.6	0.68
Medical therapy			
Beta blockers	11 (92)	14 (88)	1.0
Calcium channel blockers	5 (42)	4 (25)	0.43
Class I antiarrhythmics	4 (33)	4 (25)	0.69
Class III antiarrhythmics	2 (17)	1 (6)	0.56

Numbers in parentheses reflect percentages.

parameter, including P-wave duration, QT-interval, QTc (QT interval corrected for heart rate), PR-interval, and Tpeak-Tend interval (Figures S3 through S7). Summary results of chronic LLTS on different HRV measures, namely, SD of RR interval, root mean square of the successive differences of RR intervals, NN_{50} count, pNN_{50}, and SD2/SD1 ratio presented in Data S1 have shown mostly no differences between groups (Figures S8 through S12).

Effect of Chronic LLTS on PWA and AF Burden

Figure 3 demonstrates summary results of chronic LLTS on AF burden across all active and sham patients. AF burden was significantly lower in the active group compared with sham, after 6 months of chronic LLTS. Figure 4 demonstrates summary results of chronic LLTS on PWA burden during control conditions (no acute LLTS), across all active and sham patients. Similar to AF burden, PWA burden was significantly lower in the active group compared with sham,

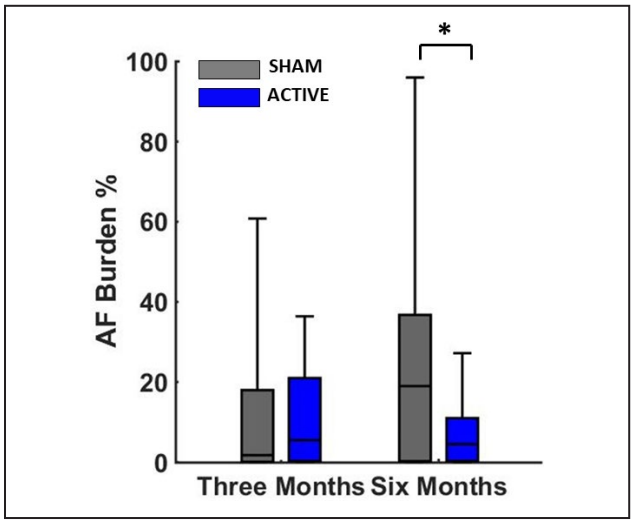

Figure 3. Chronic effects of LLTS on AF burden in active and sham patients.
There has been a statistically significant decrease of AF burden at 6 months in the active group compared with sham. The *P* value is based on a comparison of median AF burden levels at the 6-month time point after adjusting for baseline measures. "*" denotes statistical significance of $P<0.05$. AF indicates atrial fibrillation; and LLTS, low-level tragus stimulation.

after 6 months of chronic LLTS. A summary of AF burden and PWA burden at the respective time points is shown in Table 2. Summary results of chronic LLTS on PWA voltage and K_{score} are presented in Figures S13 through S16.

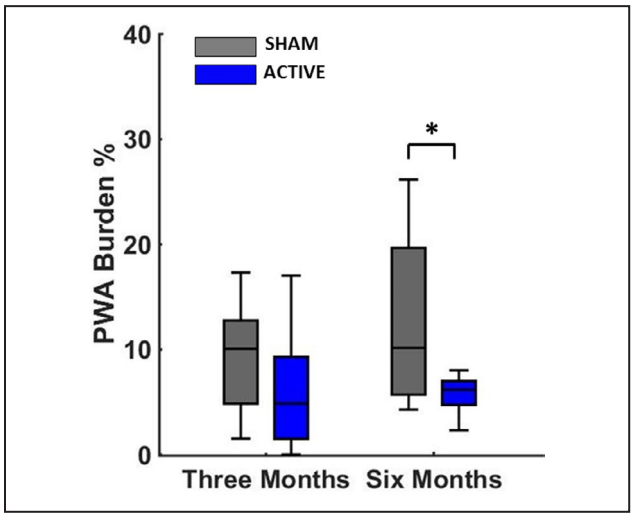

Figure 4. Chronic effects of LLTS on PWA burden in active and sham patients.
There has been a statistically significant decrease of PWA burden at 6 months in the active group compared with sham. The *P* value is based on a comparison of median PWA burden levels at the 6-month time point after adjusting for baseline measures. "*" denotes statistical significance of $P<0.05$. LLTS indicates low-level tragus stimulation; and PWA, P-wave alternans.

Table 2. Summary of AF Burden and PWA Burden at Baseline and Follow-up

	AF Burden (%)		PWA Burden (%)	
	Sham	Active	Sham	Active
Baseline	13.0±13.1	14.3±12.2	9.6±8.1	8.2±7.7
3 mo	15.2±13.9	12.7±9.2	11.2±8.9	8.1±6.0
6 mo	25.7±16.6	10.3±8.7*	13.9±9.7	7.7±3.5*

AF indicates atrial fibrillation; and PWA, P-wave alternans.
*P<0.05 vs sham.

Effect of Acute LLTS on PWA

Figures 5A and 5B demonstrate summary results of acute LLTS on PWA burden during control conditions (no stimulation) and LLTS at baseline, before commencement of chronic active LLTS. Acute LLTS led to a significant increase in PWA burden. Figure 5B demonstrates summary results of the effect of acute LLTS on ΔPWA burden at 3 and 6 months. Change in PWA burden because of acute LLTS compared with control conditions (no stimulation) was evaluated at each time point. ΔPWA burden was significantly lower in the active group compared with sham with acute stimulation at 6 months, suggesting that chronic LLTS modified the effect of acute LLTS over time.

Identifying Early Markers of Effective LLTS Treatment

Given that not all active patients had a similar response to chronic LLTS, we sought to identify whether patients who produced an early indicative response to acute LLTS also demonstrated a response to chronic therapy, hence aiding us in identifying potential biomarkers for effective LLTS treatment. Patients in the active group were categorized into 2 subgroups based on their initial response to LLTS: A1, patients with an initial increase in PWA burden during acute LLTS at baseline (n=8) and A2, patients with no increase in PWA burden during acute LLTS at baseline (n=8). Patients in group A1 had significantly lower AF burden at 6 months compared with group A2 (Figure 6A). Using logistic regression analysis, an acute increase of PWA at baseline predicted a significant decrease in AF burden at 6 months (odds ratio, 0.4; 95% CI, 0.17–0.94, P=0.03). In addition, at the individual patient level, there was a significant negative linear association between the change in AF burden and PWA burden from baseline to 6 months with acute stimulation, suggesting that an acute increase in PWA at baseline predicts lower AF burden at follow-up and may be used as a biomarker of response to chronic LLTS (Figure 6B).

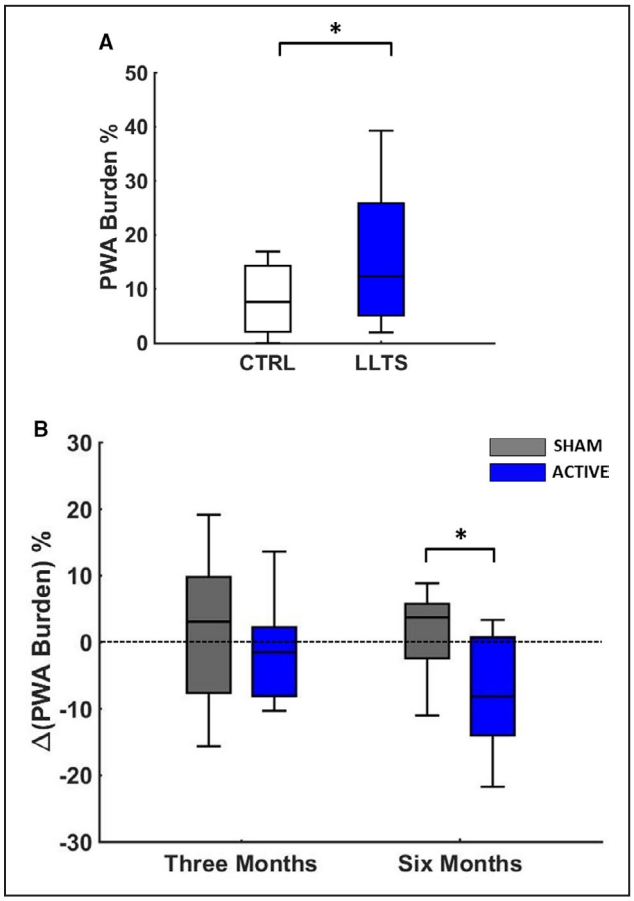

Figure 5. Effect of LLTS on PWA at baseline and follow-up. Summary results of acute LLTS on PWA burden at baseline, for all patients (**A**). In (**B**), the change in PWA burden before and after acute LLTS was compared between the 2 groups at 3 and 6 months of follow-up. At 6 months, acute LLTS in the LLTS group resulted in a favorable decrease in PWA burden, which was significantly different than the respective change in the sham group. "*" denotes statistical significance of P<0.05. CRTL indicates control; LLTS, low-level tragus stimulation; and PWA, P-wave alternans.

DISCUSSION

With increasing life expectancy and greater burden of chronic medical conditions, AF has become increasingly prevalent in the population and remains a major source of morbidity and mortality because of limited, and often ineffective treatment options.[1] Although LLTS has emerged as a promising new treatment modality, it remains hampered by the lack of a biomarker to guide patient selection and stimulation parameters. The major findings of this study are as follows: (1) chronic LLTS significantly lowers both PWA and AF burden in patients with paroxysmal AF; (2) LLTS modulates the level of PWA; acute LLTS causes an increase in PWA, while chronic LLTS leads to a decrease in PWA; and (3) PWA can serve as a marker of LLTS efficacy and guide its use in patients with AF; patients who show an early indicative increase in PWA burden because of acute LLTS demonstrate favorable effects of chronic LLTS treatment, highlighting the utility of PWA in identifying patients most likely to benefit from LLTS treatment.

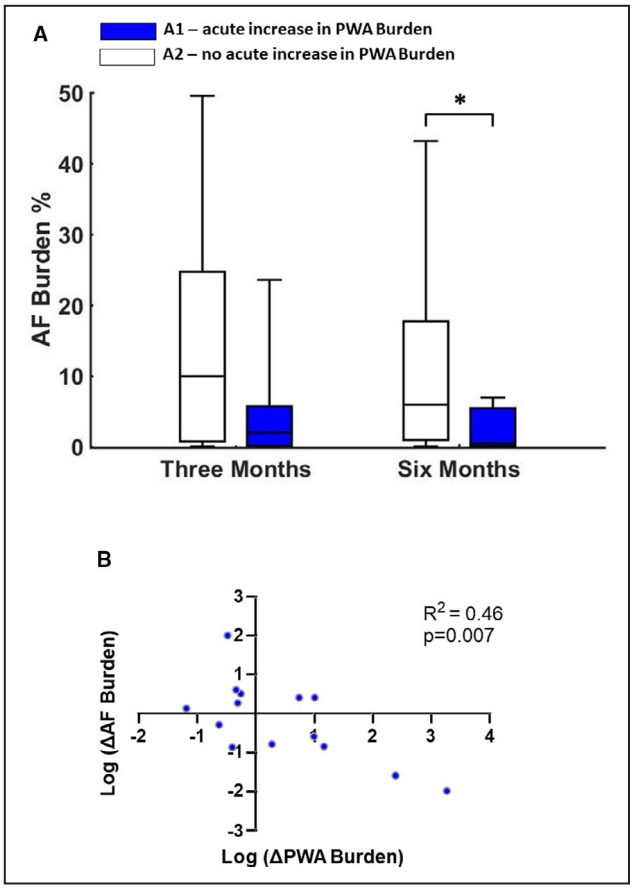

Figure 6. Response to chronic LLTS, according to the baseline acute response to LLTS.
A, Patients in the active group were categorized into 2 subgroups based on their initial response to LLTS. A1, patients with an initial increase in PWA burden during acute LLTS at baseline and A2, patients with no increase in PWA burden during acute LLTS at baseline. Patients in group A1 had a significant decrease in AF burden at 6 months, whereas AF burden did not improve with chronic LLTS in group A2. **B**, Scatter plot of change in AF burden with respect to change in PWA burden for each patient (blue dot). At the individual patient level, there was a significant negative linear association between the change in AF burden and the change in PWA burden, from baseline to 6 months, with acute stimulation. Collectively, these data suggest that acute change in PWA at baseline may be used as a biomarker of response to chronic LLTS. N=14, because 2 active patients did not complete the 6-month follow-up. "*" denotes statistical significance of $P<0.05$. AF indicates atrial fibrillation; LLTS, low-level tragus stimulation; and PWA, P-wave alternans.

The first trial that evaluated the effect of VNS in patients with heart failure, an open label, nonrandomized pilot trial, demonstrated that VNS resulted in significant improvement in functional class, quality of life, 6-minute walk test, and left-ventricular end-systolic volume, in the absence of any major side effects.[30] Despite these early promising results, 3 subsequent randomized trials of VNS in heart failure showed either neutral effects,[31,32] or only mild benefit.[33] The rather disappointing results of these trials, despite the clear rationale for decreasing sympathovagal imbalance in heart failure, highlight the notion that optimizing patient selection and stimulation parameters are crucial to elucidate the possible patient-specific favorable effects of VNS.[34]

In the current study, we observed that individual patient response to LLTS can vary greatly. While some active patients showed no observable response to chronic LLTS, in some patients a significant drop in PWA burden was observed. Additionally, response to acute and chronic LLTS was contradictory. Acute LLTS at baseline tended to increase the level of PWA in patients, yet, after 3 or 6 months of chronic LLTS, active patients showed a drop in alternans level with acute LLTS compared with control conditions (no stimulation). This is possibly indicative of chronic LLTS inducing positive changes in the atrial electrical substrate over time, making it more conducive to vagal stimulation and leading to a decrease in the overall level of alternans. Moreover, based on recent evidence that LLTS activates central vagal projections,[35] we speculate that the effect of LLTS is because of, at least in part, favorable changes in the central nervous system.

In light of the limitations associated with currently available end points pertinent to the effectiveness of VNS in patients with AF, a major advancement in this field will be achieved by determining the impact of VNS on the underlying atrial substrate and autonomic tone, and defining better metrics of immediate and long-term response.[36] In this study, we hypothesized that AA is associated with the same myocardial substrate that gives rise to AF and that modification of the AA level could serve as a marker of successful LLTS. Development of tools that can be applied in real-time to determine optimal LLTS parameters to sufficiently modify the atrial substrate is critical to improving the efficacy of this novel therapeutic modality. If validated, in future prospective studies a process to optimally select candidate patients for LLTS, based on a novel physiological biomarker, PWA, which reflects the arrhythmogenic potential of the underlying atrial substrate and its modulation by LLTS resulting in a reduction in AF burden,[7,37] could be a major improvement in the management of AF.

The effect of VNS is critically dependent on stimulation parameters.[34,36,38] Since autonomic tone differs among individual patients, it is possible that there is no single, "optimal" set of stimulation parameters for all patients. In the present study, low-level intermittent VNS was performed with stimulation strength restricted to 1 mA below an individual patient's discomfort threshold. This prevented any sudden adverse effects on heart rate in both active and sham patients because of parasympathetic overdrive, as was evident from the lack of any significant changes

in both heart rate and RR interval in all patients (Figures S1 and S2). The absence of significant effects on heart rate variability measures with chronic parasympathetic stimulation can also be possibly attributed to the selection of low-level intermittent VNS. However, since PWA is a substrate-dependent phenomenon, its physiological mechanism differs from simple HR measures and hence, in the present study, both acute and chronic LLTS significantly modulated PWA. Therefore, upon further validation, PWA may serve as a biomarker to determine customized, optimal VNS parameters for each patient and thus optimally guide the management of AF.[7,37] Furthermore, the present study could also serve as evidence for selection criteria for a broader longitudinal study of VNS in patients with AF so as to improve study outcomes in appropriately selected participants. Selecting patients who are likely to benefit from LLTS based on an acute biomarker of response will improve the overall response to LLTS and will thus increase the cost-effectiveness of this novel therapy for AF. This notion warrants further investigation in future randomized clinical trials.

Limitations

This study has several limitations. First, this was a proof-of-concept study with a limited number of patients. Therefore, further validation is required in larger studies. Second, future studies should include continuous monitoring of AF to allow a more accurate assessment of AF burden. Third, stimulation of the earlobe in the sham group may have resulted in some effect, which in turn would minimize the effect of active LLTS. However, previous studies have shown that stimulation of the earlobe, in contrast to stimulation of the tragus, does not result in activation of central vagal projections,[35] and therefore is a reasonable sham control. Finally, although other methods of AA calculation are available, the method used in this study has been previously validated by our group.[7,37]

CONCLUSIONS

Chronic, intermittent LLTS resulted in lower PWA and AF burden than did sham control stimulation. Our results support the notion that PWA can be used as a potential biomarker for guiding LLTS treatment of paroxysmal AF.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Data S1
Figures S1–S16

REFERENCES

1. Le Heuzey JY, Paziaud O, Piot O, Said MA, Copie X, Lavergne T, Guize L. Cost of care distribution in atrial fibrillation patients: the COCAF study. *Am Heart J*. 2004;147:121–126. DOI: 10.1016/S0002-8703(03)00524-6.
2. Miyasaka YBM, Bailey KR, Cha SS, Gersh BJ, Seward JB, Tsang TS. Mortality trends in patients diagnosed with first atrial fibrillation: a 21-year community-based study. *J Am Coll Cardiol*. 2007;49:986–992. DOI: 10.1016/j.jacc.2006.10.062
3. Stavrakis S, Nakagawa H, Po SS, Scherlag BJ, Lazzara R, Jackman WM. The role of the autonomic ganglia in atrial fibrillation. *JACC Clin Electrophysiol*. 2015;1:1–13. DOI: 10.1016/j.jacep.2015.01.005.
4. Sheng X, Scherlag BJ, Yu L, Li S, Ali R, Zhang Y, Fu G, Nakagawa H, Jackman WM, Lazzara R, et al. Prevention and reversal of atrial fibrillation inducibility and autonomic remodeling by low-level vagosympathetic nerve stimulation. *J Am Coll Cardiol*. 2011;57:563–571. DOI: 10.1016/j.jacc.2010.09.034.
5. Shen MJ, Shinohara T, Park H-W, Frick K, Ice DS, Choi E-K, Han S, Maruyama M, Sharma R, Shen C, et al. Continuous low-level vagus nerve stimulation reduces stellate ganglion nerve activity and paroxysmal atrial tachyarrhythmias in ambulatory canines. *Circulation*. 2011;123:2204–2212. DOI: 10.1161/CIRCULATIONAHA.111.018028.
6. Stavrakis S, Humphrey MB, Scherlag BJ, Hu Y, Jackman WM, Nakagawa H, Lockwood D, Lazzara R, Po SS. Low-level transcutaneous electrical vagus nerve stimulation suppresses atrial fibrillation. *J Am Coll Cardiol*. 2015;65:867–875. DOI: 10.1016/j.jacc.2014.12.026
7. Stavrakis S, Stoner JA, Humphrey MB, Morris L, Filiberti A, Reynolds JC, Elkholey K, Javed I, Twidale N, Riha P, et al. TREAT AF (Transcutaneous Electrical Vagus Nerve Stimulation to Suppress Atrial Fibrillation): a randomized clinical trial. *JACC Clin Electrophysiol*. 2020;6:282–291. DOI: 10.1016/j.jacep.2019.11.008.
8. Chang KC, Bayer JD, Trayanova NA. Disrupted calcium release as a mechanism for atrial alternans associated with human atrial fibrillation. *PLoS Comput Biol*. 2014;10:e1004011. DOI: 10.1371/journal.pcbi.1004011.
9. Kanaporis G, Blatter LA. Alternans in atria: mechanisms and clinical relevance. *Medicina*. 2017;53:139–149. DOI: 10.1016/j.medici.2017.04.004.
10. Narayan SM, Franz MR, Clopton P, Pruvot EJ, Krummen DE. Repolarization alternans reveals vulnerability to human atrial fibrillation. *Circulation*. 2011;123:2922–2930. DOI: 10.1161/CIRCULATIONAHA.110.977827.
11. Kim B-S, Kim Y-H, Hwang G-S, Pak H-N, Lee SC, Shim WJ, Oh DJ, Ro YM. Action potential duration restitution kinetics in human atrial

fibrillation. *J Am Coll Cardiol*. 2002;39:1329–1336. DOI: 10.1016/S0735-1097(02)01760-6.
12. Lalani GG, Schricker AA, Clopton P, Krummen DE, Narayan SM. Frequency analysis of atrial action potential alternans: a sensitive clinical index of individual propensity to atrial fibrillation. *Circ: Arrhythm Electrophysiol*. 2013;6:859–867. DOI: 10.1161/CIRCEP.113.000204
13. Holmqvist F, Platonov PG, McNitt S, Polonsky S, Carlson J, Zareba W, Moss AJ; Investigators MI. Abnormal P-wave morphology is a predictor of atrial fibrillation development and cardiac death in MADIT II patients. *Ann Noninvasive Electrocardiol*. 2010;15:63–72. DOI: 10.1111/j.1542-474X.2009.00341.x.
14. Franz MR, Jamal SM, Narayan SM. The role of action potential alternans in the initiation of atrial fibrillation in humans: a review and future directions. *Europace*. 2012;14:v58–v64. DOI: 10.1093/europace/eus273.
15. Hiromoto K, Shimizu H, Furukawa Y, Kanemori T, Mine T, Masuyama T, Ohyanagi M. Discordant repolarization alternans-induced atrial fibrillation is suppressed by verapamil. *Circ J*. 2005;69:1368–1373. DOI: 10.1253/circj.69.1368.
16. Verrier RL, Fuller H, Justo F, Nearing BD, Rajamani S, Belardinelli L. Unmasking atrial repolarization to assess alternans, spatiotemporal heterogeneity, and susceptibility to atrial fibrillation. *Heart Rhythm*. 2016;13:953–961. DOI: 10.1016/j.hrthm.2015.11.019.
17. Jousset F, Tenkorang J, Vesin JM, Pascale P, Ruchat P, Rollin AG, Fromer M, Narayan SM, Pruvot E. Kinetics of atrial repolarization alternans in a free-behaving ovine model. *J Cardiovasc Electrophysiol*. 2012;23:1003–1012. DOI: 10.1111/j.1540-8167.2012.02336.x.
18. Peuker ET, Filler TJ. The nerve supply of the human auricle. *Clin Anat*. 2002;15:35–37. DOI: 10.1002/ca.1089.
19. Merchant FM, Armoundas AA. Role of substrate and triggers in the genesis of cardiac alternans, from the myocyte to the whole heart: implications for therapy. *Circulation*. 2012;125:539–549. DOI: 10.1161/CIRCULATIONAHA.111.033563.
20. Merchant FM, Sayadi O, Puppala D, Moazzami K, Heller V, Armoundas AA. A translational approach to probe the proarrhythmic potential of cardiac alternans: a reversible overture to arrhythmogenesis? *Am J Physiol Heart Circ Physiol*. 2014;306:H465–H474. DOI: 10.1152/ajpheart.00639.2013.
21. Merchant FM, Zheng H, Bigger T, Steinman R, Ikeda T, Pedretti RF, Salerno-Uriarte JA, Klersy C, Chan PS, Bartone C, et al. A combined anatomic and electrophysiologic substrate based approach for sudden cardiac death risk stratification. *Am Heart J*. 2013;166:744–752. DOI: 10.1016/j.ahj.2013.06.023.
22. Merchant FM, Sayadi O, Sohn K, Weiss EH, Puppala D, Doddamani R, Singh JP, Heist EK, Owen C, Kulkarni K, et al. Real-time closed-loop suppression of repolarization alternans reduces arrhythmia susceptibility in vivo. *Circ Arrhythm Electrophysiol*. 2020;13:e008186. DOI: 10.1161/CIRCEP.119.008186.
23. Merchant FM, Ikeda T, Pedretti RF, Salerno-Uriarte JA, Chow T, Chan PS, Bartone C, Hohnloser SH, Cohen RJ, Armoundas AA. Clinical utility of microvolt T-wave alternans testing in identifying patients at high or low risk of sudden cardiac death. *Heart Rhythm*. 2012;9:1256–1264.e2. DOI: 10.1016/j.hrthm.2012.03.014.
24. Merchant FM, Salerno-Uriarte JA, Caravati F, Falcone S, Molon G, Marangoni D, Raczak G, Danilowicz-Szymanowicz L, Pedretti RFE, Sarzi Braga S, et al. Prospective use of microvolt T-wave alternans testing to guide primary prevention implantable cardioverter defibrillator therapy. *Circ J*. 2015;79:1912–1919. DOI: 10.1253/circj.CJ-15-0253.
25. Sohn K, Dalvin SP, Merchant FM, Kulkarni K, Sana F, Abohashem S, Singh JP, Heist EK, Owen C, Isselbacher EM, et al. Utility of a smartphone based system (cvrPhone) to predict short-term arrhythmia susceptibility. *Sci Rep*. 2019;9:1–11. DOI: 10.1038/s41598-019-50487-4.
26. Smith JM, Clancy EA, Valeri CR, Ruskin JN, Cohen RJ. Electrical alternans and cardiac electrical instability. *Circulation*. 1988;77:110–121. DOI: 10.1161/01.CIR.77.1.110.
27. Sayadi O, Puppala D, Ishaque N, Doddamani R, Merchant FM, Barrett C, Singh JP, Heist EK, Mela T, Martínez JP, et al. A novel method to capture the onset of dynamic electrocardiographic ischemic changes and its implications to arrhythmia susceptibility. *J Am Heart Assoc*. 2014;3:e001055. DOI: 10.1161/JAHA.114.001055.
28. Goldberger AL. Is the normal heartbeat chaotic or homeostatic? *News Physiol Sci*. 1991;6:87–91. DOI: 10.1152/physiologyonline.1991.6.2.87.
29. Tsuji H, Larson MG, Venditti FJ Jr, Manders ES, Evans JC, Feldman CL, Levy D. Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study. *Circulation*. 1996;94:2850–2855. DOI: 10.1161/01.CIR.94.11.2850.
30. De Ferrari GM, Crijns HJ, Borggrefe M, Milasinovic G, Smid J, Zabel M, Gavazzi A, Sanzo A, Dennert R, Kuschyk J, et al.; CardioFit Multicenter Trial I. Chronic vagus nerve stimulation: a new and promising therapeutic approach for chronic heart failure. *Eur Heart J*. 2011;32:847–855. DOI: 10.1093/eurheartj/ehq391.
31. Gold MR, Van Veldhuisen DJ, Hauptman PJ, Borggrefe M, Kubo SH, Lieberman RA, Milasinovic G, Berman BJ, Djordjevic S, Neelagaru S, et al. Vagus nerve stimulation for the treatment of heart failure: the INOVATE-HF trial. *J Am Coll Cardiol*. 2016;68:149–158. DOI: 10.1016/j.jacc.2016.03.525.
32. Zannad F, De Ferrari GM, Tuinenburg AE, Wright D, Brugada J, Butter C, Klein H, Stolen C, Meyer S, Stein KM, et al. Chronic vagal stimulation for the treatment of low ejection fraction heart failure: results of the NEural Cardiac TherApy foR Heart Failure (NECTAR-HF) randomized controlled trial. *Eur Heart J*. 2015;36:425–433. DOI: 10.1093/eurheartj/ehu345.
33. Premchand RK, Sharma K, Mittal S, Monteiro R, Dixit S, Libbus I, DiCarlo LA, Ardell JL, Rector TS, Amurthur B, et al. Autonomic regulation therapy via left or right cervical vagus nerve stimulation in patients with chronic heart failure: results of the ANTHEM-HF trial. *J Card Fail*. 2014;20:808–816. DOI: 10.1016/j.cardfail.2014.08.009.
34. Byku M, Mann DL. Neuromodulation of the failing heart: lost in translation? *JACC Basic Transl Sci*. 2016;1:95–106. DOI: 10.1016/j.jacbts.2016.03.004.
35. Frangos E, Ellrich J, Komisaruk BR. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: fMRI evidence in humans. *Brain Stimul*. 2015;8:624–636. DOI: 10.1016/j.brs.2014.11.018.
36. Qin D, Singh JP. Low-level tragus stimulation for atrial fibrillation: a glimpse of hope for neuromodulation? *JACC Clin Electrophysiol*. 2020;6:292–294. DOI: 10.1016/j.jacep.2020.01.003.
37. Stavrakis S, Kulkarni K, Singh JP, Katritsis DG, Armoundas AA. Autonomic modulation of cardiac arrhythmias: methods to assess treatment and outcomes. *JACC Clin Electrophysiol*. 2020;6:467–483. DOI: 10.1016/j.jacep.2020.02.014.
38. Ardell JL, Nier H, Hammer M, Southerland EM, Ardell CL, Beaumont E, KenKnight BH, Armour JA. Defining the neural fulcrum for chronic vagus nerve stimulation: implications for integrated cardiac control. *J Physiol*. 2017;595:6887–6903. DOI: 10.1113/JP274678.

SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL METHODS

Estimation of P-wave Alternans

The customized algorithm for the estimation of PWA begins with QRS detection. Following initial QRS detection[27], fiducial points (R-waves) are identified using a template-matching based QRS detection algorithm [26]. Using these fiducial points, an isoelectric PR segment is selected for every beat and is used as the zero amplitude reference throughout the remainder of the analysis. Then an 'average' QRS complex is estimated. For each QRS complex, a correlation coefficient with the 'average' beat is estimated and used for ventricular erroneous beat detection; a beat will be considered erroneous if its correlation coefficient is less than a threshold (CC_t) value of 0.90 or if the difference between current R-to-R (RR) waveform interval and the median RR interval from the preceding 7 beats is less/more than a threshold (RR_t) value of 20%. The process of template matching and RR interval detection is designed to eliminate erroneous beats such as premature ventricular complexes from the analysis. If a beat meets both threshold criteria, it is classified as 'good' and retained for analysis. In addition, a goodbeat percentage is calculated for all sequences as a moving average of the number of good sequences in a window of 128 beats. This is indicative of the number of consecutive good beats in the ECG recording and only sequences that meet a threshold of 80% are used for further processing.

After ventricular erroneous beat detection, erroneous atrial beats (such as premature atrial contractions) are detected in the same way that erroneous ventricular beats are detected: the correlation coefficient (CC_t) between the present beat P-wave and the average P-wave of the 128-beat sequence is set at 0.90. Once all erroneous *ventricular and atrial* beats are detected, then for each erroneous beat, the P-wave of that and the subsequent beat are removed from the sequence of beats and substituted with a median odd or even beat P-wave (estimated from all good odd or even beats), depending on whether the erroneous beat was an odd or an even one.

For purposes of PWA estimation to eliminate the effect of respiration (that may cause signal wandering), we subtract the baseline (defined as the mean value of the

electrocardiographic PQ interval) of each beat from the corresponding P-wave. The next step of the algorithm involves creating a matrix of all 128 beats in which a window that reflects the atrial depolarization for each beat is identified according to its fiducial point (Figure 2). Then, the power spectrum is estimated for each time-aligned sequence of sample points (Figure 2) within the selected atrial waveform (i.e. P-wave reflecting atrial depolarization) [26]. Subsequently, the power spectra for each sample point within the waveform are averaged and the statistical estimates of alternans (i.e. alternans voltage, noise and K-score), are obtained as previously described [26] . Briefly, P-wave alternans are estimated as follows:

$$\text{alternans voltage } (\mu V) = \sqrt{\text{alternans peak - } \mu_{noise}}$$

$$K_{score} = \frac{\text{alternans peak - } \mu_{noise}}{\sigma_{noise}}$$

where, the alternans peak is the peak of the power spectrum corresponding to 0.5 cycles/beat and the mean (μ_{noise}) and the standard deviation (σ_{noise}) of the spectral noise are estimated in a predefined noise window. The alternans voltage is a direct measure of the presence of alternans, while the K_{score} is a measure of the statistical significance of the alternans voltage.

Estimation of Heart Rate Variability Measures

To investigate the effect of chronic LLTS on heart rate variability (HRV) we evaluated several time domain and non-linear measures of HRV. First, for each sham and active patient, RR Interval values were calculated during control (no tragus stimulation) and LLTS at all three time points: baseline, 3 months and 6 months.

The Standard Deviation of RR Intervals (SDRR), a measure of long term HRV and the Root Mean Square of Successive Differences (RMSSD) of the RR intervals, a common indicator of short term HRV, was calculated. Additionally, NN_{50} count, defined as the number of times the change in consecutive RR intervals exceeds 50 ms and pNN_{50}, the percentage of consecutive RR intervals that differ by more than 50 ms ((NN_{50} Count/RR Interval Count)*100%), were also calculated.

We also estimated the SD2/SD1 ratio, where, SD1 and SD2 are non-linear measures of short (SD1) and long (SD2) term HRV calculated from Poincare maps and based on RR intervals.

SD1/SD2 ratio correlates with LF/HF ratio and is used as a measure of autonomic balance; parasympathetic nervous system (PNS) and sympathetic nervous system (SNS) activity contribute to LF power, and PNS activity primarily contributes to HF power. A low LF/HF, or high SD2/SD1 ratio reflects parasympathetic dominance. SD1 and SD2 are estimated as follows:

$SD1^2 = 0.5*SDSD^2$ (where SDSD = standard deviation of successive differences of RR intervals)

$SD2^2 = 2*SDRR^2 – 0.5*SDSD^2$

Mean values of each HRV measure were generated for all patients during control and LLTS. Comparison of HRV measures were performed at baseline, 3 months and 6 months between sham and active groups.

Effect of LLTS on P-wave Duration and QT, QT_c, PR, T_{peak}-T_{end} Intervals

After identification of fiducial points (P_{on}, P_{off}, Q_{peak} , R_{peak}, T_{peak} and T_{end}) from the ECG waveform using wavelet transform[27], P-wave duration and QT, PR and T_{peak}-T_{end} intervals were calculated for each beat. Corrected QT interval, QT_c, was calculated based on Bazett's formula as the QT interval for each beat divided by the square root of the preceding RR interval. Mean values were generated for all patients during control and LLTS. Comparison of ECG Intervals were performed at baseline, 3 months and 6 months between sham and active groups.

SUPPLEMENTAL RESULTS

Effect of Chronic Low Level Tragus Stimulation on Heart Rate, P-wave Duration and QT, QT_c, PR, T_{peak}-T_{end} Intervals

Summary results of chronic LLTS on heart rate (Figure S1), RR-interval (Figure S2), P-wave duration (Figure S3), QT-interval (Figure S4), QT_c interval (Figure S5), PR-interval (Figure S6), and T_{peak}-T_{end} interval (Figure S7), respectively, across all active and sham patients, during control and LLTS are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). At 6 months after chronic LLTS, active patients had significantly larger QT-intervals compared to sham patients during both control and LLTS. However, this effect was

not observed in the corrected QT intervals after adjustment for heart rates. Statistical comparison was performed using 1-way ANOVA.

Effect of Chronic Low Level Tragus Stimulation on Heart Rate Variability

Summary results of chronic LLTS on SDRR (Figure S8), RMSSD (Figure S9), NN_{50} count (Figure S10), pNN_{50} and SD2/SD1 ratio (Figures S11 and S12), across all active and sham patients, during control (no tragus stimulation) and LLTS are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Chronic LLTS did not lead to any significant changes in HRV in either sham or active patients.

Effect of Chronic Low Level Tragus Stimulation on P-wave Alternans

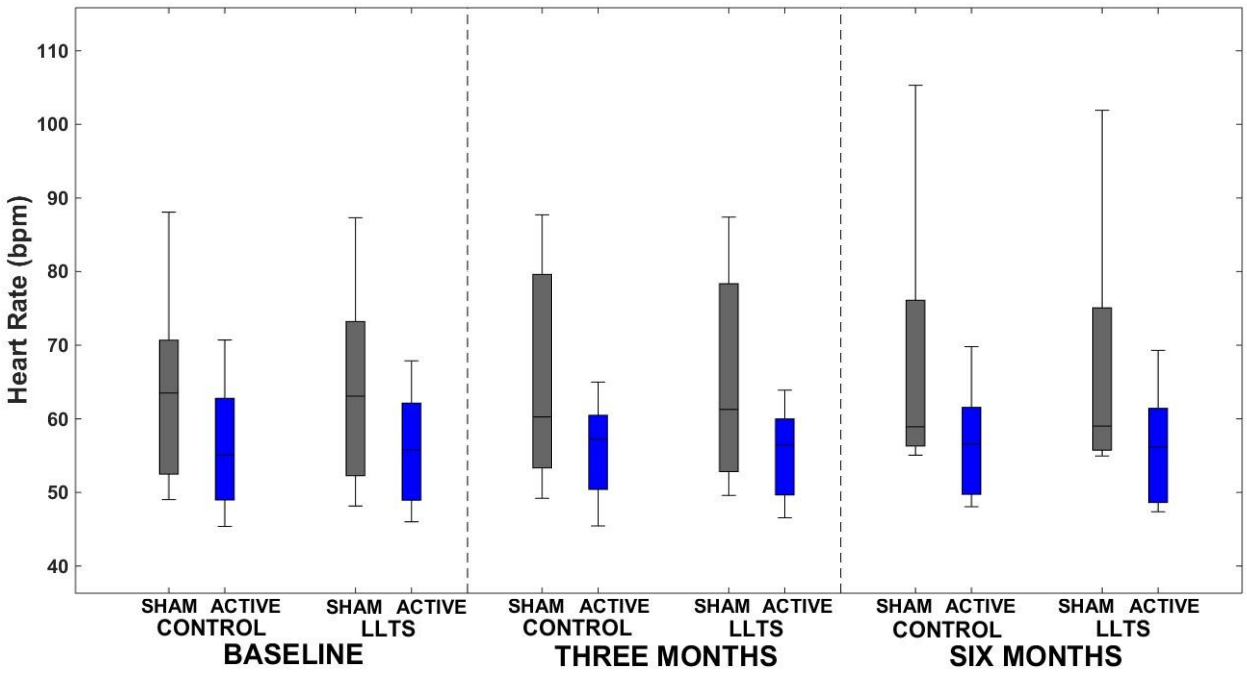
Figures OS13, OS14 and OS15 demonstrate summary results of chronic LLTS on ΔPWA voltage, ΔPWA K_{score} and ΔPWA burden across all active and sham patients, during control (no tragus stimulation) and LLTS respectively. While no significant effect of LLTS was observed in sham patients, the active group exhibited significantly lower Δalternans, ΔK_{score} and ΔPWA burden values during LLTS as compared to control.

Identifying Early Markers of Effective LLTS Treatment

Active patients were categorized into two groups based on the effect of chronic LLTS: (A1) Patients demonstrating a drop in ΔPWA voltage and K_{score} with LLTS compared to control, and (A2) Patients with no drop in ΔPWA voltage and K_{score} with LLTS compared to control, after 3 or 6 months of chronic LLTS (Figures OS16A and OS16B, respectively).

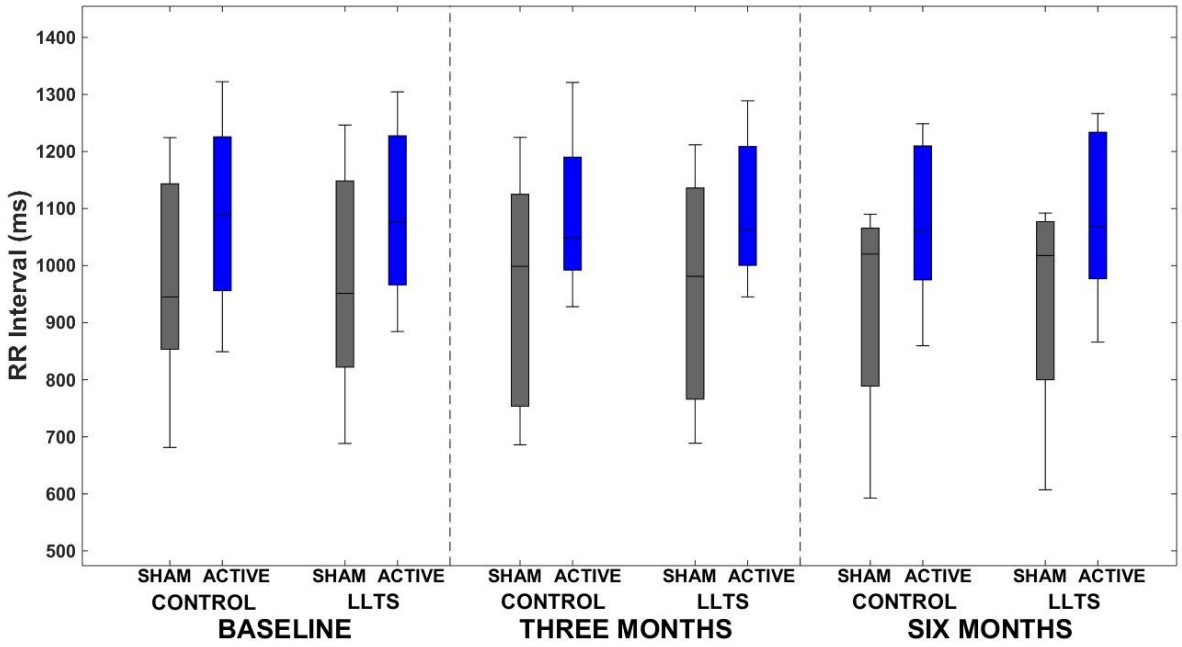
After categorizing the active patients into the two groups, for each group, the acute (at baseline) effect of LLTS on PWA voltage and K_{score} was investigated. In Figures OS16A and OS16B, we observe that active patients who demonstrate a drop in PWA voltage and K_{score} after either 3 or 6 months of chronic LLTS, show an early response to acute LLTS at baseline as well. Specifically, group A1 demonstrates an increase in PWA voltage and K_{score} with acute LLTS, while group A2 shows no change in these parameters with acute LLTS.

Figure S1. Summary results of low level tragus stimulation (LLTS) on Heart Rate across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



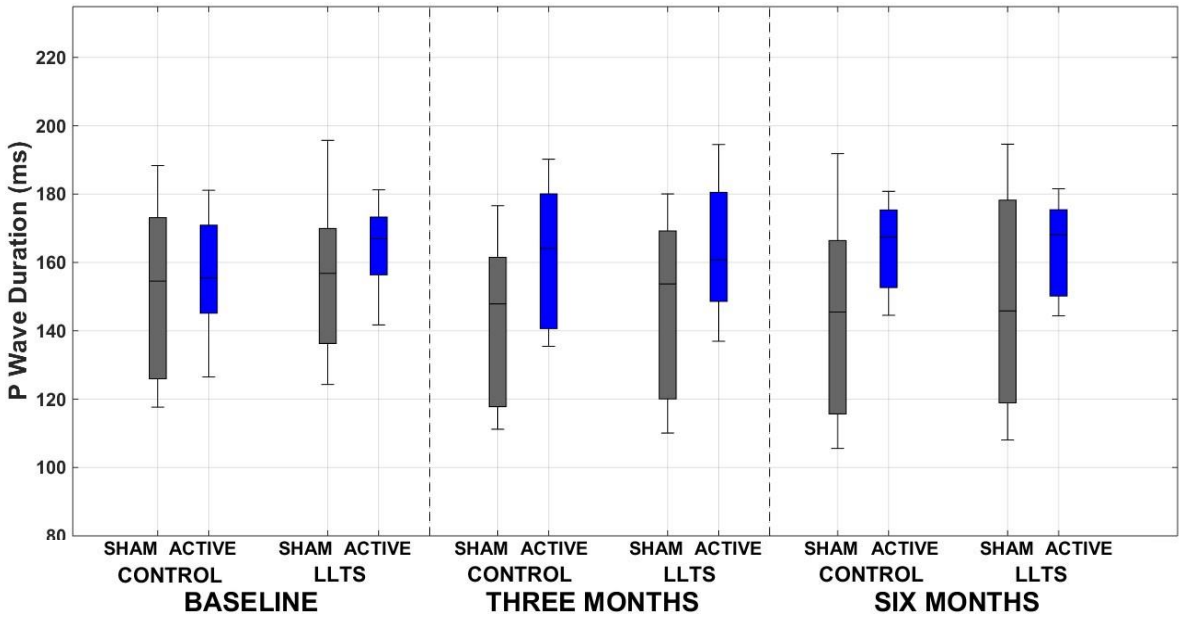
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Heart rate during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S2. Summary results of low level tragus stimulation (LLTS) on RR Interval across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



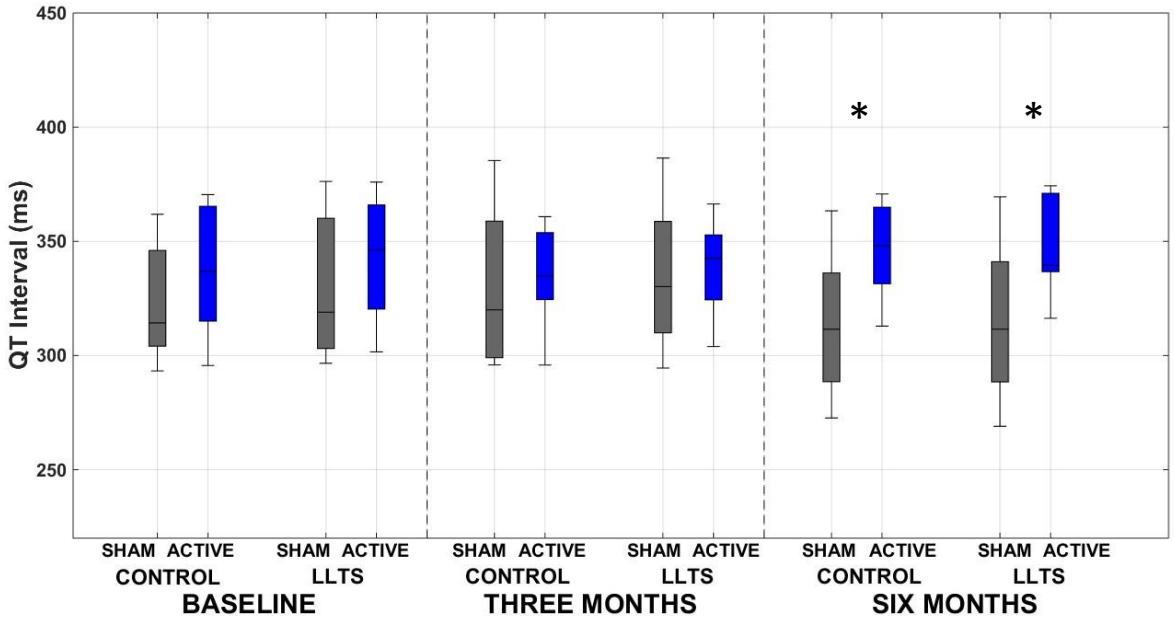
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). RR interval during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S3. Summary results of low level tragus stimulation (LLTS) on P-wave duration across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



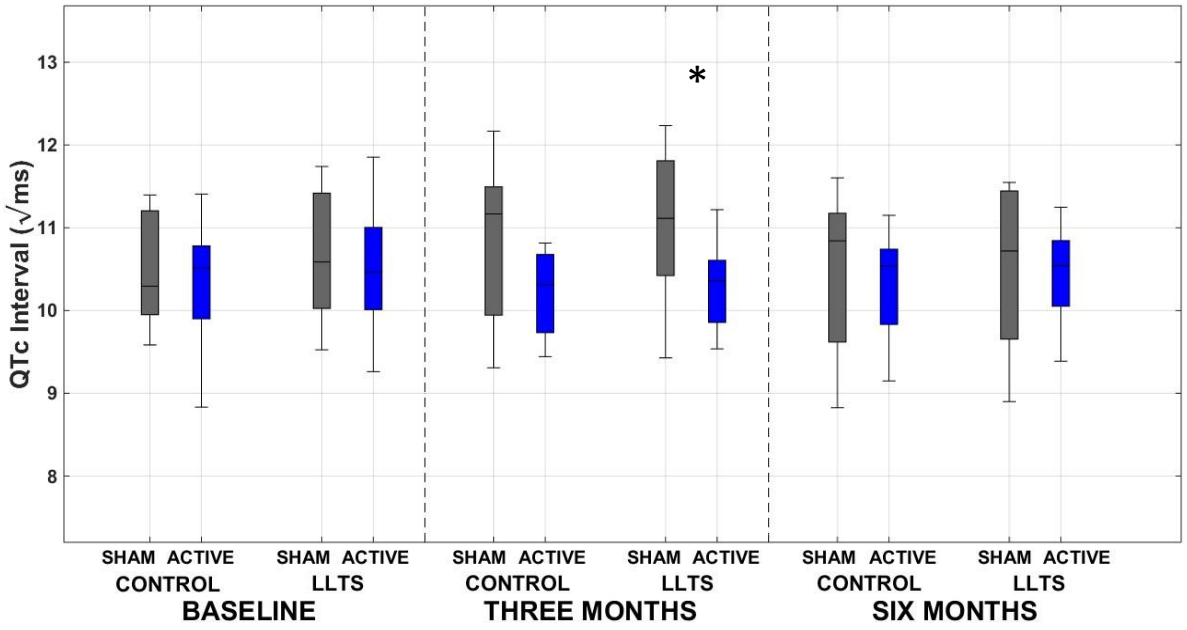
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). P-wave duration during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively.

Figure S4. Summary results of low level tragus stimulation (LLTS) on QT-interval duration across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



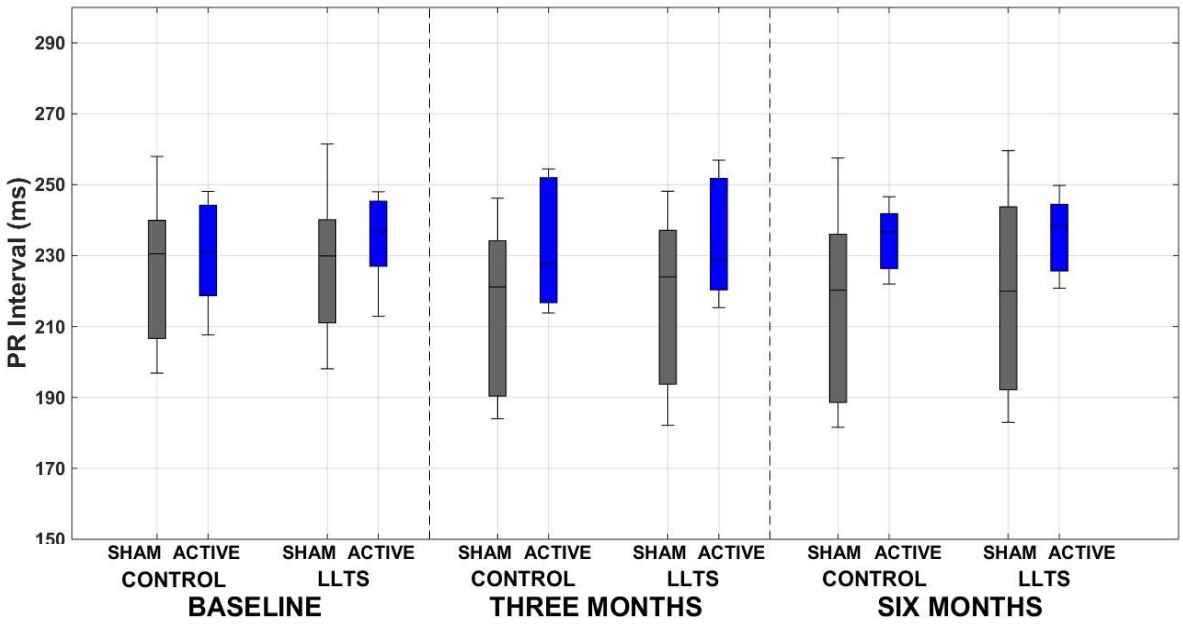
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). QT interval during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. '*' denotes statistical significance of $p < 0.05$, using 1-way ANOVA

Figure S5. Summary results of low level tragus stimulation (LLTS) on QTc-interval duration across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



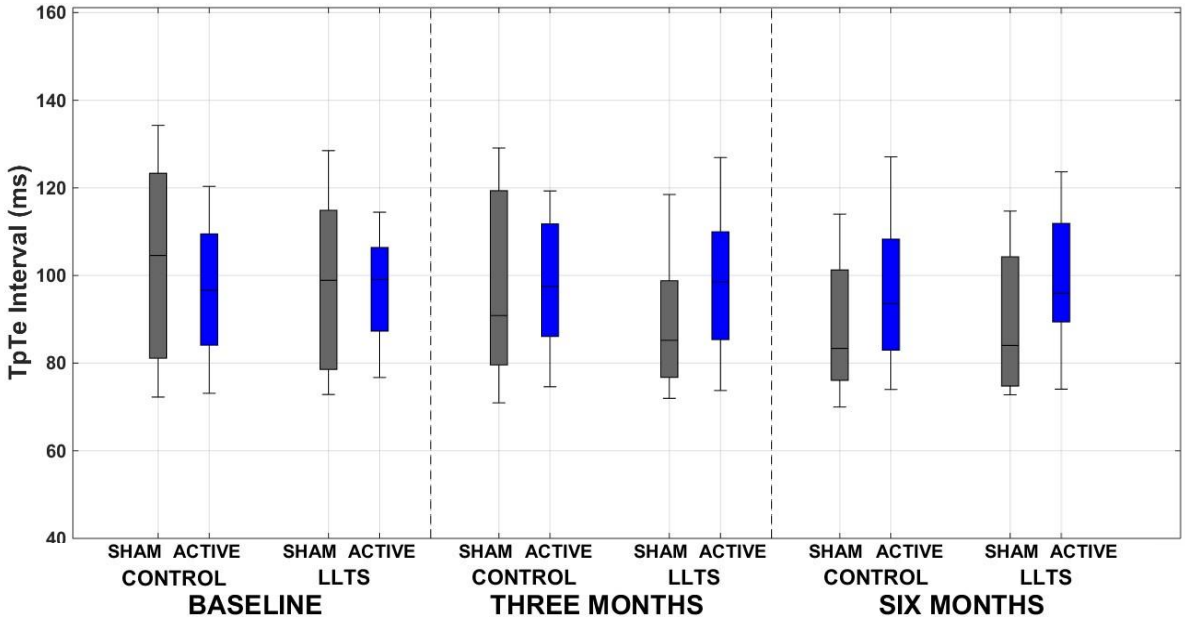
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). QTc interval during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. '*' denotes statistical significance of $p < 0.05$, using Kruskal-Wallis

Figure S6. Summary results of low level tragus stimulation (LLTS) on PR-interval duration across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



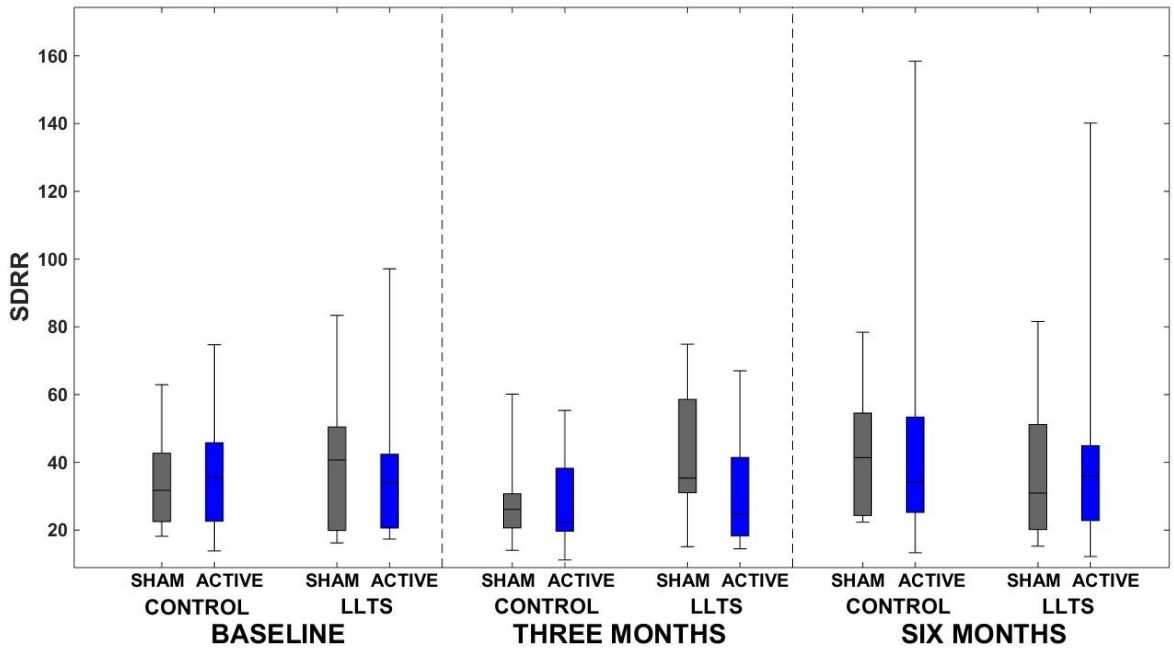
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). PR interval during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively.

Figure S7. Summary results of low level tragus stimulation (LLTS) on T_{peak}-T_{end} duration across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



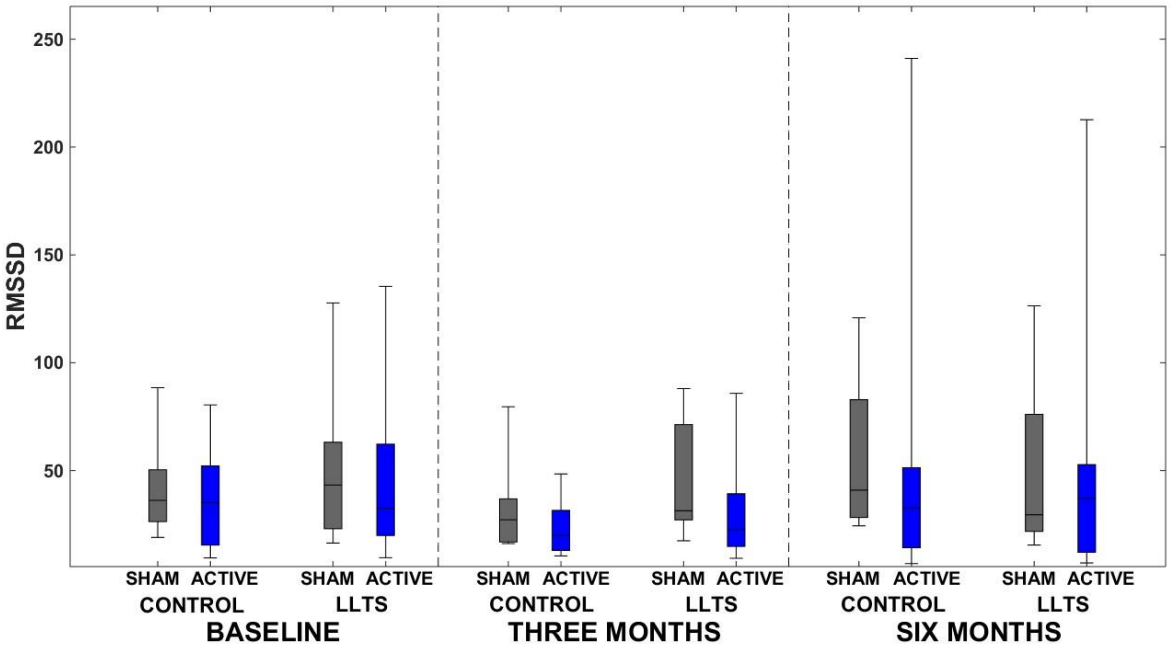
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). T_{peak}-T_{end} duration during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively.

Figure S8. Summary results of low level tragus stimulation (LLTS) on SDRR across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



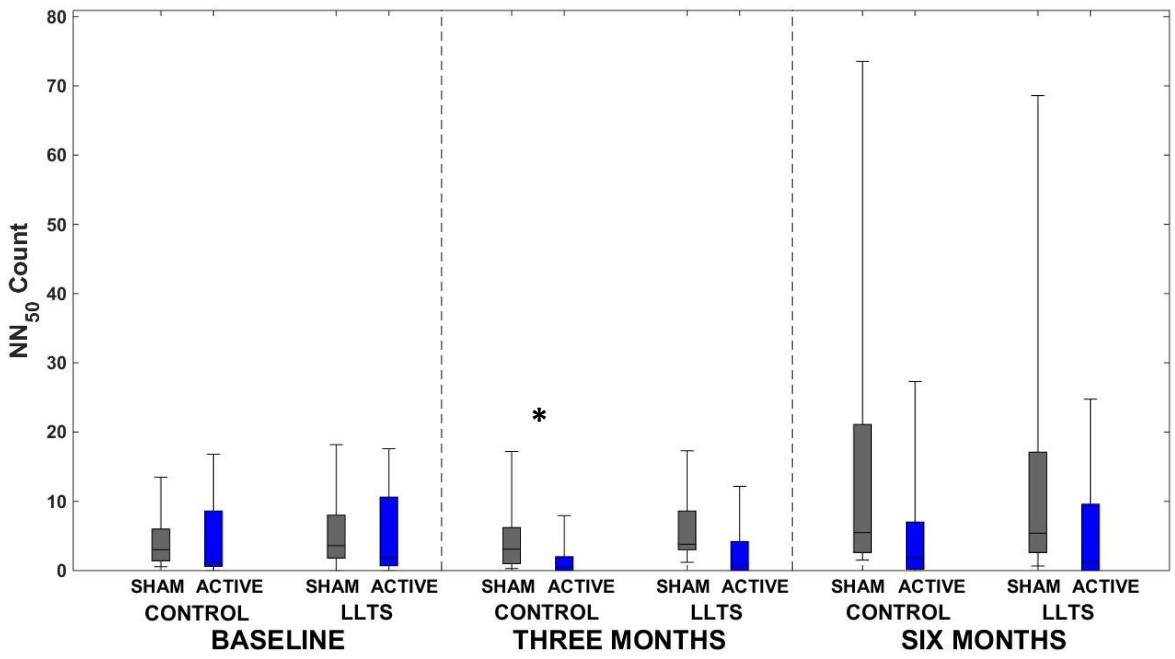
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Heart rate during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S9. Summary results of low level tragus stimulation (LLTS) on RMSSD across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



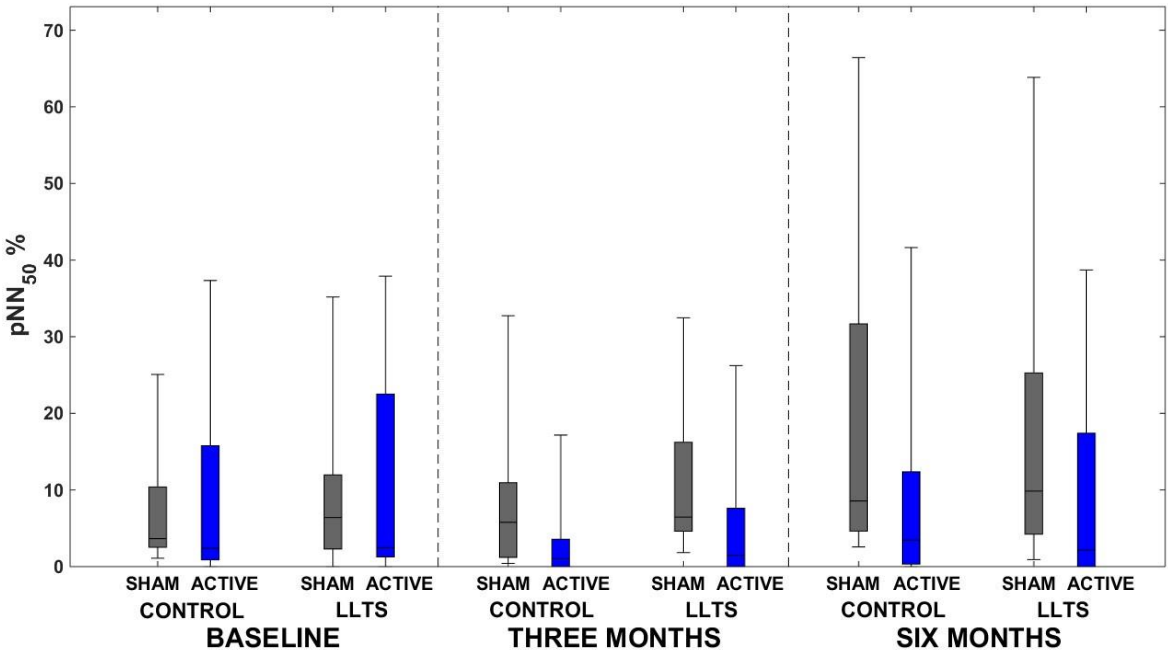
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Heart rate during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S10. Summary results of low level tragus stimulation (LLTS) on NN_{50} Count across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



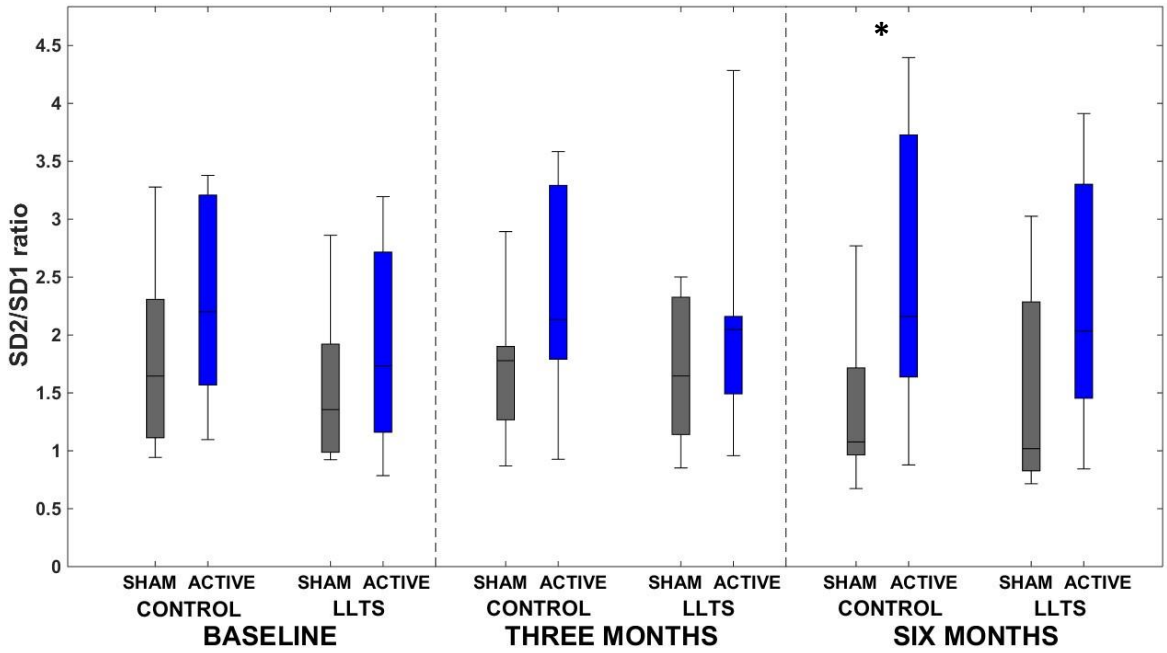
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Heart rate during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis. '*' denotes statistical significance of $p < 0.05$, using Kruskal-Wallis

Figure S11. Summary results of low level tragus stimulation (LLTS) on pNN_{50}% across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



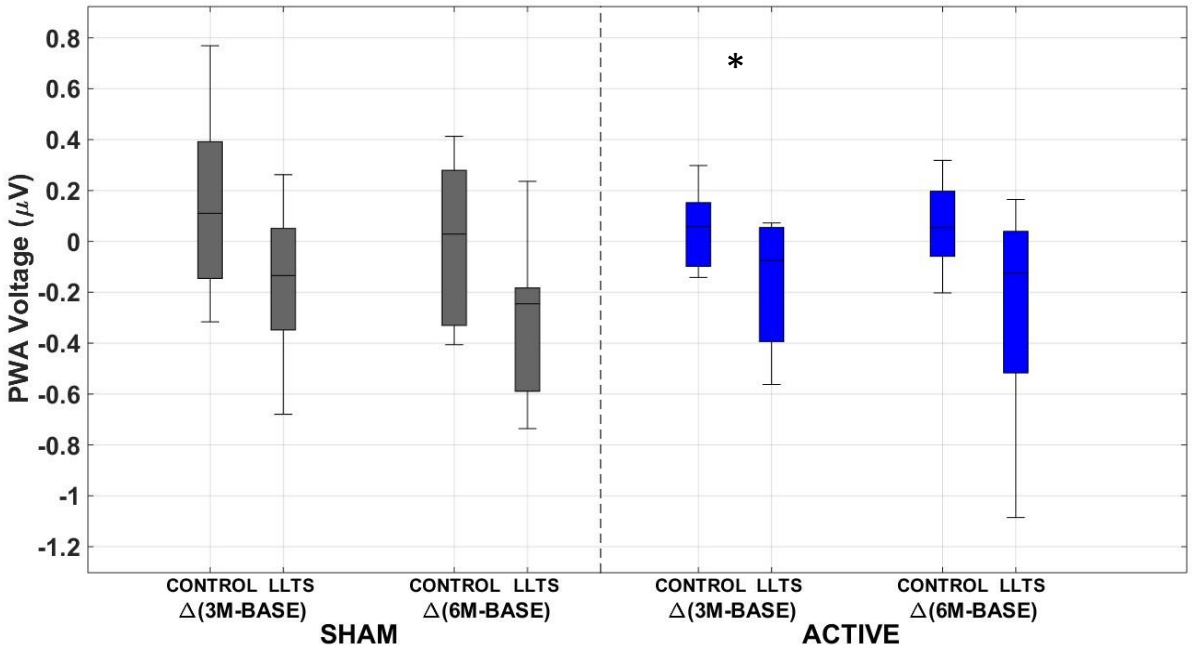
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Heart rate during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S12. Summary results of low level tragus stimulation (LLTS) on SD2/SD1 ratio across all active (with chronic LLTS) and sham (no chronic LLTS) patients.



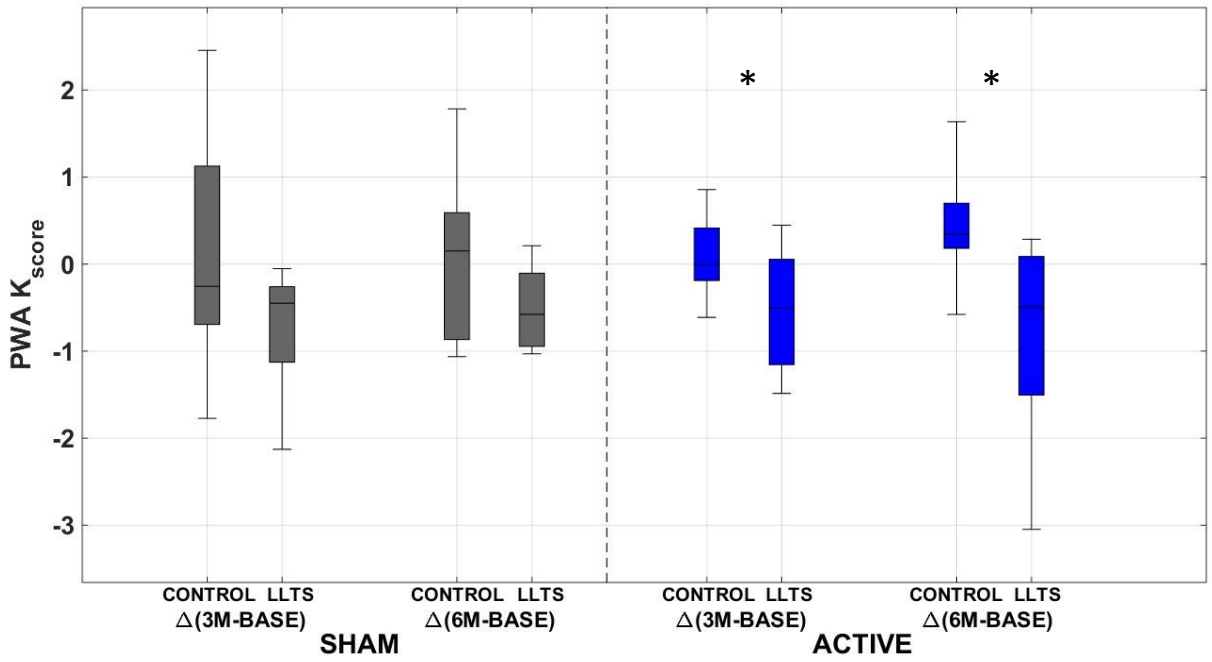
Data are presented as median (horizontal solid line), 75-25% percentiles (box) and 90-10% percentiles (error bars). Heart rate during control (no tragus stimulation) and LLTS is compared between sham and active patients at three time points: baseline, 3 months and 6 months. Sample sizes are sham (n = 12, 10, 8) and active (n= 15, 14, 14) for baseline, 3 months and 6 months, respectively. Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis. '*' denotes statistical significance of $p < 0.05$, using Kruskal-Wallis.

Figure S13. Summary results of chronic low level tragus stimulation (LLTS) on ΔP-wave alternans (ΔPWA) voltage across all active (with chronic LLTS) and sham (no chronic LLTS) patients, during control (no LLTS) and LLTS.



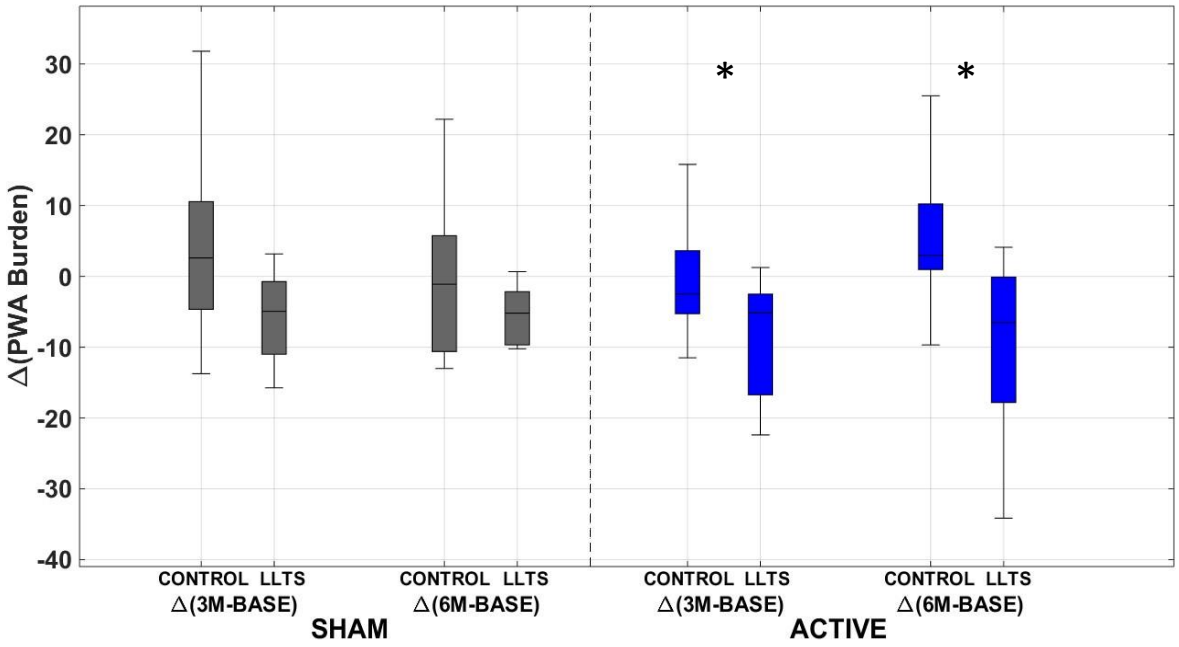
ΔPWA values are calculated between the three time points: 3 months-baseline Δ(3M-BASE) and 6 months-baseline Δ(6M-BASE). Δalternans voltage during control and LLTS is compared between sham and active patients. Statistical comparison was performed using 1-way ANOVA. '*' denotes statistical significance of $p < 0.05$. Sample sizes are sham: Δ(3M-BASE) control (n = 9), Δ(3M-BASE) LLTS (n = 9), Δ(6M-BASE) control (n = 7), Δ(6M-BASE) LLTS (n = 6); and active: Δ(3M-BASE) control (n = 11), Δ(3M-BASE) LLTS (n = 10), Δ(6M-BASE) control (n = 10), Δ(6M-BASE) LLTS (n = 8). Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S14. Summary results of chronic low level tragus stimulation (LLTS) on ΔP-wave alternans (ΔPWA) K_{score} across all active (with chronic LLTS) and sham (no chronic LLTS) patients, during control (no LLTS) and LLTS.



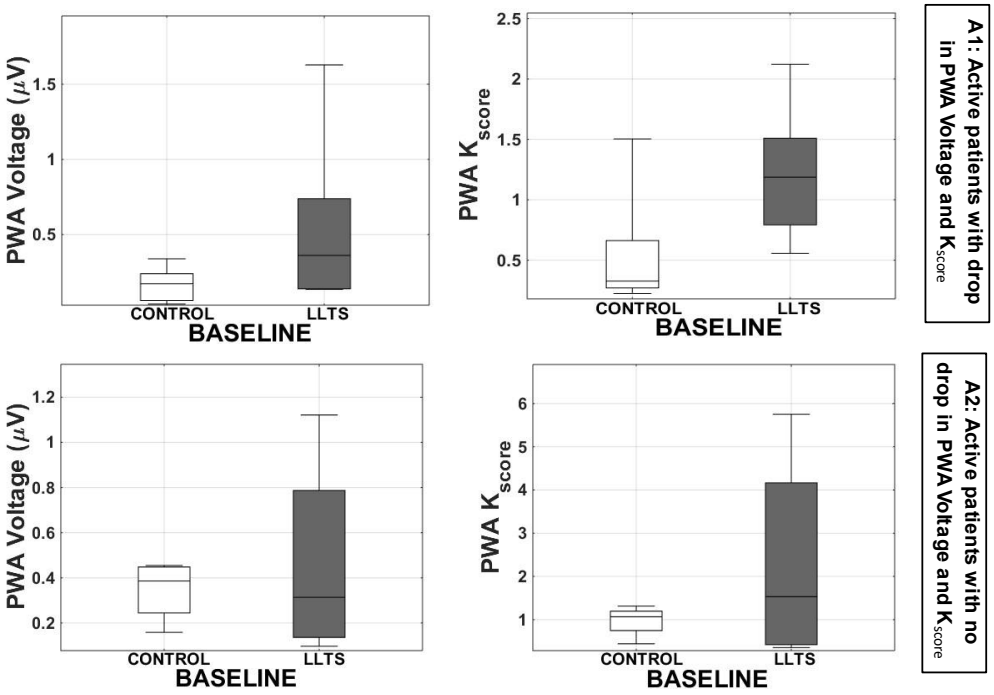
ΔPWA values are calculated between the three time points: 3 months-baseline Δ(3M-BASE) and 6 months-baseline Δ(6M-BASE). ΔPWA K_{score} during control and LLTS is compared between sham and active patients. Statistical comparison was performed using 1-way ANOVA. '*' denotes statistical significance of $p < 0.05$. Sample sizes are sham: Δ(3M-BASE) control (n = 9), Δ(3M-BASE) LLTS (n = 9), Δ(6M-BASE) control (n = 7), Δ(6M-BASE) LLTS (n = 6); and active: Δ(3M-BASE) control (n = 11), Δ(3M-BASE) LLTS (n = 10), Δ(6M-BASE) control (n = 10), Δ(6M-BASE) LLTS (n = 8). Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S15. Summary results of chronic low level tragus stimulation (LLTS) on ΔP-Wave alternans (ΔPWA) burden across all active (with chronic LLTS) and sham (no chronic LLTS) patients, during control (no tragus stimulation) and LLTS.



ΔPWA burden values are calculated between the three time points: 3 months-baseline Δ(3M-BASE) and 6 months-baseline Δ(6M-BASE). ΔPWA burden during control and LLTS is compared between sham and active patients. 1-way ANOVA was used for statistical comparison. '*' denotes statistical significance of $p < 0.05$. Sample sizes are sham: Δ(3M-BASE) control (n = 9), Δ(3M-BASE) LLTS (n = 9), Δ(6M-BASE) control (n = 7), Δ(6M-BASE) LLTS (n = 6); and active: Δ(3M-BASE) control (n = 11), Δ(3M-BASE) LLTS (n = 10), Δ(6M-BASE) control (n = 10), Δ(6M-BASE) LLTS (n = 8). Patients that experienced episodes of AF during ECG recording or had atrial pacing, AV pacing or junctional rhythm were excluded from the analysis.

Figure S16 (A) active patients are categorized into two groups based on effect of chronic LLTS: (A1; n=5) Patients demonstrating a drop in ΔP-Wave alternans (ΔPWA) voltage and K_{score} with LLTS compared to control (no tragus stimulation), and (A2; n=4) patients with no drop in ΔPWA voltage and/or K_{score} with LLTS compared to control, after three months of chronic LLTS.



(B) active patients are categorized into two groups based on effect of chronic LLTS: (A1; n=6) Patients demonstrating a drop in ΔPWA voltage and K_{score} with LLTS compared to control, and (A2; n=2) Patients with no drop in ΔPWA voltage and/or K_{score} with LLTS compared to control, after six months of chronic LLTS. For both LLTS and control, delta values are calculated using mean PWA voltage and K_{score} at 3 months (or 6 months) and baseline, Δ(3M-BASE) (or Δ(6M-BASE)). For each group, acute (at baseline) effect of LLTS on PWA voltage and K_{score} is observed. Kruskal-Wallis test was used for comparison and '*' denotes statistical significance of $p < 0.05$.