**Chronic low-level vagus nerve stimulation improves long-term survival in salt-sensitive hypertensive rats**

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Chronic hypertension (HTN) affects more than 1 billion people worldwide, and is associated with dramatically increased risk of cardiovascular disease. Despite decades of promising research, effective treatment of HTN remains challenging. Our work investigates vagus nerve stimulation (VNS) as a novel device-based therapy for HTN, and specifically evaluates the effects of VNS on long-term survival.

HTN was induced in Dahl salt-sensitive rats using a high-salt diet. Rats were implanted with cervical VNS systems and physiological recorders that acquired real-time ECG and ABP, and randomized to VNS and Sham. After 6 weeks of high-salt exposure, VNS therapy was initiated for up to 8 weeks. The endpoint for this study was a composite of all-cause death or occurrence of a serious HTN-related adverse event.

The in-vivo ECG analysis shows significant attenuation in spontaneous elevation of ABP after three weeks of VNS therapy. In addition, there is significant improvement in survival of the VNS-treated rats. Median survival improved 106%, from 17 days (Sham) to 35 days (VNS). When accounting for the influence of baseline blood pressure and heart rate on survival, VNS treatment still significantly increased survival. These results suggest that VNS has the potential to improve outcomes in subjects with severe HTN.

## I. Introduction

Hypertension (HTN) affects over 1 billion people worldwide and is the most prominent risk factor for cardiovascular disease (1, 2). In addition, these cardiovascular complications due to HTN result in over 9 million deaths annually (3). Left uncontrolled, high blood pressure (BP) can lead to remodeling of the heart, including left atrial dilation, left ventricular hypertrophy, and impaired ventricular relaxation, increasing the risk of heart failure (4). These changes make the heart more susceptible to cardiac arrhythmias, which ultimately influence the morbidity, mortality, and quality of life of patients with chronic HTN. The need to control the rise in BP while also addressing the negative effects of complex co-morbidities makes the clinical management of HTN challenging.

Currently, the majority of HTN patients are treated with antihypertensive drugs to control BP, but many limitations exist including resistant HTN, inability to tolerate therapy, and non-compliance with the medication regime (5-7). There is a need for a novel treatment option for the drug-resistant HTN patients that can also address the adverse cardiac effects of HTN. One target of new treatments is altered sympatho-vagal balance, which has been reported in HTN patients as a significant contributor to disease progression (8-10). Specifically, this typically manifests as an increase in sympathetic and a decrease in parasympathetic activities, and has been the target of novel therapies for HTN. Several of these new therapies primarily target the sympathetic nervous system with the goal of restoring balance in the autonomic nervous system. For instance, baroreflex activation therapy (BAT) indirectly suppresses sympathetic activity through activation of the arterial baroreceptor reflex, and renal denervation is an organ specific ablation approach to inhibit treat sympathetic activity to the kidney. However, both these therapies have had variable success in controlling BP in HTN patients (11-14).

Recently, we proposed to specifically target the parasympathetic nervous system using vagus nerve stimulation (VNS), and evaluated the efficacy of four weeks of VNS treatment for HTN and HTN-induced heart disease in a rat model of HTN (15). The aim of this study is to investigate the effects of low-level intermittent chronic VNS therapy on the long-term survival in HTN rats.

## II. Results

*Disease Progression*

The baseline parameters, measured prior to the start of therapy, are shown in Table 1 for Sham and VNS-treated rats, indicating no significant differences between the two groups. Average baseline HR for Sham was 371 ± 7 bpm, and for VNS, the average HR was 379 ± 9 bpm (p = N/S). MAP between groups were also similar with baseline values of 163 ± 11 mmHg and 160 ± 8 mmHg for Sham and VNS rats respectively (p = N/S).

Twenty four-hour averages of HR and BP parameters from Week 9 (21 days after the start of VNS therapy) are also shown in Table 1. By Week 9, we observed a significant reduction in the rise in systolic BP (Sham: 244 ± 7 mmHg; VNS: 224 ± 2 mmHg; $p < 0.05$), MAP (Sham: 207 ± 9 mmHg; VNS: 190 ± 3 mmHg; $p < 0.05$), and pulse pressure (Sham: 72 ± 4 mmHg; VNS: 62 ± 2 mmHg; $p < 0.05$) in the VNS-treated rats in comparison to the Sham rats. All other parameters were not significantly different between groups at Week 9.

*Long-term Survival*

Kaplan-Meier event-free survival curves for the Sham and VNS rats are shown in Figure 1A, showing a significant difference between the cumulative event-free survival of the two groups ($p < 0.05$). The dashed line indicates the start of VNS therapy at Week 6, and table below the event-free survival curve indicates the number of rats remaining at each time point. Figure 1B shows individual survival data for Sham and VNS-treated rats, indicating difference in the lifespan between two groups. The Sham rats were the first to reach the endpoint of this study with median survival of 17 (95% CI: 12 to 27) days, in contrast to the VNS-treated rats with median survival of 35 (95% CI: 23 to 42) days. In addition, two of the 6 VNS-treated rats survived to Day 56 of VNS therapy, reaching the end of the 14-week *in-vivo* protocol.

Figures 1C and 1D show the dependence of survival time on baseline MAP and HR respectively. Both VNS and Sham rats observed similar variations in MAP and HR values at baseline, however the survival effect of VNS therapy was independent of these baseline values. Table 2 shows the results of the

Cox proportional hazards regression investigating the influence of baseline MAP, baseline HR, and VNS therapy on event-free survival. The results indicate that only VNS therapy is protective and associated with improved outcome for the HTN rats, with statistically significant hazard ratio0.034 (95% CI: 0.0026-0.43), while the other two factors did not significantly impact the long-term event-free survival.

## III. Discussion

HTN remains a significant problem around the world and is accompanied with a significant increase in the risk of serious cardiovascular complications if left untreated. VNS, a novel device-based therapy, has the potential to provide an option for patients who are not responding to current pharmaceutical approaches or are unable to tolerate their side effects.

The major finding of this study was a significant improvement in the long-term survival of HTN rats treated with VNS therapy, with median event-free survival more than doubling for VNS-treated rats in comparison to Sham rats. Furthermore, the impact of VNS therapy on survival remained significant and independent of variations in baseline MAP and HR. This improvement in long-term survival could be in part due to the anti-arrhythmic electrophysiological remodeling of the hearts observed in the VNS rats over the course of therapy, as was indicated in our previous study (15). In addition, the attenuation in the rising systolic BP and MAP by Week 9, after only 3 weeks of therapy, may play a role in the delay in the onset of adverse events in HTN rats.

VNS is hypothesized to provide therapeutic effects by targeting the autonomic dysregulation evident in HTN. It is postulated that stimulation of the parasympathetic nervous system, through right cervical VNS, has the potential to rebalance the autonomic nervous system by, in addition to direct stimulation of vagal outflow, reflexively reducing sympathetic activity and increasing parasympathetic activity (16, 17). In addition, Ardell et al. recently demonstrated that VNS was able to reduce HTN-induced cardiac remodeling, specifically through a reduction in left ventricular (LV) hypertrophy (18).

Previous studies have shown the direct effect of VNS on BP in both acute and chronic studies. Several investigators have shown that through stimulation of the vagus nerve, acute drops in BP can be

observed, resulting in varying degrees of BP reduction based on the stimulation parameters (19, 20). This acute drop is mediated through activation of the aortic depressor nerve which sends signals to the brain resulting in the activation of the baroreflex and a decrease in BP (19). In addition, our previous chronic study demonstrated the impact of low-level intermittent VNS on BP through a long-term effect resulting in a reduction the rise in BP after 4 weeks of therapy in HTN rats (15). In both applications of acute and chronic VNS, significant side effects such as bradycardia and bradypnea could be avoided with appropriate stimulation parameters, demonstrating safety for VNS therapy (19, 20).

In addition to BP effects, numerous studies have shown beneficial cardiovascular effects of VNS including altering electrophysiological properties of the heart and suppression of ventricular arrhythmias (15, 21-23) presumably via activation of muscarinic receptor systems. VNS could also be positively influencing the survival rates through improvements in ventricular function and a reduction in structural remodeling. Li et al. and Zhang et al. have shown improvement in LV function through lowering LV pressures and improved ejection fraction and contractility (17, 24). Beaumont et al. recently demonstrated a reduction in LV hypertrophy in a guinea pig model of pressure overload treated with right cervical VNS (25). In addition, VNS is proposed to alter autonomic control, suppressing sympathetic activation and enhancing vagal activity. Indirect measures of autonomic balance, such as HRV and baroreceptor reflex sensitivity, have been assessed in numerous studies, showing a restoration of autonomic balance and improved prognostic indications for the VNS treated group (16, 17).

Although investigating the mechanisms of VNS was not the purpose of this study, the results warrant a discussion of additional mechanisms that could potentially contribute to improved survival in HTN rats. The hypothesized mechanism through which VNS provides therapeutic effects includes anti-adrenergic effects, anti-inflammatory pathways, activation of nitric oxide release in the heart, and inhibition of the renin-angiotensin system. Suppressing sympathetic activity through activation of vagal nerve afferent fibers can lead to a reduction in sympathetic nervous system activity resulting in reductions in HR, vascular resistance, and ABP. Several studies have demonstrated the ability of VNS to reduce systemic inflammation through the activation of the cholinergic anti-inflammatory pathway, reducing cytokine

synthesis and the inflammatory response (17, 26). Inflammation is associated with HTN, although the cause-and-effect relationship still remains unclear. It is hypothesized that inflammation can lead to endothelial dysfunction and increased oxidative stress, which promote the development and progression of HTN (27). VNS has also been shown to directly activate the nitric oxide pathway producing nitric oxide in the ventricles, altering cardiac electrophysiology and decreasing arrhythmia susceptibility (28). Another proposed mechanism of VNS is inhibition of the renin-angiotensin system through afferent vagal nerves resulting in a reduction in renin in the kidneys and marked reduction in plasm angiotensin II levels (17, 29).

Overall, our results are consistent with previous studies that show potential for VNS to provide therapy for HTN and HTN-induced heart disease. The results of this study show a strong positive effect on survival. We will continue this work by increasing the number of animals and studying varying stages of HTN. Additional studies are necessary to investigate the mechanism of VNS in treating HTN and the optimal stimulation parameters for therapeutic effects.

## IV. Methods

All experiments conform to the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and the University of Minnesota guidelines for the care and use of animals. Prophylactic antibiotic (gentamicin sulphate; 10 mg/kg, i.m.) was given prior to each surgery. During the surgeries, rats were anaesthetized with isoflurane (5% for induction, 2% or 3.5% for maintenance) in oxygen (2 L/min for induction, 1 L/min for maintenance). The rat's body temperature was maintained at 37ºC on a temperature-controlled surgery table.

### *Experimental Model of Hypertension*

Five week old male Dahl salt-sensitive rats (n=12), from Charles River Laboratories (Wilmington, MA) were used in this study. The rats were fed a high salt (8% NaCl) diet to induce HTN, and this diet was maintained for the duration of the study. The 8% NaCl diet was chosen to investigate rats in an advanced stage of HTN with increased risk of stroke. Four weeks after initiating the high salt diet, the

HTN rats were implanted with vagus nerve stimulators and physiological telemetry devices (HD S11, DSI Inc., St. Paul, MN) and divided into two groups: VNS (n = 6; functional VNS stimulators) and Sham (n = 6; non-functional VNS stimulators). At Week 6, the low-level VNS therapy was activated and applied for up to 8 weeks or until endpoints (death or severe HTN-related events) were achieved. The experimental timeline for this study is shown in Figure 2. During 8 weeks of therapy, real-time ECG and BP recordings were captured through the DSI telemeters for both Sham and VNS rats.

*Vagus Nerve Stimulator Implantation*

The VNS pulse generator and cuff electrodes were implanted as described previously (30, 31). Briefly, the surgical regions (back and the neck) were shaved. Biopolar cuff electrodes were implanted through a small incision on the neck and placed around the right cervical vagus nerve and common carotid artery bundle. The pulse generator (8 cc, 14 g, Model 103, Cyberonics Inc.) was implanted subcutaneously and positioned on the back of the rat. VNS was applied with a pulse frequency of 20 Hz, a pulse width of 500 μs, and an output current of 0.25 - 1.0 mA. The amplitude of the current was selected based on a stimulation level of sufficient strength to appear on the ECG but did not cause an acute peak reduction in heart rate (HR) greater than 10% during the active phase of the stimulation cycle. Each VNS cycle consisted of an active phase (18 sec) followed by an inactive phase (66 sec), resulting in a duty cycle of 21%.

*DSI Telemeter Implantation*

DSI transmitters were implanted as described previously (32). Briefly, the chest and inner thigh were shaved and the transmitter pressure catheter was implanted through a small incision on the inner left hind limb into the descending aorta via the left femoral artery. The two ECG leads were fixed subdermally on the chest muscle. After surgery, the rats were individually housed in cages where they were able to freely move about while receivers (model RPC1, DSI Inc.) connected to a computer via a Data Exchange Matrix

(MX2, DSI Inc.) to continuously collect data from each rat. The ECG and arterial BP data were collected at a sampling rate of 500 Hz throughout the study.

*Disease Progression*

Real-time ECG and BP data acquired from the physiological telemeter (DSI Inc.) was collected daily and averaged over 24-hour periods. Daily BP and HR values, including systolic BP, diastolic BP, mean arterial pressure (MAP), pulse pressure, HR, and heart rate variability (HRV), are collected and monitored throughout the study. HRV was defined as $HRV = \sigma^2_{HR} / \overline{HR}$, where $\sigma^2_{HR}$ is standard deviation, $\overline{HR}$ is mean over a 3-day period for baseline measures and a 24-hour period for assessing disease progress at Week 9.

Baseline parameters for the HTN rats were defined as the three-day average prior to the start of VNS therapy at Week 6. These baseline BP and HR values are used in conjunction with the treatment group to determine which factors significantly influence the long-term survival of the HTN rats. These parameters were assessed at Week 9, Day 21 of VNS therapy, between groups. Week 9 parameters were calculated as a 24-hour average, including one full light-dark cycle from 19:00 to 19:00 the following day.

*Event-Free Survival Assessment*

The 14-week survival rate was calculated to assess the effect of VNS on the event-free survival (death or HTN-related serious adverse event) of HTN rats. Throughout the duration of the *in-vivo* protocol, daily checks were performed to evaluate the health of the rats to determine if the rat had died or experienced a HTN-related serious adverse event such as a stroke or hematoma. The event-free survival times were recorded in days relative to the start of therapy beginning at Week 6.

*Data Analysis and Statistics*

BP and HR data are presented as mean ± standard error. Statistical comparisons between Sham and VNS rats were performed using one-way ANOVA. Cumulative event-free survival was presented using Kaplan-Meier curves. To determine statistical significance, the two curves were compared using the log rank test. Cox regression for event-free survival analysis was completed to assess the influence of baseline parameters and treatment on the survival of the HTN rats. A p-value of 0.05 was considered to be statistically significant.

*Data Availability*

The data collected and analyzed during this study are available from the corresponding author upon request.

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**Author Contributions:**
E.M.A. designed and performed experiments, completed the data collection and analysis, and wrote the manuscript. D.V.H. performed experiments including surgeries and animal care. I.L., B.H.K, and J.W.O. supervised the project and review the manuscript. E.G.T. designed experiments, supervised data analysis and assisted in preparation of the manuscript. All authors reviewed the manuscript.

**Competing Financial Interests:**
Drs. Libbus and KenKnight are employees of LivaNova PLC.

**Figure Legends:**

**Figure 1.** (A) Kaplan-Meier event-free survival curves for Sham and VNS rats. The dashed line at 0 indicates the start of VNS therapy at Week 6. The number of rats remaining in each group is included along the x-axis. All 6 Sham rats reach an end point by Day 33 of stimulation (during Week 10). However, for the VNS treated group, 2 out of 6 rats survive to the end of the 8-week therapy (stars). VNS long-term survival rate is significantly increased ($P < 0.05$) compared to Sham treated rats. Event-free survival times in VNS and Sham rats vs. (B) baseline MAP and (C) baseline HR values measured as a three day average prior to the start of VNS therapy.

**Figure 2.** Experimental design. HTN was induced using a high salt diet (8% NaCl) for the first 4 weeks. At Week 4, the rats were implanted with VNS pulse generator and lead systems and DSI transmitters. The rats were divided into two groups: Sham (n = 6; non-functional VNS stimulators) and VNS (n=6; functional VNS stimulators). VNS therapy was turned on at Week 6 and remained on for up to 8 Weeks of therapy or until the rat met an end point of the study.

**Tables:**

**Table 1**

| Baseline Parameter | Sham (n = 6) | VNS (n = 6) | P-value |
|---|---|---|---|
| HR (bpm) | 371 +/- 7 | 379.0 +/- 9 | 0.25 |
| HRV (%) | 9.5 +/- 0.9 | 9.9 +/- 0.4 | 0.14 |
| Systolic BP (mmHg) | 196 +/- 12 | 190.1 +/- 10 | 0.75 |

| | | | |
|---|---|---|---|
| MAP (mmHg) | 163 +/- 11 | 159.7 +/- 8 | 0.84 |
| Diastolic BP (mmHg) | 134 +/- 11 | 131.8 +/- 7 | 0.94 |
| Pulse Pressure (mmHg) | 62 +/- 2 | 58.2 +/- 4 | 0.41 |
| **Week 9 Parameters** | **Sham (n = 3)** | **VNS (n = 5)** | **P-value** |
| HR (bpm) | 408 +/- 30 | 385 +/- 5 | 0.29 |
| HRV (%) | 8.0 +/- 0.2 | 9.3 +/- 0.5 | 0.27 |
| Systolic BP (mmHg) | 244 +/- 7 | 224 +/- 2 | 0.0053* |
| MAP (mmHg) | 207 +/- 9 | 191 +/- 3 | 0.038* |
| Diastolic BP (mmHg) | 179 +/- 14 | 161 +/- 3 | 0.11 |
| Pulse Pressure (mmHg) | 72 +/- 4 | 62 +/- 2 | 0.033* |

**Table 2**

| **Factor** | **Coefficient (b)** | **SE** | **P-value** | **Hazard Ratio (95% CI)** |
|---|---|---|---|---|
| VNS Therapy | -3.4 | 1.3 | 0.0093* | 0.034(0.0026-0.43) |
| Baseline MAP | 0.052 | 0.033 | 0.11 | 1.053(0.99-1.1) |
| Baseline HR | 0.043 | 0.033 | 0.19 | 1.044(0.98-1.1) |

**Table Legends:**

**Table 1.** Baseline parameters, presented as mean +/- standard error, for VNS and Sham rats (n = 6/group) calculated as a three day average prior to the start of VNS therapy. Week 9, Day 21 of VNS therapy, parameters for VNS (n = 5) and Sham (n = 3) rats calculated as a 24 hour average. * indicates statistical significance between Sham and VNS rats ($p < 0.05$)

**Table 2.** Cox proportional-hazards regression analysis to assess the impact of treatment group, baseline BP, and baseline HR on survival. * indicates statistical significance ($p < 0.05$)