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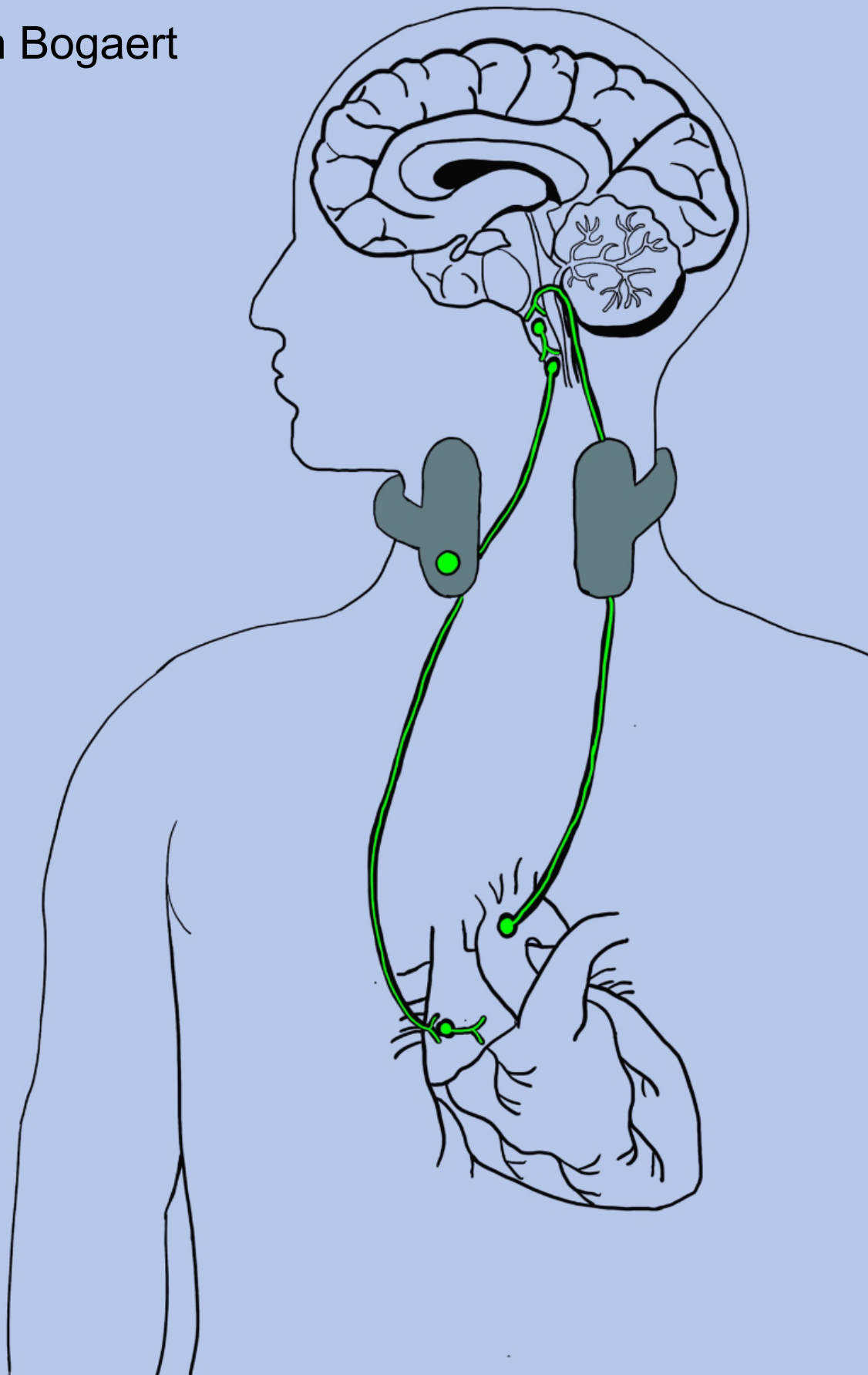

The effect of transcutaneous cervical vagus nerve stimulation on the cardiac autonomic nervous system

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Abstract

Autonomic imbalance, characterized by suppressed vagal activity and increased sympathetic activity significantly contribute to the development and progression of cardiovascular diseases. A non-invasive neuromodulation technique that may influence the cardiac autonomic nervous system (CANS) and restore autonomic imbalance is transcutaneous vagus nerve stimulation (tVNS). This thesis focuses on a novel cervical tVNS device (Pulsetto) which targets the vagus nerve through the neck. The aim of this research is to investigate the efficacy of this new device and provide insights into how cervical tVNS influences the CANS.

Two experiments were conducted: the first explored cervical tVNS in 8 atrial fibrillation (AF) patients, while the second involved 40 healthy participants, randomly assigned to either a stimulation group (n=30) or a sham group (n=10). Participants in the stimulation group received 10 minutes of stimulation. Heart rate variability (HRV) and cardiac conduction were measured via a 3-lead ECG, with data analysis focusing on HRV parameters, conduction intervals, and wave amplitude detection.

Significant HRV changes were observed during stimulation compared to pre-stimulation. Cervical tVNS significantly decreased mean HR ($P<0.001$) and LF/HF ($P=0.038$), while significantly increasing RMSSD ($P=0.001$), PNN50 ($P=0.001$) and HF power ($P=0.003$). Additionally, the QT interval and T-wave amplitude significantly increased ($P=0.001$ and $P=0.030$ respectively) in the stimulation group. None of these parameters changed in the sham group.

This thesis provides evidence that cervical tVNS can modulate cardiovascular autonomic control in healthy participants by increasing parasympathetic activity. Additionally, it is the first study to observe an increased T-wave amplitude during cervical tVNS, suggesting a novel effect on ventricular conduction. These insights indicate that cervical tVNS holds great potential for treating arrhythmias and other cardiovascular diseases.

Introduction

The autonomic nervous system (ANS) comprises a balanced interplay between the sympathetic and parasympathetic nervous systems. Imbalances within the ANS significantly contribute to the development and progression of various pathologies, particularly cardiovascular diseases (CVDs) such as rhythm disorders, heart failure, and hypertension. [1-3] CVDs, in turn, can further worsen the imbalance of the ANS, leading to a vicious cycle between autonomic dysregulation and cardiac disorders. Targeting the ANS has therefore been regarded as a crucial strategy to disrupt this vicious cycle. [4] Autonomic imbalance is characterized by suppressed vagal (parasympathetic) activity and increased sympathetic activity. There are several drugs available to restore this imbalance. However, pharmacological therapies targeting sympathetic overactivity often have numerous side effects, while those aiming to induce vagal activity demonstrate limited effectiveness. [5] This in combination with significant costs of pharmacological agents has led to increasing interest in new therapeutic approaches. Recently, non-invasive neuromodulation techniques have gained popularity in research due to their affordability, ease of use, portability and reduced risk of complications. Non-invasive neuromodulation techniques that influence the ANS include ultrasound stimulation, optogenetics, repetitive transcranial magnetic stimulation, light-emitting diode therapy and electromagnetic field (EMF) therapy. [6, 7]

The research unit Translational Electrophysiology at the Department of Cardiology of the Erasmus MC is studying another non-invasive neuromodulation technique: transcutaneous vagus nerve stimulation (tVNS). Since the vagus nerve plays an important role in regulating the ANS, stimulating the nerve may restore autonomic imbalance and potentially affect CVDs. Previous research has shown promising results for the treatment of arrhythmias, but the exact antiarrhythmic mechanisms of tVNS and the criteria for optimal patient selection remain largely unknown. [8] The researchers are therefore studying the effects of tVNS on atrial electrophysiology by performing intraoperative epicardial mapping during stimulation. tVNS is performed through the tragus of the right ear, where a branch of the vagus nerve is located. Although the initial results seem promising [9], there is significant variation in individual responsiveness. This variation might be due to anatomic differences and the fact that not everyone has a vagal nerve branch located at the tragus.

Recently, the unit Translational Electrophysiology received a new device (Pulsetto Device, Pulsetto). This device is a cervical transcutaneous vagus nerve stimulator that provides stimulation on the neck. Since every individual should have a cervical vagus nerve branch, this device may potentially overcome the issue of anatomical variation. Additionally, stimulating a different branch might lead to different effects. However, there is currently no clinical evidence for this device and there is limited knowledge about the effect of cervical tVNS on the cardiac ANS in literature.

Therefore, the aim of this thesis is to investigate the efficacy of this new device and to provide insights into how cervical tVNS influence the cardiac autonomic nervous system.

Background information

Autonomic nervous system

The autonomic nervous system (ANS) is the part of the peripheral nervous system that continuously controls involuntary physiological actions. The ANS functions below the level of consciousness and regulates processes such as heart rate, respiration rate, blood pressure and digestion. The ANS is primarily controlled by the hypothalamus and operates through a network of preganglionic and postganglionic neurons. Preganglionic neurons originate in the central nervous system (CNS) and synapse with postganglionic neurons that are located outside the CNS in autonomic ganglia. The postganglionic neurons then extend to the target organs. [10]

The ANS is divided into two main branches: the sympathetic (SNS) and parasympathetic (PSN) nervous systems. The SNS is activated during exercise and stressful situations and is therefore known as the 'fight or flight' response. The main function of the SNS is to prepare the body for physical activity by increasing well-oxygenated blood flow to the tissues that need it. This results in an increased heart rate and blood pressure, bronchodilation, pupil dilatation, sweating and inhibited digestion. In contrast, the PNS is activated during restful periods and therefore known as the 'rest and digest' response. It counteracts the SNS after a stressful event and restores the body to a state of calm. The PNS decreases heart and breathing rates, stimulates digestion and conserves energy. [11, 12]

Most organs receive simultaneous innervation from both the SNS and PNS, which are often considered to be antagonistic. The opposing effects are caused by the use of different neurotransmitters. In both systems, preganglionic neurons release acetylcholine, which binds to excitatory nicotinic receptors on the postganglionic neurons. However, the neurotransmitter released at the target tissues differ. Sympathetic postganglionic neurons release norepinephrine, while parasympathetic postganglionic neurons continue to release acetylcholine, as illustrated in Figure 1.

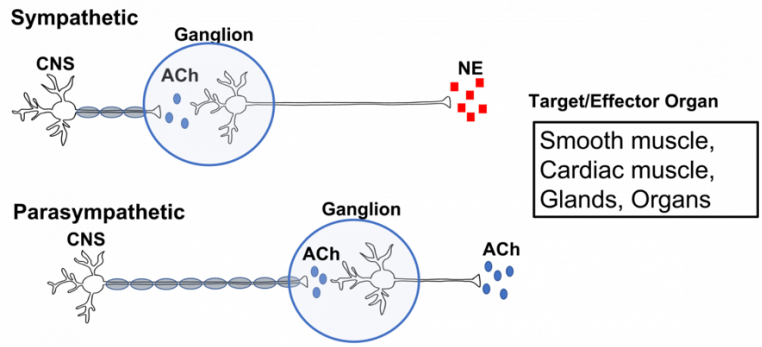

Figure 1 Illustration of the sympathetic and parasympathetic neurotransmitter of the autonomic nervous system. CNS = central nervous system, Ach = Acetylcholine, NE = Norepinephrine.

In healthy individuals, the PNS and SNS are in balance. However, factors such as chronic stress, medical conditions, medication, or lifestyle could disrupt this balance and lead to an overactive parasympathetic or sympathetic tone. This is called autonomic dysfunction or dysautonomia. Autonomic dysfunction can lead to several medical conditions, including cardiovascular diseases (CVDs), digestive disorders, respiratory issues, metabolic disorders and neurological conditions. [13, 14]

Cardiac autonomic nervous system

The heart has an intrinsic conduction system that initiates and coordinates the contraction of the heart muscle. The electrical impulse starts in the sinoatrial (SA) node (also known as the heart's natural pacemaker) and spreads throughout the heart via the atrioventricular (AV) node, the bundle of His and the Purkinje fibers. The conduction of electrical impulses in the heart induces the contraction of the surrounding cardiac muscle cells, causing the heart to beat. [15] However, with input from the intrinsic conduction system only, the fire rate of the SA will always be 100 beats per minute. To meet the body's oxygen demands it is important that the heart rate can vary under different circumstances. This is where the cardiac autonomic nervous system (CANS) plays a fundamental role.

The CANS does not only play a role in controlling the heart rate but also in modulating contractility, relaxation, conduction velocity, excitability and myocardial blood flow. It therefore influences both the electrophysiology and hemodynamics of the heart. The anatomy of the CANS is complex and can be divided into the intrinsic and extrinsic CANS. The extrinsic system consists of sympathetic and parasympathetic components. An overview of the extrinsic CANS is provided in Figure 2.

Sympathetic innervation originates from the upper thoracic segments of the spinal cord, specifically between T1 and T4. The preganglionic neurons arise from the lateral gray matter of the spinal cord and synapse onto postganglionic nerve fibers in the cervical or thoracic ganglia of the sympathetic chain. The cardiac cervical and thoracic nerves then travel to the heart alongside the epicardial vascular structures and innervate both the atrial and ventricular myocardium. The main effects of sympathetic innervation are an increased heart rate, increased conduction velocity and increased myocardial contractility. Parasympathetic innervation primarily originates from the dorsal motor nucleus of the medulla oblongata. The preganglionic fibers travel almost entirely within the vagus nerve and its intrathoracic branches. These fibers synapse in cardiac ganglia from where postganglionic nerves innervate the SA node and AV node. This results in a slower activity in the SA node and AV node, resulting in a decreased heart rate, delayed conduction and decreased contractility. In contrast to sympathetic activation, parasympathetic fibers are mainly distributed to the atria rather than the ventricles. Parasympathetic activation does therefore not affect intraventricular conduction and contractility in the ventricles. [16-19]

The intrinsic cardiac nervous system is a very complex neural network that contains numerous cardiac ganglia. The ganglia are organized within the epicardial fat on the surfaces of the atria and ventricles. Each ganglia contains 200 to 1000 local circuit neurons that play a crucial role in responding to and maintaining beat-to-beat regulation of cardiac function. [20] The intrinsic cardiac system can act entirely independent from external influences. However, it also integrates the opposing parasympathetic and sympathetic inputs and coordinates the heart's response to maintain optimal function. [21]

This mechanism with several interacting feedback loops relies on the delicate balance of the sympathetic and parasympathetic CANS. Autonomic disbalance can therefore have significant consequences and lead to the development and progression of many CVDs.

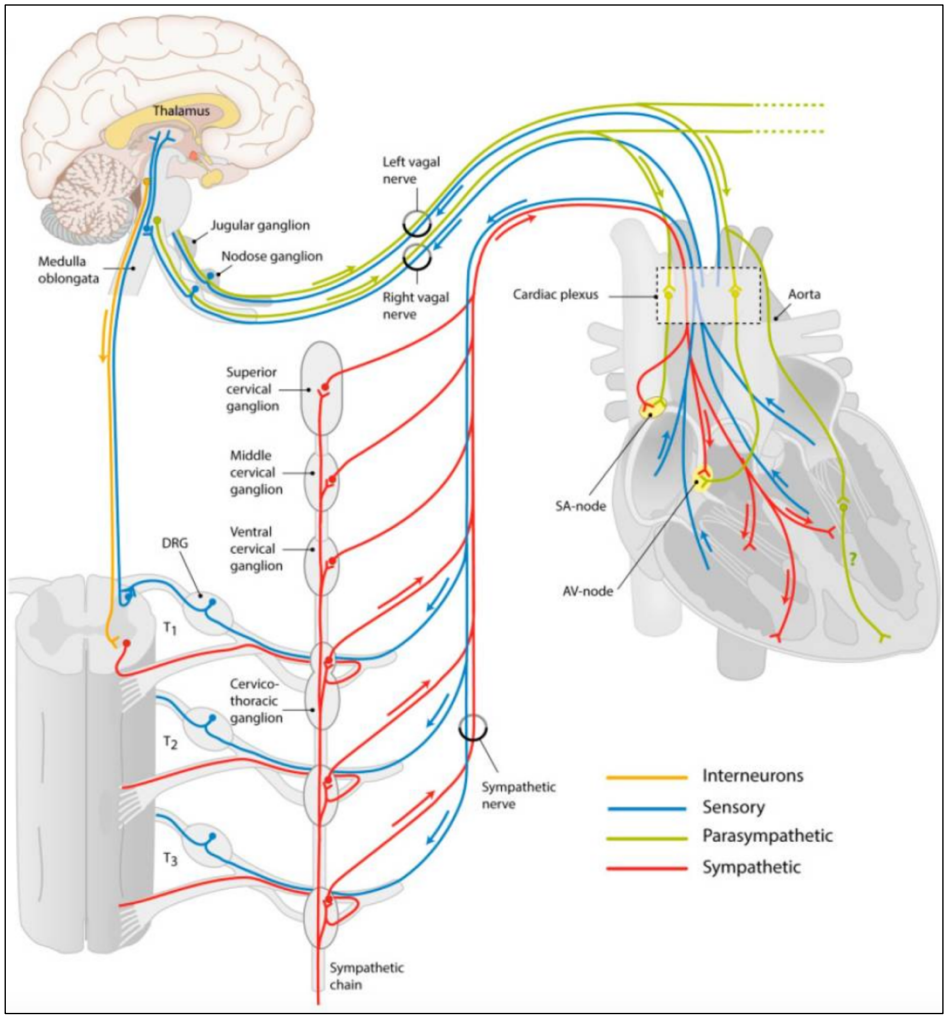

Figure 2: Overview of the extrinsic cardiac autonomic nervous system (CANS)

Vagus nerve

The vagus nerve (cranial nerve X) is the longest and most important nerve of the parasympathetic nervous system; 75% of all parasympathetic nerve fibers are carried by the vagus nerve. It is a mixed nerve, consisting of 20% efferent fibers and 80% afferent fibers. Efferent fibers transmit motor signals from the brain to the body and afferent fibers transport sensory information from the body to the brain. As shown in Figure 3, the vagus nerves (left and right) originate from the medulla oblongata in the brainstem and exit the skull through the jugular foramen. It passes down the neck between the internal jugular vein and the carotid artery, surrounded by the carotid sheath. After entering the thorax, the left vagus nerve travels anterior to the aortic arch behind the primary left bronchus and into the esophagus, while the right vagus nerve travels behind the esophagus and primary right bronchus. Both nerves then enter the abdomen through the esophageal hiatus of the diaphragm and follow distinct paths to their terminal branches. Throughout this path, the vagus nerve has many branches that innervate structures including the ear, larynx, pharynx, heart, lungs, and gastrointestinal tract. [22, 23]

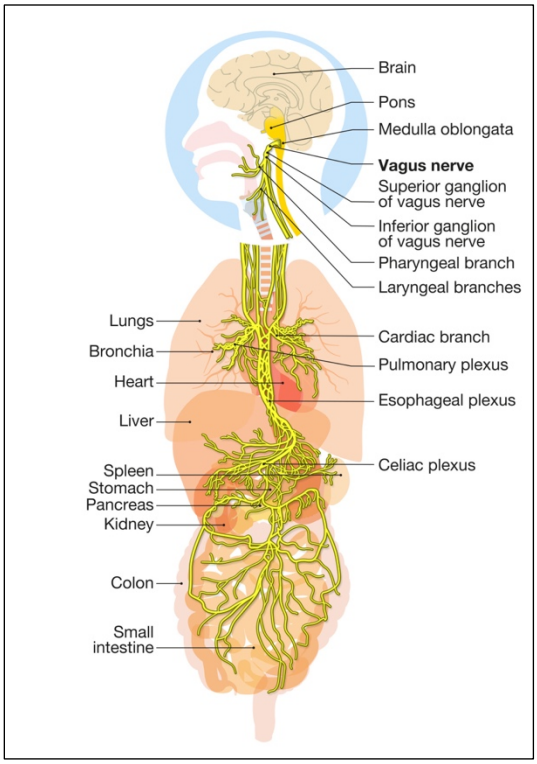

Figure 3: Anatomical pathway and branches of the vagus nerve

The vagus nerve contains three types of fibers: A-fibers (Aα, Aβ, Aγ, and Aδ), B-fibers, and C-fibers, which are classified based on their conduction velocities and diameters. [24] Aα-, Aβ-, and Aγ-fibers are thick, myelinated fibers that contribute to both sensory input and motor output with the fastest conduction velocities. Aδ fibers are smaller, thinly myelinated fibers that primarily carry sensory signals, including those related to pain and temperature. B-fibers are moderately myelinated and smaller than A-fibers, primarily providing efferent parasympathetic preganglionic innervation. Finally, C-fibers are the thinnest and unmyelinated, with the slowest conduction velocities and primarily carrying afferent visceral information. [25, 26]

At the cervical level, the vagus nerve is made up of about 20% A- and B- fibers and 80% C-fibers. [27] At the auricular level, the vagus nerve branch is predominantly composed of A-fibers, with fewer C-fibers compared to the cervical level. Approximately 50% of the myelinated axons in the auricular branch belong to the Aδ group, while nearly 20% are Aβ fibers. [28]

Vagus nerve stimulation

Vagus nerve stimulation (VNS) is a neuromodulation therapy that delivers electrical impulses to the vagus nerve. The therapy involves an implantable pulse generator and an electrode that is wrapped around the vagus nerve. It is an FDA-approved treatment for drug-resistant epilepsy (since 1997) and depression (since 2005). [29] To date, over 125.000 patients have been implanted with a VNS system worldwide. [30] However, despite its clinically meaningful antidepressant and anti-seizure effects, VNS remains a costly and invasive therapy with various side effects. [31] Transcutaneous vagus nerve stimulation (tVNS) is a non-invasive alternative that has been developed to overcome those limitations. There are two primary approaches: auricular and cervical. In the auricular approach, the auricular branch of the vagus nerve is stimulated via surface skin electrodes applied at the tragus. In the cervical approach, the electrodes are applied to the side of the neck and target the cervical branch of the vagus nerve. Stimulating the afferent fibers of these vagus nerve branches causes them to send signals to the nucleus tractus solitarius (NTS) in the brainstem. The NTS then activates the caudal ventrolateral medulla (CVM) and dorsal motor nucleus (DMN). When the DMN is highly active, it sends signals through the efferent fibers of all vagus nerve branches which results in enhanced parasympathetic activity. [32]

Although the precise mechanisms of tVNS are still poorly understood, it has been explored as a potential therapy for a wide range of conditions, including inflammation, Alzheimer's disease, headache, chronic pain, and tinnitus. [6, 33] Recent studies have also investigated the effects on the CANS, where it has shown potential in treating arrhythmias, acute and chronic ischemia diseases, and heart failure induced by autonomic imbalance. However, it should be mentioned that many studies report heterogeneous results. This variability may be due to differences in stimulation parameters (pulse width, frequency, intensity, duration) and electrode placement areas.

For electrode placement, it is notable that tVNS is almost exclusively applied to the left vagus nerve. This stems from safety concerns arising from dog studies where right-sided VNS resulted in bradycardia, leading to the belief that right-sided VNS should not be attempted in clinical settings. [34] This concern is due to the asymmetrical innervation of the heart, where the right vagus nerve predominantly innervates the SA node and the left predominantly innervates the AV node. [35] However, it can be questioned whether these concerns are justified, as the anatomy of the cervical vagus nerve differs between dogs and humans. In addition, for auricular tVNS, cardiac effects seen through stimulation are mediated through the NTS and dorsal motor nucleus, which deliver signals to the heart bilaterally via the efferent cervical vagus nerves. It is therefore unlikely that right-side stimulation causes cardiac adverse events. [36] It is also notable that very little research has been conducted on the effects of bilateral cervical neurostimulation on the CANS, which may be due to these concerns.

As mentioned earlier, the stimulation parameters vary significantly between studies. The most used waveforms are monophasic rectangular waveforms or sinusoidal wave bursts. Stimulation intensities typically range from 0.5 to 12 mA, with frequencies generally between 20 and 30 Hz. [26] When selecting the optimal stimulation parameters, it is crucial to activate the A and B fibers of the vagus nerve, as these are responsible for parasympathetic activity, while avoiding activation of the C fibers. [37]

Pulsetto

The Pulsetto device (Pulsetto, Lithuania) is a wearable non-invasive cervical vagus nerve stimulator. It delivers electrical impulses through the skin of the neck to stimulate both the left and right cervical branches of the vagus nerve. It has been commercially available since 2022 and is marketed as a product that can reduce stress and anxiety and improve mental health. However, there is currently no clinical evidence supporting these or any other effects. The device offers five settings with different stimulation parameters, as listed in Table 1. The intensity of the stimulation can be self-controlled by 9 levels with a maximum of 37.8V and 60mA (Table 2).

Figure 4: Pulsetto device

Setting	Signal	Frequency	Shape output signals	Pulse Width
Stress	Five 4500 Hz pulses	25 Hz	Polyphasic rectangular	100 µS
Anxiety	Five 4750 Hz pulses	25 Hz	Polyphasic rectangular	100 µS
Sleep	Five 4750 Hz pulses	25 Hz	Polyphasic rectangular	100 µS
Burnout	Five 5200 Hz pulses	25 Hz	Asymmetrical biphasic balanced rectangular	80 µS
Pain	Five 4900 Hz pulses	30 Hz	Asymmetrical biphasic balanced rectangular	120 µS

Table 1: The five settings of the Pulsetto device with the corresponding stimulation parameters.

Level	1	2	3	4	5	6	7	8	9
Intensity [V]	4.1	6.2	8.4	10.8	14.5	16.8	21.4	28.6	37.8
Intensity [mA]	6.5	9.8	13.3	17.1	23.0	26.7	34.0	45.4	60.0

Table 2: The intensity levels of the Pulsetto device expressed in voltages and milliamperes.

To clarify the meaning of the signal parameters described in Table 1, the signal for the 'stress' setting is visualized in Figure 5.

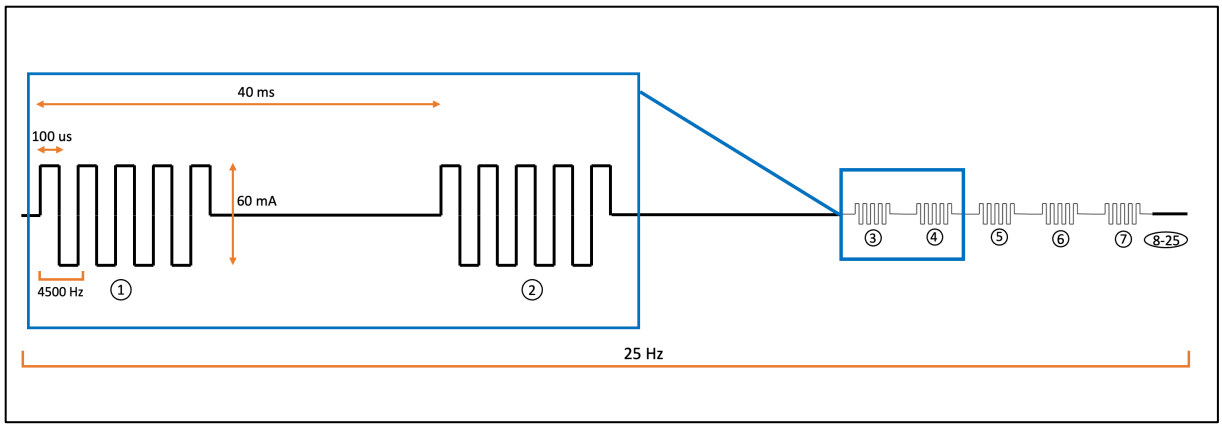

Figure 5: Schematic representation of the electrical parameters of the Pulsetto device in the ‘stress’ setting: 4500 Hz polyphasic rectangular wave burst of 5 rectangular pulses repeated at a frequency of 25 Hz generating an output of 60 mA at 37.8 V.

Heart rate variability

The most objective and validated indicator for the cardiovascular autonomic function is the heart rate variability (HRV). HRV is the variation in time between heart beats. A low HRV suggests a less reactive and adaptive autonomic system and thus a more impaired autonomic cardiovascular control. HRV can be estimated by using frequency-domain or time-domain parameters. [38] Time-domain analyses concentrate on the temporal variability in heart rate that might be affected by parasympathetic dominance. The most widely used time-domain parameters are the mean HR, SDNN, RMSSD and pNN50. [39] These parameters are all derived from normal-to-normal (NN) intervals, which are the intervals between heartbeats excluding ectopic beats. SDNN is the standard deviation of NN intervals and can be calculated as:

$$SDNN = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(NN_i - \overline{NN})^2} \qquad (1)$$

SDNN reflects all the cyclic components responsible for variability in the period of recording. It is the estimate of the overall HRV and reflects the heart's intrinsic ability to hormonal influences. The SDNN value is also highly dependent on the duration of the recording and is more accurate in 24-hour recordings than in shorter ones. RMSSD is the root mean square of successive NN interval differences and is derived by the following equation:

$$RMSSD = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}\Delta NN_i{}^2} \qquad (2)$$

The RMSSD measures beat-to-beat variability and is the primary time-domain parameter used to estimate parasympathetically mediated changes in HRV. RMSSD is correlated with pNN50, which is the percentage of successive NN intervals that differ from each other by more than 50 ms:

$$NN50 = \sum_{i=1}^{n}\{|\Delta NN_i| > 50ms\}$$

$$pNN50 = \frac{NN50}{n} \cdot 100 \qquad (3)$$

pNN50 is also based on high-frequency variations and reflects parasympathetic nervous system activity. However, RMSSD has better statistical properties then pNN50 and is therefore preferred in clinical use. [40]

HRV analysis in the frequency-domain is more commonly used to determine cardiovascular autonomic function. By analyzing different frequency components, it is possible to determine the activity of the ANS branches. The very low frequency (VLF) ranges from 0.0033-0.04 Hz and is influenced by thermal and hormonal controls. The low frequency (LF) component ranges from 0.04-0.15 Hz and is related to both sympathetic and parasympathetic effects, whereas the high frequency (HF) component ranging from 0.15-0.4 Hz is exclusively influenced by parasympathetic activity. The ratio of LF to HF power (LF/HF ratio) is therefore considered as an indicator of autonomic balance with a high ratio indicating sympathetic dominance and a low ratio indicating parasympathetic dominance. [40] Taken together, higher RMSSD, SDNN, and HF values and lower LF and LF/HF ratio values are typically associated with improved cardiovascular ANS control. [41] An overview of HRV analysis in time and frequency domain is visualized in Figure 6.

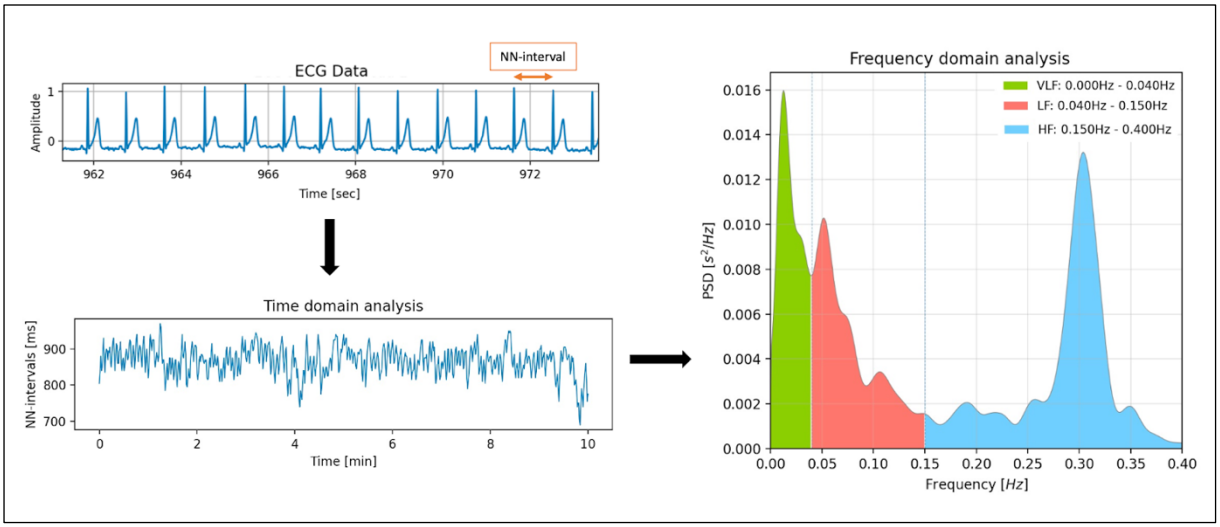

Figure 6: HRV analysis using time-domain and frequency-domain methods. The top left panel shows the ECG) data, with the NN-intervals marked between successive R-wave peaks. The left bottom panel presents the NN-intervals plotted over time, demonstrating variability in heartbeats. The right panel displays the frequency-domain analysis, highlighting the power spectral density across three frequency bands: Very Low Frequency (0.000Hz - 0.040Hz), Low Frequency (0.040Hz - 0.150Hz), and High Frequency (0.150Hz - 0.400Hz).

ECG characteristics

An electrocardiogram (ECG) is a graphic representation of the electrical activity of the heart over time. The electrical signals generated by the cardiac muscle can be detected by electrodes placed on the body's surface. The potential difference between two electrodes then creates the electrical waveform that reflects the heart's activity. As shown in Figure 7, the ECG is typically represented as a series of waves, each corresponding to a specific phase of the cardiac cycle.

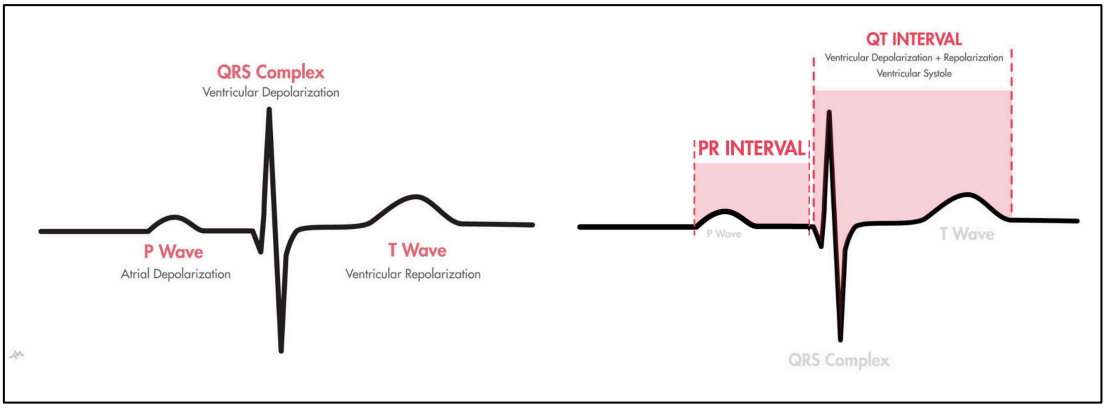

Figure 7: The basic pattern of electrical activity across the heart

The P-wave represents the depolarization of the atria, indicating atrial contraction. The QRS complex follows the P-wave and represents the depolarization of the ventricles. The T-wave reflects the repolarization of the ventricles, signifying the recovery phase after contraction. The PR-interval is the time from the onset of the P-wave to the beginning of the QRS complex, representing the delay between atrial and ventricular depolarization. The QT-interval measures the total time for ventricular depolarization and repolarization. [42]

Experiment 1

The first experiment was conducted during the yearly ‘atrial fibrillation patient day’ of the AFIP Foundation, focusing on patients diagnosed with paroxysmal or persistent AF. The primary objective was to evaluate the immediate effects of cervical tVNS on heart rhythm in these patients.

Methods
Measurements were performed throughout the day during breaks in the day program. Patients willing to participate received an explanation about the research and then signed an informed written consent form. Characteristics including age, gender, and type of atrial fibrillation (persistent/paroxysmal) were collected.

An electrically conductive gel was applied on the neck and the electrodes of the Pulsetto device were placed at the location of the vagus nerve. Participants received 10 minutes of stimulation with 4.5-kHz bursts of 5 rectangular pulses repeated at a frequency of 25 Hz (‘stress’ setting). The intensity level was set to 16.8 V as this was the common threshold before stimulation became uncomfortable.

Heart rate measurements were conducted using the MyDiagnostick device (Applied Biomedical Systems BV, Maastricht, Netherlands). This device is a rod with metal handles on both ends that the patient needs to hold. It records single-lead electrocardiograms of one minute with a sampling rate of 200 Hz. Heart rate measurements were taken at four moments: the minute before stimulation, after 5 minutes of stimulation, after 10 minutes of stimulation and the minute directly after stimulation (Figure 8).

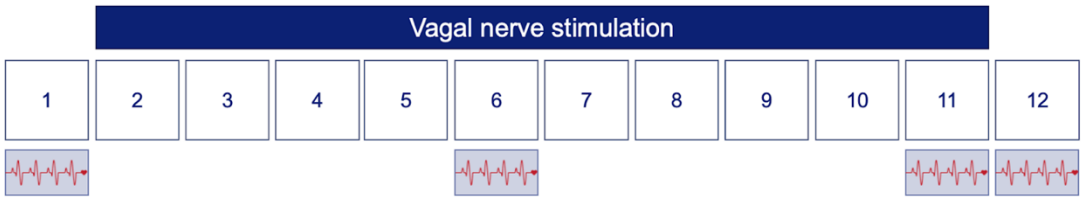

Figure 8: Measurement protocol for Experiment 1, with each block representing one minute. The ECG blocks show when one-minute heart rate measurements were taken.

The primary goal was to evaluate the difference between the measurement taken before and during the last minute of stimulation. If any beneficial effect was observed, the other two measurements were used to determine whether the effect already began after 5 minutes and whether it persisted once the stimulation had stopped.

During the protocol, patients were instructed to remain seated and avoid talking to minimize external influences on the heart rhythm. They were also instructed not to squeeze in the MyDiagnostick to avoid muscle artifacts.

Obtained data was imported in Python (version 3.10.14) as binary files and processed to isolate the ECG signals. A median filter with a kernel size of 3 was applied to reduce noise in the ECG signal. This was followed by a 5th order low-pass Butterworth filter with cutoff frequency of 30 Hz to remove high-frequency noise and smooth the data. R-peaks were detected using a peak detection function with a manually adjusted threshold for each data segment and a minimum distance between peaks. All ECG segments were inspected to ensure all R peaks were detected. The RR intervals were determined by calculating the time between consecutive R-peaks. R-peaks were marked as premature beats when the preceding RR interval was shorter than the mean RR intervals minus two standard deviations and followed by an interval more than 1.4 times longer than the short interval. When a

participant was in AF during the recording, premature beats could not be detected using RR intervals due to the irregular rhythm. Instead, premature beats in AF originate from the ventricles and were identified by their higher amplitude compared to normal QRS complexes using a threshold. After detecting premature beats, the surrounding intervals were removed to create a sequence of normal-to-normal (NN) intervals for further HRV analysis.

HRV analysis was performed on the NN-intervals in both the time domain and frequency domain. The time-domain parameters SDNN, RMSSD, and pNN50 were calculated using the previously described equations (1-3). For frequency-domain analysis, Welch's method was applied to estimate the power spectral density of the NN intervals. The absolute power of the LF (0.04-0.15 Hz) and HF (0.15-0.4 Hz) components were determined. LF and HF power were normalized as a percentage of the total power to calculate the LF/HF ratio. It was chosen not to include the VLF (0.003-0.04 Hz) component because it is not influenced by the ANS. [43]

No statistical analyses were performed due to the limited number of patients and the lack of a sham group. To visualize the effects of the stimulation, figures were created for each HRV parameter, the mean heart rate, and the number of ectopic beats, showing the results for all participants at the four time points. Distinctions were made between participants who were in AF and those in sinus rhythm at the time of measurement.

Results

Eight participants (1 male, 7 females, mean age: 68.1±7.1 years) were enrolled in the experiment. Two of them had persistent AF and the other six had paroxysmal AF. Except for the two participants with persistent AF, all others were in sinus rhythm during the experiment. The absolute differences between the HRV parameters measured before and during the tenth minute of stimulation were calculated and are presented in Table 3. Figures showing the outcomes of all measurements can be found in Appendix A.

	Rhythm	Mean HR	SDNN	RMSSD	PNN50	HF power	LF power	LF/HF
1	AF	76.7 (+4.1)	214 (-12.9)	294 (-35.5)	80.6 (-0.0)	24311 (-10811)	15603 (-3716)	0.6 (+0.2)
2	SR	77.3 (+1.4)	28.2 (-4.0)	16.7 (-3.9)	0.0 (0)	100.1 (-80.9)	496 (-150)	5.0 (+13.0)
3	SR	73.4 (+6.4)	23.0 (-2.6)	8.3 (-0.8)	0.0 (0)	10.3 (-4.6)	80.4 (+62.1)	7.8 (+17.2)
4	SR	64.4 (+4.8)	15.3 (+3.6)	11.9 (+5.5)	0.0 (+3.0)	37.6 (-12.6)	67.1 (+116.2)	1.8 (+5.5)
5	SR	53.0 (+0.5)	26.8 (-5.9)	38.3 (-5.7)	15.7 (-3.9)	312.1 (-143.9)	225.3 (-137.7)	0.7 (-0.2)
6	SR	68.2 (-0.8)	25.5 (-7.0)	35.0 (-21.4)	4.4 (-4.4)	49.1 (+5.5)	68.8 (+100.4)	1.4 (+1.7)
7	AF	113.4 (-9.9)	97.2 (+10.8)	157.0 (-2.2)	83.2 (-3.6)	1981 (+1411)	433.9 (+221.8)	0.2 (-0.03)
8	SR	59.1 (+0.1)	13.5 (+2.7)	14.2 (-3.6)	0.0 (0)	65.3 (-33.9)	52.0 (-6.8)	0.8 (+0.6)

Table 3: Changes in HRV parameters before and during the tenth minute of stimulation for eight participants. The table shows the baseline value (+- absolute change during tenth minute of stimulation).

Two participants had multiple extra beats during the minute before stimulation, all of which were premature ventricular beats. This number was significantly reduced after stimulation, as shown in Figure 9. One of them mentioned having skipped beats throughout the entire morning but no longer experiencing them after stimulation. This reduction in symptoms persisted for the rest of the day according to this participant.

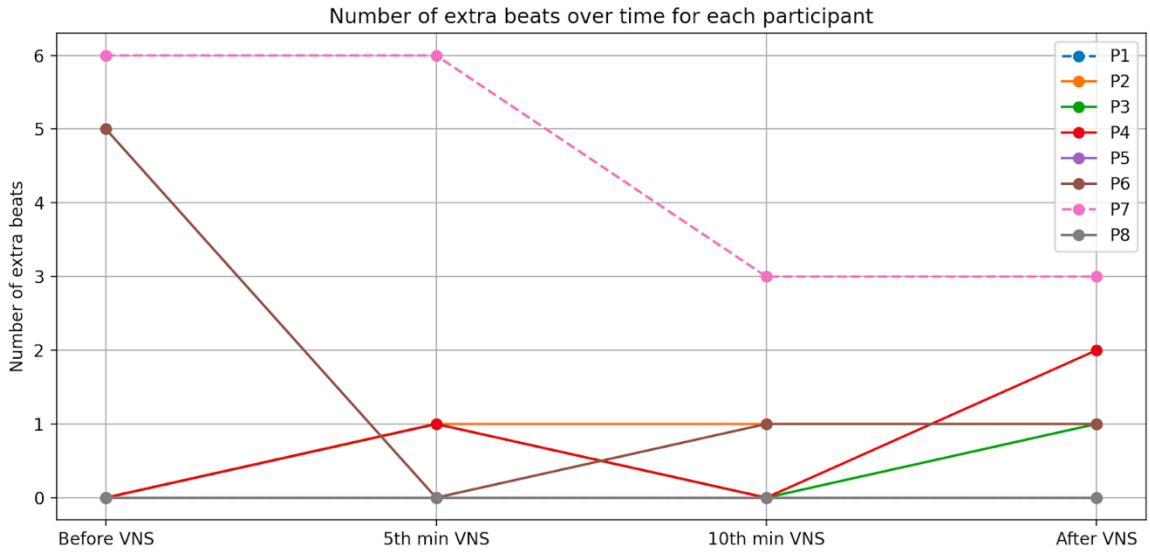

Figure 9: Number of extra beats in 1-minute ECG recordings at 4 time points: before VNS, the 5th minute of VNS, the 10th minute of VNS and the minute directly after VNS in all 8 participants. Dotted graphs are the participants that were in AF during the measurement.

None of the participants experienced any side effects during the stimulation and all participants were comfortable with the initial intensity setting.

Experiment 2

The aim of the second experiment was to determine the effect of cervical tVNS on the CANS in healthy individuals with different settings of the Pulsetto device.

Methods
40 healthy subjects were recruited to participate in this experiment. Participants were randomly assigned to either the stimulation group (n=30) or sham group (n=10). Within the stimulation group, three different stimulation settings of the Pulsetto device were used: ‘stress’ (n=10), ‘burnout’ (n=10) and ‘pain’ (n=10). The main differences between these groups are the stimulation frequency (25 or 30 Hz) and the signal shape; an overview is provided in Table 1.

The protocol consisted of a 30-minute heart rate measurement, with no stimulation during the first 10 minutes and (sham) stimulation during the second 10 minutes. The final 10 minutes were again without stimulation to observe whether any effects persisted after the stimulation. Three equal-length recordings were chosen, because it is inappropriate to compare HRV time domain measures (especially SDNN) obtained from recordings of different durations. [40] Heart rate measurements were performed with the SpiderView Holter recorder (MicroPort, Italy). Electrodes were placed as shown in Figure 10, resulting in a 3-lead electrocardiogram (X,Y,Z) with a sample rate of 200 Hz.

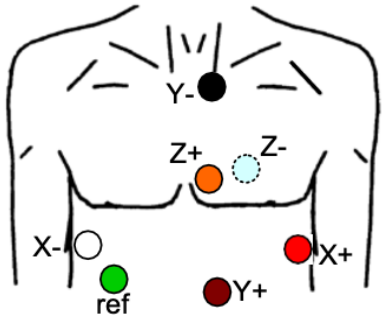

Figure 10: Electrode placement resulting in a 3-lead electrocardiogram (X,Y,Z)

After applying the Holter recorder and the Pulsetto device (including conductive electrode gel) on the neck, participants were instructed to work on their computer for the remaining duration of the protocol. This choice was made because the effect of tVNS may be more pronounced during periods of focus. Participants were instructed to sit quietly, stay awake and not to eat during the experiment. They were also asked not to speak, except when the stimulation started to describe how it felt. It sometimes happened that the electrodes were positioned on the sternocleidomastoid muscle, causing it to tremble a lot. If this occurred, or when the stimulation felt uncomfortable or painful, the electrodes were repositioned until participants felt only a mild vibration. The intensity level was initially set to 16.8 V (26.7 mA). If the stimulation remained uncomfortable after repositioning, the intensity was lowered to a tolerable level. For participants in the sham group, the stimulator was intentionally placed over the sternocleidomastoid muscle to induce tremors, giving them the impression that they were going to receive stimulation. The stimulator was then turned off during the repositioning process without the participants being aware of it.

All experiments were conducted in a quiet room between 9:00 and 12:00. To account for variations in circadian rhythms, the experiments were performed exactly four hours after the participants woke up. As illustrated in Figure 11, this timing was chosen because HRV is typically lowest in the morning. This approach ensures that any increase in HRV can be attributed to the stimulation rather than the circadian pattern. Participants were instructed not to drink coffee on the morning of the experiment because this could influence the ANS. [44]

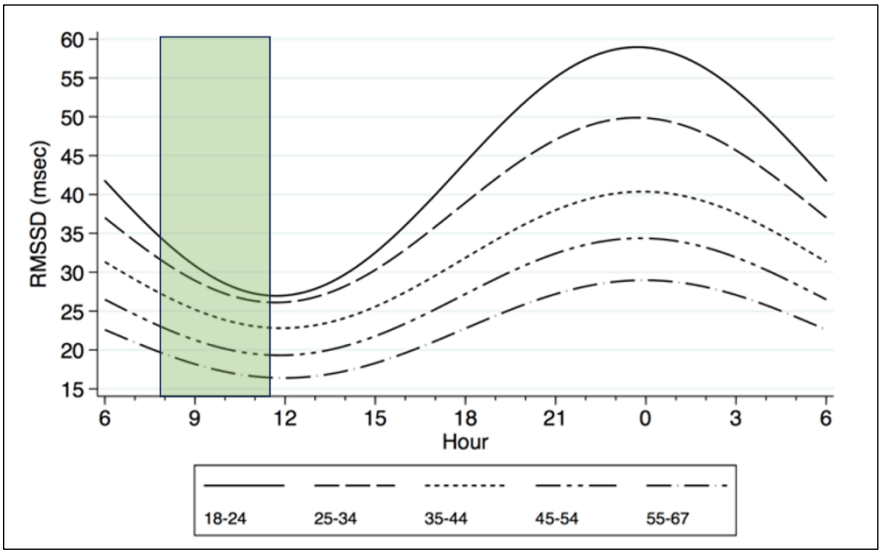

Figure 11: Circadian pattern of RMSSD by age-group. [45] The green block indicates the time range during which participants were measured. Within this range, there is no increase in RMSSD due to the time of day.

Data analysis

Data obtained from the Holter recorder was imported in Python and the lead with the most prominent R-peaks and the fewest artifacts was selected for further analysis (typically lead X). Filtering the signal was avoided because the quality was sufficient for analysis, and filtering could potentially affect the precise location of the R-peaks. Ten-minute segments were created (pre-, during-, and post-stimulation) ensuring that the 'during' segment started at the time point where repositioning of the stimulation device was completed. The determination of NN-intervals and the calculation of HRV parameters were performed using the same method described in Experiment 1.

In addition to Experiment 1, this experiment also examined changes in conduction times and the morphology of the ECG signal. Individual heartbeat segments were extracted by centering an 800 ms time window around each detected R-peak. These segments were averaged to produce a representative heartbeat signal for each experimental condition (pre-, during-, and post-stimulation).

QRS complex detection

Windows of 150 ms around the already located R-peaks were defined to isolate the QRS complexes. QRS complexes can have different morphologies, such as the presence or absence of a Q-peak and/or an S-peak. Therefore, multiple approaches were used to accurately determine the onset and offset of the QRS complex. The first approach to detect the onset was by identifying local minima's (negative peaks) in the second derivative of the ECG signal. The last negative peak before the R-peak was then selected as the initial onset. The second approach searched for the first significant positive gradient (>0.002) within a window before the R-peak as a potential onset. If this onset corresponded to a higher signal amplitude compared to the initial detection, the onset was updated accordingly. The Q-peak was identified by the minimum value in the signal between the onset and the R-peak, but only if this minimum was lower than the signal at the onset; otherwise no Q-peak was present.
The first approach to find the QRS offset was similar to the onset and detected the first negative peak of the second derivative of the ECG signal after the R-peak. The second approach looks for the first point after the R-peak where the absolute value of the first derivative of the ECG signal is lower than a threshold (0.02). The final QRS offset is the maximum of the two detected offsets, or the one detected offset if only one is found. If there was a value between the R-peak and the offset that was lower than the signal amplitude at the offset, it was identified as the S-peak.

P-wave detection
The peak of the P-wave was first identified by the highest peak within a 200 ms search window before the QRS onset. Then, a window of 150 ms was centered around this peak, but constrained to end no later than the QRS onset. Within this window, the onset and offset of the P-wave were detected using the second derivative of the signal. The maximum values of the second derivative before and after the P-wave peak were respectively marked as the P-wave onset and offset.

T-wave detection
The T-wave peak was identified as the point with the maximum amplitude in a search window of 100 ms after the R-peak until the end of the signal. A segment starting from the T-wave peak was analyzed, and the steepest downward slope was determined using the first derivative of the signal. The point where this slope intersected the isoelectric line (average signal value of the last 50 seconds of the signal) was identified as the T-wave offset. The offset was then rounded to the nearest sample point for precise determination.

An example of the peak-, onset-, and offset- detections of an average heartbeat from one participant is illustrated in Figure 12.

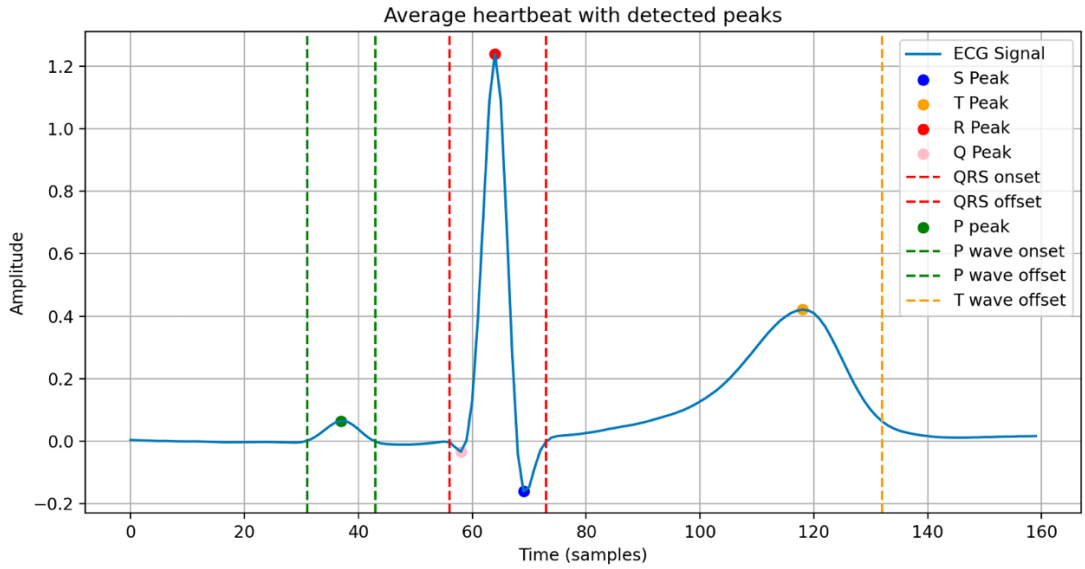

Figure 12: Peak-, onset-, and offset- detections of a 10-minute segment averaged heartbeat from one participant.

Conduction intervals and wave amplitudes were defined as visualized in Figure 13, and calculated for each averaged heartbeat (pre, during and post). Because the QT interval is dependent on heart rate, the corrected QT (QTc) interval was calculated by dividing the QT interval by the square root of the mean NN interval to allow for comparison of QT values over time. [46]

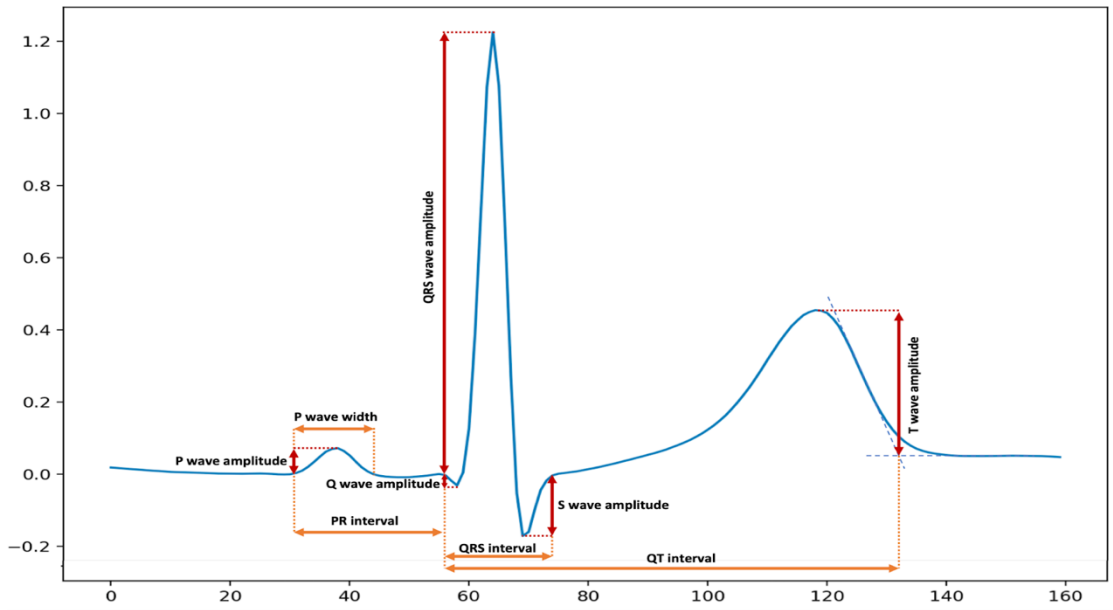

Figure 13*:* Determination of intervals and amplitudes with the values of the previous detected peaks, onsets and offsets.

Responder/non-responder

Within the stimulation group, an individual-level analysis was conducted to identify 'responders' and 'non-responders.' The focus was on HRV parameters that showed a significant difference between pre- and during- stimulation. To determine what 'normal' changes (not due to stimulation) in these parameters are, the relative changes in the sham group were analyzed. The range of normal variation for each parameter was then defined by the interval that contained 95% of the data. Specifically, the 2.5th percentile represents the lower bound, and the 97.5th percentile represents the upper bound. For each participant in the stimulation group was then determined for which parameters the relative change between pre- and during- stimulation exceeded these limits. Participants were classified as 'responders' or 'non-responders' based on the number of parameters that fell outside the normal range.

Additionally, all 'non-responders' were measured and analyzed again to determine whether the lack of response was due to measurement issues (for example incorrect placement of the tVNS device) or individual factors (such as anatomical variations).

Statistical analysis

The Shapiro-Wilk test was used to test the normality of the distribution of each parameter. Normally distributed data are presented as mean (± SD) and non-normally distributed data are presented as median [Q1-Q3]. To determine whether the difference during and after stimulation was statistically significant compared to before stimulation, a paired t-test was applied to normally distributed data and a Wilcoxon signed-rank test to non-normally distributed data. Subsequently, it was examined whether differences existed within the stimulation group across the different settings (‘stress’, ‘burnout’ or ‘pain’) and between genders. This analysis was performed by comparing the delta pre-during and delta pre-post values. Depending on the normality of the distribution, an unpaired t-test or a Mann-Whitney U test was conducted. A Bonferroni correction was applied to account for multiple comparisons when analyzing the different settings. All statistical analyses were performed using Python and a P value of ≤0.05 was considered statistically significant.

Results

40 participants were enrolled in the experiment and randomly assigned to either the sham group (3 males, 7 females, mean age: 28.5±7.6 years) or the stimulation group (11 males, 19 females, mean age: 28.6±5.3 years). Except for one participant with frequent premature ventricular beats, none of the participants had a history of cardiac issues. Ten participants reported that the stimulation was too uncomfortable or painful at intensity level 6. For these participants, the intensity was reduced to a more tolerable level: level 5 (n=8), level 4 (n=1), and level 3 (n=1). Of these 10 participants, 6 received stimulation with the 'stress' setting, 2 with the 'burnout' setting, and 2 with the 'pain' setting.

The results of the HRV values and the significance tests are displayed in Table 9. During stimulation, mean HR decreased (P<0.001), while RMSSD (P=0.001), PNN50 (P=0.001), HF power (P=0.003) and LF/HF (P=0.038) increased compared to pre-stimulation. SDNN and LF power did not show significant changes during stimulation compared to pre-stimulation (P=0.871 and P=0.516, respectively). None of the parameters post-stimulation were significantly different from pre-stimulation. In the sham group, there were no significant changes in any HRV values between the pre-during and pre-post periods. The only exception was the SDNN parameter, which showed a significant increase during stimulation (P = 0.022), but this change did not persist post-stimulation (P = 0.102). Figure 14 shows the distribution of the data displayed in box plots.

Group	Parameter	Pre	During	Post	P value pre-during	P value pre-post
tVNS (n=30)	Mean HR	71.0 (±11.1)	68.5 (±10.1)	69.6 (±10.0)	**<0.001**	0.053
	RMSSD	35.6 [27.8–51.1]	39.7 [27.7–61.7]	38.7 [27.2–53.0]	**0.001**	0.253
	SDNN	56.6 [50.5–69.0]	57.5 [49.1–75.5]	57.6 [52.0–72.1]	0.871	0.262
	PNN50	13.0 [5.96–26.9]	18.2 [5.18–33.1]	16.4 [4.21–33.5]	**0.001**	0.404
	LF power	789 [486–1430]	945 [527–1485]	884 [600–1509]	0.516	0.092
	HF power	375 [254–940]	479 [295–1370]	417 [259–1026]	**0.003**	0.253
	LF/HF	1.47 [1.12–3.15]	1.69 [1.13–2.24]	1.93 [1.30–3.23]	**0.038**	0.808
Sham (n=10)	Mean HR	68.9 (±8.4)	68.9 (±7.8)	70.0 (±8.6)	0.953	0.148
	RMSSD	41.9 [31.0–63.8]	40.1 [31.1–56.3]	41.8 [32.5–52.9]	0.557	0.322
	SDNN	60.3 (±23.5)	67.4 (±30.3)	67.6 (±21.0)	**0.022**	0.102
	PNN50	23.9 (±20.3)	25.0 (±21.7)	24.6 (±21.0)	0.437	0.792
	LF power	887 (±517)	1028 (±706)	1117 (±747)	0.245	0.125
	HF power	639 [448–1402]	609 [410–1570]	713 [371–1102]	0.557	0.695
	LF/HF	1.04 [0.71–1.22]	1.10 [1.00–1.19]	1.03 [0.96–1.50]	0.105	0.232

Table 9: Statistical test results for HRV parameters in the stimulation and sham groups. Data are mean (± SD) for paired t-tests and median [Q1–Q3] for Mann-Whitney U tests. A P-value <0.05 is considered statistically significant.

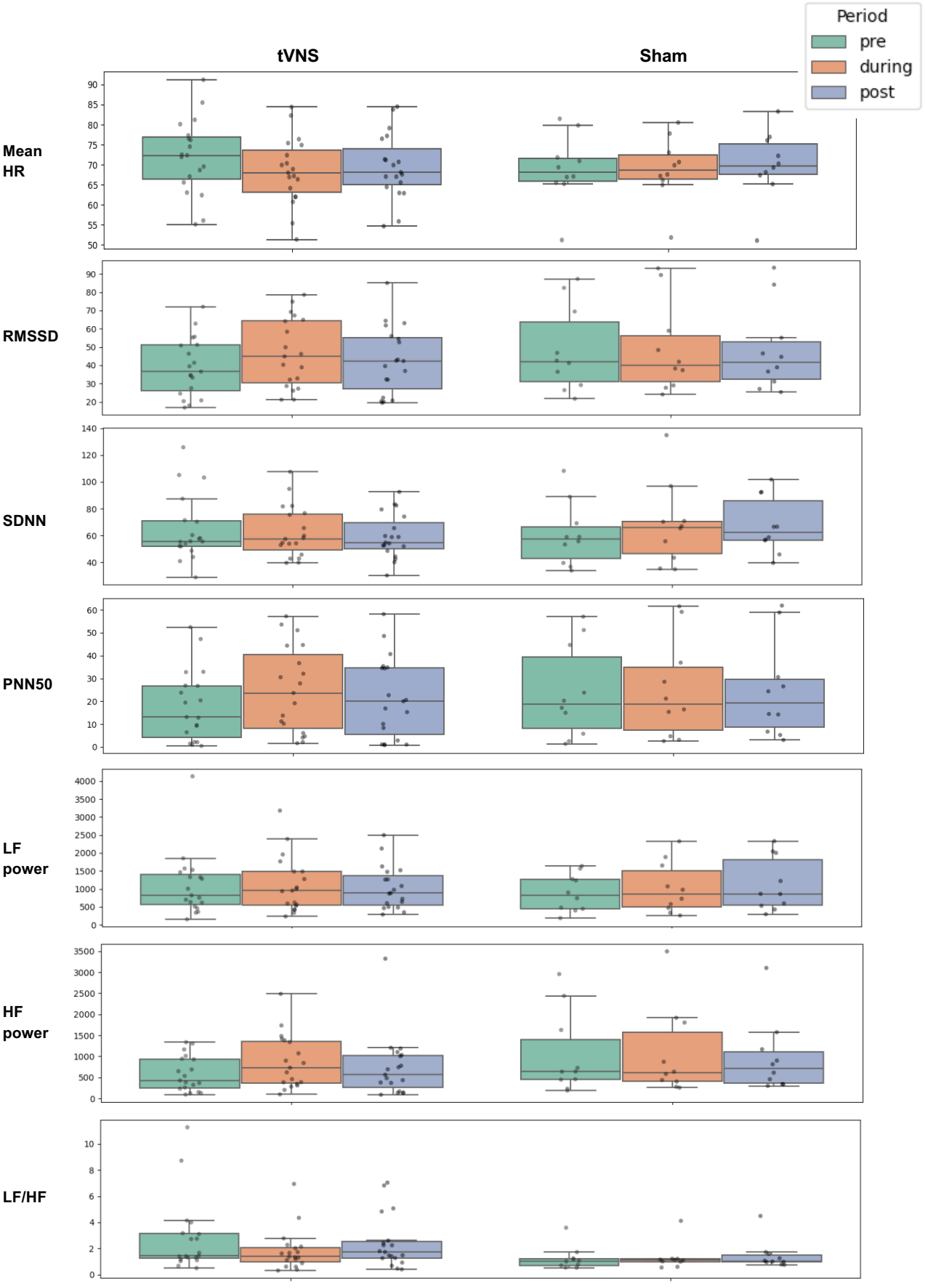

Figure 14: Box plots of heart rate and HRV parameters (Mean HR, RMSSD, SDNN, PNN50, LF power, HF power, LF/HF) for stimulation (tVNS) and sham groups across pre-, during-, and post- stimulation periods.

No statistically significant differences were found within the stimulation group across the different settings (‘stress’, ‘burnout’, or ‘pain’) or between genders. An overview of the results and corresponding P values can be found in Appendix B.

The relative differences between pre- and during-stimulation for each participant were calculated for mean HR, RMSSD, HF power, and LF/HF. For PNN50, the absolute difference was used as it is already a percentage. The range of normal variation of the sham group's data for those parameters can be found in Table 10. The green colored value represents the limit used to determine responder or non-responder status, depending on the hypothesis (mean HR and LF/HF decreasing, and the other parameters increasing). Figure 15 shows an example of one of the parameters (mean HR), where the relative changes of all individuals are plotted alongside the range of normal variation.

	Mean HR	RMSSD	PNN50	HF power	LF/HF
Mean	0.13 %	1.25 %	1.08	6.38 %	9.18 %
Lower limit	-2.33 %	-13.4 %	-6.24	-22.87 %	-25.07 %
Upper limit	1.84 %	10.53 %	7.23	33.39 %	55.57 %

Table 10: Range of normal variation of the sham group's data.

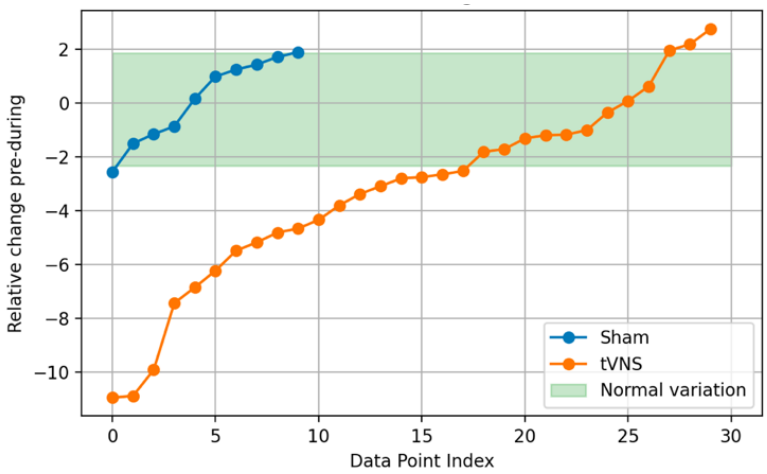

Figure 15: Line plot of relative changes in mean heart rate during stimulation compared with baseline of all participants. The range of normal variation is 95% of the sham’s group data and is displayed as a green block.

Eight participants in the stimulation group had no parameters where the change exceeded the normal variation limits, and three participants had one such parameter. These 11 participants (5 males, 6 females, 5 from the ‘stress’ group, 4 from the ‘burnout’ group, and 2 from the ‘pain’ group) are therefore considered ‘non-responders’. The remaining 19 participants had 2 (n=6), 3 (n=4), 4 (n=7), or 5 (n=2) parameters outside the normal range limits and are therefore considered responders. An overview of all results and scores per participant can be found in Appendix C.

After excluding the non-responders, additional statistical analyses were performed on the stimulation group and shown in Table 11. In addition to the significant difference in Mean HR, RMSSD, PNN50, and HF power between the pre- and during-stimulation periods ($P<0.001$ for all), there is now also a significant difference between the pre- and post-stimulation periods and between the during- and post-stimulation periods. For the LF/HF parameter, a significant difference is observed between the pre- and during-stimulation periods and between the during- and post-stimulation periods, but not between the pre- and post-stimulation periods.

Parameter	Pre	During	Post	P value pre-during	P value pre-post	P value during-post
Mean HR	71.9 (±9.3)	68.4 (±8.3)	69.5 (±8.1)	**<0.001**	**0.016**	**0.025**
RMSSD	39.0 (±16.0)	46.7 (±19.0)	42.6 (±18.5)	**<0.001**	**0.007**	**0.005**
SDNN	55.7 [51.9–70.8]	57.4 [49.3–76.0]	54.7 [50.2–69.8]	0.798	0.096	0.060
PNN50	13.1 [4.3–28.8]	23.6 [8.2–40.5]	20.0 [5.6–34.7]	**<0.001**	**0.036**	**0.003**
LF power	819 [567–1398]	952 [545–1485]	886 [550–1370]	0.650	0.679	0.709
HF power	427 [247–937]	732 [365–1360]	563 [268–1020]	**<0.001**	**0.049**	**0.003**
LF/HF	1.43 [1.24–3.13]	1.38 [0.99–2.08]	1.73 [1.26–2.51]	**<0.001**	0.241	**<0.001**

Table 11: Statistical test results for HRV parameters in the tVNS group after removal of the 'non-responders'. Data are mean (± SD) for paired t-tests and median [Q1–Q3] for Mann-Whitney U tests. A P-value <0.05 is considered statistically significant.

Six of the 'non-responders' were measured again. Two of them had more than two parameters exceeding the normal range limits and therefore now considered 'responders,' while the other four remained 'non-responders. Appendix D presents all the results from the second measurements.

Conduction parameters

The results of the conduction times and significance tests are presented in Table 12. The QT interval significantly increased during stimulation (P = 0.001), whereas the QTc interval significantly decreased (P = <0.001). However, neither change persisted post-stimulation (P = 0.132 and P = 0.351, respectively). The PQ interval also significantly increased during stimulation (P = 0.009) and remained significantly higher than baseline post-stimulation (P = 0.017). No statistically significant differences were observed in the QRS interval or P-wave width. In the sham group, there were no significant changes in any intervals between the pre-during and pre-post periods.

Group	Parameter	Pre	During	Post	P value pre-during	P value pre-post
tVNS (n=30)	PQ interval	121.2 (±25.1)	123.8 (±24.8)	122.7 (±24.8)	**0.009**	**0.017**
	QRS interval	88.8 (±8.7)	88.8 (±8.9)	89.0 (±8.7)	1	0.573
	QT interval	360 [343–378]	365 [345–384]	360 [341–385]	**0.001**	0.132
	QTc interval	393.2 (±22.3)	390.2 (±21.1)	392.2 (±21.4)	**<0.001**	0.351
	P wave width	65 [60–75]	68 [60–84]	65 [60–79]	0.537	0.441
Sham (n=10)	PQ interval	127 (±15.1)	126.5 (±15.5)	128.5 (±17.0)	0.343	0.193
	QRS interval	86.5 (±10.6)	87 (±9.5)	86.5 (±10.6)	0.343	
	QT interval	367.5 (±30.3)	368.5 (±29.3)	367 (±31.0)	0.343	0.780
	QTc interval	392.0 (±24.5)	393.4 (±24.2)	394.4 (±25.9)	0.152	0.140
	P wave width	69.5 (±17.2)	68.5 (±16.0)	69.5 (±18.0)	0.343	1

Table 12: Statistical test results for conduction intervals in the stimulation and sham groups. Data are mean (± SD) for paired t-tests and median [Q1–Q3] for Mann-Whitney U tests. A P-value <0.05 is considered statistically significant.

After closer examination of the data at individual level, it was observed that there was a noticeable change in P-wave morphology in two participants. Further inspection of the ECG shows that these participants have frequent alternation between sinus rhythm and atrial rhythm. Since atrial beats originate from a different focus within the atria, they have distinct P-wave morphologies. Figure 16 shows segments of the ECG containing both morphologies, along with the average heartbeats (+95%

confidence interval) for the pre-, during-, and post-stimulation periods. Based on the P-wave morphologies of the atrial beats (negative in participant 1 and enlarged in participant 2) combined with the morphologies of the averaged heartbeats, it appears that there are fewer atrial beats during stimulation compared with pre- and post- stimulation. To ensure that these two participants did not influence the statistical analysis of the PQ interval, the analysis was recalculated after removing them from the dataset. There was still a significant difference in the PQ intervals between the pre-during and pre-post stimulation periods (pre-during: P = 0.005, pre-post: P = 0.009).

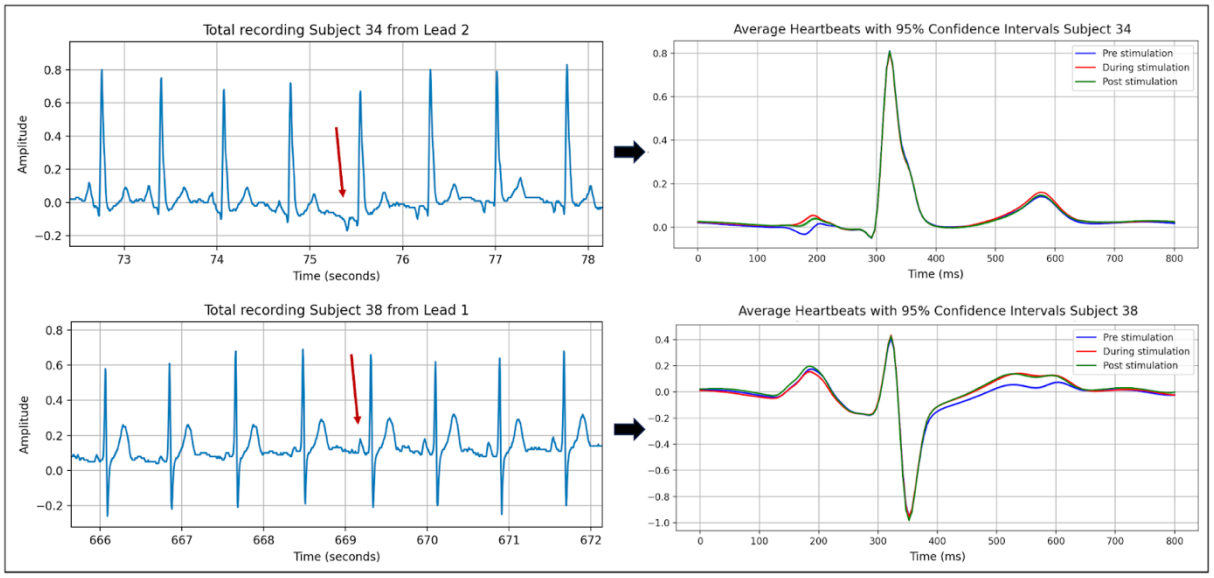

Figure 16: Segment of ECG recordings from two participants showing alternation between sinus rhythm and atrial rhythm. The red arrows point to the P-wave of the first atrial beats following sinus rhythm. On the right are the corresponding average heartbeats from 10-minute recordings taken pre-, during-, and post-stimulation, with 95% confidence intervals, where a change in P-wave morphology is visible.

Since the y-axes were not consistent across the measurements for different participants, changes in the amplitudes of the P-, Q-, R-, S-, and T-waves between pre-during and pre-post stimulation were calculated using the relative difference and are presented in Table 13. The T-wave amplitude significantly increased during stimulation compared to pre-stimulation (P= 0.030). None of the other amplitudes showed a significant difference.

Parameter		Δ pre-during	Δ pre-post	P value Δ pre-during	P value Δ pre-post
P amplitude	tVNS sham	-0.30 [-5.33–8.27] 3.26 [-0.08–7.10]	1.45 [-6.10–8.52] 1.09 [-1.43–8.27]	0.241	0.743
Q amplitude	tVNS sham	-2.47 [-12.93–1.90] -1.01 [-3.54–4.15]	1.95 [-6.50–11.22] 3.65 [-8.53–8.25]	0.494	0.900
R amplitude	tVNS sham	1.49 [-0.85–3.65] -0.57 [-1.11–2.25]	1.09 [-1.69–5.45] -0.87 [-2.38–3.16]	0.373	0.463
S amplitude	tVNS sham	-1.42 [-6.57–4.11] 2.45 [0.49–6.17]	2.91 [-3.73–11.74] 7.23 [-2.81–9.08]	0.248	0.624
T amplitude	tVNS sham	3.36 [0.01–8.81] 0.23 [-0.52–1.47]	2.61 [-1.57–10.18] 1.11 [-2.98–3.65]	**0.030**	0.325

Table 13: Results of Mann-Whitney U tests for relative changes in wave amplitudes in the tVNS and sham groups. Data are presented as median [Q1–Q3]. A P-value <0.05 is considered statistically significant.

Similar to the HRV parameters, the relative differences between pre- and during-stimulation were calculated for each participant for the parameters with significant differences (QT interval, QTc interval, and T-wave amplitude). The range of normal variation was determined based on the 95% range of the sham group data, and it was assessed whether each participant exceeded those limits for any of the parameters. The normal ranges and individual scores for each participant are provided in Appendix E. A total of 17 participants had an increase in QT interval, 9 had a decrease in QTc interval, and 15 had an increase in T-wave amplitude, all of which were outside the normal range. Four examples of the changing T-wave morphology are provided in Figure 17.

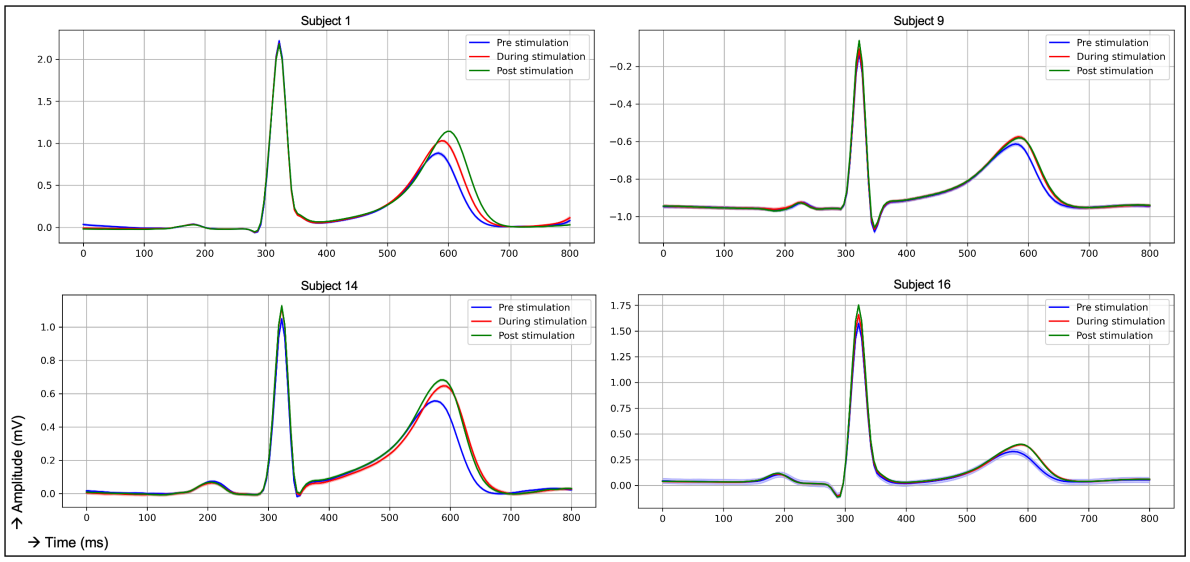

Figure 17: Average heartbeats recorded over a 10-minute period before, during, and after stimulation, with 95% confidence intervals. This figure highlights the morphological changes in the T-wave observed in four subjects.

A score of zero for the conduction parameters defined a participant as a non-responder, resulting in six non-responders. After excluding them, additional statistical analyses on conduction intervals were performed on the stimulation group, which are shown in Table 14. In addition to the significant difference in QT interval between the pre- and during-stimulation periods (P<0.001), there is now also a significant difference between the pre- and post-stimulation (P=0.029). This effect was already in PQ interval and did not change after removal of the non-responders.

Group	Parameter	Pre	During	Post	P value pre-during	P value pre-post	P value during-post
tVNS (n=30)	PQ interval	119.2 (±25.3)	122.1 (±25.0)	121.0 (±25.3)	**0.020**	**0.009**	0.307
	QT interval	359.4 (±24.7)	364.0 (±25.1)	362.7 (±24.8)	**<0.001**	**0.029**	0.207
	QTc interval	396 [385–408]	393 [381–407]	396 [383–407]	**<0.001**	0.197	**0.021**

Table 14: Statistical test results for conduction parameters in the tVNS group after removal of the 'non-responders'. Data are mean (± SD) for paired t-tests and median [Q1–Q3] for Mann-Whitney U tests. A P-value <0.05 is considered statistically significant.

After removal of participants whose T-wave amplitude was outside the range of normal variation (n=15), an increase in T-wave amplitude was found between pre and post stimulation (P=0.004).

	Δ pre-during	Δ pre-post	Δ during-post	P value Δ pre-during	P value Δ pre-post	P value Δ during-post
tVNS	9.29 [6.34–16.5]	10.5 [3.55–23.9]	0.08 [-2.12–5.68]	**<0.001**	**0.004**	0.677
sham	0.03 [-0.51–1.36]	2.24 [-2.67–3.71]	0.19 [-2.36–4.21]			

Table 15: results of Mann-Whitney U tests for T wave amplitude in the tVNS group after removal of the 'non-responders'. Data Is presented as median [Q1–Q3]. A P-value <0.05 is considered statistically significant.

Discussion

Experiment 1

Key findings

The aim of the first experiment was to explore the potential effects of cervical tVNS on the CANS in patients with (paroxysmal) AF. The hypothesis was that cervical tVNS would decrease the mean HR, LF power, and LF/HF ratio, while increasing RMSSD, SDNN, PNN50, and HF power. However, the results did not show any of the expected effects on HRV parameters after 5 or 10 minutes of stimulation compared with the baseline. A noteworthy observation was the reduction in ectopic beats in two participants following tVNS. This suggests that tVNS may have an acute stabilizing effect which leads to a reduction in excessive excitability in the heart. However, since only two participants had multiple ectopic beats at baseline, the generalizability of these findings is limited. Further research with larger cohorts would be necessary to draw more definitive conclusions.

Limitations

The lack of the expected effect in HRV parameters could potentially be attributed to several limitations in this experiment. First, the ECG recordings were limited to 1-minute segments because this was the maximum recording time of the MyDiagnostick device. However, for a reliable HRV measurement, a recording time of at least 5 minutes is typically required. [39, 40] Another limitation of the recording device was that the quality was not sufficient to analyze the morphology and conduction times of the ECG. Only the R-peaks could be detected. Additionally, the patients were in a noisy environment surrounded by many other curious people. This could have caused stress and excitement, potentially affecting the HRV measurements by influencing the sympathetic nervous system. Finally, the small sample size (n=8) and the absence of a sham group prevented any statistical analyses from being conducted.

Experiment 2

Key findings HRV

The second experiment was aimed to overcome the limitations encountered in the first study and to establish a more reliable measurement protocol. Longer ECG recordings were used and a sham control group was implemented. The initial results of the second experiment showed a significant reduction in mean HR, and a significant increase in RMSSD, PNN50 and HF power during tVNS compared with the baseline. These effects were not observed in the sham group, supporting the hypothesis that cervical tVNS is able to increase parasympathetic activity. However, LF/HF showed a significant increase during stimulation, contrary to the expected decrease. Upon further inspection of the data, it can be seen that while the median value increased significantly, the overall distribution shifted downward. This suggests that individuals with a high LF/HF baseline value (indicative of an imbalanced autonomic nervous system) did experience a decrease in LF/HF after stimulation, leading to a more balanced system. This is further supported by the fact that after removal of the ‘non responders’ from the data, there was a significant decrease in LF/HF during stimulation compared to pre-stimulation.

Currently, there are very few studies that have investigated the effect of cervical tVNS on the CANS. One pilot study conducted on dogs demonstrated a significant increase in HRV (SDNN) and a decrease in heart rate. [47] Another preliminary study applied 4 minutes of cervical tVNS to 20 healthy subjects, resulting in a significant increase in cardiac vagal tone after 90 minutes. [48] Additionally, Muthilungham et al. examined the effects of two weeks of cervical tVNS and found a significant reduction in heart rate compared to sham treatment. [49]

On the other hand, the effect of auricular tVNS on the CANS has been studied more extensively. Some similar studies in healthy people receiving auricular tVNS resulted in significant changes in heart rate and HRV parameters that were in line with the results from this experiment. [50, 51] However, a

recent meta-analysis on HRV changes with auricular tVNS also indicated that there is insufficient evidence to support the hypothesis that auricular tVNS alters HRV parameters compared to sham. [52] The main reason for this inconsistency might be due to anatomic differences and the fact that not everyone has a vagal nerve branch located at the tragus. Cervical tVNS might therefore be a promising alternative for auricular tVNS.

Key findings conduction system
The results of this study also demonstrated the effect of cervical tVNS on the heart's electrical conduction system. A significant increase in the QT interval was observed during stimulation. Since the QT interval is affected by heart rate, this increase might be attributed to the decrease in heart rate. After correcting for heart rate, it was found that the QTc interval significantly decreased. Since the QRS duration remained unchanged, this decrease in QTc interval suggests that ventricular repolarization became relatively quicker during stimulation.

Additionally, the T-wave amplitude significantly increased during stimulation, indicating stronger or more synchronized repolarization. [53] This could be due to increased potassium ion (K^+) outflow during the repolarization phase or more uniformly repolarization across the ventricular myocardium. A quicker and more synchronized repolarization reduces the likelihood of reentrant arrhythmias to develop. [54] These changes in ion flow and the resulting T-wave morphology alteration could therefore contribute to a lower risk of arrhythmias during cervical tVNS. These findings are particularly interesting, as it is currently believed that parasympathetic postganglionic nerves innervate the SA node and AV node but do not affect ventricular conduction.

There are no other studies that have investigated the effect of tVNS on the conduction times and wave amplitudes. However, there is some evidence that sympathetic activation leads to a decrease in T-wave amplitude. This effect has been observed in studies where sympathetic activation was induced through stimulation of the right stellate sympathetic ganglia [55], infusions with noradrenaline [56], and the administration of nonselective beta agonists. [57] In addition, decreases in T-wave amplitude could be reversed by beta-blockade with propranolol, further supporting the evidence that the T-wave amplitude reduction is mediated by sympathetic activity. [58] Besides that, a study by Annilla et al. showed that parasympathetic blockade also decreased the amplitude of T-wave, indicating that the T-wave amplitude not only reflects sympathetic activity, but the balance of sympathetic and parasympathetic nervous activity. [59] Therefore, the observed increase in T-wave amplitude following cervical tVNS may suggest a shift in the autonomic balance by enhancing parasympathetic activity or reducing sympathetic dominance.

This study also demonstrated a small but significant increase in the PQ interval between pre-during- and pre-post- stimulation. Since the P-wave duration remained unchanged, the increase in the PQ interval must be due to a delay in conduction through the AV node. Slowing of AV nodal conduction is a typical response to increased parasympathetic tone [60], further supporting the beneficial effect of cervical tVNS on the CANS.

Notably, several parameters showed significant differences between pre- and during-stimulation, but not between pre- and post-stimulation. However, after repeating the analyses following the removal of the ‘non-responders,’ the differences between pre- and post-stimulation values became significant, indicating that the effect of tVNS persists after stimulation. However, it was also observed that the during- and post-stimulation values differed significantly from each other. The data suggest that the inducing or increasing effect seen during stimulation diminishes after stimulation. Although the effect does not immediately return to baseline, it begins to trend in that direction within ten minutes.

Another interesting finding was the reduction in atrial beats during stimulation compared to pre- and post-stimulation periods. This suggests a potential benefit in modulating autonomic balance and reducing the occurrence of rhythm disorders, particularly atrial arrhythmias. However, since only two

participants had frequent atrial beats at baseline, further research is necessary to determine the generalizability and underlying mechanisms of this effect.

Responders/non-responders
The beneficial effects of tVNS were not observed in every participant, leading to the classification of individuals as 'responders' and 'non-responders'. This is a well-known phenomenon in previous studies on auricular tVNS, where this is often attributed to the absence of the auricular branch of the vagus nerve in the tragus. Given that every person has a cervical branch of the vagus nerve, it raises the question of whether it is truly possible to be a non-responder to cervical tVNS. After remeasurement of the non-responders some participants were found to become responders, suggesting that the stimulation device may not have been placed correctly during the initial measurement. However, some participants remained non-responders and a closer examination of their data revealed that they either had a very high HRV at baseline leaving little room for further effect from the stimulation, or they were men with a broader neck circumference. In these cases, the distance from the device to the vagus nerve might have been too large. These participants also reported feeling only very little sensation during the stimulation.

It is important to note that the method for determining whether someone is a responder or non-responder in this study was self-developed, and there is no established guideline for this in the literature. For example, in the study by Kang et al., a responder was defined as a subject whose pNN50 increased by more than 12% after tVNS, based on the average increase seen in another study. [61] However, this approach does not account for the fact that this average likely includes non-responders, nor does it consider the normal variability overall. The method used in this thesis, which incorporates a sham group to distinguish between normal variability and changes specifically caused by stimulation, is therefore a more robust way of determining a responder.

The parameters for which most subjects fell outside the normal range after stimulation were mean HR (60%) and QT time (57%), followed by RMSSD and T-wave amplitude (both 50%). The parameters with the fewest subjects outside the normal range were PNN50 (27%) and QTc (30%). Overall, the determination of a responder was consistent between HRV scores and conduction scores, but not always. Sometimes a subject was classified as a responder based on HRV parameters but not conduction parameters, or vice versa. This makes it difficult to determine which method is superior. It is possible that conduction parameters are more robust, as they may be less influenced by external factors than HRV.

Stimulation intensity
Another point of discussion is the intensity of the stimulation. Not all participants were comfortable with a stimulation intensity of 26.7 mA. Notably, 60% of these participants received stimulation with the 'stress' setting, which differs from the other two settings in its output waveform (polyphasic rectangular instead of asymmetrical biphasic balanced rectangular). This waveform may be more likely to activate C-fibers, which are associated with pain, leading to discomfort and reduced tolerance.

Limitations
A limitation of this study is that it was conducted on a sample of healthy young adults with generally well-balanced autonomic nervous systems (ANS). A larger effect of tVNS might be observed in elderly individuals or in patients with an imbalanced ANS, particularly those with a shift toward sympathetic predominance, such as in conditions like heart failure, hypertension, and arrhythmias.

Another constraint of this experiment was the sampling frequency of the Holter recording, which was 200 Hz, resulting in a resolution of 5 ms. This frequency is insufficient for precise measurements when analyzing differences in conduction times. For example, the maximum difference in the PQ interval between pre-during and pre-post stimulation was 5 ms, equivalent to just one sample. A higher sampling frequency would therefore provide more accurate measurements.

Another limitation of the study is the uncertainty regarding the precise stimulation settings of the Pulsetto device. The settings mentioned in this thesis are those provided by the company. However, a neurologist from Erasmus MC recently tested the device with an oscilloscope and obtained different values. He noted that there was no difference in the signal between the various settings, such as 'stress,' 'pain,' and 'burnout.' Additionally, his measurements indicated that the device's intensity range is between 1.5 and 3.5 mA, contrary to the maximum of 65 mA claimed by the company. Therefore, it is crucial to investigate the actual settings before proceeding with further research.

Future perspective

For the next steps, several recommendations can be made. First, it is important to determine the actual settings of the Pulsetto device before conducting further research. Additionally, this study has shown that it is not always clear whether the stimulation is precisely targeting the correct location. Future research could start by locating the carotid artery to accurately position the vagus nerve for stimulation. Another approach is to measure the activity of the pharyngeal muscles (a collective term for several small muscles in the throat) which are innervated by motor fibers of the vagus nerve. The assumption is that stimulation of the nerve also leads to motor activation. This can be relatively easily measured and could therefore be an indicator for vagal nerve activation and help confirm correct placement of the stimulator.

Future studies should also aim to refine the optimal stimulation parameters for tVNS, including intensity, frequency, waveform, and duration. Given the variability in responses across participants in this study, future research could focus on personalizing tVNS settings for greater efficacy. In addition, this study focused on acute responses to cervical tVNS, long-term studies are needed to evaluate the chronic effects of repeated stimulation. Research could explore whether prolonged use of cervical tVNS leads to sustained autonomic improvements and reduction in arrhythmias. An interesting outcome in both experiments was the reduction in ectopic beats. Although this could only be observed in a few individuals, it is certainly something worth exploring further in future research as this could potentially reduce arrhythmias.

The broader future perspective of this thesis is that tVNS could potentially treat CVDs, such as rhythm disorders. However, there remains a knowledge gap regarding the exact antiarrhythmic properties of tVNS and the ability to effectively select suitable patients for this treatment. As mentioned in the introduction, the Translational Electrophysiology research unit at the Department of Cardiology, Erasmus MC, is currently investigating the effect of auricular tVNS on atrial electrophysiology through intraoperative epicardial mapping. The challenges they are encountering (potentially due to the anatomy of the ear) could possibly be addressed with cervical tVNS. Therefore, a recommendation would be to replicate their study using cervical stimulation.

Additionally, this study is the first to demonstrate that tVNS influences T-wave amplitude, suggesting that the stimulation affects ventricular conduction and not just the SA and AV nodes. It would be interesting to conduct epicardial mapping studies on the ventricles of the heart to explore the precise electrophysiological effects during tVNS.

Conclusion

This thesis provides evidence that cervical tVNS can modulate cardiovascular autonomic control in healthy participants. The results show that cervical tVNS affects both HRV and cardiac conduction, with a significant increase in parasympathetic activity. Additionally, the novel finding of increased T-wave amplitude during tVNS suggests a previously unrecognized effect on ventricular conduction. The insights suggest that cervical tVNS could be more effective than auricular tVNS, highlighting the need for further investigation. With its potential to treat arrhythmias and other cardiovascular diseases, cervical tVNS represents a significant step forward in non-invasive cardiac therapies.

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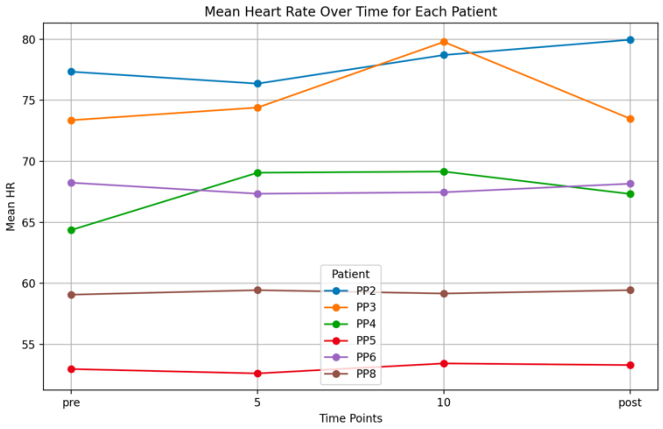
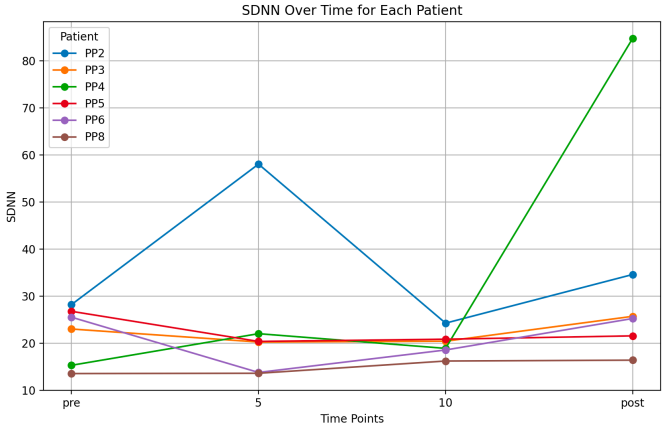
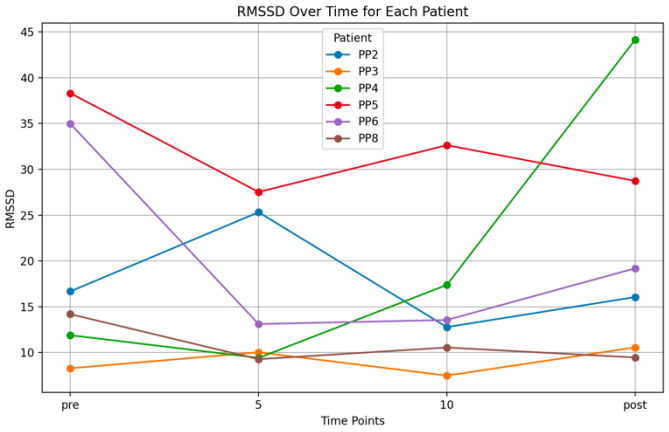
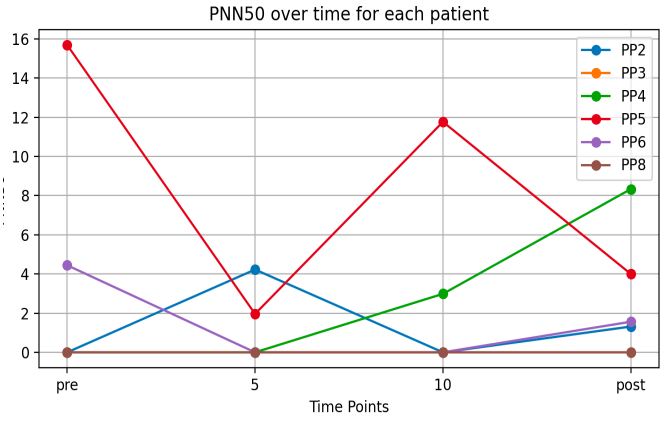
Appendix A

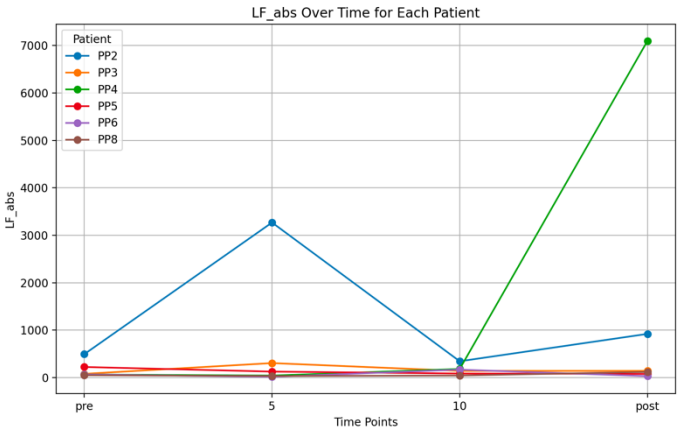
Participants in SR during the experiment:

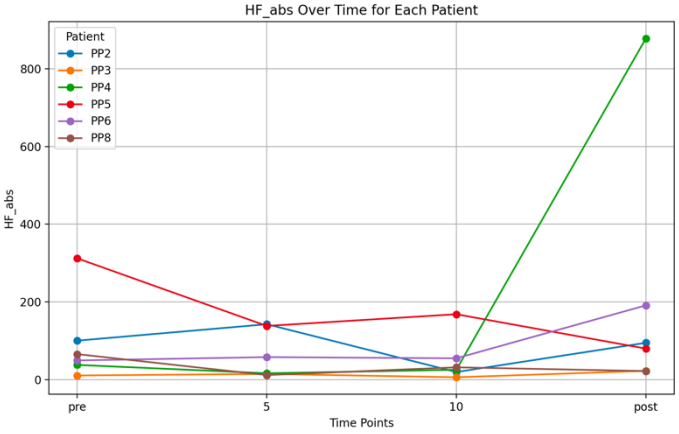


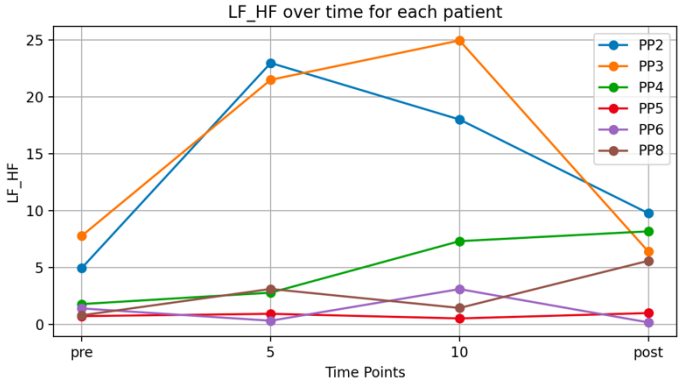




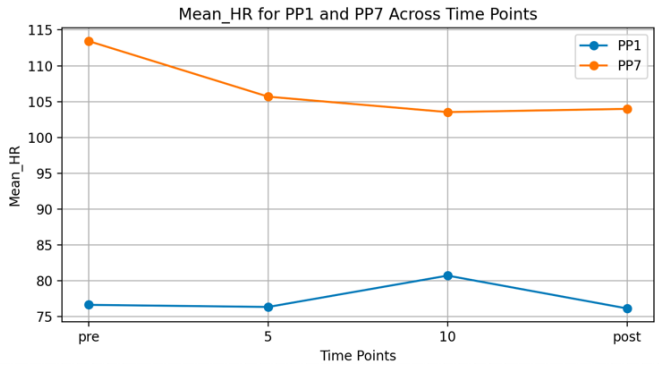


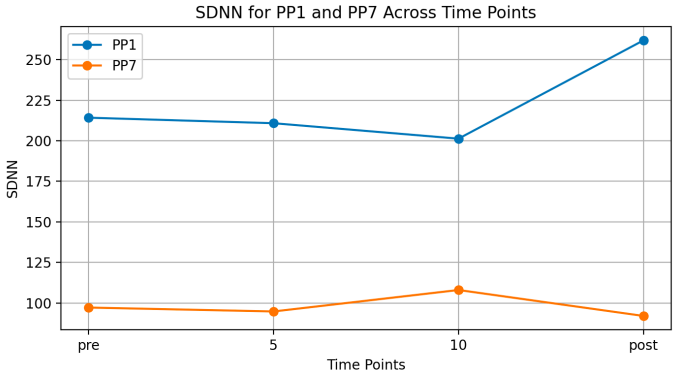


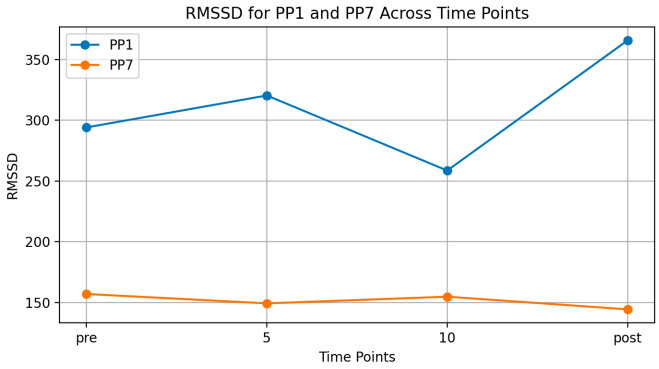


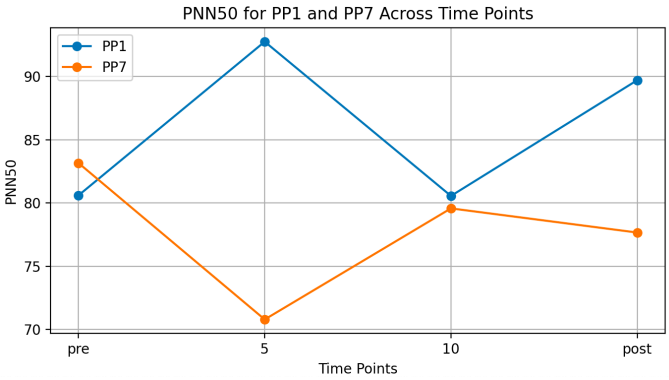


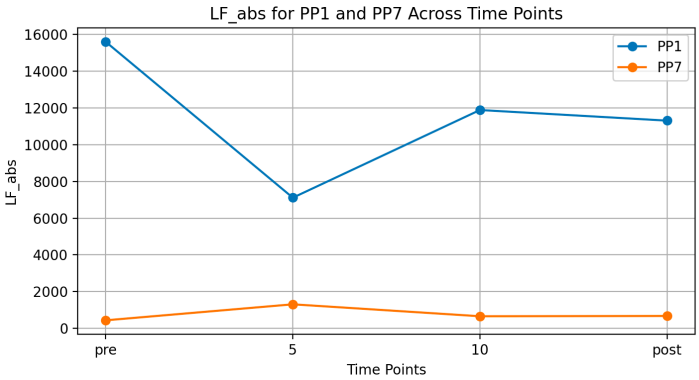
Participants in AF during the experiment:

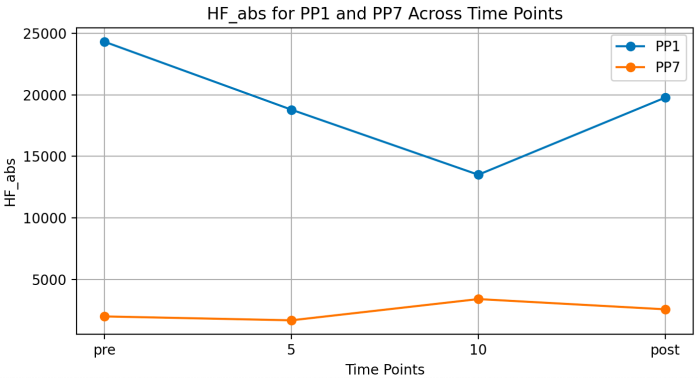


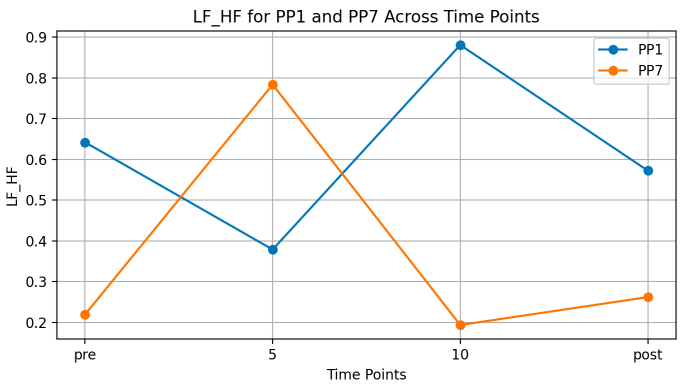












Appendix B

Parameter	M/F	Δ pre-during	Δ pre-post	P value Δ pre-during	P value Δ pre-post
Mean HR	M F	-1.00 [-3.09–0.01] -2.36 [-4.01– -1.24]	-0.46 [-2.18–1.65] -0.57 [-3.13–0.41]	0.156	0.439
RMSSD	M F	2.68 [-0.01–6.26] 4.72 [-0.25–7.90]	0,22 [-3.06–4.49] 1,49 [-1.15–3.86]	0.667	0.731
SDNN	M F	4.13 [-3.95–6.86] -2.14 [-7.37–9.11]	-0.78 [-4.47–3.02] -3.36 [-9.81–4.58]	0.576	0.576
PNN50	M F	2.56 [-0.39–4.83] 3.88 [0.70–7.88]	-0.66 [-2.94–1.39] 0.65 [-0.62–2.95]	0.491	0.245
LF power	M F	-1.00 [-45.6–247.9] 46.5 [-127.6–251.1]	65.8 [-124.3–215.1] 126.4 [-40.4–254.8]	0.829	0.699
HF power	M F	11.1 [-29.3–134.9] 128.3 [21.5–263.2]	15.6 [-80.4–103.4] 40.6 [-16.6–124.4]	0.212	0.863
LF/HF	M F	0.29 [-0.86–0.49] -0.18 [-0.67– -0.02]	0.31 [-0.86–0.95] 0.12 [-0.31–0.44]	0.389	0.966

Table 16: Results of the Mann-Whitney U test within the stimulation group between genders. Relative changes in the HRV parameters between pre-during and pre-post stimulation are presented in as median [Q1-Q3]. A P-value <0.05 is considered statistically significant.

Parameter	Group	Δ **pre-during**	Δ **pre-post**		**P value Δ pre-during**	**P value Δ pre-post**
Mean HR	1 2 3	-2.66 [-4.14– -1.16] -2.33 [-3.73– -0.51] -1.55 [-2.38– -0.70]	-1.46 [-3.40–0.26] -0.76 [-2.63–0.48] 0.13 [-0.51–2.20]	1 vs 2 2 vs 3 1 vs 3	0.850 0.385 0.273	0.734 0.140 0.104
RMSSD	1 2 3	-0.01 [-2.14–5.11] 4.77 [1.16–6.40] 4.79 [2.71–9.09]	1.05 [-2.14–2.75] 2.09 [-0.55–5.04] -0.43 [-2.48–3.29]	1 vs 2 2 vs 3 1 vs 3	0.241 0.623 0.140	0.473 0.427 0.910
SDNN	1 2 3	3.12 [-6.12–7.43] -4.21 [-8.18–3.69] 4.97 [-4.87–10.28]	-1.64 [-7.50–3.62] -1.14 [-12.16-6.57] -1.67 [-7.49–0.87]	1 vs 2 2 vs 3 1 vs 3	0.521 0.273 0.571	0.850 0.791 0.910
PNN50	1 2 3	0.18 [-1.16–3.07] 4.02 [1.36-7.44] 4.21 [1.56–10.06]	0.37 [-0.58–2.17] 1.19 [-1.09–6.36] -0.33 [-2.11–1.28]	1 vs 2 2 vs 3 1 vs 3	0.186 0.571 0.076	0.571 0.521 0.678
LF power	1 2 3	5.7 [-243.1–238.8] -31.2 [-252.2–232.8] 22.7 [-25.9–346.2]	-28.8 [181.0–137.3] 297.3 [3.0–368.2] 106.6 [19.8–140.1]	1 vs 2 2 vs 3 1 vs 3	0.791 0.427 0.571	0.054 0.345 0.427
HF power	1 2 3	-7.7 [-39.2–183.9] 110.1 [15.1–164.8] 135.6 [64.6–448.4]	26.3 [-22.2–115.7] 54.3 [-56.3–126.9] 12.0 [-55.4–51.8]	1 vs 2 2 vs 3 1 vs 3	0.571 0.345 0.140	0.791 0.678 0.910
LF/HF	1 2 3	-0.06 [-1.25–0.24] -0.26 [-0.72– -0.02] -0.24 [-0.67–0.23]	-0.42 [-0.89–0.08] 0.26 [-0.41–0.45] 0.43 [-0.08–1.08]	1 vs 2 2 vs 3 1 vs 3	0.970 0.791 0.850	0.104 0.571 0.026

Table 17: Results of the Mann-Whitney U test within the stimulation group across the different settings (1 = 'stress', 2 = 'burnout', 3= 'pain'). Relative changes in the HRV parameters between pre-during and pre-post stimulation are presented as median [Q1-Q3]. After Bonferroni correction, a P-value of <0.017 is considered significant.

Appendix C1

		Intensity	Group	ΔMean HR [%]	ΔRMSSD [%]	ΔPNN50	ΔHF power [%]	ΔLF/HF [%]	**Score**
1	F	6	2	-10,89	17,55	8,33	29,96	-0,76	3
2	M	6	3	2,18	-4,44	-0,92	-13,02	14,75	0
3	M	5	1	-2,66	1,81	0,21	2,89	-50,22	2
4	M	6	2	1,94	0,62	-2,28	-1,56	65,16	0
5	F	4	2	-2,53	11,64	4,20	29,87	-4,87	2
6	F	3	1	-6,24	46,01	20,80	113,35	-45,76	5
7	M	6	2	-6,86	9,08	3,84	13,56	-32,27	2
8	F	6	3	-3,39	10,20	4,74	52,53	-44,00	3
9	F	5	1	-4,67	13,63	7,43	37,03	-0,87	4
10	F	6	3	-2,81	-1,28	0,97	18,89	-38,79	2
11	F	5	1	0,62	-11,61	-3,33	-21,97	-14,30	0
12	F	6	3	-1,20	45,18	17,76	100,43	-37,56	4
13	M	6	2	-4,35	17,41	9,54	25,32	-17,44	3
14	M	6	3	-9,92	36,38	17,72	134,43	-58,56	5
15	F	5	3	-3,10	17,73	1,23	62,80	-33,03	4
16	F	6	2	-10,96	14,78	9,92	13,59	-28,42	4
17	M	5	1	-1,32	-7,75	-1,40	-8,44	6,08	0
18	M	6	3	2,75	6,81	5,82	2,26	127,04	0
19	F	6	1	-2,76	12,44	0,64	45,52	-48,17	4
20	M	6	1	-1,18	-1,78	0,15	-19,64	48,62	0
21	F	6	2	-0,36	-0,21	0,76	2,43	-32,00	1
22	M	6	3	-1,01	10,93	2,56	32,09	-25,81	2
23	F	6	2	-5,49	17,19	4,78	49,00	-56,72	4
24	F	5	2	0,07	10,09	3,18	10,90	37,69	0
25	F	6	1	-5,19	-7,73	-5,22	-18,78	61,63	1
26	F	6	2	-1,82	-8,82	-2,29	-14,69	-1,74	0
27	F	6	3	-1,72	15,32	11,47	32,49	0,02	2
28	F	6	1	-4,82	-8,33	-0,44	-16,81	-1,56	1
29	M	6	3	-3,81	55,13	3,68	127,62	67,39	3
30	F	6	1	-7,44	40,71	3,88	117,57	-38,45	4

Green = value outside the normal range
Orange = Score <2 and thus considered as a ‘non-responder’

Appendix D

Results of the second measurements of the ‘non-responders’

		Intensity	Group	ΔMean HR [%]	ΔRMSSD [%]	ΔPNN50	ΔHF power [%]	ΔLF/HF [%]	**Score**
2	M	6	3	-3,85	0,62	-2,04	12,68	16,79	1
11	F	5	1	1,25	-10,65	-1,97	-21,33	-24,75	0
17	M	5	1	-7,64	40,46	8,99	82,67	-51,83	5
18	M	6	3	-3,96	12,96	0,16	74,81	-51,20	4
20	M	6	1	-4,50	8,76	1,25	24,56	-22,45	1
28	F	6	1	1,77	-22,49	26,08	-45,08	10,28	1

Green = value outside the normal range
Orange = Score <2 and thus considered as a ‘non-responder’

Appendix E

		Intensity	Group	ΔQT [%]	ΔQTc [%]	ΔT amp [%]	**Score**	**Score HRV**
1	F	6	2	2,70	-3,05	15,18	3	3
2	M	6	3	0,00	1,08	2,50	0	0
3	M	5	1	0,00	-1,34	-1,42	1	2
4	M	6	2	-1,16	-0,21	-0,30	0	0
5	F	4	2	0,00	-1,27	1,82	1	2
6	F	3	1	2,67	-0,59	-1,02	1	5
7	M	6	2	1,22	-2,31	-10,12	2	2
8	F	6	3	1,30	-0,43	47,21	2	3
9	F	5	1	1,41	-0,99	11,36	2	4
10	F	6	3	0,00	-1,41	7,01	2	2
11	F	5	1	0,00	0,31	2,77	0	0
12	F	6	3	1,19	0,58	-4,23	1	4
13	M	6	2	1,54	-0,69	-3,03	1	3
14	M	6	3	4,29	-1,02	17,76	2	5
15	F	5	3	0,00	-1,56	3,95	2	4
16	F	6	2	2,74	-3,05	22,31	3	4
17	M	5	1	1,49	0,82	7,37	2	0
18	M	6	3	-1,43	-0,08	5,67	1	0
19	F	6	1	1,32	-0,09	2,48	1	4
20	M	6	1	1,52	0,91	-2,68	1	0
21	F	6	2	0,00	-0,18	2,02	0	1
22	M	6	3	0,00	-0,51	0,94	0	2
23	F	6	2	1,39	-1,43	2,57	2	4
24	F	5	2	0,00	0,03	4,01	1	0
25	F	6	1	1,37	-1,29	7,20	3	1
26	F	6	2	0,00	-0,91	5,34	1	0
27	F	6	3	0,00	-0,86	-0,39	0	2
28	F	6	1	1,47	-1,01	14,07	2	1
29	M	6	3	1,54	-0,41	9,29	2	3
30	F	6	1	3,03	-0,88	21,50	2	4

Green = value outside the normal range
Orange = Score <1 and thus considered as a 'non-responder

	ΔQT	ΔQTc	ΔT amp
Lower limit	-0,99	-1,03	-4,60
Upper limit	1,49	0,93	3,20

Table 18 Range of normal variation of the sham group's data.