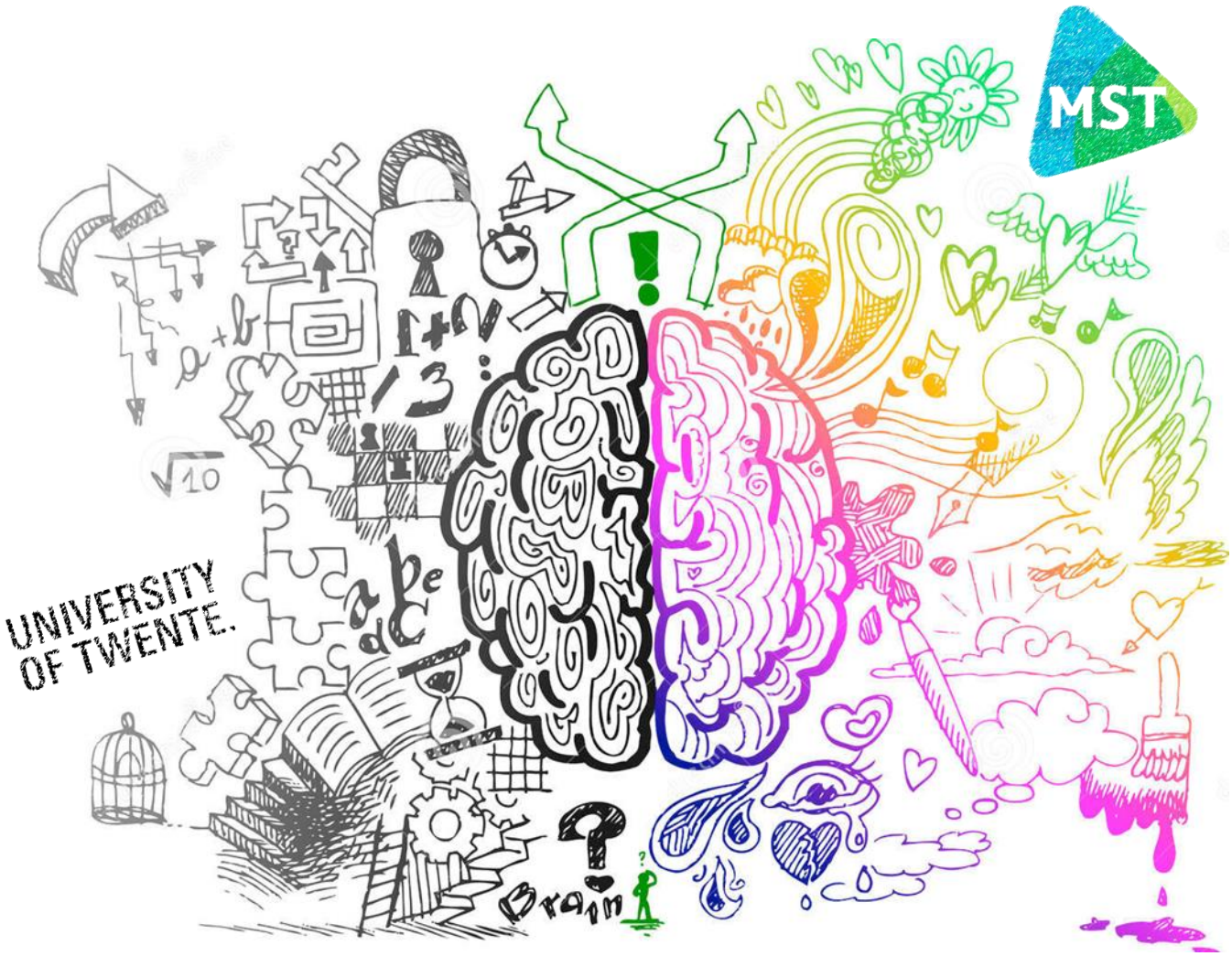
Technical Medicine – Master thesis

The effects of transcutaneous vagus nerve stimulation in patients diagnosed with epilepsy and healthy subjects



Author:
Dieuwke van Dartel, BSc

Committee:
Prof. Dr. Ir. M.J.A.M. van Putten
Drs. K.H. Kho
Dr. Ir. C.C. de Vos
Dr. M. Groenier
Prof. Dr. Ir. P. H. Veltink

Colofon

Author: Dieuwke van Dartel, BSc.
Student number: s1216937
E-mail address: dieuwke.vandartel@student.utwente.nl

Master thesis
Education: Technical Medicine
Organisation: University of Twente and Medisch Spectrum Twente
Graduation period: January 2017 – February 2018
Graduation date: February 23, 2018

Committee:
Prof. Dr. Ir. M.J.A.M. van Putten
Drs. K.H. Kho
Dr. Ir. C.C. de Vos
Dr. M. Groenier
Prof. Dr. Ir. P. H. Veltink

The figure shown on the front page is based on a figure obtained from www.shutterstock.com. I edited the figure by myself to make the figure for the front page. All the other figures used in this thesis are obtained from articles or provided by myself.

Abstract

Introduction: Vagus nerve stimulation (VNS) is a form of neurostimulation used for treating patients suffering from refractory epilepsy. The exact mechanism underlying the effects of VNS is still not fully understood and unfortunately, not all epilepsy patients respond well to VNS. In addition to the anti-seizure effect of VNS, afferent stimulation of the vagus nerve could also affect the autonomic nervous system. The autonomic activities do not play a role in the anti-seizure effect, but they could be a marker for the stimulation effects of VNS.
Recently, a transcutaneous VNS (tVNS) has become available, which could be used as a possible predictor for the effects of an implanted VNS system in epilepsy patients. The tVNS stimulates the auricular branch of the vagus nerve (ABVN) and therefore, it might show similar effects as VNS. The exact working mechanism of tVNS however is not completely understood. Therefore, the aim of this master thesis is to provide more insight into the effects of tVNS.

Method: The effect of tVNS is assessed for 21 healthy subjects and 4 epilepsy patients by measuring the effect of tVNS on the pupil diameter, heart rate and heart rate variability (HRV). Two different stimulation locations (cymba conchae and earlobe stimulation) are used to assess the effects of tVNS. The measurement conditions are no tVNS stimulation, tVNS stimulation at a low intensity level, and tVNS stimulation at a maximal intensity level.
The pupil diameter is measured during low intensity stimulation and maximal intensity stimulation using trials of 6 seconds, which provoked a pupillary light reflex. The heart rate was measured continuously throughout the measurement. HRV was measured during low intensity continuous tVNS stimulation.

Results: The overall results for the healthy subjects show a statistically significant increase in the pupil diameter parameters, a statistically significant decrease in the heart rate and a statistically significant increase in the R-R interval when comparing the ON phase of tVNS stimulation with the OFF phase. No statistically significant differences are shown in the effects of cymba conchae stimulation on the study parameters compared with the effects of earlobe stimulation on the study parameters. Both low and maximal stimulation show a significant increase in the pupil diameter parameters and a significant decrease in the heart rate during the ON phase compared with the OFF phase. Due to the small number of epilepsy patients, no overall results can be described.

Conclusion: The main finding of this study is that tVNS shows a statistically significant increase in the pupil diameter and a statistically significant decrease in the heart rate during the ON phase compared with the OFF phase. However, these results are shown during both cymba conchae stimulation and earlobe stimulation. Therefore, it cannot be concluded from this study that the effects of cymba conchae stimulation on the pupil diameter and the heart rate are caused by the activation of the ABVN.

Contents

1. Introduction

After cerebrovascular disease, epilepsy is the most common neurological disorder in incidence and prevalence and affects approximately 0.5-1% of the world's population (1–5). Epilepsy is defined as a disorder of the brain characterized by an enduring predisposition to generate seizures that are associated with neurobiological, psychological, cognitive and social problems (1,5–7). According to the International League Against Epilepsy (ILAE) a seizure is defined as a transient occurrence of signs and/or symptoms and is caused by abnormal excessive or synchronous neuronal activity in the brain (6–8). The manifestation of seizures are diverse among patients and these depend on the region of the brain that is affected (9). The manifestation also depends on the spatial distribution of the abnormal synchronization, which classifies seizures as focal seizures or generalized seizures (1,8,10). Aetiology also plays a role in the classification of seizures (6,10).

The management of epilepsy can be challenging. The most common form of treatment is pharmacological treatment. Daily intake of fixed doses of anti-epileptic drugs (AEDs) are used to control seizures (9). Unfortunately, AED therapy fails for some epilepsy patients and approximately one third of the patients are considered to be drug resistant (1,4,7,9,11–14). In these patients the quality of life is severely impacted and they are also at a significantly increased risk of sudden unexplained death in epilepsy. Therefore many patients with drug-resistant epilepsy are treated with invasive procedures (15). Invasive procedures are epilepsy surgery, vagus nerve stimulation (VNS) and deep brain stimulation (DBS) (11,16).

1.1 Vagus nerve stimulation

The vagus nerve is the 10th cranial nerve and is a mixed parasympathetic nerve, containing both afferent sensory (80%) and efferent motor (20%) fibers (17–20). Besides the afferent and efferent fibers, the vagus nerve also contains A-fibers, B-fibers and C-fibers. These fibers are defined in accordance to the classification that subdivides the peripheral nerve fibers according to their conduction velocity (21). The vagal A-fibers are the largest and consist of myelinated fibers carrying afferent visceral information and efferent input. The B-fibers are small myelinated fibers carrying parasympathetic efferent fibers. The C-fibers are small and unmyelinated. These fibers are carrying afferent visceral information (21,22).

VNS delivers electrical stimulation to the vagus nerve to decrease the excitability of the brain and thereby decrease the frequency or duration of seizures. VNS requires the implantation of a pulse generator, which is surgically implanted in the anterior chest wall. Leads are running from the generator and electrodes are wrapped around the left vagus nerve at the carotid bifurcation (Figure 1) (13,23,24). The electrodes stimulate the vagus nerve in a regular cycle. The pulses are generated by the pulse generator (25).

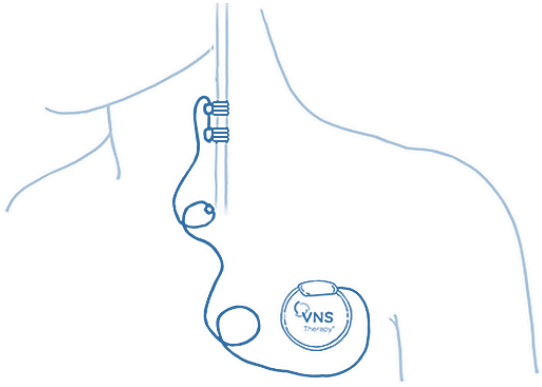


Figure 1: Schematic view of the electrode of the VNS wrapped around the vagus nerve (24).

The exact mechanism underlying the effects of VNS are still not fully understood and unfortunately, not all epilepsy patients respond well to VNS. Long term studies that assessed the effectiveness of VNS, concluded that over 50% seizure reduction was accomplished in 20-63% of the patients after treatment for six months to six years (2,9,16,17,26,27). The proposed mechanisms of action are: unidirectional stimulation of thick myelinated afferent vagus nerve fibers, access to the nucleus tractus solitarius (NTS) in the brainstem, and elicitation of a typical cerebral activation pattern (11,18,25). The NTS is considered to play the most important role in the mechanism of VNS and projects among others to the locus coeruleus (LC) in the brainstem. The projection of the NTS to the LC is suggested to be important, because LC is the main source for norepinephrine (NE) in the forebrain and the LC-NE system plays a crucial role in the anti-seizure effect of VNS (28,29).

1.2 Predictors in the success of VNS

Despite the growing application of VNS, it is still not possible to predict which patients respond to what extent to VNS therapy. Investigating the working mechanism of VNS and determining the success of VNS is important to inform patients and give them information about the expected seizure reduction with VNS. If it is possible to predict which patient is a responder and which patient is a non-responder of VNS therapy makes it possible to prevent patients with a low likelihood to respond from undergoing surgery and having an expensive VNS system implanted. Biomarkers to assess VNS efficacy pre-operatively would be high valuable and could reduce the number of non-responders.

Several researches are performed to find a predictive value for the success of VNS. Bodin et al. (30) evaluated the impact of VNS on the synchronicity of interictal electroencephalography (EEG) rhythms and found that responders of VNS therapy (seizure reduction of >50%) have a lower global level of synchronization than non-responders. This effect was particularly significant in the delta and alpha bands. Jansky et al. (31) attempted to predict the success of VNS based upon the localization of the seizure focus. They found that the absence of bilateral interictal epileptiform discharge is correlated independently with successful VNS treatment. Despite the research that is already performed, it is still not possible to predict which patients respond to what extent to VNS therapy.

1.3 Transcutaneous vagus nerve stimulation

Transcutaneous vagus nerve stimulation (tVNS) is an alternative strategy to stimulate the vagus nerve in a non-invasive manner, and could possibly play a role in the prediction of the effects of VNS. The tVNS uses an intra-auricular electrode (like an earphone) which stimulates the auricular branch of the vagus nerve (ABVN) at the level of the cymba conchae (figure 2) (17,32). To activate the ABVN fibers, the stimulus intensity is adjusted to a level just above the patient's detection threshold (32).

Only a few studies have been performed on the effectiveness of tVNS in seizure reduction of epilepsy patients (4,33–36). In these studies epilepsy patients were included with either focal or generalized epilepsy. Stefan et al. (35) assessed the effectiveness of tVNS in epilepsy patients using tVNS for nine months, and reported a reduction in seizure frequency in five of the seven patients, but non reached 50% reduction of seizure frequency. Aihua et al. (4) found an effective reduction of seizure frequency of approximately 40% after 12 months with tVNS in 26 patients who underwent ABVN stimulation. From these patients approximately 40% of the patients reached a seizure reduction of 50% or more. Aihua et al. also found a reduced seizure severity and a significantly improved quality of live and mood. The studies from He et al. (34), Bauer et al. (33), and Rong et al. (36) also found a reduction in seizure frequency, which ranged from 34% after 20 weeks in the study from Bauer et al. to 54% after 24 weeks in the study of He et al. Rong et al. showed a seizure reduction of 47%

after 24 weeks. The responder rate was approximately 30% in the study of Bauer et al. and approximately 54% in the study of He et al. Rong et al. did not report responder rates.

Figure 2: transcutaneous vagus nerve stimulation

Recently, He et al. (37) showed that there was no significant difference in animals between VNS and tVNS with respect to the anticonvulsive effect, but still a little is known about the possible effects of tVNS in humans and there is also not much evidence that the ABVN projects to the same parts of the brain as the central vagus nerve. However, Frangos et al. (38) have shown with fMRI that electrical stimulation of the left cymba conchae produces significant activation of "classical" central vagal projections including widespread activity in the ipsilateral NTS, bilateral spinal trigeminal nucleus, LC, dorsal raphe nucleus (DRN), contralateral parabrachial nucleus (PBN), amygdala and nucleus accumbens (38). It is also shown that the human ABVN consists of somatosensory afferent fibers (A- and C-fibers) (20,39). Safi et al. (40) demonstrated that the human ABVN also comprises thick myelinated axons of the Aβ class, which are only five to six times less numerous than those in the cervical vagus nerve. The thick myelinated axons of the Aβ-fibers project to the NTS of the brainstem and it is presumed that these fibers are involved in the effects of both VNS and tVNS (20,40,41). This may present an anatomical basis for the clinical effectiveness of tVNS in the cymba conchae as a valuable alternative to VNS, which means that it may be possible to use the tVNS as a predictor for the effect of VNS. In order to gain more information about the effectiveness of tVNS, research should be performed on the reflex and sensory responses elicited by tVNS. This can provide functional indicators of the type of fibers recruited and provide more evidence that tVNS really projects on the ABVN.

1.4 Autonomic effects of the vagus nerve stimulator

In addition to the anti-seizure effect of VNS, it has been shown by different studies that the afferent stimulation of the vagus nerve also affects the autonomic nervous system (ANS) (39,42–46). The autonomic activities caused by stimulation of the vagus nerve do not play a role in the anti-seizure effect, but they could be a marker for the stimulation effects of VNS.

One of the autonomic activities caused by afferent stimulation of the vagus nerve is a change in the pupillary size. Desbaumes Jodoin et al. (42) and Bianca and Komisaruk (43) showed that VNS causes an increase in resting pupil diameter during VNS. Another effect of VNS on the autonomic activities are the changes in the heart rate and heart rate variability (HRV). It has been demonstrated that VNS has an influence on the cardiac function. Changes in heart rate and HRV have been reported by several investigators (22,26,44,47). Mulders et al. found a decreased heart rate in about 80% of the patients during VNS episodes in both rest and exercise (26). Frei and Osorio found a clear effect of left VNS on the heart rate and the HRV. They demonstrated the occurrence of bradycardia as well as a combination of bradycardia and tachycardia during VNS (47). HRV was either increased or decreased during stimulation, depending on the subject and on

the stimulation parameters. Zaaimi et al. showed complex effects of VNS on the heart rate in children (44).

Pupil diameter, heart rate and HRV changes could be caused by VNS due to stimulation of the LC and NTS (28,39,42,45–50). Despite the fact that these changes do not play a role in the anti-seizure effect, they could be considered as markers for LC and NTS stimulation, which are important for the anti-seizure role of VNS. To assess if the tVNS can be used as a predictor of the effect of VNS, it is important that tVNS has similar effects as VNS. Therefore, it is expected that variations in pupil diameter and cardiac function due to tVNS are markers that tVNS really stimulates the ABVN and consecutively the NTS. This would be an important indicator for the anti-seizure effects of tVNS.

1.5 Aim of this master thesis

The aim of this master thesis is to provide more insight into the effects of tVNS: to assess if the ABVN and consecutively the NTS are really stimulated by tVNS. This information is helpful in determining the predictive role of tVNS for the success of VNS.

The aim of this master thesis is to study the effect of tVNS on the pupil diameter, heart rate and HRV. These effects are assessed in both epilepsy patients and healthy subjects. Two different stimulation intensities are used, to assess the differences between tVNS stimulation at low intensity and maximal intensity. Also, two stimulation locations are used, to assess the differences between real tVNS stimulation at the cymba conchae and sham stimulation at the earlobe.
Furthermore, the effect of an already implanted VNS on the pupil diameter and heart rate is assessed in epilepsy patients with an implanted VNS.

Beside this study I have also performed two other studies about epilepsy and the effects of tVNS. These researches are not included in the main body of this master thesis, but are added to the Appendix.
The first study is a clinical pilot study in eight patients to assess the effect of tVNS on the seizure frequency, quality of life and mood in patients with refractory epilepsy (Appendix I).
The second study is conducted in over 100 patients with epilepsy and control subjects to assess if epilepsy can be detected with the use of breath analysis via an electronic nose (Appendix J).

1.6 Hypotheses

The first hypothesis is that there is an increase in pupil diameter and HRV, and a decrease in heart rate during tVNS stimulation compared with periods of no tVNS stimulation.
The second hypothesis is that these effects will only occur during cymba conchae stimulation and not during sham stimulation.
The third hypothesis is that both low tVNS stimulation and maximal tVNS stimulation show an increase in pupil diameter. The heart rate only decreases during maximal tVNS stimulation.
The last hypothesis is that an implanted VNS shows an increase in pupil diameter and a decrease in heart rate during VNS stimulation compared with periods of no VNS stimulation.

2. Background information

2.1 Vagus nerve

The vagus nerve is the 10th cranial nerve and is a mixed parasympathetic nerve, containing both afferent sensory (80%) and efferent motor (20%) fibers (17–20). The cell bodies for the efferent fibers are located in two subnuclei: 1. the dorsal motor nucleus of the vagus nerve, which projects to the pharynx, larynx, and gastrointestinal tract, and 2. the nucleus ambiguous, which innervate the skeletal muscles of the neck and face and modifies the heart rate and the heart rate variability, providing parasympathetic control of the heart (18–20). The vagus nerve carries afferent fibers from the thoracic, abdominal viscera and from aortic baroreceptors and chemoreceptors of the aortic arch. These afferent fibers play a crucial role in the reflex regulation of digestive, respiratory and cardiovascular functions, such as slowing of breathing, bradycardia, and vasoconstriction (18,21). The cell bodies of these afferent fibers are located in the nodose ganglion and transmit to the caudal part of the NTS. Another part of the afferent fibers carries touch, pain and temperature information from the ear and parts of the pharynx and larynx. The cell bodies of these afferents are located in the jugular ganglion and transmit to the spinal trigeminal nucleus.

2.2 Anticonvulsive effects of vagus nerve stimulation

During VNS the different vagal nerve fibers are depolarized and the anticonvulsant effect of VNS may depend on the type of sensory afferent fibers stimulated by the device (51,52). The ability of VNS to depolarize nerve fibers depends on the intensity and duration of the stimulus. In other words, it depends on the amplitude of the current and the width of the pulse (53). If the width of the pulse is kept constant and the stimulus intensity is increased stepwise, large low-threshold nerve fibers will be depolarized first. As the intensity increase, higher-threshold smaller fibers would be gradually recruited (53–55).

The large myelinated vagal A-fibers have the lowest amplitude-duration threshold required for VNS to excite action potentials (ranging from 0.02 to 0.2 mA). The excitation thresholds of the B-fibers are a little higher (ranging from 0.04 to 0.6 mA) and the excitation threshold of the small unmyelinated C-fibers are the highest (more than 2.0 mA) (21,55). When the excitation threshold for the C-fibers is reached, the A- and B-fibers are also recruited (55).

Recent studies suggests that the anti-seizure effects of VNS depends on the activation of A- and B-fibers, and not on the activation of C-fibers, because destruction of the C-fibers in animals does not alter the anti-seizure effects (21,22,40,53,54,56,57). Activation of C fibers is therefore unnecessary and probably also undesirable, because animal studies show autonomic effects such as bradycardia during stimulation of the C-fibers (52,53,58). Because epilepsy is a disease with a cortical origin, it is preferred to stimulate unidirectional, which means only the activation of the afferent vagal fibers (57).

Stimulation of the afferent vagal fibers of the vagus nerve projects to the NTS. The NTS is considered to play an important role in the mechanism of VNS and projects to the dorsal raphe nucleus (DRN), parabrachial nucleus (PBN), and the LC in the brainstem as well as to the amygdala, hypothalamus, thalamus, and cortex, see figure 3 (25,42). The projection of the NTS to the LC is suggested to be important for the anti-seizure effect of VNS, because the LC is the main source for norepinephrine (NE) in the forebrain (28). Animal studies showed that VNS causes an enhancement of the NE concentrations in the cortex, nucleus accumbens, and hippocampus (59–61). Data supporting the crucial role of the LC-NE system in the anti-seizure effect of VNS show that when the LC is lesioned, the anti-seizure effect of VNS is blocked (29). Furthermore, it appears

that the activity of the LC is critical in limiting the spreading and duration of seizures and impairment of the LC-NE system increases the neuronal damage (62,63).

The activation of the LC-NE system is not the only process that plays an important role in the anti-seizure effect of VNS. The anti-epileptic effects of VNS have also been attributed by an increased synaptic activity in the thalamus and thalamo-cortical projection pathways, which would result in an increased arousal and possibly a decreased synchrony of synaptic activities between and within cortical regions (25).

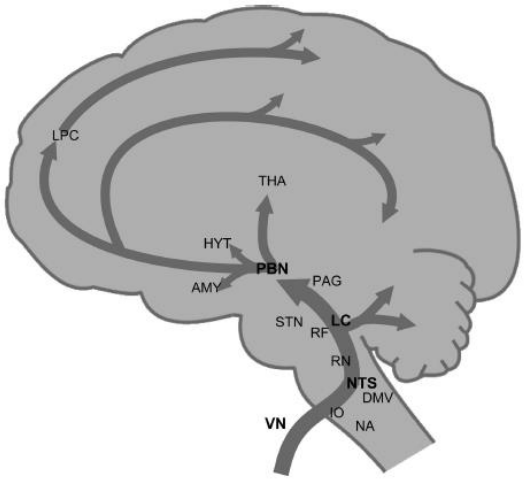


Figure 3: Anatomic overview of the projections of the vagus nerve. (A) Schematic representation of the brain region innervated directly or indirectly by the vagus nerve. VN = Vagus Nerve, IO = Inferior Olive, NA = Nucleus Ambiguous, DMV = Dorsal Motor Nucleus of the Vagus, NTS = Nucleus Tractus Solitatrius, RN = Raphe Nuclei, RF = Medial reticular formation of the medulla, LC = Locus Coeruleus, STN = Spinal Trigeminal Nucleus, PAG = Peri-Aqueductal Gray, PBN = Parabrachial Nucleus, AMY = Amygdala, HYT = Hypothalamus, THA = Thalamus, LPC = Lateral Prefrontal Cortex (25)

2.3 Pupil response

Pupillary response is a response that varies the size of the pupil, via the optic and oculomotor cranial nerve. Pupil diameter is under a dual autonomic innervation. The sympathetic part of the ANS innervates the dilator muscle of the iris. The preganglionic sympathetic neurons are located in the C8-T1 segments of the spinal cord. The axons of these preganglionic neurons project to the sympathetic chain and travel in the sympathetic trunk to the superior cervical ganglion (64). Within the superior cervical ganglion, the preganglionic axons form nicotinic, cholinergic synapses with postganglionic neurons. The axons of these postganglionic neurons project from the superior cervical ganglion to the orbit, where they enter the eye via the short and long ciliary nerves and travel to the iris (figure 4). Here they release norepinephrine, which acts on the adrenoreceptors of the dilator muscle (64,65).

The parasympathetic component of the autonomous nervous system innervates the sphincter muscle of the iris. The preganglionic parasympathetic fibers controlling the sphincter originate from neurons in the Edinger–Westphal nucleus (EW) and travel via the third cranial nerve to the ciliary ganglion,

which is located within the orbit of the eye (Figure 4). Within the ciliary ganglion, the preganglionic constriction neurons form nicotinic, cholinergic synapses with the postganglionic neurons. The axons of these postganglionic neurons leave the ciliary ganglion to enter the eye via the short ciliary nerves and travel to the iris. Here they release acetylcholine, which acts on the muscarinic receptors of the sphincter muscle (64,65).

VNS could affect resting pupil diameter through several routes. The main target of the vagus nerve, the NTS, has widespread projections to nuclei affecting autonomic function including the hypothalamus, nucleus paragigantocellularis, LC, and PBN. The LC can modulate both sympathetic and parasympathetic outflow to the pupil, and the PBN and LC both projects to the cholinergic part of the EW nucleus, controlling the pupil size (42,48–50). The pupil size is closely linked to the LC (28).
During tVNS it is shown that the sensory vagus also projects on the NTS and LC (38). This suggests that the pupil diameter could provide information about the effect of tVNS on the autonomic nervous system.

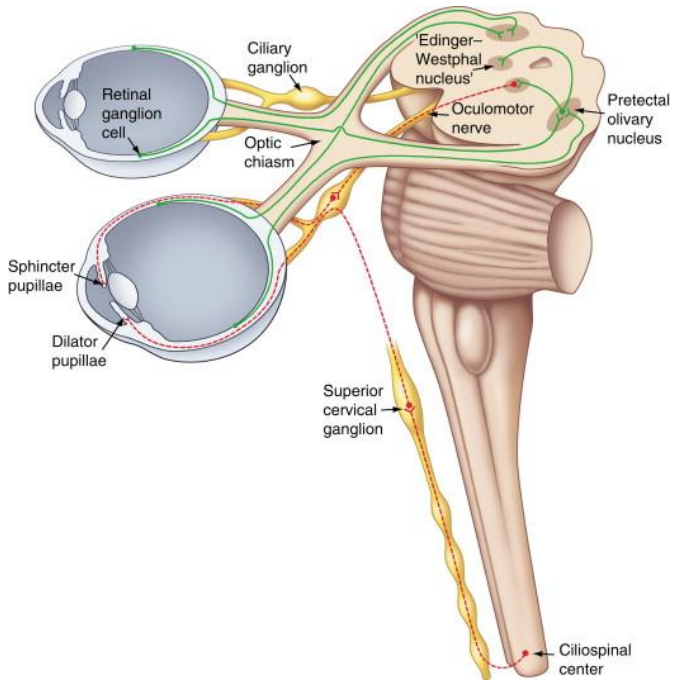


Figure 4: Anatomical drawing showing the parasympathetic and sympathetic innervation of the iris (64).

2.4 Cardiac response

The heart itself is capable of generating its own electrical impulses, which results in coordinated and rhythmic contractions of its chambers. The rhythmic and spontaneous contraction of the heart is initiated by the periodic electrical discharge of the sinoatrial (SA) node, which is the heart's pacemaker (66,67). In addition to this intrinsic regulation of the electrical activity of the heart, the control of the ANS is also essential for the regulation of the heart rate (67–69).
The parasympathetic part of the ANS arises from the dorsal motor nucleus or the nucleus ambiguous within the medulla oblongata in the brainstem, and efferent outflow occurs via the vagus nerve (68,69). From the vagus nerve they synapse with postganglionic cells on the epicardial surface and releases acetylcholine. The right and left vagi are distributed differentially to the various cardiac structures. The right vagus nerve affects the SA node predominantly and the left vagus nerve affects

the atrioventricular (AV) node predominantly (69). The parasympathetic part of the ANS decreases the heart rate (67–70).
The sympathetic part of the ANS arises from the upper thoracic region of the spinal cord. Short preganglionic enter the paravertebral chains of the ganglia (68,69). These preganglionic fibers synapse with postganglionic fibers. Long sympathetic efferent fibers extend to the SA and AV nodes in the heart and releases the neurotransmitter NE. This results an increase in the heart rate (67–70).

VNS could have an influence on the cardiac effects via the autonomic way or via the efferent way. VNS may result in direct excitation of efferent cardiac vagal fibers. If the stimulus threshold for a total anodal block of the VNS is not reached, the VNS can propagate in a bidirectional way to the brainstem and the heart. Depending on the circumstances efferent vagal stimulation can produce either a decrease or an increase in the heart rate (44).

VNS can also affects the autonomic activity (39,44–46). Afferent stimulation of the vagus nerve may cause reflex autonomic activation via the sympathetic chain and dorsal root ganglia, but it can also cause an increase in the parasympathetic efferent outflow (46). The afferent stimulation of the vagus nerve synapse in the NTS, which could activate the dorsal motor nucleus of the vagus and the nucleus ambiguous to increase the parasympathetic activity (39,45,46). Stimulation of the NTS by VNS sends also excitatory projections to the caudal ventrolateral medulla (CVLM). The CVLM inhibits the rostroventrolateral medulla (RVLM) which is the primary source of excitatory drive to sympathetic preganglionic neurons in the intermediolateral column (IML) of the spinal cord. This inhibition would decrease the sympathetic activity (39,45).

When VNS changes the cardiac response via the autonomic pathway, it also influences the HRV. HRV is generally used for the evaluation of the ANS (71). HRV represents the difference between successive heartbeats, and is caused by the interaction between the parasympathetic nervous system and the sympathetic nervous system (20,72,73). Activity of the sympathetic pathway increases the heart rate and decrease the HRV, whereas activity of the parasympathetic pathway decreases the heart rate and increases the HRV (74,75). Normally the HRV is high due to several physiological factors, for example respiration, day- or nighttime, exercise or psychological conditions (76).
To analyze the separate sympathetic and parasympathetic contributions to the ANS, the frequency domain of the HRV is analyzed. High frequency (HF) component in the frequency domain reflects the parasympathetic modulation of the heart rate and the low frequency (LF) component reflects the sympathetic modulation of the heart rate, but some studies suggest that vagal activity plays a role as well (71,74,75,77). The LF/HF ratio provides information about the autonomic balance, such that a decrease in LF/HF ratio indicates a shift in cardiac autonomic balance toward parasympathetic predominance and improvement in HRV (decrease heartrate) (45). This could either be due to a decrease in sympathetic activity or an increase in parasympathetic activity.

Clancy et al. (45) have shown that tVNS may also influence the activity of the sympathetic nervous system. In healthy human volunteers, tVNS significantly improved the HRV. For the tVNS it is expected that it influence the cardiac parameter via the afferent way through the NTS (39,45). This suggests that the heart rate and heart rate variability could provide information about the effect of tVNS on the autonomic nervous system.

3. Method

3.1 Subjects

Three groups of subjects were recruited for this study. The first group consisted of healthy participants, the second group consisted of patients who were diagnosed with epilepsy, and the third group consisted of patients who were both diagnosed with epilepsy and implanted with a VNS. Patients diagnosed with epilepsy were recruited via the department of neurosurgery at the Medical Spectrum Twente, the Netherlands. Flyers were used to recruit the healthy subjects. Additionally, some relatives of epilepsy patients volunteered as healthy control.

The inclusion criteria for all subjects were: eighteen years or older; and physically and cognitively capable of participating the study. For the two patient groups, it was also important that they were diagnosed with epilepsy. An additional inclusion criterion for the epilepsy patients with an implanted VNS was that the patients were actively being treated with the VNS. Exclusion criteria included: implanted with a pacemaker; presence of an eye disease; eye surgery in the past; history of cardiovascular disease; diabetes; use of beta blockers; and use of anticholinergic or psychostimulant medication. Subjects were asked not to smoke and not to consume caffeine or alcohol three hours before participation.

The study followed the principles laid out in the Declaration of Helsinki and ethical approval was obtained from the Medical Research Ethics Committee Twente. All subjects gave written informed consent.

3.2 Measurement procedure

Measurements were performed at the department of neurosurgery at the Medical Spectrum Twente. Prior to the measurement subjects were asked about their demographics and lifestyle-related factors. For the patients with epilepsy information was also obtained about their epilepsy. Appendix A shows the questionnaires for the different group of subjects.
The effect of tVNS on the study parameters: pupil diameter, heart rate and HRV was assessed for the healthy subjects and the patients with epilepsy (figure 5). For the epilepsy patients with a VNS, the effect of their own VNS on the study parameters pupil diameter and heart rate was assessed (figure 5).

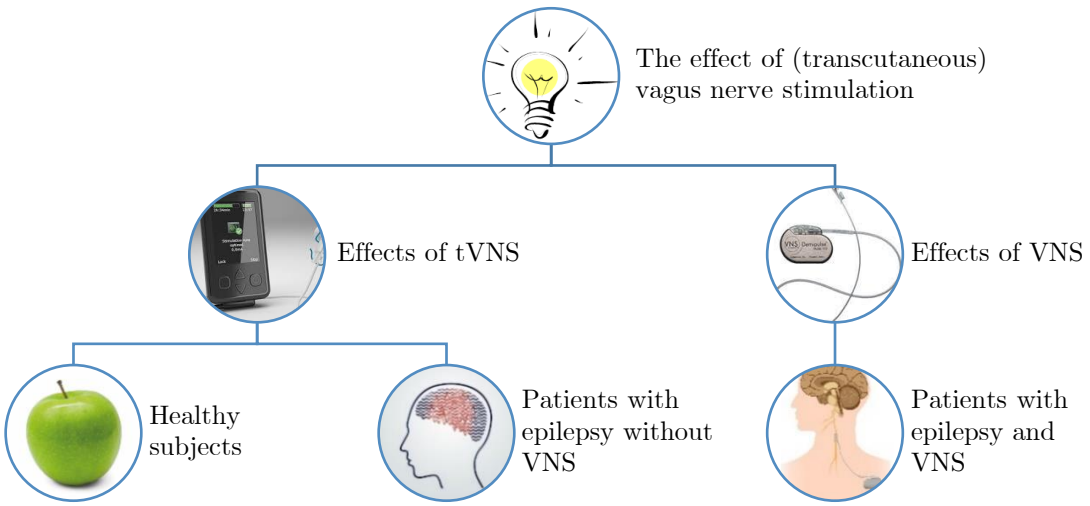


Figure 5: An overview of the different measurement groups. The effect of tVNS is studied for the healthy subjects and the patients with epilepsy without VNS. The effect of VNS is studied for the patients with epilepsy and VNS.

3.2.1 tVNS measurements

tVNS was administered through a tVNS device, which was obtained from Cerbomed GmbH (Erlangen, Germany). The tVNS device stimulated with a stimulation frequency of 25 Hz, 30 seconds ON/OFF phases, and stimulation intensities adjusted for the individual subjects.

Measurements took place in a quiet room with a controlled ambient lighting level (340 lux) from above the subject's head. One measurement consisted of eight periods with a total duration of 30 minutes (figure 6) and during these 30 minutes subjects had to sit in front of a computer screen at a distance of 50 cm. The full measurement (all eight periods) had been conducted twice in each subject, with the tVNS administered once at the earlobe and once at the cymba conchae.

The measurement conditions during the eight periods of a measurement were:

- No tVNS stimulation,
- tVNS stimulation at a low intensity level, and
- tVNS stimulation at a maximal intensity level.

During period 2, 3, 5, 6, and 8 of the measurement, tVNS stimulation was applied. During period 2,3, and 8 tVNS stimulation was applied with a low intensity stimulation: stimulation intensity just above the subject's detection threshold (first tingling sensation). During period 5 and 6 tVNS stimulation was applied at a maximal intensity level: just below the subject's pain threshold.

During period 2, 3, 5, and 6, the tVNS stimulated with a 30 second ON phase and a 30 second OFF phase. Period 8 consisted of continuous tVNS stimulation of 5 minutes.
Period 1, 4 and 7 were resting periods in which no tVNS stimulation was applied and subjects just looked at the computer screen.

The study parameters that were assessed during the tVNS measurements were the pupil diameter, the heart rate and the HRV.
The pupil diameter was measured during period 3 and period 6 of the measurement. The pupil diameter recordings were performed using trials of 6 seconds. When a trial started, a fixation cross appeared on the black computer screen for 2 seconds. After 2 seconds a with disk (23 lux, 15 cm diameter) appeared in the centre of the screen for 1.5 seconds to provoke a pupillary light reflex. The trial terminated 2.5 seconds after the end of a stimulus. A 5 second pause was inserted at the end of each trial to let the pupil recover from constriction and to allow the subject time to blink. A maximum of 2 trials was presented during each 30 second stimulation period (ON phase) and each 30 seconds of no stimulation (OFF phase). Appendix B shows a figure of the trials that were shown during the measurement.
The heart rate was measured continuously throughout the measurement. HRV was measured during rest (period 7) and during the period of continuous stimulation at a low intensity level (period 8).

The tVNS measurements were performed at two locations to assess the differences in effects of tVNS stimulation at the cymba conchae and sham stimulation at the earlobe. Cymba conchae stimulation was applied according to figure 7A. Earlobe stimulation was conducted by positioning the earpiece of the tVNS upside down (figure 7B). The sequence of stimulation location was varied among the subjects. Subjects were unaware of the fact that stimulation at the earlobe was supposed to be sham stimulation.

More information about the measurement procedure and information about the used measurement equipment can be found in Appendix C and D.

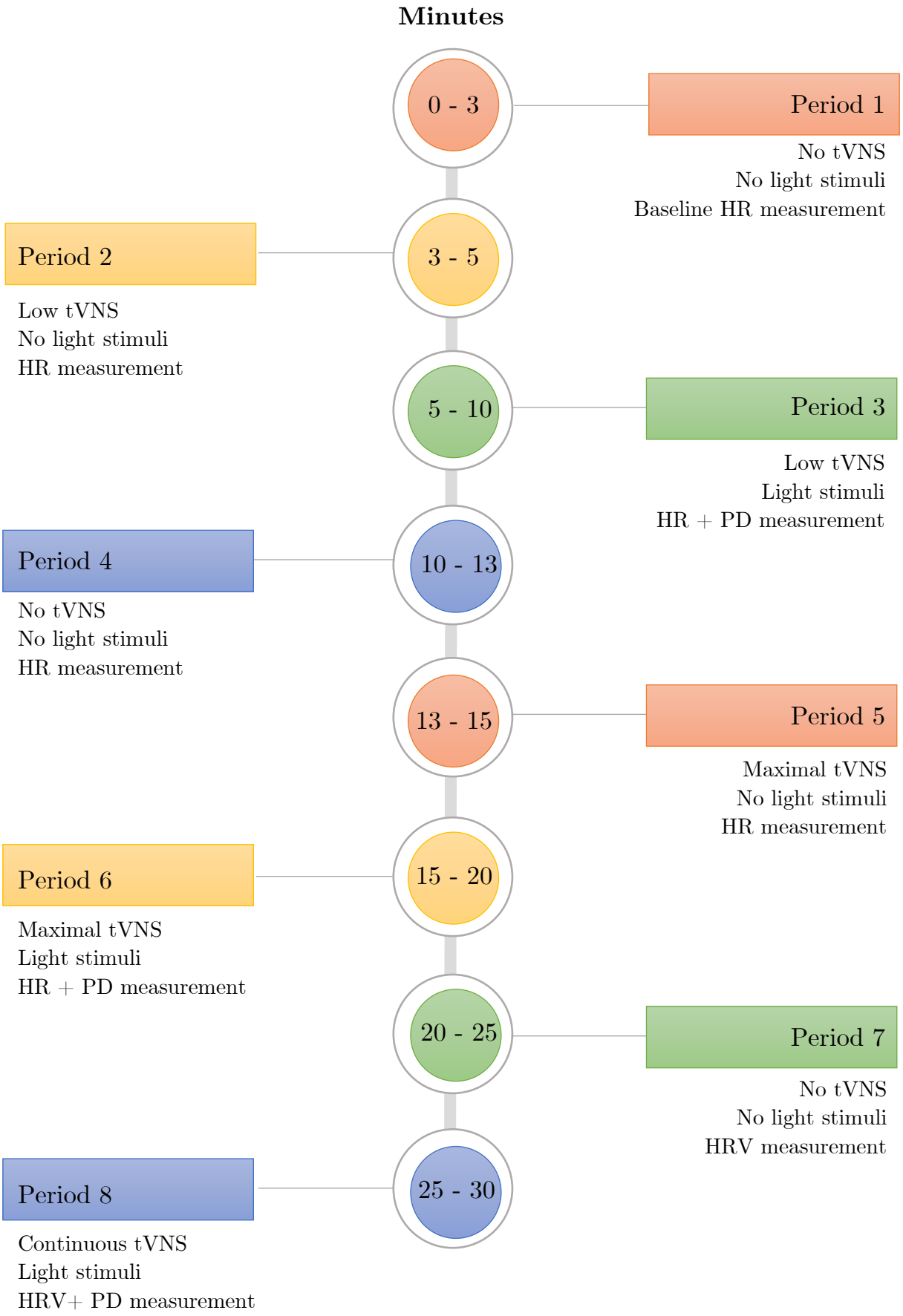


Figure 6: An overview of the measurement procedure of the tVNS measurements. HR = heart rate, HRV = heart rate variability, PD = pupil diameter.

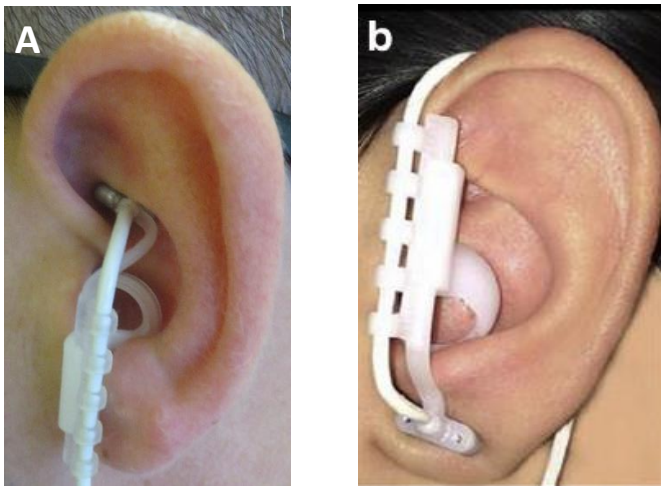


Figure 7: Two different stimulation locations used for the measurements. A shows cymba conchae stimulation. Earlobe stimulation is shown in B.

3.2.2 VNS measurements

During the measurements with VNS the only measurement conditions were with VNS stimulation (ON phase) and without VNS stimulation (OFF phase). The study parameters that were measured were the pupil diameter and the heart rate.

The VNS measurement set-up was identical to the tVNS measurement set-up, but because of the different stimulation settings for VNS compared with tVNS, the periods were slightly different. The stimulation settings of the VNS had a 30 second ON phase and a five minute OFF phase. The duration of the measurement was 22 minutes and consisted of eight periods. Period 2, 4, 6 and 8 contained the ON phase of the VNS. The other four periods contained the five minutes OFF phase of the VNS. The pupil diameter was measured during period 1, 2, 3, 4, 6 and 7. Pupil diameter recordings were performed with the use of the same stimuli as used during the measurement with tVNS. The heart rate was measured continuously throughout the measurement. The HRV was not measured, because of a too short stimulation time of the VNS (30 seconds).

3.3 Analysis

3.3.1 Pupil diameter analyses

Pupil diameter recording were performed during period 3 (low stimulation intensity) and period 6 (maximal stimulation intensity). During each period 10 trials with light stimuli were shown during the ON phase and 10 trials during the OFF phase. The onset of all the trials were marked in the Tobii Pro Lab 1.76 analysing software (Tobii Technology) and loaded into Matlab (MATLAB, 2014b, the MathWorks, Inc), together with the time and pupil diameter data.

In Matlab the pupil diameter data was interpolated with the use of linear interpolation to correct for missing pupil diameter data, and for missing time data. The pupil diameter recordings of the 10 trials from each of the stimulation conditions (ON phase low stimulation, OFF phase low stimulation, ON phase maximal stimulation, OFF phase maximal stimulation) were extracted from the overall pupil diameter data and checked for corrupt data. When the pupil diameter recording of a trial was affected with artefacts or errors due to missing data, the trial was excluded. When at least 4 trials with pupil diameter recordings per stimulation condition were available, an average was calculated from the pupil diameter recordings for that stimulation condition, which resulted in an average pupil diameter recording for each of the four stimulation conditions: the ON phase of

low stimulation, the OFF phase of low stimulation, the ON phase of maximal stimulation and the OFF of maximal stimulation. Pupil diameter recording have been done for both eyes.

The resting pupil diameter was calculated by measuring the average pupil diameter 1 second prior to the onset of the light reflex stimulus.
The contraction amplitude was defined as the difference between minimal pupil diameter after the onset of the light stimulus and the resting pupil diameter.
Resting pupil diameter and constriction amplitudes were calculated for the average pupil diameter recording of (at least) four stimulation conditions for each eye and for each subject.

For the measurement with the VNS, 14 trials were measured during the OFF phase and 4 trials during the ON phase. The average resting pupil diameter and constriction amplitude were calculated for the ON and OFF phase for each eye and each subject.

3.3.2 Heart rate analyses

The lead II ECG was chosen for the analysis of the heart rate, because it detects the most prominent R peak (figure 8) (45). Obtained ECG data was filtered with a bandpass Butterworth filter from 0.5 to 50 Hz with the use of Matlab. For every subject epochs of 25 seconds were selected during the periods of no stimulation, low stimulation, and maximal stimulation. Two epochs were selected during the period of no stimulation (period 1), which contained the baseline heart rate. Fourteen epochs were selected during each period of low stimulation (period 2 and 3) and the period of maximal stimulation (period 5 and 6). Seven of the fourteen epochs were during the ON phase of the tVNS and seven epochs were during the OFF phase. The epochs from the ON phase were separated from the epochs with the OFF phase.

For every epoch the heart rate was calculated. R-peaks from the QRS-complex of the ECG were detected using the Matlab function findpeaks and were checked visually for each epoch for missing peaks. When all peaks were correctly defined, the time between the peaks was calculated (R-R interval) and subsequently the heart rate. If all peaks were not defined correctly within an epoch, the epoch was excluded from further analysis.

Heart rate values were averaged for the epochs for baseline, ON phase, and OFF phase for each subject and for both low and maximal stimulation.
For the measurements with the VNS, the difference in heart rate was only assessed between the epochs of the ON phase and the epochs of the OFF phase.

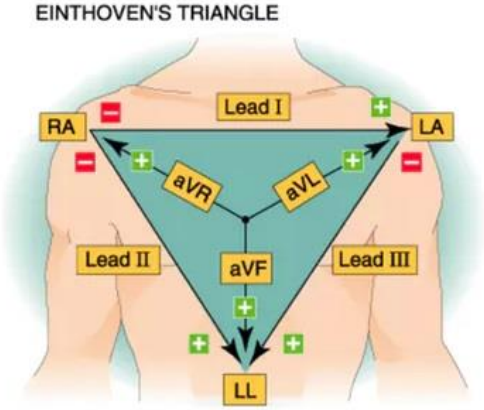


Figure 8: Three lead wire ECG system. A lead II ECG was obtained from the negative electrode of the right arm, which was placed below the clavicle of the right shoulder, and the positive electrode of the left leg, which was placed below the left pectoral muscle near the apex of the heart.

3.3.3 Heart rate variability analyses

The HRV was obtained from the lead II ECG from the last ten minutes of the measurement with the tVNS. The last ten minutes existed of five minutes of no stimulation (period 7) and five minutes of continuous tVNS stimulation at low stimulation intensity (period 8). With the use of Matlab the R-peaks of the QRS-complex of the ECG were detected and the R-R intervals were calculated. The R-R intervals were loaded into the Kubios HRV standard software (version 3.0.2) to analyse the HRV and to calculate the time-domain parameters and frequency-domain parameters. Artefacts in the R-R interval due to ectopic beats or missed peak detection were corrected in Kubios by choosing an appropriate correction level which removed the artefacts. When corrections were applied, detected artefact beats were replaced using cubic spline interpolation (75,77).

The default settings of Kubios were used for the further analysis, because those preference values were designed for short-term human HRV recordings (75). In the time-domain the standard deviation of the normal-to-normal R-R interval (SDNN) and the root mean square of successive differences (RMSSD) were calculated. The R-R intervals were represented in Kubios in the form of a discrete event series (DES), which was the plot of R-R interval vs time. For the frequency-domain, the spectrum of the HRV signal was generally calculated by interpolating the DES. It was advised to calculate the Power Spectral Density (PSD) of the DES with the non-parametric method (77). In Kubios the spectrum is estimated with the non-parametric Welch's periodogram (75). Subsequently, the low frequency (LF) component (0.04 to 0.15 Hz), the high frequency (HF) component (0.15 to 0.4 Hz) and the LF/HF ratio were calculated (20,75,77,78).

3.4 Statistical analysis

To test if there are differences in the effect of tVNS on the study parameters during the ON phase compared to periods of no stimulation, paired-samples t-tests were used. For pupil diameter parameters and the heart rate the mean ON phase between low intensity stimulation and maximal intensity stimulation was compared with the mean OFF phase between low intensity and maximal intensity stimulation. For the heart rate the mean ON phase was also compared with the baseline. For HRV parameters the ON phase was compared with the OFF phase. The paired-samples t-tests were performed for each stimulation location individually.

To test if the effects of tVNS only occur during cymba conchae stimulation and not during earlobe stimulation, also paired-samples t-tests were performed. For each study parameter the difference was calculated between the mean ON phase and the mean OFF phase for each subject. The differences during cymba conchae stimulation were compared with differences during earlobe stimulation with the use of a paired-samples t-test.

The differences between low intensity tVNS and maximal intensity tVNS were only assessed for the pupil diameter parameters and the heart rate. To test for these differences also paired-samples t-tests were performed. The difference between the ON phase and OFF phase during low stimulation were calculated for the pupil diameter parameters and for the heart rate for each subject and were compared with the difference between the ON phase and the OFF phase during maximal stimulation. For the heart rate the ON phase of both stimulation intensity was also compared with the baseline.

For the effect of VNS on the heart rate and pupil diameter, the ON phase was compared with its OFF phase with the use of a paired-samples t-test.

4. Results

4.1 Subjects

A total of 29 subjects are included in this study. The group of healthy subjects consist of 21 subjects with a mean age of 34 (19-55) years, the group of epilepsy patients without a VNS consist of 4 subjects with a mean age of 47 (33-64) years, and the group of epilepsy patients with a VNS also consist of 4 patients, with a mean age of 51 (34-59). An overview of the subject characteristics together with the used (t)VNS settings during the measurements are shown in table 1. No subjects are excluded based on the exclusion criteria.

Table 1: Subject characteristics. Subjects with an asterisk are patients who started first with cymba conchae stimulation and thereafter with earlobe stimulation.

Subjects	Sex	Age (y)	Intensity tVNS cymba conchae [low(mA)/max(mA)]	Intensity tVNS earlobe [low(mA)/max(mA)]	Duration epilepsy (y)
Healthy subjects					n/a
C01	F	24	1.5/4.5	1.6/4	
C02	M	54	1.8/3.6	0.5/1.8	
C03	M	25	1.2/3.2	1.3/3.5	
C04	F	33	1.3/5	1.2/4.4	
C05	M	29	2.7/4.8	1.3/4.7	
C06*	F	22	0.7/4.2	1.4/4.2	
C07*	F	55	1.3/4.6	1.9/3.5	
C08	F	53	0.9/2.5	1.7/4.5	
C09	M	25	0.8/2.9	1/3.9	
C10	F	37	0.8/5	0.6/5	
C11	M	29	1.1/5	1.7/5	
C12	F	22	2.5/3.7	0.4/1.8	
C13	M	19	1.3/1.7	1.7/2.9	
C14	M	21	2.9/5	1.8/3.5	
C15	F	54	2.7/5	1.9/5	
C16*	F	25	3/5	1.8/5	
C17*	M	52	2.2/5	2/4.6	
C18*	M	25	1.6/3.8	1/2.1	
C19*	F	50	1/5	0.9/5	
C20*	F	43	1.5/5	1.4/3	
C21	F	29	1.3/5	1.3/5	
Epilepsy patients without VNS					
P01	M	33	2/5	0.9/5	5
P02	F	64	1.5/4.5	1.5/5	28
P03	M	36	2.5/5	1.2/4	34
P04	F	56	1.2/5	2.5/5	1
Epilepsy patient with VNS			VNS settings [mA/Hz/µs/s ON/min OFF]		
PV01	M	34	1.75/30/500/30/5		23
PV02	F	53	1/30/250/30/5		53
PV03	M	59	1.25/30/250/30/5		9
PV04	F	56	1.75/30/250/30/5		10

4.2 Pupil diameter

Due to some technical problems with the recording software of the Tobii Glasses Eyetracker Device, the pupil diameter recordings for the earlobe measurement are missing for healthy subject C16 and healthy subject C21. Due to too many missing data points in the pupil diameter recordings, the cymba conchae measurement is excluded for C21, for P04 and for PV03. The earlobe measurement is excluded for P04. For other subjects a part of the measurement is excluded, due to too many missing data. Appendix E show which subjects had missing data.

From the pupil diameter recordings of the 10 trials during each stimulation condition, the average pupil diameter recording is calculated. Figure 9 shows an example of the pupil diameter recordings of the 10 trails together with the average of those trials. This recording is from one the healthy subject (C01) during the ON phase of low intensity tVNS stimulation. More figures of the used data during the analysis are shown in Appendix F.

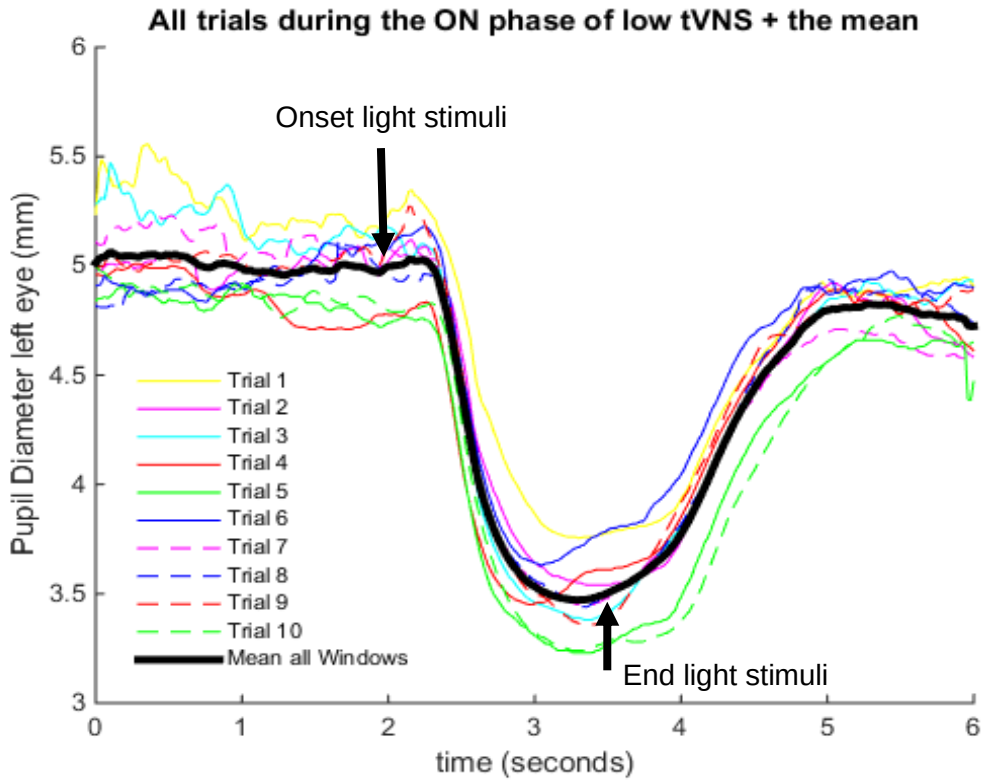


Figure 9: The pupillary response of the left eye of subject C01 during the ON phase of low cymba conchae stimulation. The pupil diameter recordings of the ten trials are plotted. Also, the mean of the ten pupil diameter recordings is calculated and is pictured with the black line. Two seconds after the start of a trial, the white disk appears on the computer screen for 1.5 second, which provokes a pupillary light reflex.

Figure 10 shows the average pupil diameter recordings of all the healthy subjects during the ON phase of tVNS stimulation and the OFF phase of tVNS stimulation at the cymba conchae and the earlobe for both eyes. This figure shows that there is an increase in the resting pupil diameter during the ON phase compared with the OFF phase for both stimulation locations and both eyes. The figure also shows that the constriction amplitude increases during tVNS stimulation.

The paired-samples t-test shows a statistically significant increase in the resting pupil diameter during the ON phase of tVNS compared with the OFF phase of tVNS for the healthy subjects

(p<0.05). Both the left eye and the right eye show this statistically significant increase ($p < 0.05$) and the increase is shown for both stimulation locations ($p < 0.05$). The statistical results are shown in table 2. With regard to the constriction amplitude a statistically significant increase is shown in both eyes during cymba conchae stimulation ($p < 0.05$) (table 2) and only in the left eye during earlobe stimulation ($p < 0.05$) (table 2). Appendix F shows a table with the average values fort the pupil diameter parameters.

Statistically significant increases in the pupil diameter parameters are shown for cymba conchae stimulation and earlobe stimulation (table 2). When comparing the effect of cymba conchae stimulation with the effect of earlobe stimulation, the paired-samples t-test show that there is no statistically significant difference in the effect of stimulation location for the resting pupil diameter (left: t(15)=1.31, p=0.210, right z=-1.26, p=0.21) and the constriction amplitude (left: t(15)=0.68, p=0.51, right: t(13)=1.66, p=0.12). During cymba conchae stimulation also no statistically significant differences in the pupil diameter parameters are shown between the left eye and right eye during the ON phase (resting pupil diameter: t(16)=-0.46, p = 0.65, constriction amplitude: t(16)=-0.47, p=0.65) and during the OFF phase (resting pupil diameter: t(16)=-0.53, p = 0.61, constriction amplitude: t(16)=-0.54, p=0.6). During earlobe stimulation also no statistically

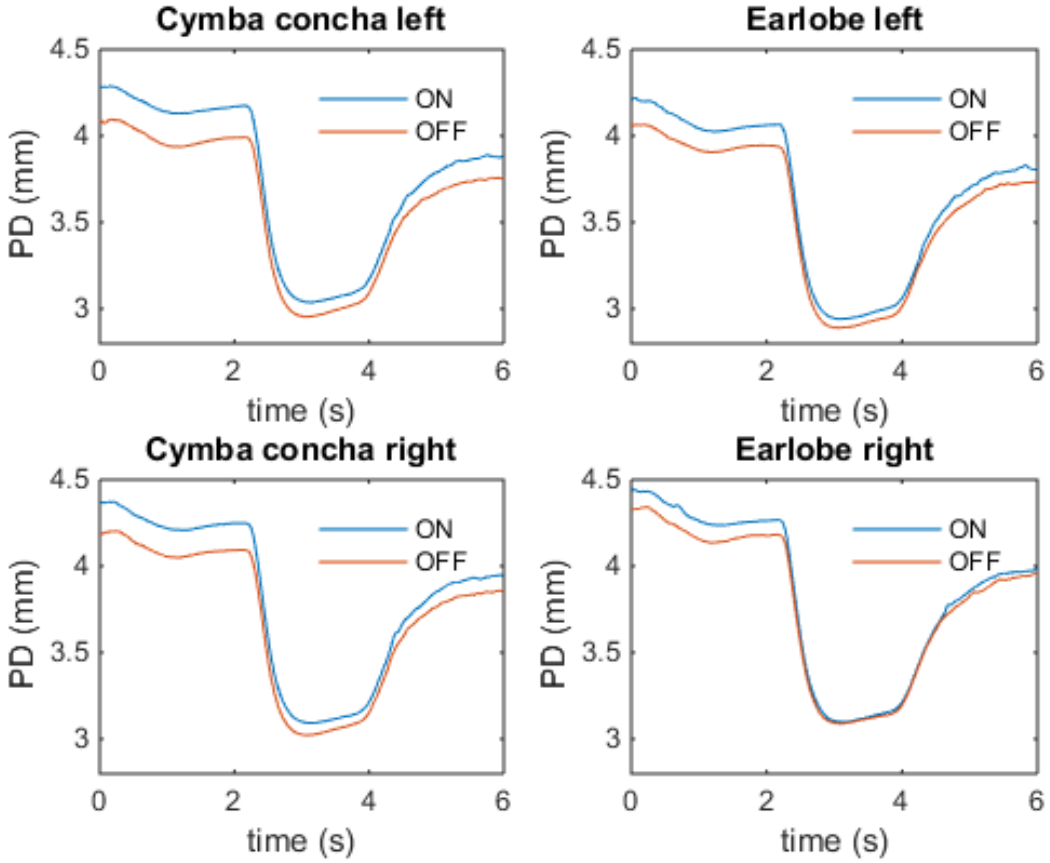


Figure 10: Pupil diameter recording during the ON and the OFF phase of both eyes and both stimulation locations. The figure in the top left shows the pupil diameter recording for the left eye during cymba conchae stimulation. Top right shows the pupil diameter recording for the left eye during earlobe stimulation. The two bottom figures show the pupil diameter recordings for the right eye. The bottom lefts show the results during cymba conchae stimulation and the bottom right during earlobe stimulation. The time of 6 seconds is the duration time of a trial. At 2 seconds the light stimulus is given to provoke a pupillary light reflex. The resting pupil diameter is calculated 1 second prior to the onset of the light stimuli. The constriction amplitude is the difference between the minimal pupil diameter after the onset of the light stimuli and the resting pupil diameter. PD = pupil diameter.

significant differences in the pupil diameter parameters are shown between the left eye and the right eye during the OFF phase (resting pupil diameter: t(12)=-1.46, p = 0.17, constriction amplitude: t(11)=-1.99, p=0.07). During the ON phase no statistically significant difference is shown between the left eye and the right eye for the constriction amplitude (t(13)=-1.64, p = 0.12). However, a statistically significant difference is shown for the resting pupil diameter (z=-2.56, p=0.01) between the right and left eye. The resting pupil diameter is larger for the right eye.

Looking at the results more detailed, the ON and OFF phase during low intensity tVNS stimulation are separated from the ON and OFF phase during maximal intensity tVNS stimulation. Figure 11 shows the average pupil diameter recording for the healthy subjects during low intensity stimulation and maximal intensity stimulation during both cymba conchae stimulation and earlobe stimulation. The results are shown for the left eye and show that there is an increase in the resting pupil diameter and the constriction amplitude during both low intensity and maximal intensity stimulation for both stimulation locations. The same results are shown for the right eye. A figure with the average values for all subjects during the different measurement conditions is shown in Appendix F

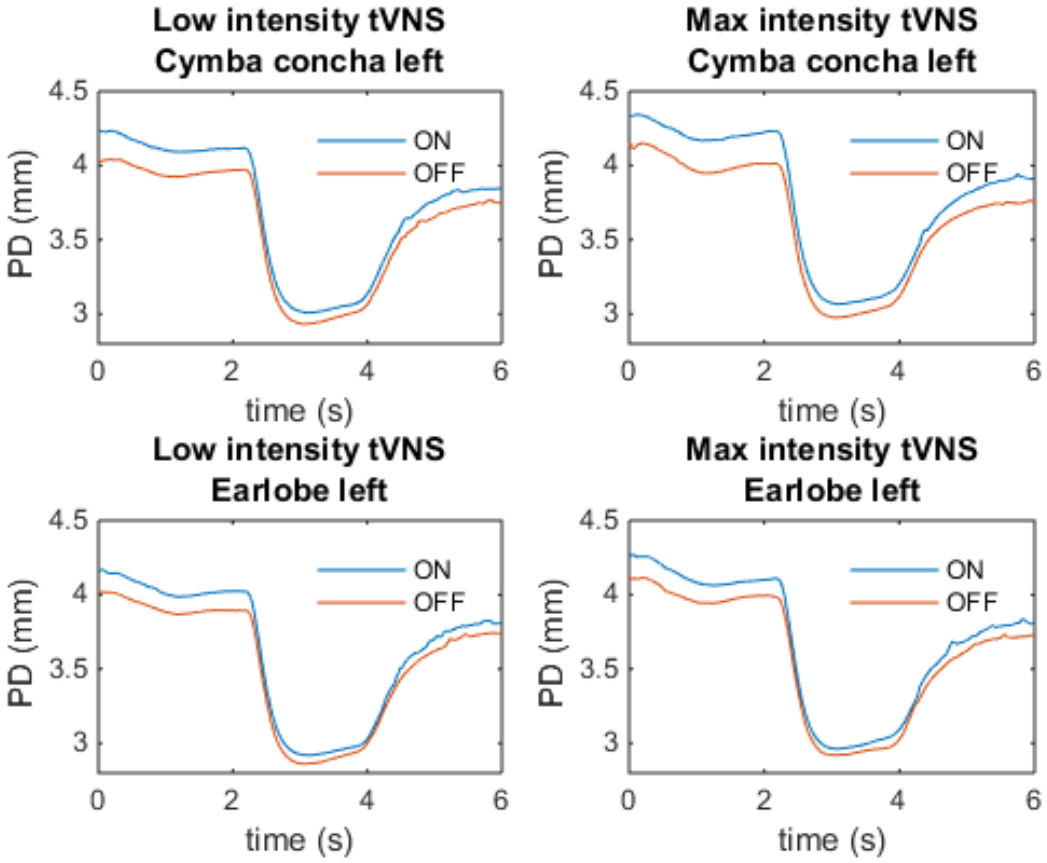


Figure 11: Pupil diameter recording during the ON and the OFF phase of the left eye during both low intensity stimulation and maximal intensity stimulation. The figure in the top left shows the pupil diameter recording for the left eye during low intensity cymba conchae stimulation. Top right shows the pupil diameter recording for the left eye during maximal intensity cymba conchae stimulation. The two bottom figures show the pupil diameter recordings during earlobe stimulation. The bottom left shows the results during low intensity and the bottom right during maximal intensity. The time of 6 seconds is the duration time of a trial. At 2 seconds the light stimulus is given to provoke a pupillary light reflex. The resting pupil diameter is calculated 1 second prior to the onset of the light stimuli. The constriction amplitude is the difference between the minimal pupil diameter after the onset of the light stimuli and the resting pupil diameter. PD = pupil diameter.

Comparing the effect of low intensity stimulation between the ON phase and the OFF phase with the effect of maximal intensity stimulation between the ON phase and the OFF phase, the paired samples t-test shows no statistically significant differences in the effects for the resting pupil diameter for both cymba conchae stimulation (left: t(17)=-0.75, p=0.46, right: t(16)=-0.3, p=0.77) and earlobe stimulation (left: t(16)=0.28, p=0.78, right: t(13)=0.33, p=0.75). Also no statistically significant differences are shown for the constriction amplitude (cymba conchae: left: t(17)=-0.39, p=0.71, right: t(16)=0.05, p=0.96. Earlobe: left: t(16)=-0.09, p=0.93, right: t(13)=-0.04, p=0.97).

The paired-samples t-test show a statistically significant increase in the resting pupil diameter and the constriction amplitude during the ON phase compared with the OFF phase for both stimulation intensities at the cymba conchae and both eyes. Table 2 shows the statistical results. For earlobe stimulation, a statistically significant increase in the resting pupil diameter and constriction amplitude is shown during low tVNS stimulation for both eyes (table 2). Maximal stimulation only shows a statistically significant increase in the resting pupil diameter of the left eye.

With regard to the patients without a VNS, the results show almost no differences between the ON phase and the OFF phase during cymba conchae stimulation for both eyes. During earlobe stimulation the resting pupil diameter and the constriction amplitude slightly increase during the ON phase compared OFF phase.
The results of cymba conchae stimulation also shows almost no differences in rest pupil diameter and constriction amplitude of both eyes during the ON phase compared to the OFF phase of low stimulation. For maximal stimulation a decrease in pupil diameter and constriction amplitude is shown for both eyes during the ON phase compared to the OFF phase. Earlobe stimulation also shows almost no differences in the rest pupil diameter and constriction amplitude for both eyes during the ON phase of low and maximal tVNS stimulation compared with its OFF phase. Mean values are shown in Appendix F. No statistical tests are performed because of the low number of subjects and due to too much missing data.

Epilepsy patient with a VNS shows an increase in resting pupil diameter and constriction amplitude for both eyes during the ON phase compared with the OFF phase. Appendix F shows the mean results these patients. For this group no statistical tests are performed.

Table 2: The results of the paired-sampled t-test for the pupil diameter parameters for the different measurement conditions.

	Cymba conchae stimulation		Earlobe stimulation	
Resting PD	Left	Right	Left	Right
ON vs OFF	t(17)=4.8, p=0.0002	t(16)=4.9, p=0.0002	z=-3.3, p=0.001	z=-2.3, p=0.02
ON low vs OFF low	t(18)=4.1, p=0.001	t(16)=3.9, p=0.001	t(16)=3.4, p=0.004	t(16) = 2.3, p=0.04
ON max vs OFF max	z= -3.6, p=0.0003	z= -3.3, p=0.001	z= -2.1, p=0.03	t(13)=1.5, p=0.15
Constriction amplitude	Left	Right	Left	Right
ON vs OFF	t(17)=3.8, p=0.001	t(16)=3.8, p=0.002	z= -2.9, p=0.004	t(13)=1.8, p=0.09
ON low vs OFF low	t(18)=3.1, p=0.006	t(16)=3.1, p=0.007	z = -2.7, p=0.006	z= -2.1, p=0.04
ON max vs OFF max	t(17)=3.4, p=0.003	t(17)=2.8, p=0.013	z= -1.8, p=0.07	t(13)=1.9, p=0.08

4.3 Heart rate

For every subject the heart rate was calculated for each epoch of 25 seconds over the complete measurement, which resulted in 30 calculated heartrates. All the epochs were visually inspected for missing peaks and noise. No measurements or stimulation conditions had to be excluded due to too much missing epochs.

Figure 12 shows the results for the heart rate for the healthy subjects during the baseline, the ON phase of tVNS stimulation and the OFF phase of tVNS stimulation. Overall, the healthy subjects show a decrease in heart rate during the ON phase of tVNS stimulation compared with the heart rate during the baseline and the OFF phase. These results are obtained for both cymba conchae stimulation and earlobe stimulation. Appendix G shows the individual results for the heart rate during the baseline, ON phase and OFF phase in a table.

The paired-samples t-test show a statistically significant decrease in the heart rate during the ON phase of tVNS stimulation compared with the OFF phase of tVNS stimulation. These results are obtained during both cymba conchae stimulation ($t(20) = -5.63$, $p = 0.00002$) and earlobe stimulation ($t(20) = -5$, $p = 0.00007$). No statistically significant differences in the heart rate are shown between the baseline and the ON phase of tVNS stimulation during both cymba conchae stimulation ($t(20) = -1.93$, $p = 0.07$) and earlobe stimulation ($t(20) = -1.92$, $p = 0.07$).

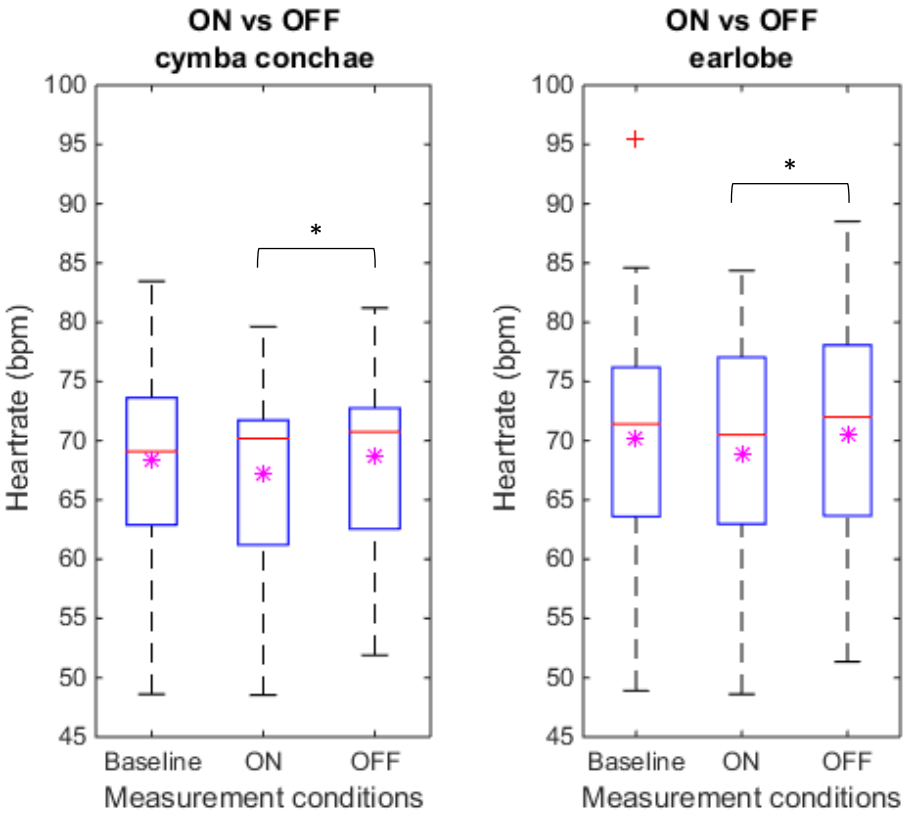


Figure 12: The average heart rate of all the healthy subjects during the baseline, the ON phase of tVNS stimulation and the OFF phase of tVNS stimulation. The mean heart rate in beats per minute (bpm) in the three measurement conditions is given as the purple dot. The red plus sign is an outlier. The left figure shows the results during cymba conchae stimulation and the right figure shows the results during earlobe stimulation. The red line is the median. * means that there is a statistically significant difference with $p < 0.05$.

Cymba conchae and earlobe stimulation shows the same effects on the heart rate during tVNS stimulation compared with the OFF phase and the baseline (figure 12). The paired-samples t-test do not show significant differences between the effect of cymba conchae stimulation and earlobe stimulation on the heart rate when comparing the ON phase with the OFF phase ($t(20)=0.11$, $p=0.91$) and comparing the ON phase with the baseline ($t(20)=0.14$, $p=0.89$).
With regard to the stimulation sequence, 7 out of 21 subjects are measured first with cymba conchae stimulation and then with earlobe stimulation (table 1). A carry-over effect of cymba conchae stimulation is shown, which result in a statistically significant decreased heart rate during the baseline of earlobe stimulation ($t(19)=-2.71$, $p=0.01$).

Looking at the results in more detail, the ON phase during low stimulation intensity is now separated from the ON phase during maximal stimulation intensity. Figure 13 shows the results of the heart rate for all the measurement conditions separately. Both low intensity stimulation and maximal intensity stimulation show a decrease in the heart rate during the ON phase compared with the OFF phase and compared with the baseline. Appendix G also shows the results of the heart rate for each subject individually for low and maximal intensity separately (table G3).

When comparing the effect of low intensity stimulation between the ON phase and the OFF phase with the effect of maximal intensity stimulation between the ON phase and the OFF phase, the paired-samples t-test shows a statistically significant difference. The effect of maximal intensity stimulation on the heart rate shows a statistically significant larger decrease in the heart rate than the effect of low intensity stimulation during both cymba conchae stimulation ($t(20)=3.13$, $p=0.005$) and earlobe stimulation ($t(20)=2.95$, $p=0.008$). Despite the statistically significant difference in the effect of the two stimulation intensities, both low stimulation and maximal stimulation show a statistically significant decrease in the heart rate during the ON phase compared with the OFF phase, see table 3.

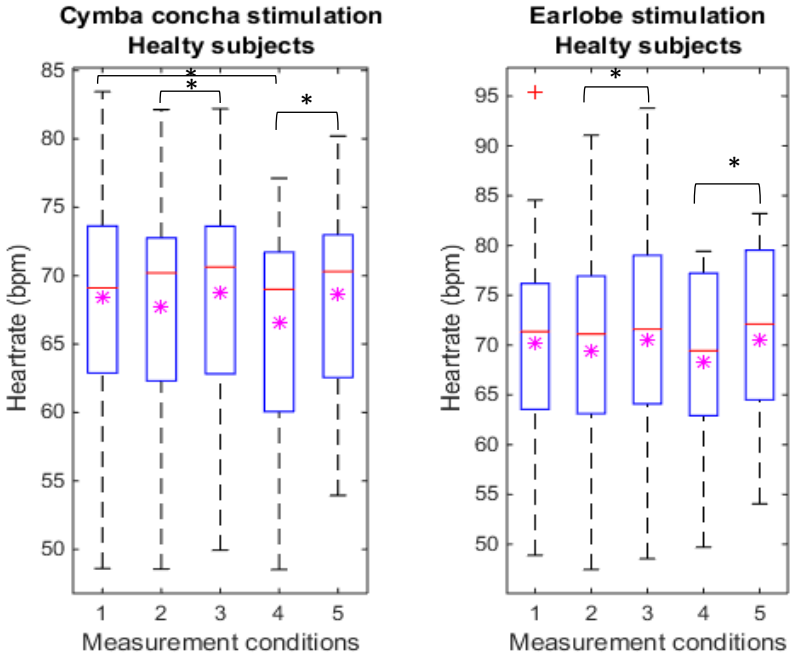


Figure 13: The heart rate results of all the healthy subjects during the different measurement conditions. 1 = the baseline, 2 = ON phase during low intensity stimulation, 3 = OFF phase during low intensity stimulation, 4 = ON phase during maximal intensity stimulation, 5 = OFF phase during maximal intensity stimulation. The purple dot represents the mean heart rate for each stimulation condition. * means that there is a statistically significant difference with $p < 0.05$.

Table 3: The results of the paired-sampled t-test for the heart rate during the different measurement conditions.

Measurement condition	Cymba conchae stimulation	Earlobe stimulation
ON vs OFF	t(20) = -5.63, p = 0.00002	t(20) = -5, p = 0.00007
ON vs baseline	t(20) = -1.93, p = 0.07	t(20) = -1.92, p = 0.07
Low vs Max	t(20) = 3.13, p = 0.005	t(20) = 2.95, p = 0.008
ON low vs OFF low	t(20) = -3.85, p = 0.001	t(20) = -4.1, p = 0.001
ON low vs baseline	t(20) = 1.2, p = 0.24	t(20) = 1.92, p = 0.07
ON max vs OFF max	t(20) = -5.61, p = 0.00002	t(20) = -4.842, p = 0.0001
ON max vs baseline	t(20) = 2.32, p = 0.03	z = -1.72, p = 0.09

Comparing the effects of low stimulation between the ON phase and the baseline with the effects of maximal simulation between the ON phase and the baseline, the paired samples t-test shows also a statistically significant difference during cymba conchae stimulation (t(20)=-2.72, p=0.01), which results in a statistically significant larger decrease in heart rate during maximal intensity stimulation compared with low intensity stimulation. The paired-samples t-test show a statistically significant decrease in the heart rate during maximal intensity stimulation when comparing the ON phase with the baseline (t(20)=2.32, p=0.031). No statistically significant difference was shown during low intensity stimulation (t(20)=1.2, p=0.244). During earlobe stimulation no statistically significant differences in the effect of both stimulation intensities between the ON phase and the baseline are shown (z=-1.17, p=0.24). Both stimulation intensities do not show a statistically significant difference in the heart rate during the ON phase and the baseline (table 3).

With regard to the epilepsy patients without a VNS, the results show no differences in the heart rate during the ON phase compared with the baseline and a very small decrease (0.2 bpm) during the ON phase compared with the OFF phase. These results are obtained for cymba conchae stimulation. During earlobe stimulation an increase is shown in the heart rate when comparing the ON phase with the baseline and also a small increase is shown during the ON phase compared with the OFF phase. The individual results of the heart rate are shown in Appendix G.
Comparing low intensity tVNS stimulation separately from tVNS stimulation at maximal intensities during cymba conch stimulation, the results show that during low stimulation the heart rate slightly increases compared with the baseline (0.3 bpm) and do not change compared with the OFF phase. During maximal stimulation the heart rate is lower compared with the baseline and the OFF phase. For earlobe stimulation, an increase in the heart rate is seen during the low stimulation compared with the baseline and the OFF phase. During maximal stimulation the heart rate decreases compared with the baseline and the OFF phase. The individual results are shown in figure 14.

With regard to the patients with epilepsy and a VNS, the VNS decreases the heart rate during the ON phase compared to the OFF phase (figure 15).

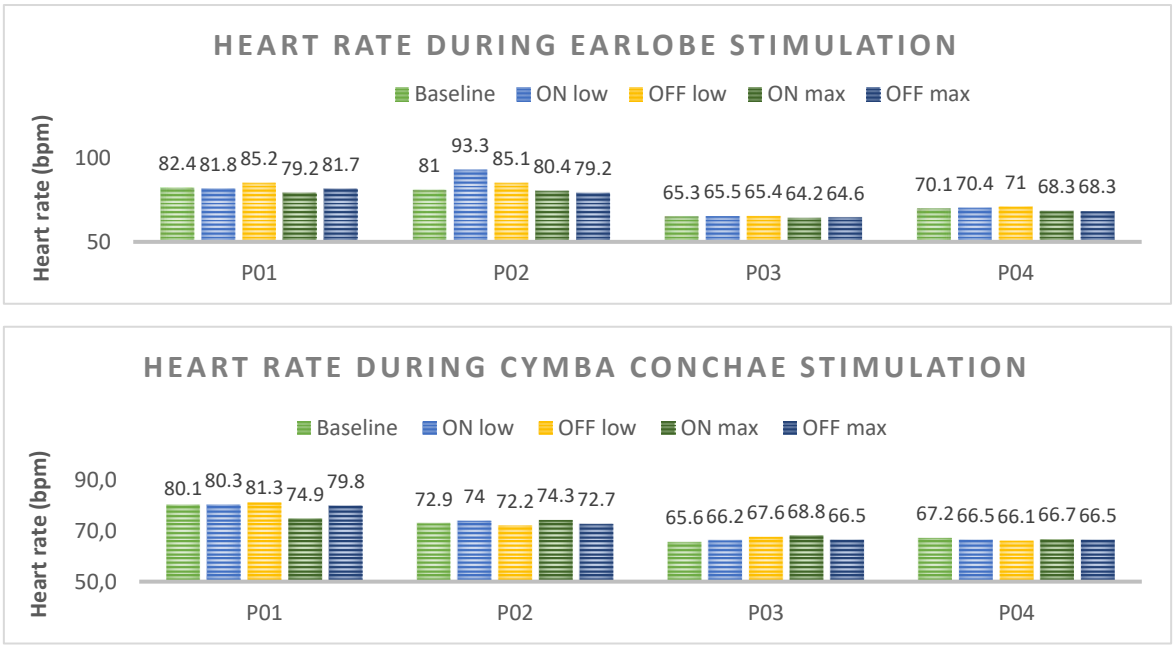


Figure 14: The results of the tVNS measurement on the heart rate for the epilepsy patients without a VNS. The results are shown for each of the four epilepsy patients and for all measurement conditions. The top figure shows the effects of earlobe stimulation. The bottom shows the effects of cymba conchae stimulation. The heart rate is given in beats per minute.

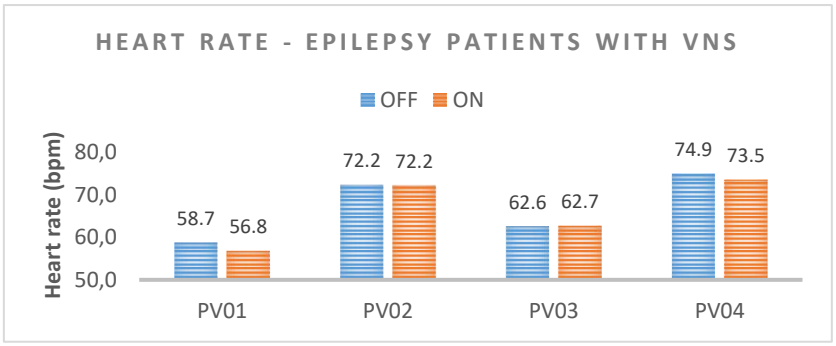


Figure 15: The results of the measurements with the VNS. Results are shown for each patient individually. The heart rate is given in beats per minute.

4.4 Heart rate variability

The HRV was measured only for the two groups of subjects which were measured with the tVNS. The R-R intervals were analysed with the use of Kubios HRV standard software. Appendix H show some figures of the used data and the obtained results given by Kubios. No measurements had to be excluded.

Figure 16 show the results for the HRV parameters for the healthy subjects during cymba conchae stimulation. Figure 17 show the results for the HRV parameters during earlobe stimulation. The mean HRV parameters are for the healthy subjects are shown in Appendix H. Overall, for the time-domain, the results show an increase in the R-R interval for both stimulation locations during continuous tVNS stimulation compared with the rest phase. SDNN shows a small decrease during the continuous tVNS stimulation of cymba conchae compared with the rest phase and a small increase during earlobe stimulation. The RMSSD slightly increases during tVNS compared to the rest phase during cymba conchae stimulation as well as during earlobe stimulation.

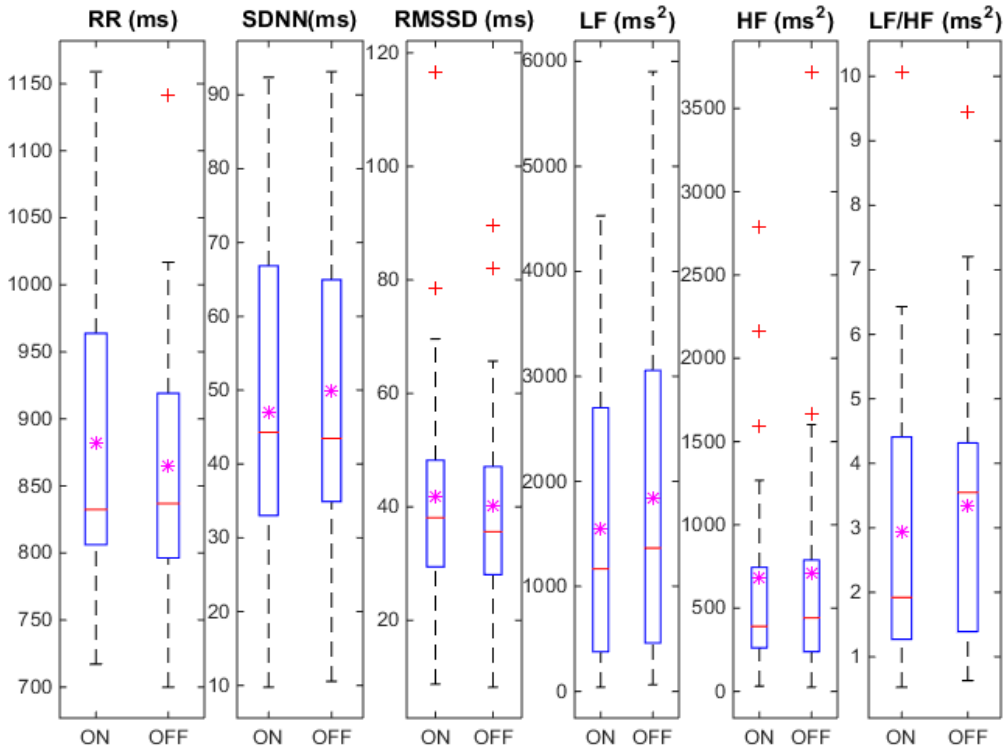


Figure 16: The results from the HRV during **cymba conchae** stimulation for the healthy subjects. The ON phase is the phase of continuous tVNS stimulation. The OFF phase is the rest phase prior to continuous stimulation. The purple dots are the mean values for each stimulation parameter.

The results for the frequency-domain parameters shows a decrease in LF, HF and the LF/HF ratio during the tVNS stimulation compared to the rest phase during cymba conchae stimulation (figure 16). During earlobe stimulation an increase is shown for LF, HF and LF/HF ratio during tVNS stimulation compared with the rest phase (figure 17).

The paired-samples t-test shows a statistically significant increase in the R-R interval during continuous tVNS stimulation compared with the rest phase during both cymba conchae stimulation ($t(20)=-3.43$, $p=0.003$) and earlobe stimulation ($t(20)=-3.74$, $p=0.001$). SDNN, RMSSD, LF, HF, and the LF/HF-ratio do not show significant differences between continuous stimulation compared with the rest phase for both stimulation locations, see table 4.

With regard to the effects of cymba conchae stimulation and earlobe stimulation, no differences are shown between the effect of cymba conchae stimulation during the continuous stimulation phase compared with the rest phase and earlobe stimulation during the continuous stimulation phase compared with the rest phase. The paired samples t-test also shows no differences between the effect of cymba conchae stimulation and earlobe stimulation for the R-R interval ($z=-1.2$, $p=0.23$), the SDNN ($z=-0.12$, $p=0.903$), the RMSSD ($z=-1.2$, $p=0.23$), the LF ($z=-0.85$, $p=0.39$), the HF ($z=-0.92$, $p=0.36$) and the LF/HF ratio ($z=-0.85$, $p=0.39$).

The results for the patients with epilepsy show a decrease in RR-interval, SDNN, RMSSD, LF, HF and LF/HF ratio during the period of cymba conchae tVNS compared to the rest period (figure 18). Earlobe stimulation shows an increase in RR-interval, SDNN, RMSSD, LF and LF/HF ratio during the ON phase compared to the OFF phase. Only for the HF it shows a decrease during the ON phase (figure 19). The mean results are shown in Appendix H.

Table 4: Results from the paired-samples t-test for the HRV parameters.

	Cymba conchae – ON vs OFF	Earlobe – ON vs OFF	Cymba conchae vs earlobe
RR	t(20)=-3.43, p=0.003	t(20)=-3.74, p=0.001	z=-1.2, p=0.23
SDNN	z = -1.34, p=0.18	t(20)= -0.16, p=0.88	z=-0.12, p=0.903
RMSSD	z = -0.78, p=0.43	z = -1.78, p = 0.08	z=-1.2, p=0.23
LF	t(20)= 1.48, p=0.15	z = -0.33, p= 0.74	z=-0.85, p=0.39
HF	z = 0.37, p=0.72	z = -1, p=0.31	z=-0.92, p=0.36
LF/HF	t(20)= 1.5, p=0.15	z = -0.09, p=0.93	z=-0.85, p=0.39

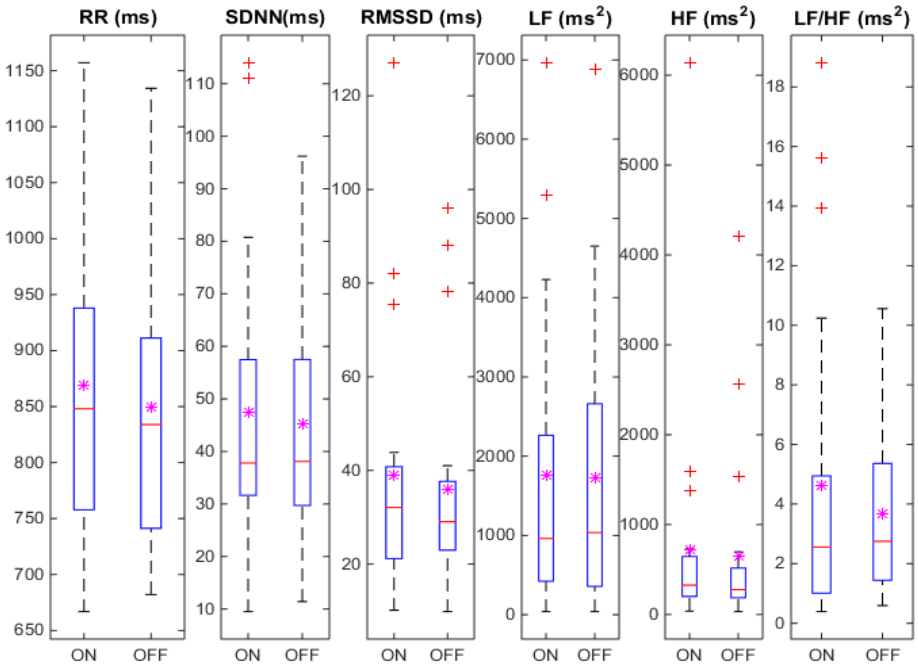


Figure 17: The results from the HRV during **earlobe** stimulation for the healthy subjects. The ON phase is the phase of continuous tVNS stimulation. The OFF phase is the rest phase prior to continuous stimulation. The purple dots are the mean values for each stimulation

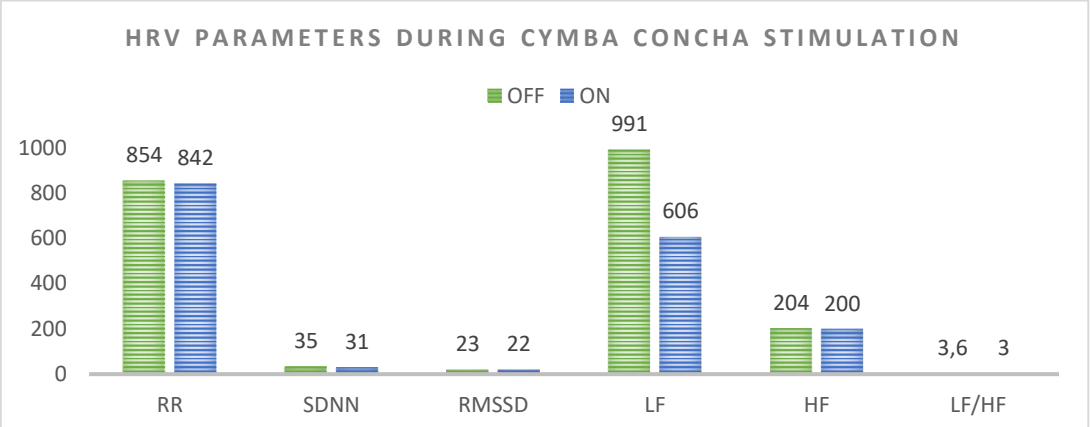


Figure 18: The HRV parameter during cymba conchae stimulation for the patients with epilepsy without a VNS. LF/HF is the LF/HF ratio. The left number is during the OFF phase and the right number is during the ON phase of tVNS stimulation.

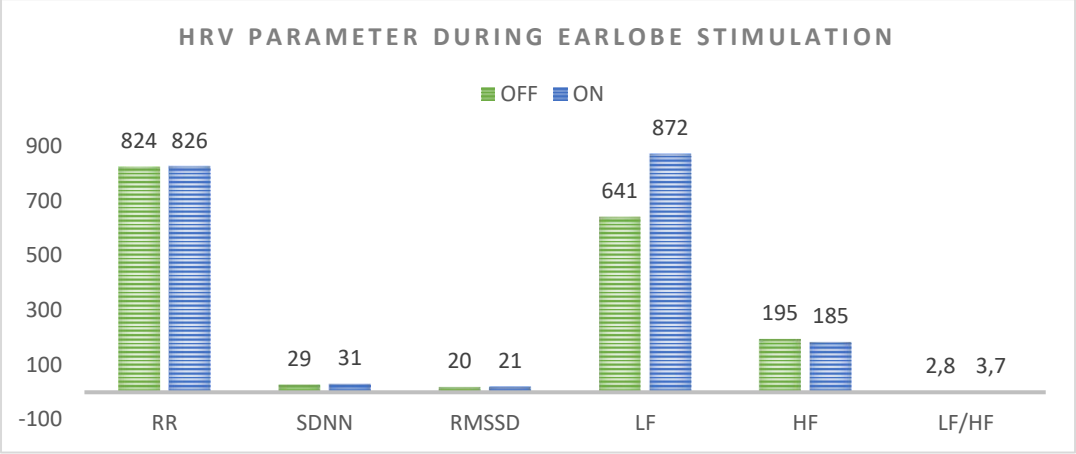


Figure 19: The HRV parameter during earlobe stimulation for the patients with epilepsy without a VNS. LF/HF is the LF/HF ratio. The left number is during the OFF phase and the right number is during the ON phase of tVNS stimulation.

5. Discussion

Here we discuss the results from our study about the effect of tVNS on the pupil diameter, heart rate and heart rate variability in healthy subjects and epilepsy patients. Three hypotheses were tested for the effects of tVNS.

With regard to the first hypothesis, the overall results of the healthy subjects show a statistically significant increase in the pupil diameter parameters, a statistically significant decrease in the heart rate and a statistically significant increase in the R-R interval when comparing the ON phase of tVNS stimulation with the OFF phase.

With regard to the second hypothesis, no statistically significant differences are shown in the effects of cymba conchae stimulation on the study parameters compared with the effects of earlobe stimulation on the study parameters.

With regard to the third hypothesis, the results show no statistically significant differences between the effect of low stimulation and maximal stimulation on the pupil diameter parameters, and overall both stimulation intensities show a significant increase in the pupil diameter parameters during the ON phase compared with the OFF phase. With regard to the heart rate, maximal intensity tVNS stimulation show a statistically significant larger decrease in heart rate compared with low intensity stimulation. The overall results show a significant decrease in the heart rate for both stimulation intensities during the ON phase compared with the OFF phase.

5.1 Effect of cymba conchae stimulation compared with earlobe stimulation

Earlobe stimulation was supposed to be sham stimulation during our measurements, because the earlobe is 100% innervated by the greater auricular nerve and because several studies show that stimulation of the earlobe does not significantly projects to the same brain regions as the vagal projections (23,38,79). Therefore, it was hypothesized that stimulation of the earlobe would not have any influence on our study parameters. In our results earlobe stimulation do show significant effects on the pupil diameter and heart rate.

One of the reasons that earlobe stimulation does affect the pupil diameter and heart rate could be due to the stimulation sequence. Seven healthy subjects were measured first with cymba conchae

stimulation and then with earlobe stimulation (table 1). This sequence of stimulation could have an influence on the results, because of a carry-over effect of cymba conchae stimulation. Regarding the heart rate, a carry-over effect is seen in the subjects who started with cymba conchae stimulation. These subjects started with a significant lower heart rate during the baseline of earlobe stimulation. Despite this decrease in heart rate during the baseline, the heart rate still decreases significantly during the ON phase of tVNS stimulation compared with the OFF phase. This is due to the earlobe stimulation itself, because during the ON phase the only condition that changes is the switch from the tVNS to the ON phase.
Furthermore, it is seen that for the subjects who are measured first with earlobe stimulation (no carry-over effect), the earlobe stimulation also shows an overall decrease in the heart rate and increase in the pupil diameter parameters. For these reasons I do not think that the carry over effect causes the effects of earlobe stimulation on the study parameters.

Another explanation of the effects of earlobe stimulation on the study parameters could be due to the effect of arousal. During the stimulation with the tVNS, subjects feel the stimulation during both low and maximal stimulation. During both stimulation intensities all the subjects felt a tingling sensation during stimulation. The sensation of stimulation could create an arousal which could have an influence on the results. With regard to the pupil diameter, an arousal produces a pupillary dilation, which is mediated via the hypothalamus (64). The hypothalamus causes a systemic increase in the sympathetic tone, which results in dilatation of the pupil diameter. Our results also show an increase in rest pupil diameter and constriction amplitude, so it may be possible that the effect of earlobe stimulation on the pupil diameter is caused by an arousal. This arousal could also play an important role during stimulation of the cymba conchae, which could bias the results of cymba conchae stimulation. I think that the arousal should be considered as a possible cause of the increase in the pupil diameter parameters during earlobe stimulation.
However, if the stimulation sensation causes an arousal, the effect of earlobe stimulation would also result in an increase of the heart rate (64). Our results show a decrease in the heart rate during earlobe stimulation, which is conflicting. Therefore, I believe that there is more than only an arousal which causes the effect of earlobe stimulation on the study parameters.

A study from Frangos et al. shows that earlobe stimulation projects to the caudal ventrolateral medulla (38). This projection could explain why earlobe stimulation decreases the heart rate. Projection to the caudal ventrolateral medulla could inhibit the rostroventrolateral medulla, which is the primary source of excitatory drive to preganglionic neurons in the intermediolateral column of the spinal cord. This inhibition would decrease the sympathetic activity, which results in a decrease of the heart rate (39,45).

5.2 Pupil diameter healthy subjects

Results show that tVNS during cymba conchae stimulation significantly increases the rest pupil diameter and the constriction amplitude during the ON phase of tVNS stimulation compared with the OFF phase of tVNS stimulation. This was also expected in our hypothesis. If the results are really due to tVNS stimulation (and not due to an arousal), they can be caused by an activation of the sympathetic ANS or by an inhibition of the parasympathetic ANS due to tVNS stimulation. Our results show a significant mean increase of 0.2 mm for the resting pupil diameter for both eyes during cymba conchae stimulation. This result is comparable with the results of Desbaumes Jodoin et al. for the effect of VNS stimulation on the pupil diameter. They showed a significant mean increase in resting pupil diameter of 0.23 mm. However, they did not show a significant increase in the constriction amplitude. This may be linked to the different light parameters used during the measurements.

5.3 Pupil diameter epilepsy patients

The results for the epilepsy patients without a VNS shows similarities and differences compared with the healthy subjects. During earlobe stimulation a slight increase in the pupil diameter parameters is seen during tVNS stimulation compared with the OFF phase, which is comparable with the healthy subjects. For all the other measurement conditions differences are shown. During maximal tVNS stimulation at the cymba conchae a decrease in the pupil diameter parameters is shown during the ON phase compared with the OFF phase. For all the other measurement conditions almost no differences are shown in the pupil diameter recordings between the ON phase and the OFF phase. I believe that the differences between this patient group and the healthy subjects is due to the low number of patients in the patient group, because only pupil diameter recordings were available for 3 patients and from those 3 patients also parts of the measurement were missing.

Epilepsy patients with a VNS all show an increase in pupil diameter and constriction amplitude for both eyes during the ON phase compared with the OFF phase. These results are similar with the healthy subjects. Desbeaumes Jodoin et al. also show an increase in the resting pupil diameter within the same range of stimulation intensities as the VSN patients in this study (42). This result could be caused by a shift of autonomic balance toward a sympathetic activation or a parasympathetic inhibition.

5.4 Cardiac parameters healthy subjects

As expected from the hypotheses, tVNS stimulation shows a decrease in the heart rate during the ON phase compared with the OFF phase, from which I believe that it is caused by the stimulation of the ABVN and subsequently by an increase in the parasympathetic ANS or an inhibition of the sympathetic ANS.

Maximal intensity tVNS stimulation was supposed to also stimulate the C-fibers of the vagus nerve, because the stimulation intensity during maximal stimulation was above the 2.0 mA. In animal data is was shown that activation of the C-fibers show autonomic effects such as bradycardia (52,53,58). Therefore, it was hypothesized that the effects of tVNS on the heart rate only occurs during maximal intensity tVNS stimulation. However, our results do also show a decrease in heart rate during low intensity tVNS stimulation when comparing the ON phase with the OFF phase. The intensities used in the healthy subjects ranged from $0.7 - 3$ mA during low stimulation, from which 6 out of 21 subjects had a stimulation intensity above the 2.0 mA during low stimulation. It is possible that the relatively high intensities used during low stimulation could cause the results. Another explanation is that the A-fibers, which could be stimulated during low stimulation, could also cause a decrease in the heart rate. The A-fibers project to the NTS, and stimulation of the NTS could subsequently decrease the heart rate by the activation of the parasympathetic ANS or the inhibition of the sympathetic ANS.

The decreases in heartrate during cymba conchae stimulation were statistically significant during the ON phase of tVNS stimulation compared with the OFF phase. There was only a significant difference between the baseline and the ON phase during maximal intensity stimulation. The fact that there is an effect between the ON phase and the OFF phase and less effects between the ON phase and the baseline, could possibly be explained by the fact that tVNS could cause a poststimulation tachycardia (47).
A tachycardia after stimulation gives higher heartrates during the OFF phase and could be the reason that there is a significant difference between the ON phase and OFF phase and that there is no significant difference between the baseline and the ON phase. However, when comparing the mean heart rate of the baseline during cymba conchae stimulation with the OFF phase during tVNS

stimulation, it shows small differences between those heart rates (baseline: 68.4 bpm, OFF phase: 68.7). It seems unlikely that poststimulation tachycardia is present after tVNS stimulation in our results, but there still is a possibility. I also think that the intensity plays a role in the results. Low intensity stimulation does show statistically significant differences between the ON and the OFF phase, but more stimulation at a maximal intensity also show a statistically significant decrease during the ON phase compared with the baseline. I think the effect between the ON phase and the baseline is more important than the effect between the ON phase and the OFF phase, because the baseline is measured prior to stimulation, so there is no possibility that the baseline could be influenced by stimulation.

After the measurement some subjects indicated that maximal stimulation gave an unpleasant or painful feeling when the stimulator was turned ON. This pain experience during maximal stimulation could affect heart rate, which would result in an increase in heart rate (80). However, when regarding the results, these subjects still show a decrease in the heart rate during the ON phase compared with the OFF phase.

Except for the R-R interval, none of the HRV parameters show a statistically significant difference when comparing the rest phase with the phase of continuous tVNS stimulation. There is a statistically significant increase in the R-R interval during tVNS stimulation, which means that there is a decrease in the heart rate. This is same as for the results mentioned above. However, when the R-R interval shows statistically significant changes due to stimulation of the ABVN, it is expected that also more HRV parameter shows statistically significant differences, because the autonomic nervous system is activated. According to the significant increase in RR-interval, it is expected that the LF/HF ratio would also decreases significantly which indicates a shift in cardiac autonomic balance toward parasympathetic/vagal predominance and thus an increase in HRV (45). However, this was not the case according to our results. The results show a decrease in the LF/HF ratio, but this was not significant.

5.5 Cardiac parameters epilepsy patients

With regard to the epilepsy patients without a VNS, the results show no differences in the heart rate during the ON phase compared with the baseline and a very small decrease (0.2 bpm) during the ON phase compared with the OFF phase. This similar with the effect of the heathy subjects. However, the patients group is very low.

Two out of four VNS patients show a decrease in heart rate during the ON phase compared to the OFF phase. The result can either occur due to the afferent stimulation of the vagus in the direction of the NTS or due to efferent stimulation of the parasympathetic vagus nerve. It should me marked that these results are obtained in the two patients with a VNS intensity of 1.75 mA. The other two patients have lower intensities (1 and 1.25 mA), so this may indicate again that the stimulation intensity could play a role in the results.

5.6 Limitations

The measurement consisted of two parts of 30 minutes in which the subjects were looking at the computer screen. During one part the effect of cymba conchae stimulation were assessed and during the other part the effects of earlobe stimulation were assessed. During the course of the study it became clear the measurement was very exhausting for some subjects. Some subject had difficulties with not falling asleep and other subjects were doing fine during the measurement. When asking the fatigue, almost all subjects give higher scores for exhaustion after the measurement compared to prior to the measurement. The extend of tiredness could have an influence on the pupil diameter recordings with the Tobii Glasses Eye Tracker. When subjects almost fall asleep, they blink a lot

and they have more difficulties with looking at the screen on focus on the light stimuli. This could cause more missing data in our pupil diameter recordings. Falling asleep could also influence the heart rate, because during sleep the heart rate decreases. A decrease is also shown in our results. However, I do not think that our results are affected by sleepiness, because there were no subjects that really felt asleep during the measurement. During our measurements we also have tried to control for the effect of tiredness by varying the location sequence between the subjects, so not all subjects were measured first with earlobe stimulation and then with cymba conchae stimulation. However, it should be considered to adjust the duration of the measurement to reduce the extend of tiredness.

Another point with regard to the measurement procedure, is that the start of tVNS stimulation was not automatically linked with the times of the ECG recordings. Our electrodes were also not capable of detecting tVNS stimulation. Therefore, the electrode in the neck was pushed manually to create an artefact in one of the ECG leads, which could be linked to our ECG data. It was tried to push the electrode as accurate as possible, but there still is some inaccuracy. To control for this during the heart rate analyses, it was chosen to take the epochs a little bit smaller. Therefore, it was chosen to take epochs of 25 seconds instead of 30 seconds. However, it is preferred for the future to capture the stimulation of the tVNS in a more automatic way. For example, if we are able to start the measurement with the eye tracker at the exact same time as the ECG recordings, the data from the eye tracker could be used to determine very accurate when tVNS was started. During this study we were not able to start the all the measurement equipment at the same time.

During this study the statistical analyses are performed with the use of paired-samples t-test. It was also considered to use a Mixed model test to test for the effects of tVNS on the pupil diameter, heart rate and HRV. However, this model compares all the different measurement conditions with each other and also makes a correction for all the comparisons. During our analysis we do not compare all the measurement conditions with each other. For example, we do not compare the OFF phase of low intensity stimulation during earlobe stimulation with the ON phase of maximal stimulation during cymba conchae stimulation. The mixed model does make this comparison. Therefore, we have decided that the mixed model test has too much power to use for our analysis an instead of the mixed model test, paired-samples t-test were used.

Further, the carry-over effect. A carry-over effect was seen during the heart rate analysis. Despite the carry-over effect, we used all subjects for our analysis. The carry-over effect was not calculated for the pupil diameter and the HRV, because we did not have the baseline values for these two parameters.

The overall results are statistically significant for the healthy subjects. For the epilepsy patients there are effects of (t)VNS on the pupil diameter, heart rate and HRV, but due to the low number of patients it could not be tested if these effects are significant of not. More patients should be included to investigate the significant differences of (t)VNS stimulation on the pupil diameter, heart rate and HRV in epilepsy patients.

6. Conclusion

The main finding of this study is that tVNS shows a statistically significant increase in the pupil diameter recordings and a statistically significant decrease in the heart rate during the ON phase compared with the OFF phase. However, these results are shown during both cymba conchae stimulation and earlobe stimulation. Therefore, it cannot be concluded from this study that the effects of cymba conchae stimulation on the pupil diameter and the heart rate are caused by the activation of the ABVN and subsequently of the NTS.

7. References

1. de Vries EE, van den Munckhof B, Braun KPJ, van Royen-Kerkhof A, de Jager W, Jansen FE. Inflammatory mediators in human epilepsy: A systematic review and meta-analysis. Neurosci Biobehav Rev. 2016;63:177–90.
2. Galbarriatu L, Pomposo I, Aurrecoechea J, Marinas A, Agundez M, Gomez JC, et al. Vagus nerve stimulation therapy for treatment-resistant epilepsy: A 15-year experience at a single institution. Clin Neurol Neurosurg. 2015;137:89–93.
3. Greco A, Rizzo MI, De Virgilio A, Conte M, Gallo A, Attanasio G, et al. Autoimmune epilepsy. Autoimmun Rev. 2016;15:221–5.
4. Aihua L, Lu S, Liping L, Xiuru W, Hua L, Yuping W. A controlled trial of transcutaneous vagus nerve stimulation for the treatment of pharmacoresistant epilepsy. Epilepsy Behav. 2014;
5. Srivastava A, Dixit AB, Banerjee J, Tripathi M, Sarat Chandra P. Role of inflammation and its miRNA based regulation in epilepsy: Implications for therapy. Clin Chim Acta [Internet]. 2016;452:1–9. Available from: http://dx.doi.org/10.1016/j.cca.2015.10.023
6. Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, Lee P, et al. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia. 2005;46(4):470–2.
7. Duun-Henriksen J, Bjarup Dissing H, Kjær TW., Thomsen CE. Detection and Prediction of Epileptic Seizures. Technical University of Denmark; 2013.
8. Fisher RS, Cross JH, French JA, Higurashi N, Hirsch E, Jansen FE, et al. Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. Epilepsia. 2017;58(4):522–30.
9. Varsavsky A, Mareels I, Cook M. Epileptic seizures and the EEG: measurement, models, detection and prediction. CRC Press; 2012.
10. van Putten MJAM. Essentials of Neurophysiology: Basic Concepts and Clinical Applications for Scientists and Engineers. 1st ed. Springer Publishing Company, Incorporated; 2009.
11. Ellrich J. Transcutaneous Vagus Nerve Stimulation. Eur Neurol Rev. 2011;6(4):254–6.
12. Schmidt D, Schachter SC. Drug treatment of epilepsy in adults. BMJ. 2014;348(March):g254.
13. van Straten AF., Jobst BC. Future of epilepsy treatment : integration of devices. Future Neurol. 2014;9:587–99.
14. Ghani S, Vilensky J, Turner B, Tubbs RS, Loukas M. Meta-analysis of vagus nerve stimulation treatment for epilepsy: correlation between device setting parameters and acute response. Child's Nerv Syst. 2015;31(12):2291–304.
15. Nune G, DeGiorgio C, Heck C. Neuromodulation in the Treatment of Epilepsy. Curr Treat Options Neurol. 2015;17(10):0–5.
16. Elliott RE, Morsi A, Kalhorn SP, Marcus J, Sellin J, Kang M, et al. Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: Long-term outcomes and predictors of response. Epilepsy Behav. 2011;20(1):57–63.
17. Ben-Menachem E, Revesz D, Simon BJ, Silberstein S. Surgically implanted and non-invasive vagus nerve stimulation: A review of efficacy, safety and tolerability. Eur J Neurol. 2015;22(9):1260–8.
18. Krahl S, Clark K. Vagus nerve stimulation for epilepsy: A review of central mechanisms. Surg Neurol Int. 2012;3(5):255.
19. DeGiorgio CM, Krahl SE. Neurostimulation for drug-resistant epilepsy. Contin Lifelong Learn Neurol. 2013;19(3, Epilepsy):743–55.
20. De Couck M, Cserjesi R, Caers R, Zijlstra WP, Widjaja D, Wolf N, et al. Effects of short and prolonged transcutaneous vagus nerve stimulation on heart rate variability in healthy subjects. Auton Neurosci. 2016;
21. Ruffoli R, Giorgi FS, Pizzanelli C, Murri L, Paparelli A, Fornai F. The chemical neuroanatomy of vagus nerve stimulation. J Chem Neuroanat. 2011;42(4):288–96.
22. Banzett RB, Guz A, Paydarfar D, Shea SA, Schachter SC, Lansing RW. Cardiorespiratory variables and sensation during stimulation of the left vagus in patients with epilepsy. Epilepsy Res. 1999;35(1):1–11.

23. Yakunina N, Kim SS, Nam E-C. Optimization of Transcutaneous Vagus Nerve Stimulation Using Functional MRI. Neuromodulation Technol Neural Interface. 2016;
24. How VNS Therapy Works [Internet]. Available from: https://us.livanova.cyberonics.com/learn-more/how-it-works
25. Beekwilder J, Beems T. Overview of the clinical applications of vagus nerve stimulation. J Clin Neurophysiol. 2010;27(2):130–138.
26. Mulders DM, De Vos CC, Vosman I, Van Putten MJAM. The effect of vagus nerve stimulation on cardiorespiratory parameters during rest and exercise. Seizure. 2015;33:24–8.
27. Terry RS. Vagus Nerve Stimulation Therapy for Epilepsy. Epilepsy Top. 2014;139–60.
28. Schevernels H, Bochove ME Van, Taeye L De, Bombeke K, Vonck K, Roost D Van, et al. Epilepsy & Behavior The effect of vagus nerve stimulation on response inhibition. 2016;64:171–9.
29. Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. Epilepsia. 1998;39(7):709–14.
30. Bodin C, Aubert S, Daquin G, Carron R, Scavarda D, McGonigal A, et al. Responders to vagus nerve stimulation (VNS) in refractory epilepsy have reduced interictal cortical synchronicity on scalp EEG. Epilepsy Res. 2015;
31. Janszky J, Hoppe M, Behne F, Tuxhorn I, Pannek HW, Ebner A, et al. Vagus nerve stimulation: predictors of seizure freedom. J Neurol Neurosurg Psychiatry. 2005;76:384–9.
32. Yuan H, Silberstein SD. Vagus Nerve and Vagus Nerve Stimulation, a Comprehensive Review: Part II. Headache. 2016;56(2):259–66.
33. Bauer S, Baier H, Baumgartner C, Bohlmann K, Fauser S, Graf W, et al. Transcutaneous Vagus Nerve Stimulation (tVNS) for Treatment of Drug-Resistant Epilepsy: A Randomized, Double-Blind Clinical Trial (cMPsE02). Brain Stimul. 2016;9:356–63.
34. He W, Jing X, Wang X, Rong P, Li L, Shi H, et al. Transcutaneous auricular vagus nerve stimulation as a complementary therapy for pediatric epilepsy: a pilot trial. Epilepsy Behav. 2013;28(3):343–6.
35. Stefan H, Kreiselmeyer G, Kerling F, Kurzbuch K, Rauch C, Heers M, et al. Transcutaneous vagus nerve stimulation (t-VNS) in pharmacoresistant epilepsies: A proof of concept trial. Epilepsia. 2012;53(7):115–8.
36. Rong P, Liu A, Zhang J, Wang Y, He W, Yang A, et al. Transcutaneous vagus nerve stimulation for refractory epilepsy: a randomized controlled trial. Clin Sci. 2014;CS20130518.
37. He W, Zhu B, Rong P. A new concept of transcutaneous vagus nerve stimulation for epileptic seizure. In: Abstr Soc Neurosci. 2009. p. 4.
38. Frangos E, Ellrich J, Komisaruk BR. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: FMRI evidence in humans. Brain Stimul. 2015;8:624–36.
39. Murray AR, Atkinson L, Mahadi MK, Deuchars SA, Deuchars J. The strange case of the ear and the heart: The auricular vagus nerve and its influence on cardiac control. Auton Neurosci. 2016;199:48–53.
40. Safi S, Ellrich J, Neuhuber W. Myelinated Axons in the Auricular Branch of the Human Vagus Nerve. Anat Rec. 2016;299(9):1184–91.
41. Ellrich J. Transcutaneous access to the vagus nerve: A new treatment option in CNS diseases. 2012;
42. Desbeaumes Jodoin V, Lespérance P, Nguyen DK, Fournier-Gosselin M-P, Richer F. Effects of vagus nerve stimulation on pupillary function. Int J Psychophysiol. 2015;98(3):455–9.
43. Bianca R, Komisaruk BR. Pupil dilatation in response to vagal afferent electrical stimulation is mediated by inhibition of parasympathetic outflow in the rat. Brain Res. 2007;1177(1):29–36.
44. Zaaimi B, Grebe R, Berquin P, Wallois F. Vagus nerve stimulation therapy induces changes in heart rate of children during sleep. Epilepsia. 2007;48(5):923–30.
45. Clancy JA, Mary DA, Witte KK, Greenwood JP, Deuchars SA, Deuchars J. Non-invasive Vagus nerve stimulation in healthy humans reduces sympathetic nerve activity. Brain Stimul. 2014;7(6):871–7.

46. Vaseghi M. Cardiac Parasympathetic Neural Remodeling and Electrophysiological Effects of Vagal Nerve Stimulation. 2016;
47. Frei M, Osorio I. Left vagus nerve stimulation with the neurocybernetic prosthesis has complex effects on heart rate and on its variability in humans. Epilepsia. 2001;42(8):1007–16.
48. Akert K, Glicksman MA, Lang W, Grob P, Huber A. The Edinger-Westphal nucleus in the monkey. A retrograde tracer study. Brain Res. 1980;184(2):491–8.
49. Berridge CW, Waterhouse BD. The locus coeruleus--noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res Rev. 2003;42(1):33–84.
50. Breen LA, Burde RM, Loewy AD. Brainstem connections to the Edinger-Westphal nucleus of the cat: a retrograde tracer study. Brain Res. 1983;261(2):303–6.
51. Evans MS, Verma-Ahuja S, Naritoku DK, Espinosa JA. Intraoperative human vagus nerve compound action potentials. Acta Neurol Scand. 2004;110(4):232–8.
52. Woodbury DM, Woodbury JW. Effects of Vagal Stimulation on Experimentally Induced Seizures in Rats. Epilepsia. 1990 Jun;31(s2):S7–19.
53. Tecoma ES, Iragui VJ. Vagus nerve stimulation use and effect in epilepsy: What have we learned? Epilepsy Behav. 2006;8(1):127–36.
54. Verlinden TJM, Rijkers K, Hoogland G, Herrler A. Morphology of the human cervical vagus nerve: Implications for vagus nerve stimulation treatment. Acta Neurol Scand. 2016;133(3):173–82.
55. Groves DA, Brown VJ. Vagal nerve stimulation: A review of its applications and potential mechanisms that mediate its clinical effects. Neurosci Biobehav Rev. 2005;29(3):493–500.
56. Krahl SE, Senanayake SS, Handforth A. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia. 2001;
57. Vonck K, Herdt V de, Sprengers M, Ben-Menachem E. Neurostimulation for epilepsy. 1st ed. Vol. 108, Handbook of Clinical Neurology. Elsevier B.V.; 2012. 956-970 p.
58. Woodbury JW, Woodbury DM. Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: use of a cuff electrode for stimulating and recording. Pacing Clin Electrophysiol. 1991;14(1):94–107.
59. Raedt R, Clinckers R, Mollet L, Vonck K, El Tahry R, Wyckhuys T, et al. Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. J Neurochem. 2011;117(3):461–9.
60. Manta S, El Mansari M, Debonnel G, Blier P. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. Int J Neuropsychopharmacol. 2013;16(2):459–70.
61. Roosevelt RW, Smith DC, Clough RW, Jensen RA, Browning RA. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. Brain Res. 2006;1119(1):124–32.
62. Giorgi FS, Ferrucci M, Lazzeri G, Pizzanelli C, Lenzi P, AlessandrÏ MG, et al. A damage to locus coeruleus neurons converts sporadic seizures into self-sustaining limbic status epilepticus. Eur J Neurosci. 2003;17(12):2593–601.
63. Giorgi FS, Mauceli G, Blandini F, Ruggieri S, Paparelli A, Murri L, et al. Locus coeruleus and neuronal plasticity in a model of focal limbic epilepsy. Epilepsia. 2006;47(SUPPL. 5):21–5.
64. McDougal DH, Gamlin PDR. Pupillary control pathways. senses A Compr Ref. 2008;1:521–36.
65. Joos KM, Melson MR. Control of the Pupil. Prim Auton Nerv Syst. 2012;239–42.
66. Moore KL, Dalley AF, Agur AMR. Clinically oriented anatomy. In Lippincott Williams & Wilkins; 2013. p. 135–60.
67. Zarzoso M, Noujaim SF. 39 - Pulmonary Vein Ganglia and the Neural Regulation of the Heart Rate. In: Zipes DP, Jalife JBT-CEFC to B (Sixth E, editors. Philadelphia: W.B. Saunders; 2014. p. 393–8. Available from: http://www.sciencedirect.com/science/article/pii/B978145572856500039X
68. Drew RC, Sinoway LI. Chapter 36 - Autonomic Control of the Heart. In: Robertson D, Biaggioni I, Burnstock G, Low PA, Paton JFRBT-P on the ANS (Third E, editors. San Diego: Academic Press; 2012. p. 177–80.

69. Pappano AJ, Gil Wier W. 5 - Regulation of the Heartbeat BT - Cardiovascular Physiology (Tenth Edition). In Philadelphia: Content Repository Only!; 2013. p. 91–118.
70. Cole L, Kramer PR. Chapter 3.2 - The Heart and Lungs. In: Cole L, Kramer Biochemistry and Basic Medicine PRBT-HP, editors. Boston: Academic Press; 2016. p. 79–83.
71. Acharya UR, Joseph KP, Kannathal N, Lim CM, Suri JS. Heart rate variability: A review. Med Biol Eng Comput. 2006;44(12):1031–51.
72. Metelka R, others. Heart rate variability-current diagnosis of the cardiac autonomic neuropathy. A review. Biomed Pap. 2014;158(3):327–38.
73. Kranjec J, Beguš S, Geršak G, Drnovšek J. Non-contact heart rate and heart rate variability measurements: A review. Biomed Signal Process Control. 2014;13:102–12.
74. Tarvainen MP, Lipponen J, Niskanen J, Ranta-aho PO. Kubios HRV USER ' S GUIDE. 2017; Available from: www.kubios.com
75. Tarvainen MP, Niskanen JP, Lipponen JA, Ranta-aho PO, Karjalainen PA. Kubios HRV - Heart rate variability analysis software. Comput Methods Programs Biomed. 2014;113(1):210–20.
76. Reed MJ, Robertson CE, Addison PS. Heart rate variability measurements and the prediction of ventricular arrhythmias. Qjm. 2005;98(2):87–95.
77. Guidelines Heart rate variability. European Heart Journal. 1996. 354-381 p.
78. Huston JM, Tracey KJ. The pulse of inflammation: heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy. J Intern Med. 2011;269(1):45–53.
79. Peuker ET, Filler TJ. The nerve supply of the human auricle. Clin Anat. 2002;15(1):35–7.
80. Terkelsen AJ, Mølgaard H, Hansen J, Andersen OK, Jensen TS. Acute pain increases heart rate: Differential mechanisms during rest and mental stress. Auton Neurosci Basic Clin. 2005;121(1–2):101–9.

Appendix

A: Questionnaires – Healthy subjects

PERSONALIA			
Patiënt			
Studie-nummer		IC getekend	
Leeftijd			
Geslacht (m/v)			
Lengte (m)			
Gewicht (kg)			

ANAMNESE	
Roken? Zo ja, wanneer voor het laatst?	
Alcohol? Zo ja, wanneer voor het laatst?	
Drugs? Zo ja, wanneer voor het laatst?	
Koffie? Zo ja, wanneer voor het laatst?	
Heeft u een ziekte? Zijn er belangrijke medische gebeurtenissen in uw voorgeschiedenis?	
Gebruikt u medicatie? Zo ja, welke medicatie?	

A: Questionnaires – Epilepsy patients without VNS

PERSONALIA			
Patiënt			
Studie-nummer		IC getekend	
Leeftijd			
Geslacht (m/v)			
Lengte (m)			
Gewicht (kg)			

ANAMNESE	
Roken? Zo ja, wanneer voor het laatst?	
Alcohol? Zo ja, wanneer voor het laatst?	
Drugs? Zo ja, wanneer voor het laatst?	
Koffie? Zo ja, wanneer voor het laatst?	
Heeft u nog andere ziekten? Zijn er nog belangrijke medische gebeurtenissen in uw voorgeschiedenis?	
Gebruikt u medicatie? Zo ja, welke medicatie?	

Epilepsie	
Hoe lang hebt u al epilepsie?	
Hoe werken de anti-epileptica?	
Wat voor soort epilepsie hebt u?	
Wat voor soort aanvallen hebt u?	

Hoe vaak hebt u aanvallen?		
Wanneer was uw laatste aanval?		
Hoe lang duurt een gemiddelde aanval?		
Nervus vagus stimulator	Ja	Nee

A: Questionnaires – Epilepsy patients with VNS

PERSONALIA			
Patiënt			
Studie-nummer		IC getekend	
Leeftijd			
Geslacht (m/v)			
Lengte (m)			
Gewicht (kg)			

ANAMNESE	
Roken? Zo ja, wanneer voor het laatst?	
Alcohol? Zo ja, wanneer voor het laatst?	
Drugs? Zo ja, wanneer voor het laatst?	
Koffie? Zo ja, wanneer voor het laatst?	
Heeft u nog andere ziekten? Zijn er nog belangrijke medische gebeurtenissen in uw voorgeschiedenis?	
Gebruikt u medicatie? Zo ja, welke medicatie?	

Epilepsie	
Hoe lang heeft u al epilepsie?	
Hoe werken de anti-epileptica?	
Wat voor soort epilepsie heeft u?	
Wat voor soort aanvallen heeft u?	
Hoe vaak heeft u aanvallen?	

Wanneer was uw laatste aanval?		
Hoe lang duurt een gemiddelde aanval?		
Nervus vagus stimulator (NVS)	Ja	Nee
Hoe lang heeft u al een NVS?		
Wat zijn uw ervaringen met een NVS?		
Wat zijn de instellingen van uw NVS?		

B: Trials presented for the pupil diameter recordings

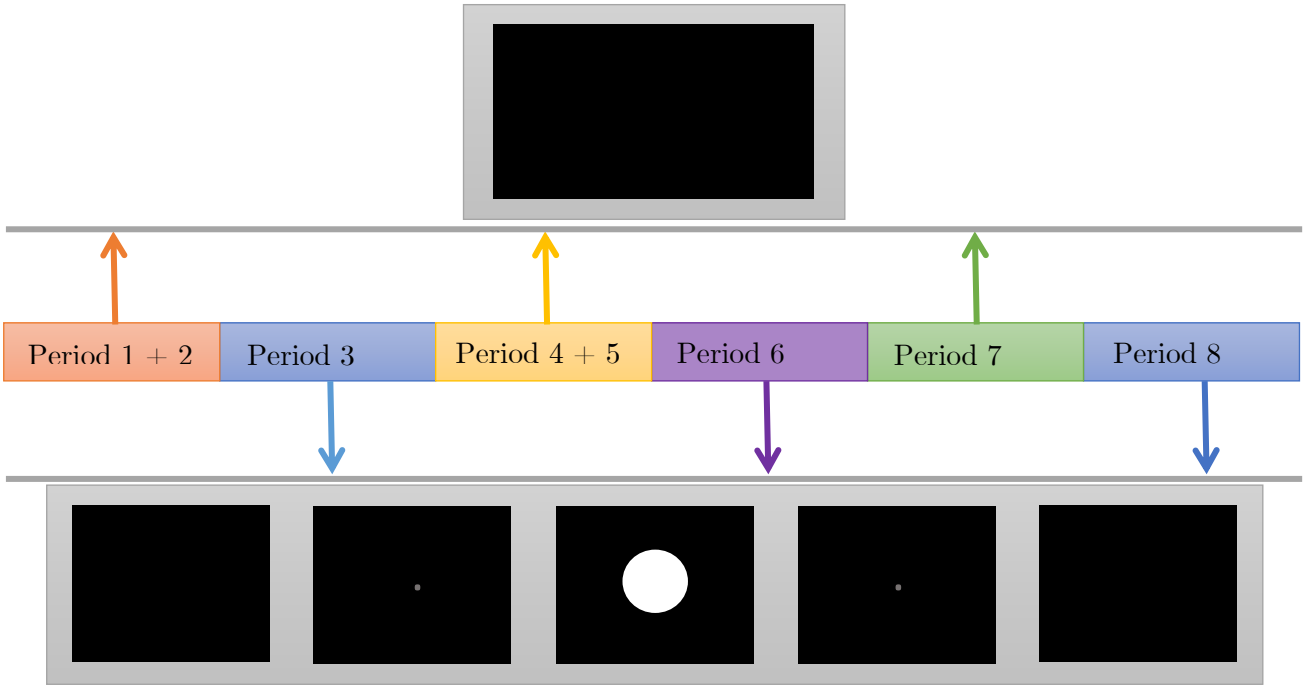


Figure B1: An overview of what is happening on the computer screen during the measurement. During period 1,2,4,5 and 7 subjects were looking at a black computer screen. During period 3,6 and 8 the trials with the light stimuli were shown to provoke a pupillary light reflex. Between the trials there were pauses in which the screen was black.

C: Measurement set-up

The measurements were performed in a quiet room with an ambient lighting level op 340 lux. A controlled ambient lighting level was chosen instead of complete darkness to avoid a ceiling effect in the baseline pupil size (1). If the room was completely dark, the pupil has its maximal size and has no opportunity to become larger due to tVNS. If the room was not completely dark, it could also be studied if the tVNS increased the baseline pupil diameter.

Subjects were also asked not to talk during the measurement, because talking could influence the blood pressure and heart rate (2,3). Listening could also influence the blood pressure and heart rate. Therefore it was chosen to perform the measurements in a quiet room (2).

Figure C1 shows the measurement set-up of the measurement. The pupil diameter was measured with the Tobii Glasses Eye Tracker Device (Tobii Technology) (4) from the BMSlab powered by Tech4People from the University of Twente. The Tobii Glasses consisted of a wearable eye tracker (glasses), which was the head unit, and a recording unit. The recording unit was connected to the head unit via a HDMI cable. The recording unit held the battery and stored the recorded data on a SD memory card. The recording unit was controlled from a tablet running controller software. Pupil diameters were recorded with a sample frequency of 50Hz or 100Hz, depending on which Tobii Glasses Eye Tracker Device was provided by the BMSlab. More information about the Tobii Glasses Eye Tracker Device can be found in Appendix D.

To facilitate the heart rate and HRV analysis, electrocardiogram (ECG) recordings were made with the Philips IntelliVue MP30 monitor, and recorded and stored with the use of ixTrend Express software (ixellence GmbH, Germany) and a sample frequency of 500Hz. Electrodes were placed below the clavicle of the right shoulder, below the clavicle of the left shoulder, below the pectoral muscle near the apex of the heart, and at the back of the right hand of the subject. Also, two electrodes were placed in the neck of the subject. For the patients with epilepsy and a VNS, these two electrodes were placed over the VNS electrode on the left side of the neck to record episodes of

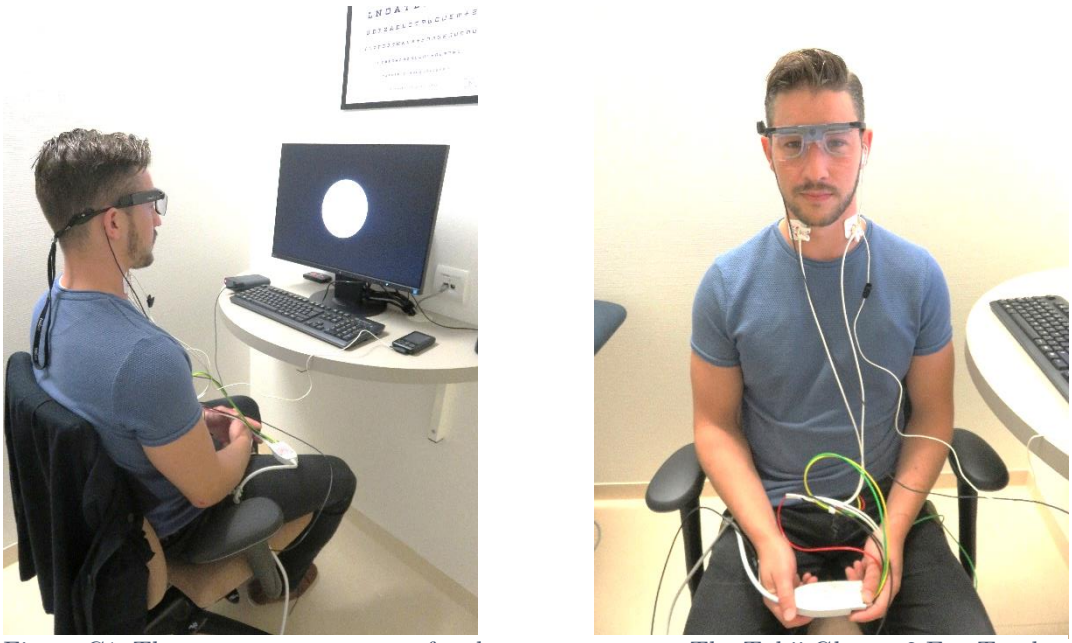


Figure C1: The measurement set-up for the measurements. The Tobii Glasses 2 Eye Tracker Device was placed on the subject's head. The ECG electrode were all applied. In this case it was a measurement with the tVNS, so electrode in the neck were place on both sides of the neck. Subjects sat in front of a computer screen situated at a distance of 50 cm.

stimulation. For the other two group of subjects, one electrode was placed at the sternocleidomastoid muscle on the left side of the neck and one at the sternocleidomastoid muscle on the right side of the neck.

During the measurement pressure was applied on one of the electrodes when a new period started. Applying pressures created an artefact in the ECG signal, which was used as a marker for period transition during the analysis. According to the literature it is not possible to evoke a baroreflex by pressing the electrode in the neck, so it is assumed that pressing the electrode does not affect the heart rate or HRV (5,6). To create a baroreflex pressure must be applied for at least five seconds at the carotid sinus (5). That was not the case in this study

References

1. Desbeaumes Jodoin V, Lespérance P, Nguyen DK, Fournier-Gosselin M-P, Richer F. Effects of vagus nerve stimulation on pupillary function. Int J Psychophysiol. 2015;98(3):455–9.

2. Liehr P. Uncovering a hidden language: The effects of listening and talking on blood pressure and heart rate. Arch Psychiatr Nurs. 1992;6(5):306–11.

3. Lynch JJ, Long JM, Thomas SA, Malinow KL, Katcher AH. The effects of talking on the blood pressure of hypertensive and normotensive individuals. Psychosom Med. 1981;43(1):25–33.

4. Tobii Technology (2013) Mobile Eye Tracking [Internet]. Available from: http://www.tobiipro.com/product-listing/tobii-pro-glasses-2/

5. Pasquier M, Clair M, Pruvot E, Hugli O, Carron P-N. Carotid Sinus Massage. N Engl J Med. 2017 Oct 11;377(15):e21.

6. Lloyd MG, Wakeling JM, Koehle MS, Drapala RJ, Claydon VE. Carotid sinus hypersensitivity: block of the sternocleidomastoid muscle does not affect responses to carotid sinus massage in healthy young adults. Physiol Rep. 2017 Oct 1;5(19).

D: Tobii Glasses Eye Tracker Device

During this study the Tobii Glasses Eye Tracker Device (Tobii Technology) from the BMSlab powered by Tech4People from the University of Twente was used. The Tobii Glasses consists of a wearable eye tracker (glasses), which is the head unit (figure D1), and a recording unit (1). The recording unit is connected to the head unit via a HDMI cable. The recording unit hold the battery and stores the recorded data on a SD memory card. The recording unit was controlled from a tablet running controller software.

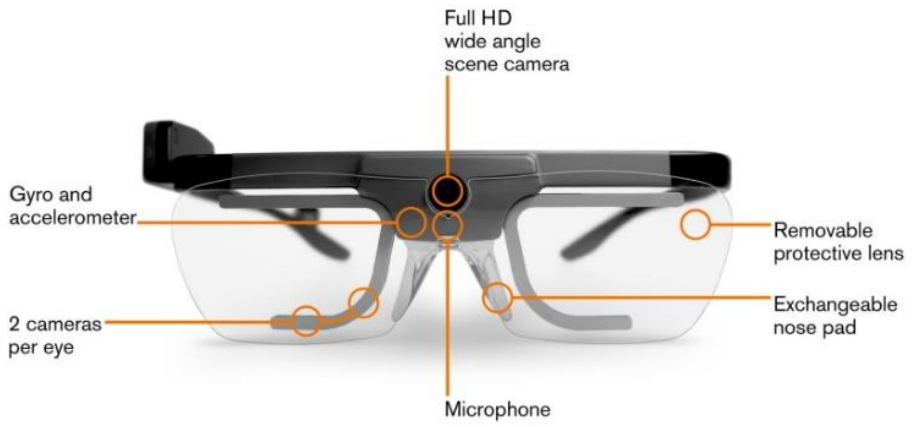


Figure D1: The Tobii Glasses Eye Tracker Device head unit (1).

There are several different techniques to detect and track the movements of the eyes. The Tobii eye tracker uses near-infrared illumination to create reflection patterns on the cornea and pupil of the eye of the subject and optical image sensors are used to capture images of the eyes and the reflection patterns (2). Advanced image-processing algorithms and a physiological 3D model of the eye are then used to estimate the position of the eye in space and the point of gaze with high accuracy. Figure D2 shows how the wearable Tobii eye tracker works (2).

Before an eye tracking measurement is started, a calibration procedure has to be performed. During this calibration procedure, the eye tracker measures characteristics of the subject's eyes and uses them together with the internal, physiological 3D eye model to calculate the gaze data (3). This physiological 3D model included information about shapes, light refraction and reflection properties of the different parts of the eyes (3).
During the calibration the subject is asked to look at a specific point on the screen of the tablet which runs the controller software. During the period of calibration several images of eyes are collected and analyzed. The resulting information is then integrated in the eye model and the gaze point for each image sample is calculated (3). During the calibration the dark pupil method is tested. The dark pupil method is an illumination setup where an illuminator is placed away from the optical axis causing the pupil to appear darker than the iris. Ethnicity is an factor that affect the dark pupil response (4).

The Tobii Glasses Eye Tracker Device measures the pupil diameter with the use of the optical sensors. The optical image sensors register an image of the eyes which is then used to calculate parameters to adjust the eye model algorithms (5). The eye model provides information about the distance between the eye and the sensors. With the use of that information the firmware can calculate the pupil size by measuring the diameter of the pupil on the image and multiply it with a scaling factor (5).

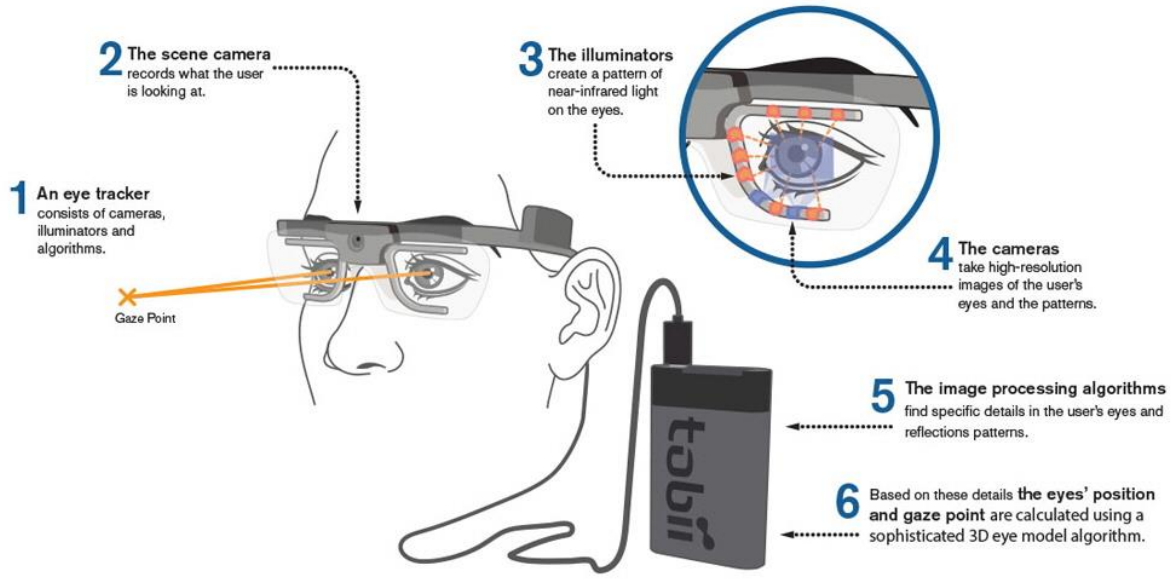


Figure D2: The working mechanism of a wearable Tobii Glasses Eye Tracker Device (2).

References

1. Tobii Pro Glasses 2 [Internet]. Available from: https://www.tobiipro.com/product-listing/tobii-pro-glasses-2/
2. How do Tobii Eye Trackers work? [Internet]. Available from: https://www.tobiipro.com/learn-and-support/learn/eye-tracking-essentials/how-do-tobii-eye-trackers-work/
3. What happens during the eye tracker callibration? [Internet]. Available from: https://www.tobiipro.com/learn-and-support/learn/eye-tracking-essentials/what-happens-during-the-eye-tracker-calibration/
4. Dark and bright pupil tracking [Internet]. Available from: https://www.tobiipro.com/learn-and-support/learn/eye-tracking-essentials/what-is-dark-and-bright-pupil-tracking/
5. Are pupil size calculations possible with Tobii Eye Trackers? [Internet]. Available from: https://www.tobiipro.com/learn-and-support/learn/eye-tracking-essentials/is-pupil-size-calculations-possible-with-tobii-eye-trackers/

E: Missing parts of the pupil diameter recordings

Table E1: The excluded parts of the measurements for the concerning subjects. C = healthy control, P = patients without a VNS, PV = patients with a VNS, (R) = the right eye, (L) = the left eye.

Part of the data that has to be excluded	Subjects from which this part is excluded	
	Cymba conchae	Earlobe
Measurement of the left eye	PV03	C12, C18
Measurement of the right eye	C20	C01, C20
ON phase low stimulation	C15 (R)	P02, P03
OFF phase low stimulation	C15 (R), C16	P02, P03
ON phase maximal stimulation	C15, C16 (L)	C15 (R)
OFF phase maximal stimulation	C15, C16 (L), P02	C05 (R), C13 (R), C15 (R)

F: Pupil diameter results

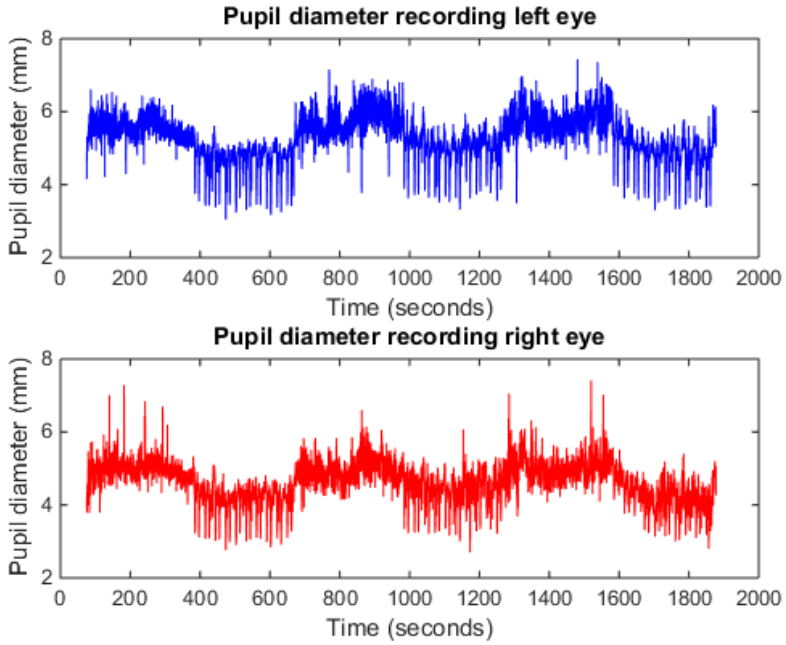


Figure F1: This figure shows the pupil diameter recording for the left eye (upper figure) and the right eye (bottom figure) for 1 subject. This data is obtained after interpolating the data with the use of linear interpolation. Three phases during the measurement shows decreases in the pupil diameter. During these phase pupillary light reflexes were provoked.

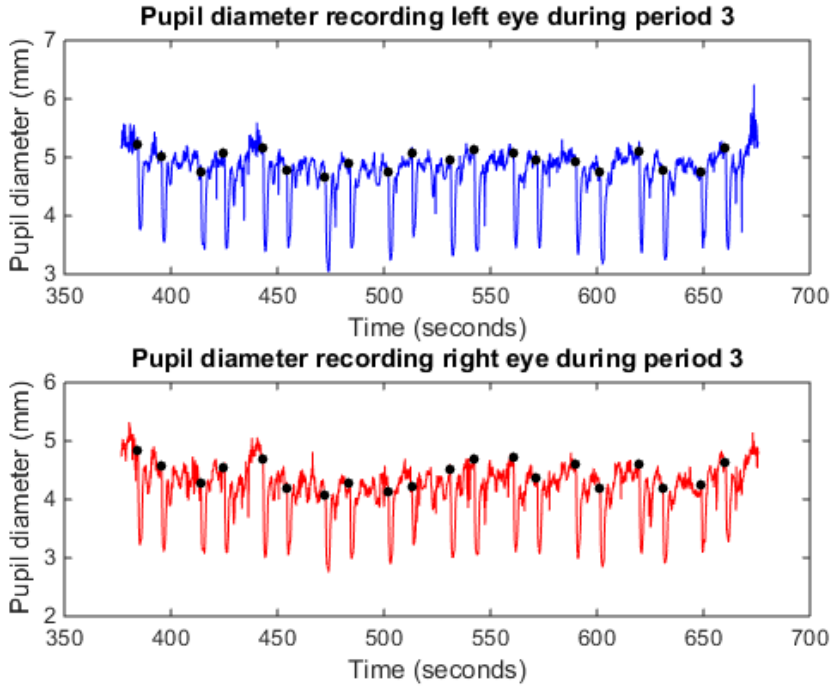


Figure F2: After interpolation of the pupil diameter recordings (F1) the different periods with tVNS stimulation are plotted. This figure shows the pupil diameter recordings during period 3 of the measurement for the left eye (upper figure) and the right eye (bottom figure). The black dots in the figure represents the onset of the light stimuli. 20 light stimuli were given during this period. 10 during the ON phase of tVNS stimulation and 10 during the OFF phase.

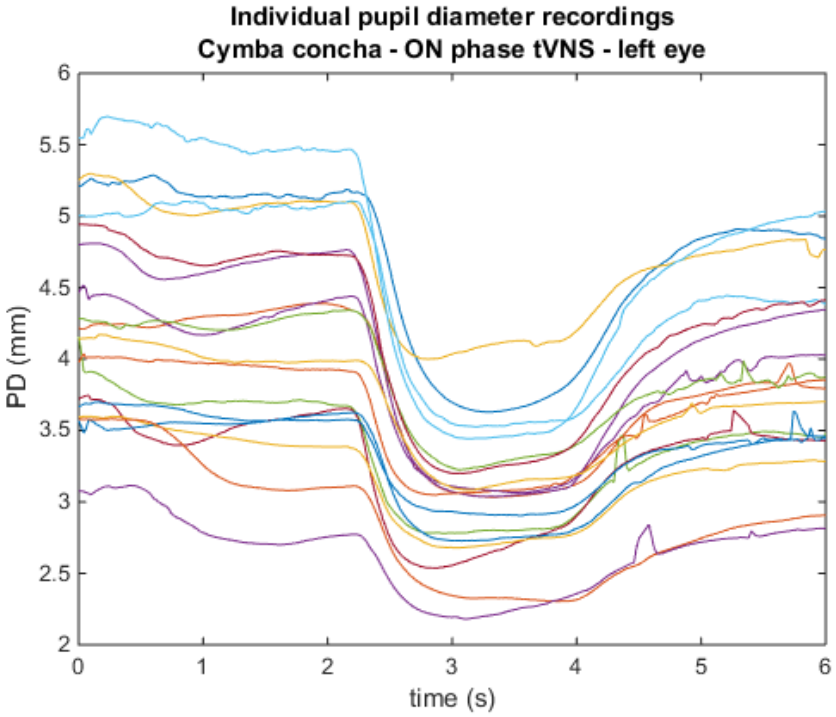


Figure F3: From the 10 different trials during a measurement conditions, the average is calculated. This figure shows the average during the ON phase of tVNS stimulation of the left eye for all the measured subjects separately. Each line represents a subject. This figure shows that there are some variances between the different measured subjects.

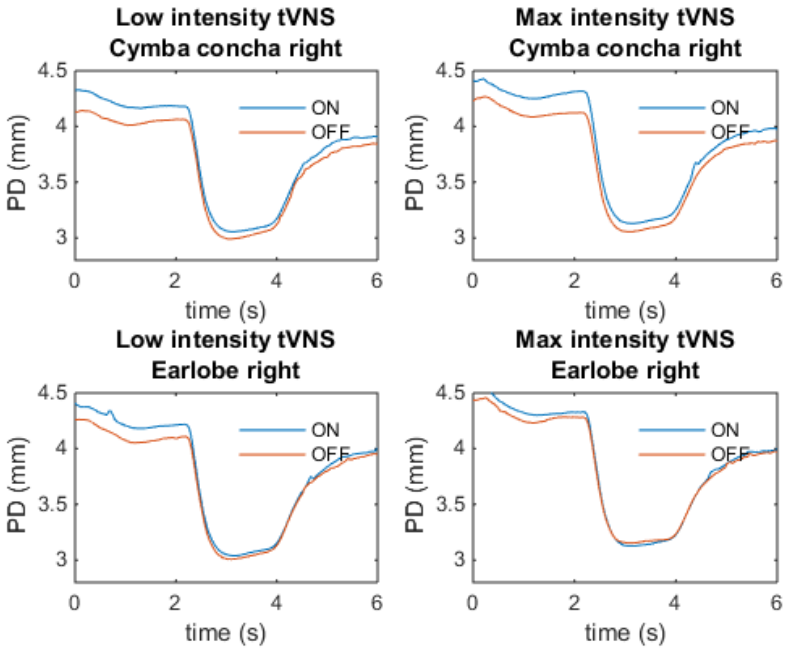


Figure F4: Pupil diameter recording during the ON and the OFF phase of the right eye during both low intensity stimulation and maximal intensity stimulation. The figure in the top left shows the pupil diameter recording for the right eye during low intensity cymba conchae stimulation. Top right shows the pupil diameter recording for the right eye during maximal intensity cymba conchae stimulation. The two bottom figures show the pupil diameter recordings during earlobe stimulation. The bottom left shows the results during low intensity and the bottom right during maximal intensity.

Table F1: The mean rest pupil diameter (PD) and the mean constriction amplitude (amplitude) together with their standard deviation for the healthy subjects and the epilepsy patients without a VNS. Mean are show for the both cymba conchae stimulation, both eye, and all the stimulation phases. For the epilepsy patients without a VNS no standard deviation is shown for the pupil diameters during the ON and OFF phase of low earlobe stimulation. This is because there is only one subject left for the analysis.

Stimulation phase	Healthy subjects				Patients with epilepsy without a VNS			
	Rest PD (mm)		Amplitude (mm)		Rest PD (mm)		Amplitude (mm)	
Cymba conchae	Left	Right	Left	Right	Left	Right	Left	Right
Mean ON	4.1±0.73	4.2±0.53	1.11±0.36	1.15±0.31	3.41±0.3	3.7±0.5	0.89±0.1	0.98±0.22
Mean OFF	3.9±0.74	4±0.54	1±0.37	1.04±0.31	3.45±0.49	3.7±0.58	0.95±0.21	1±0.26
ON phase low	4.14±0.73	4.20±0.56	1.12±0.40	1.15±0.33	3.42±0.39	3.74±0.60	0.92±0.15	1.05±0.31
OFF phase low	3.97±0.73	4.05±0.55	1.03±0.38	1.06±0.31	3.45±0.49	3.67±0.55	0.97±0.24	1±0.26
ON phase max	4.24±0.83	4.30±0.53	1.16±0.42	1.18±0.34	3.41±0.22	3.67±0.41	0.86±0.05	0.91±0.15
OFF phase max	4.02±0.80	4.07±0.56	1.05±0.43	1.05±0.34	3.8	4.10±0.02	1.05	1.16±0.03
Earlobe								
Mean ON	4.1±0.68	4.2±0.55	1.14±0.35	1.17±0.25	3.86±0.66	3.91±0.45	1.13±0.31	1.1±0.24
Mean OFF	3.9±0.71	4±0.56	1.05±0.35	1.07±0.28	3.77±0.63	3.79±0.47	1.1±0.29	1.05±0.21
ON phase low	4.05±0.67	4.21±0.53	1.12±0.36	1.18±0.27	4.02	4.11	1.19	1.16
OFF phase low	3.90±0.68	4.12±0.54	1.04±0.36	1.13±0.28	4.08	4.12	1.26	1.18
ON phase max	4.13±0.73	4.30±0.54	1.16±0.39	1.20±0.26	3.87±0.66	3.93±0.48	1.13±0.31	1.10±0.25
OFF phase max	4.00±0.79	4.23±0.53	1.08±0.41	1.15±0.26	3.80±0.61	3.81±0.53	1.12±0.25	1.07±0.22

Table F2: The mean rest pupil diameter and the mean constriction amplitude together with their standard deviation for the epilepsy patients with a VNS.

Stimulation phase	Rest pupil diameter (mm)		Constriction amplitude (mm)	
	Left	Right	Left	Right
ON phase of VNS	4.21±1.19	4.17±1.03	1.42±0.81	1.34±0.59
OFF phase of VNS	4.06±1.07	4.08±0.92	1.27±0.69	1.24±0.47

G: Heart rate results

Table G1: The heart rate results for each healthy subject during the baseline, the mean ON phase and the mean OFF phase. Also, the mean is given and the standard deviation (SD).

	Heart rate (bpm) – Cymba conchae				Heart rate (bpm) - Earlobe		
Subject	**Baseline**	**ON**	**OFF**		**Baseline**	**ON**	**OFF**
C01	80,7	74,2	78,5		82,7	77,5	81,7
C02	64,1	61,7	62,8		65,2	64,1	65,6
C03	56,7	53,5	56,6		55,2	55,8	56,8
C04	58,0	58,8	61,3		63,6	62,6	63,8
C05	69,4	70,6	71,0		68,3	72,8	73,2
C06	83,5	79,6	81,2		80,3	78,8	79,6
C07	72,6	70,2	70,8		75,1	71,1	72,4
C08	72,1	69,6	69,6		71,4	73,1	72,9
C09	60,2	64,9	66,7		71,9	69,4	72,0
C10	71,4	70,8	72,0		66,4	66,4	66,9
C11	69,1	70,2	71,9		84,6	80,9	82,6
C12	77,3	75,0	75,0		79,5	78,8	77,6
C13	69,1	70,9	70,9		74,7	75,9	77,5
C14	83,0	76,9	80,6		95,5	84,4	88,5
C15	61,5	59,7	61,8		65,4	64,0	63,3
C16	63,4	56,7	59,7		54,5	50,9	54,2
C17	65,0	64,2	65,9		63,6	63,1	65,2
C18	48,6	48,5	51,9		48,9	48,6	51,4
C19	63,8	66,3	66,7		60,4	60,8	63,3
C20	76,9	76,6	77,1		72,9	70,5	71,4
C21	69,9	70,9	71,6		74,1	76,9	80,4
Mean	68,4	67,1	68,7		70,2	68,8	70,5
SD	9	8,2	7,8		11	9,8	9,8

Table G2: The heart rate results for the epilepsy patients during the baseline, the mean ON phase and the mean OFF phase. Also, the mean is given and the standard deviation (SD).

	Heart rate (bpm) – Cymba conchae				Heart rate (bpm) - Earlobe		
Subject	**Baseline**	**ON**	**OFF**		**Baseline**	**ON**	**OFF**
P01	80,1	77,6	80,6		82,4	80,5	83,5
P02	72,9	74,1	72,4		80,95	86,8	82,2
P03	65,6	67,3	67,1		65,25	64,8	65
P04	67,2	66,6	66,3		70,09	69,3	69,7
Mean	71,4	71,4	71,6		74,7	75,4	75,1
SD	6,6	5,4	6,6		8,3	10,1	9,2

Table G3: the heart rate results for the healthy subjects during the baseline, the ON and OFF phase during low intensity tVNS stimulation, and the ON and OFF phase during maximal intensity tVNS stimulation. Also, the mean is given and the standard deviation (SD).

Cymba conchae stimulation						Earlobe stimulation				
Subject	**Baseline**	**ON Low**	**OFF Low**	**ON Max**	**OFF Max**	**Baseline**	**ON low**	**OFF low**	**ON max**	**OFF max**
C01	80,7	75,2	79,3	73,2	77,6	82,65	78,9	82,5	76,0	80,9
C02	64,1	63,1	63,3	60,4	62,3	65,2	64,6	65,5	63,6	65,7
C03	56,7	54,5	57,6	52,4	55,5	55,15	54,8	56,7	56,7	56,8
C04	58,0	59,2	60,0	58,4	62,6	63,55	62,2	63,8	63,0	63,8
C05	69,4	70,2	71,6	70,9	70,3	68,3	71,5	72,5	74,0	73,9
C06	83,5	82,1	82,2	77,1	80,2	80,3	78,7	79,3	78,8	79,9
C07	72,6	71,0	70,6	69,3	70,9	75,1	71,1	71,9	71,0	72,9
C08	72,1	70,1	70,4	69,0	68,8	71,35	74,6	73,7	71,6	72,1
C09	60,2	63,0	65,0	66,8	68,3	71,9	69,8	71,4	68,9	72,6
C10	71,4	70,4	71,4	71,2	72,6	66,4	66,6	67,0	66,1	66,7
C11	69,1	72,0	72,8	68,5	71,0	84,55	82,3	84,4	79,4	80,8
C12	77,3	75,2	76,0	74,8	74,0	79,45	80,3	78,9	77,3	76,2
C13	69,1	71,7	71,5	70,1	70,3	74,65	74,5	75,5	77,2	79,4
C14	83,0	78,2	80,9	75,6	80,2	95,45	91,1	93,8	77,6	83,2
C15	61,5	60,2	61,5	59,2	62,1	65,4	64,9	64,2	63,0	62,3
C16	63,4	57,4	59,8	55,9	59,5	54,45	51,8	52,8	49,9	55,6
C17	65,0	64,9	66,1	63,5	65,6	63,55	63,4	64,4	62,8	65,9
C18	48,6	48,6	49,9	48,5	53,9	48,9	47,5	48,6	49,7	54,1
C19	63,8	65,3	65,7	67,3	67,7	60,35	59,4	61,8	62,1	64,7
C20	76,9	78,6	77,6	74,7	76,6	72,85	71,6	71,6	69,4	71,2
C21	69,9	71,2	71,0	70,6	72,2	74,05	76,3	79,3	77,5	81,4
Mean	68,4	67,7	68,8	66,6	68,7	70,2	69,3	70,5	68,4	70,5
SD	9,0	8,5	8,3	8,0	7,4	11,0	10,7	10,9	9,1	8,9

Table G4: the heart rate results for the epilepsy patients during the baseline, the ON and OFF phase during low intensity tVNS stimulation, and the ON and OFF phase during maximal intensity tVNS stimulation. Also, the mean is given and the standard deviation (SD).

Cymba conchae stimulation						Earlobe stimulation				
Subject	**Baseline**	**ON low**	**OFF low**	**ON max**	**OFF max**	**Baseline**	**ON low**	**OFF low**	**ON max**	**OFF max**
P01	80,1	80,3	81,3	74,9	79,8	82,4	81,8	85,2	79,2	81,7
P02	72,9	74,0	72,2	74,3	72,7	80,95	93,3	85,1	80,4	79,2
P03	65,6	66,2	67,6	68,3	66,5	65,25	65,5	65,4	64,2	64,6
P04	67,2	66,5	66,1	66,7	66,5	70,09	70,4	71,0	68,3	68,3
Mean	71,4	71,7	71,8	71,0	71,4	74,7	77,7	76,7	73,0	73,5
SD	6,6	6,8	6,9	4,1	6,3	8,3	12,4	10,1	8	8,3

H: Heart rate variability results

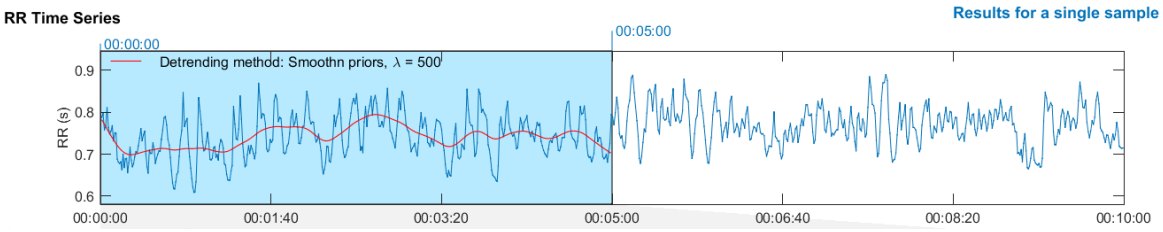


Figure H1: This figure shows the R-R intervals plotted over the time for one healthy subject. The R-R intervals are calculated with the use of the ECG's in Matlab and loaded into Kubios. The time axis has a duration of 10 minutes. The R-R intervals during the first 5 minutes are shown in the blue plane and contains the five minutes of rest. The white plane shows 5 minutes of continuous tVNS stimulation. The R-R interval is given in seconds.

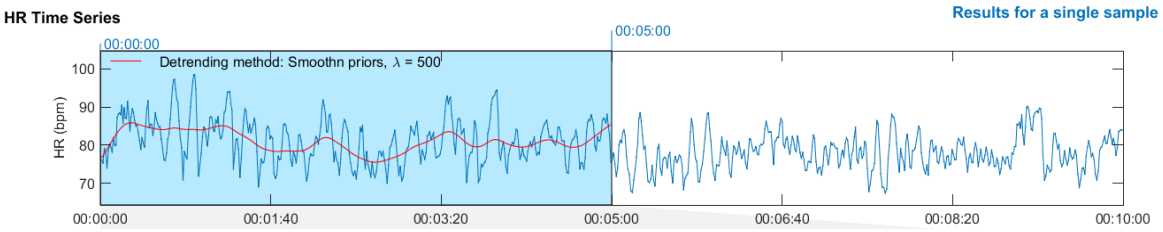


Figure H2: This figure shows heart rate (HR) plotted over the time for one healthy subject. The heart rate is calculated from the R-R intervals, which are obtained from the ECG's in Matlab and loaded into Kubios. The time axis has a duration of 10 minutes. The heart rate during the first 5 minutes are shown in the blue plane and contains the five minutes of rest. The white plane shows 5 minutes of continuous tVNS stimulation. The heart rate is given in beats per minute.

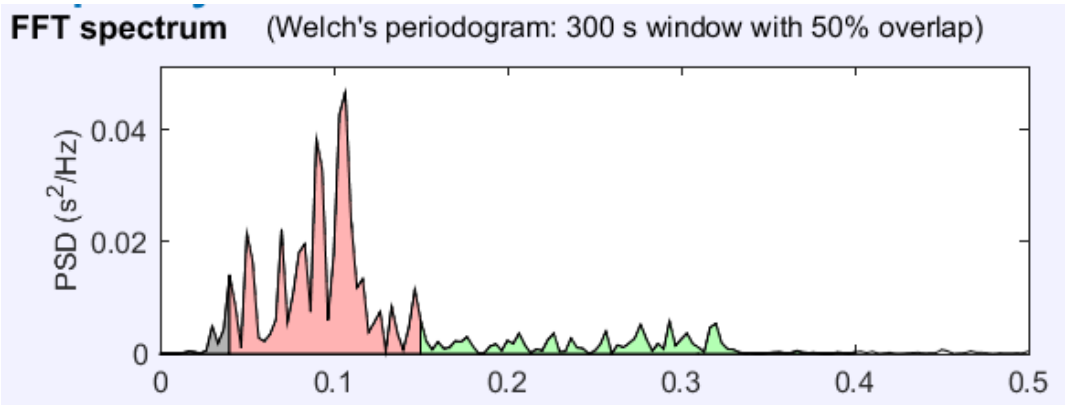


Table H3: This figure shows the obtained FFT spectrum, which was calculated with the non-parametric Welch's periodogram. The red area represents the LF and the green area represents the HF.

Table H1: The mean heart rate variability parameters together with the standard deviation (SD) for the healthy subjects and the epilepsy patients without a VNS.

HRV parameters	Cymba conchae stimulation		Earlobe stimulation	
Healthy subjects	Rest	tVNS	Rest	tVNS
RR (ms)	865 ± 105	882 ± 117	849 ± 119	869 ± 127
SDNN (ms)	50 ± 22	47 ± 21	45 ± 24	47 ± 27
RMSSD (ms)	40 ± 20	42 ± 23	36 ± 23	38 ± 26
LF (ms^2)	1834± 1669	1552 ± 1368	1719 ± 1841	1752 ± 1908
HF (ms^2)	706 ± 835	681 ± 717	646 ± 997	722 ± 1304
LF/HF (ms^2)	3.35 ± 2.38	2.93 ± 2.38	3.68 ± 2.68	4.64 ± 5.40
Epilepsy patients				
RR (ms)	854 ± 60	842 ± 42	824 ± 79	826 ± 67
SDNN (ms)	35 ± 18	31 ± 9	29 ± 13	31 ± 15
RMSDD (ms)	23 ± 9	22 ± 6	20 ± 6	21± 7
LF (ms^2)	991 ± 1341	605 ± 336	641 ± 687	872 ± 1197
HF (ms^2)	204 ± 150	200 ± 48	195 ± 138	185 ± 173
LF/HF (ms^2)	3.61 ± 2.47	3.03 ± 1.50	2.84 ± 1.28	3.66 ± 2.05

I: The effect of tVNS on seizure reduction, quality of life, and mood in patients suffering from refractory epilepsy

3.1 Rationale

Vagus nerve stimulation (VNS) is a form of neurostimulation used for treating patients suffering from refractory epilepsy. However, this implementation does not always yield a positive result. Most long term studies that were done to assess the efficacy of VNS concluded that a more than 50% seizure reduction was accomplished in 20-63% of the patients after treatment for six months to six years (1–8).
Despite the growing application of VNS, it is still not possible to predict which patient respond to what extend to VNS therapy. Recently, transcutaneous VNS (tVNS) has become available, which stimulates the auricular branch of the vagus nerve via an earpiece. This might be an effective method of reducing seizure frequency in epilepsy patients.

The main objective of this part of the master thesis is to gain more insight in the effects of tVNS in patients suffering from refractory epilepsy. Therefore, the research question is:

- What is the effect of tVNS on the seizure reduction, quality of life, and mood in patients who are suffering from refractory epilepsy?

The research question is a part of a larger study, which studies the predictive value of tVNS for the effect of a VNS. However, for my master thesis I only study the effect of tVNS on the seizure reduction, quality of life, and mood in epilepsy patients. The other parts of the larger study will not be included in this master thesis.

3.2 Hypothesis

tVNS results in a seizure reduction after 3 months of stimulation and causes an improvement in mood and quality of life.

3.3 Method

3.3.1 Subjects

At this moment ten epilepsy patients were recruited in the larger study to assess the predicting value of tVNS for the effects of VNS. Subjects that were recruited for this study were patients that are referred for an implantable VNS system to the department of the neurosurgery at the Medisch Spectrum Twente (MST) in Enschede, the Netherlands. Inclusion criteria were: eighteen years or older, without severe cognitive impairment; diagnosed with epilepsy; scheduled for an implantable VNS system; and physically and cognitively capable of using the tVNS device.

From the ten recruited patients, eight patients were recruited for this study. Two patients from the larger study were not included, because one patient experienced some difficulties using the tVNS, so this patient decided to stop with the study. The other patient was just recently included, so not all the measurement data was available yet.

The study followed the principles laid out in the Declaration of Helsinki and ethical approval was obtained from the Medical Research Ethics Committee Twente. All subjects gave written informed consent.

3.3.1 Measurement procedure

The total duration of the whole study was fifteen months for each participating patient and consisted of seven visits to the MST (figure I1). The effects of tVNS in epilepsy patients was assessed during the first three visits. The effect of the VNS was assessed during the last four visits. The VNS was surgically implanted between the third and fourth visit.

The first visit (at t = -3m) was considered as the intake. During the intake the baseline parameters of the patients were acquired and explanation was given to the patients about the tVNS device. Baseline parameter that were acquired included:

a) Demographics and lifestyle-related factors (e.g. smoking, exercise, diet);
b) Seizure frequency from patient's seizure diary;
c) Medication use;
d) EEG features measured by 24-hour (in-home) EEG;
e) Cardiorespiratory parameter measured by 24-hour ECG;
f) Quality of life (QOLIE31P), and mood (MADRS);
g) Blood cytokine levels;
h) Cortical excitability.

When the baseline parameters were acquired, patients started with the use of tVNS. tVNS was administered through a tVNS device, which was obtained from Cerbomed GmbH (Erlangen, Germany). In 2010, the company received a CE mark for the tVNS. The tVNS stimulated with a stimulation frequency of 25 Hz, 30 second ON and OFF phase, and stimulation intensity which was just above the patient's threshold (first tingling sensation). tVNS was applied at the cymba conchae to stimulate the ABVN. The recommended daily dose of the tVNS was four hours a day. Patients had to take this daily stimulation dose in several sessions spread evenly throughout the day. Six weeks after the intake patients had a new visit to the hospital, to assess the effects of tVNS (t= -6wk, figure I1). During this visit the parameter a) to g) were acquired again, and in addition there was also information obtained about:

i) Stimulation parameters;
j) Perceived side effects;
k) Patient global impression of change (PGIC), which was a questionnaire considering the effect of (t)VNS on the patient's seizures.

At time point t=0 in figure I1 patients were using the tVNS for twelve weeks and another visit was scheduled. During this visit the parameters a) to k) were acquired again and patients had to return the tVNS. After t=0 all patients were implanted with the VNS. After VNS implantation the patients had to visit the hospital less frequent than during the period with tVNS. The effects of VNS on seizure frequency, seizure characteristics, quality of life, EEG features, side-effects, blood cytokine levels and cortical excitability will be assessed every three months (t=3, 6, 9, 12). During

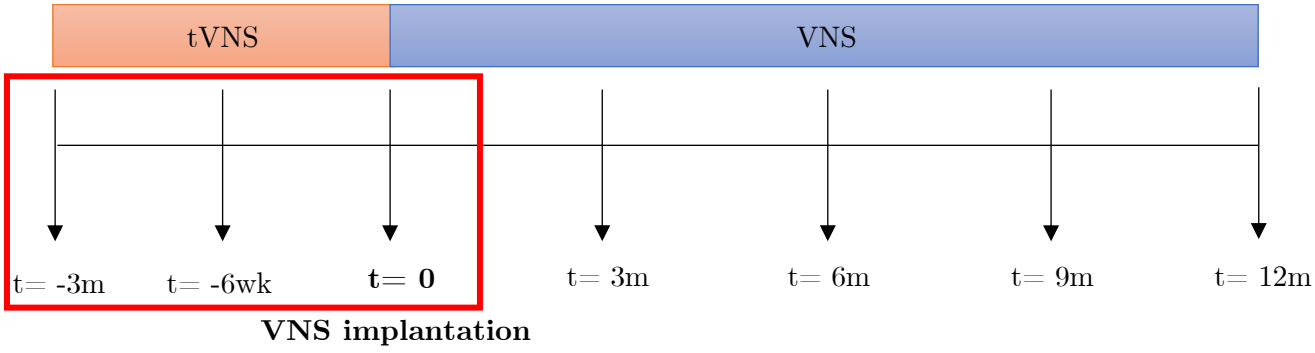


Figure I1: Flowchart of the experimental setup for the epilepsy patients. The red box shows the period of tVNS stimulation. Patients were implanted with the VNS after t=0.

these visits the parameters a) to k) were acquired and additionally information was obtained about the seizure supressing effect of the magnet and degree of magnet use. Cortical excitability (h) was only assessed at t=3 and 12 months.

3.3.3 Analysis

Section 3.2.2 described the whole measurement procedure for patients who were included in the study. For this master thesis only the effect of tVNS was studied (figure I1, the red box). The effects of tVNS is on the seizure reduction (parameter b, k), the quality of life (parameter f) and the mood (parameter f) were studied. The quality of life was assessed with the QOLIE31P, which is a standardized questionnaire to assess the patient's health-dependent quality of life. The total score ranged from 0 to 100 and higher scores representing better quality of life. The mood was assessed with the Montgomery-Asberg Depression Rating Scale (MADRS), which assessed the severity of patient's depressive symptoms using a series of ten questions. The maximal total score is 60. Higher scores indicated a more severe depression. For the quality of life and the mood, mean scores together with the standard deviation are given. Due to the limited number of patients, no statistical analysis was performed on the result

3.4. Results

3.4.1 Subjects

At this moment ten epilepsy patients are included in the larger study to assess the predicting value of tVNS for the effect of VNS. From those ten patients, eight patients are included for this thesis. The mean age of these eight patients is 47 years (31-59 years). Subject characteristics from the included patients are shown in table I1.

Table I1: Subject characteristics

Subjects	Gender	Age	Type of epilepsy
tVNS01	M	34	Focal epilepsy
tVNS02	M	31	Focal epilepsy
tVNS03	F	56	Focal epilepsy
tVNS04	F	53	Focal epilepsy
tVNS05	M	59	Focal epilepsy
tVNS07	M	53	Generalized epilepsy
tVNS08	F	43	Focal epilepsy
tVNS09	F	50	Focal epilepsy

3.4.2 Seizure reduction of tVNS

Patients keep seizure diaries. Seizure frequency is determined at baseline, 6 weeks after the start of tVNS and twelve weeks after the start of tVNS. The results of the seizure frequency are shown in table I2 and show that there is a decrease in seizure frequency after six weeks of tVNS for tVNS02, tVNS08 and tVNS09. However, according the PGIC patient tVNS02 and tVNS09 do not report an improvement in seizure frequency. tVNS09 do reports an improvement of seizure frequency in the PGIC.

After twelve weeks of tVNS the decrease in seizure frequency was still shown for tVNS08 and tVNS09 compared to the baseline measurement (table I2). The data of tVNS02 is missing. Both tVNS08 and tVNS09 reports an improvement in seizure frequency.

Overall, the mean results of all the patients show a decrease in seizure frequency during the 6 weeks tVNS measurement (mean 22 ± 20) compared with the baseline (mean 51 ± 99). A decrease is also shown after twelve weeks of using tVNS (mean 24 ± 25) compared with the baseline. However, tVNS09 is a big outlier in the data, which has a great impact on the results. When removing the data of tVNS09 almost no differences are shown between the seizure frequency at the baseline (mean 16 ± 18) compared with the 6 week use of tVNS (mean 16 ± 13) and compared with the 12 week use of tVNS (mean 15 ± 11).

Besides the seizure reduction, patients also report a positive effect of tVNS on the intensity of the seizures and the recovery period after a seizure. After twelve weeks of VNS, three out of the eight patients reported a decrease in seizure intensity due to tVNS stimulation. tVNS02 reports a shorter recovery after a seizure because of tVNS stimulation during the six week measurement. Data of the 12 week measurement is missing for this patient.

Table I2: Seizure frequency at the baseline, after 6 weeks of using tVNS and after 12 weeks of using tVNS. The seizure frequency is given as the number of seizure during 6 weeks. The for all the three periods the mean seizure frequency is given together with the standard deviation (SD).

Patient	Baseline	6 weeks tVNS	12 weeks tVNS
tVNS01	6	16	26
tVNS02	42	30	
tVNS03	10	10	11
tVNS04	3	6	6
tVNS05	3	5	8
tVNS07	9	9	10
tVNS08	42	38	34
tVNS09	294	63	77
Mean	51	22	24
SD	99	20	25

3.4.3 Quality of life and mood

Positive effects of tVNS on the quality of life and the mood have been reported by the patients (figure I3 and I4). Six out of eight patients show a decrease in the MADRS score after 6 weeks of using tVNS compared with the baseline. For the baseline compared with using the tVNS for 12 weeks, also six out eight patients show a decrease (figure I3).
With regard to the quality of life, five out of eight patients show an increase after using the tVNS for 6 weeks compared with the baseline (figure I4). After using the tVNS for 12 weeks, also 5 out of eight patients show an increase in the quality of life compared with the baseline.

Overall, the quality of life shows an increase after six weeks of tVNS stimulation (mean 58 ± 14) and after 12 weeks of tVNS stimulation (mean 63 ± 17) compared to the baseline (mean 53 ± 14). Furthermore, the MADRS score also show positive results, because the MADRS score decreases after six weeks of tVNS stimulation (mean 7.5 ± 4) and after 12 weeks of tVNS stimulation (6.9 ± 4) compared to the baseline (mean 8.3 ± 3). However, one patient shows opposite results compared with the other patients. When removing this patient from the data, the overall results improves. The Quality of life now show a mean value of 55 ± 14 at the baseline, a mean value of 61 ± 12 after 6 weeks of using tVNS, and a mean value of 68 ± 10 after 12 weeks of using tVNS. For the MADRS results the mean values are 8.7 ± 3, 7.1 ± 5, and of 5.5 ± 3, respectively.

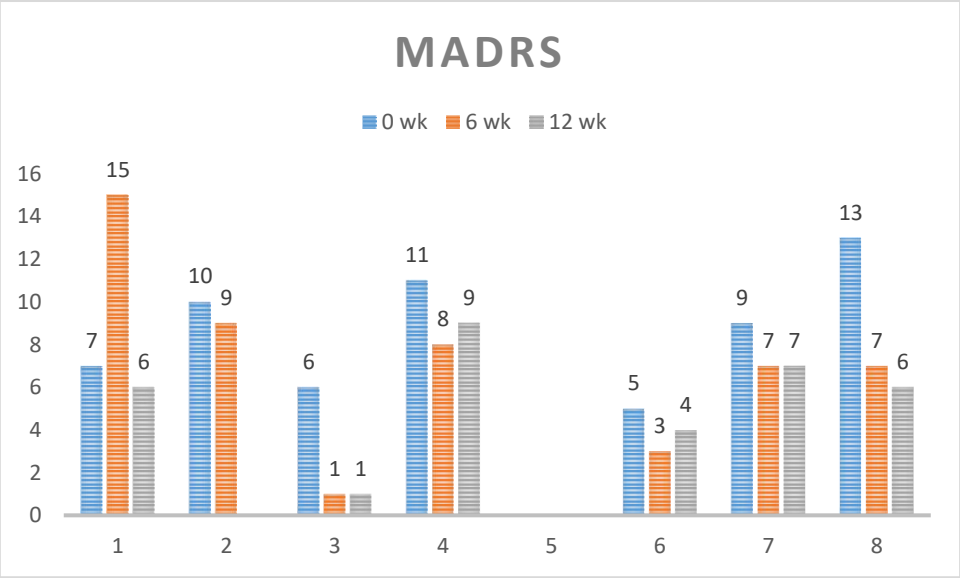


Figure I3: The results of the MADRS scores for each patient individually. Blue represents the baseline, red represents using tVNS for 6 weeks, and grey represents using tVNS for 12 weeks.

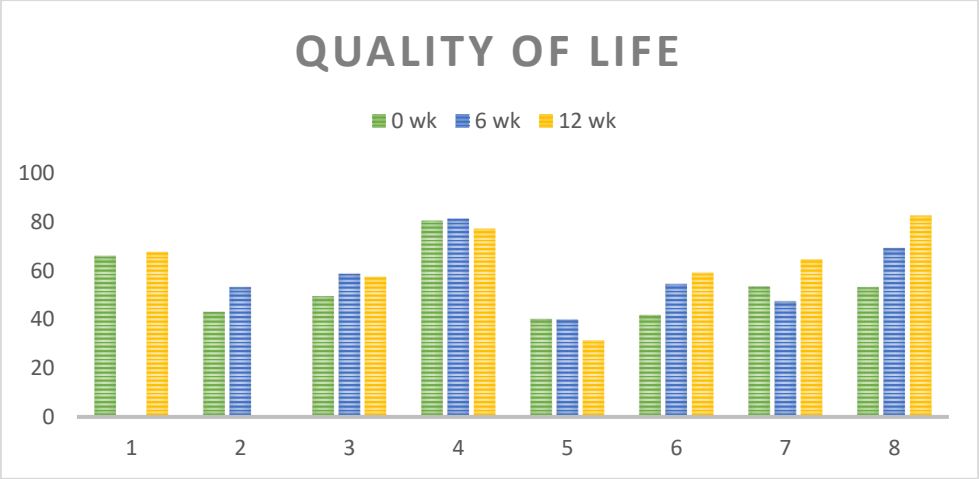


Figure I4: The results of the quality of life for each patient individually. Green represents the baseline, blue represents using tVNS for 6 weeks, and yellow represents using tVNS for 12 weeks.

Patient also reports a positive effect of tVNS on alertness and concentration. Four out of eight patients describe those effects. These effects are also seen in the MADRS and quality of life scores, because these patients do also show an improvement in these scores.

3.4.4 Side effects

Two of the eight patients reported the occurrence of a headache during the period of tVNS stimulations. One patients reported ear pain during the stimulations.

3.5 Discussion

During this study the effect of tVNS on seizure reduction, quality of life and mood was studied in patients with refractory epilepsy. The overall results show that the tVNS has a positive effect on the quality of life and on the mood in the epilepsy patients and a les positive effect on the seizure reduction, because only three out of the eight patients showed a little difference in seizure frequency.

No effects due to medication were expected on these results, because none of patient had a change in medication during the measurement.

The effect of tVNS on the seizure frequency was low in the included patients. This could be due to the fact that not every patient is a responder to tVNS, which results in different reaction among patients on tVNS stimulation. Another possibility that could cause the low effect of tVNS on the seizure frequency is that tVNS was only applied for three months. Other studies uses a longer period of stimulation to achieve result from tVNS stimulation (9,10).

The seizure frequency results could also be influenced by the reporting on the seizure diaries. At the baseline measurement no diaries were available and the patients were asked about the seizure frequency. This method was less reliable than using seizure diaries from the period of 6 weeks prior to the baseline measurement. For example, when regarding tVNS09. The seizure frequency is much higher at the baseline compared with the other two measurement moments. During the 6 week measurement and the 12 week measurement, the seizure diaries were used. However, the patient herself reported much high seizure frequencies.
Furthermore, patients also report that they had some difficulties with the seizure diary and sometimes the forgot that they had to write down their seizures. This also makes the seizure diaries less reliable. It is difficult to get reliable results for the seizure frequency.

The result of tVNS on the quality of life and the mood are very positive. The results were not caused by the anti-seizure effect of the tVNS alone during this study, but also because tVNS had a positive influence on the alertness and concentration of the patients. Patient who did not show any differences in seizure frequency still show an improvement in mood and quality of life due to the fact the they feel more alert and had a better concentration. According to literature it is also shown that tVNS is used for patients with a depression and that is has positive effects in these patients (11,12). Therefore, it is possible that during this study the tVNS has a positive effect on the mood and the quality of life without improving the seizure frequency.

3.6 Conclusion

tVNS has a positive effect on the quality of life and the mood of patients with refractory epilepsy. However, tVNS shows little effects of the seizure frequency after stimulation the cymba conchae for a period of 12 weeks, because only 2 out of 8 patients show a decrease in seizure frequency after 12 weeks of tVNS stimulation.

3.7 References

1. Mulders DM, De Vos CC, Vosman I, Van Putten MJAM. The effect of vagus nerve stimulation on cardiorespiratory parameters during rest and exercise. Seizure. 2015;33:24–8.

2. Elliott RE, Morsi A, Kalhorn SP, Marcus J, Sellin J, Kang M, et al. Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: Long-term outcomes and predictors of response. Epilepsy Behav. 2011;20(1):57–63.

3. Ben-Menachem E, Revesz D, Simon BJ, Silberstein S. Surgically implanted and non-invasive vagus nerve stimulation: A review of efficacy, safety and tolerability. Eur J Neurol. 2015;22(9):1260–8.

4. Galbarriatu L, Pomposo I, Aurrecoechea J, Marinas A, Agundez M, Gomez JC, et al. Vagus nerve stimulation therapy for treatment-resistant epilepsy: A 15-year experience at a single institution. Clin Neurol Neurosurg. 2015;137:89–93.

5. Terry RS. Vagus Nerve Stimulation Therapy for Epilepsy. Epilepsy Top. 2014;139–60.

6. Varsavsky A, Mareels I, Cook M. Epileptic seizures and the EEG: measurement, models, detection and prediction. CRC Press; 2012.

7. Ardesch JJ, Buschman HPJ, Wagener-Schimmel LJJC, van der Aa HE, Hageman G. Vagus nerve stimulation for medically refractory epilepsy: A long-term follow-up study. Seizure. 2007;16(7):579–85.

8. De Vos CC, Melching L, Van Schoonhoven J, Ardesch JJ, De Weerd AW, Van Lambalgen HCE, et al. Predicting success of vagus nerve stimulation (VNS) from interictal EEG. Seizure. 2011;20(7):541–5.

9. Aihua L, Lu S, Liping L, Xiuru W, Hua L, Yuping W. A controlled trial of transcutaneous vagus nerve stimulation for the treatment of pharmacoresistant epilepsy. Epilepsy Behav. 2014;

10. Bauer S, Baier H, Baumgartner C, Bohlmann K, Fauser S, Graf W, et al. Transcutaneous Vagus Nerve Stimulation (tVNS) for Treatment of Drug-Resistant Epilepsy: A Randomized, Double-Blind Clinical Trial (cMPsE02). Brain Stimul. 2016;9:356–63.

11. Fang J, Rong P, Hong Y, Fan Y, Liu J, Wang H, et al. Transcutaneous Vagus Nerve Stimulation Modulates Default Mode Network in Major Depressive Disorder. Biol Psychiatry. 2018 Feb 13;79(4):266–73.

12. Rong P, Liu A, Zhang J, Wang Y, He W, Yang A, et al. Transcutaneous vagus nerve stimulation for refractory epilepsy: a randomized controlled trial. Clin Sci. 2014;CS20130518.

J: Detecting epilepsy via breath analysis using an electronic Nose

Another study I performed during my master thesis was about the detection of epilepsy with the use of an electronic nose. I started this study during my master 2 internship at the neurosurgery department at the MST and I really liked to continue with this study during my graduation year.

The study was about the detection of epilepsy via breath analysis using an electronic nose. There is not yet a quick test, nor a non-invasive method to identify subjects with epilepsy. The Aeonose™ (electronic nose) is a diagnostic test device to detect patterns of volatile organic compounds (VOC's) in exhaled air. These VOC's are related to metabolic activities in the body. It is assumed that the disease-specific metabolic pathways may give rise to specific VOC patterns and therefore aid in the diagnostic process. Pilot studies have already indicated that electronic noses, consisting of an array of VOC-sensors, may be used to detect diseases in different diagnostic areas. Our goal was to study whether the eNose can detect VOC patterns that distinguish between patients with confirmed diagnosis of epilepsy and healthy subjects without any suspicion of epilepsy.

The progress and the results of the study at this moment are presented in the poster below. To control for the fact that most epilepsy patients use anti-epileptic drugs (AEDs) additional patient groups were measured: patients (temporarily) not using AEDs and patients using AEDs but who are not diagnosed with epilepsy.

We also presented the poster at the American Epilepsy Society annual meeting in Washington D.C. past December.

E-mail: d.vandartel@mst.nl

Detecting epilepsy via breath analysis using an electronic Nose

Dieuwke van Dartel[1,3] , Kuan H. Kho[1] , H.Jurgen Schelhaas[2] , Albert Colon[2] , Cecile C. de Vos[1,3,4]

1: Department of Neurology and Neurosurgery, Medisch Spectrum Twente, Enschede, The Netherlands. 2: Academic centre for Epileptology, Kempenhaeghe, Heeze, the Netherlands 3: Stichting The NeurobBionics Foundation, Enschede, The Netherlands, 4: Montreal Neurological Institute, McGill University, Canada.

Rationale

Electronic nose (eNose) technology enables to measure and classify a pattern of volatile compounds (VOC's) in exhaled breath.

An eNose allows for online recognition of complex exhaled breath mixtures using sensor arrays and learning algorithms. The eNose is trained by analysing exhaled breath of patients with a specific disease and control subjects without the disease. So that it learns which pattern of VOC's or 'breath-prints' match with a disease. Over the last few decades eNose measurements have demonstrated to be a potential diagnostic tool in diseases like asthma, tuberculosis, and lung cancer, but also e.g. colorectal cancer.

Several studies have reported a release of prototypical inflammatory cytokines, as well as danger signals in epileptic brain tissue [1-4].

Based on these increased levels of cytokines, we hypothesise that an eNose is able to identify a breath-print for epilepsy.

Method

Patients with epilepsy and control subjects were measured to train the eNose for identifying epilepsy breath-prints. To control for the fact that most epilepsy patients use anti-epileptic drugs (AEDs), additional patient groups were measured: patients (temporarily) not using AEDs and patients using AEDs but not diagnosed with epilepsy.

An Aeonose™ (eNose Company, the Netherlands) was used, which is a non-invasive, handheld device, and has no side effects (Figure 1). During the measurement subjects were asked to breathe in and out through the Aeonose™ for five minutes. This period is followed by 10 minutes of breath analysis (Figure 2). The conductivity values of the exhaled breath measurements were stored in the Aeonose™ and then uploaded to a database. Pattern-recognition software (Aethena™, eNose Company, the Netherlands) was used for finding the best model to distinguish between the breath prints of afflicted and healthy subjects [5]. This model is then used to evaluate relevant parameters like sensitivity, specificity, negative and positive predictive values.

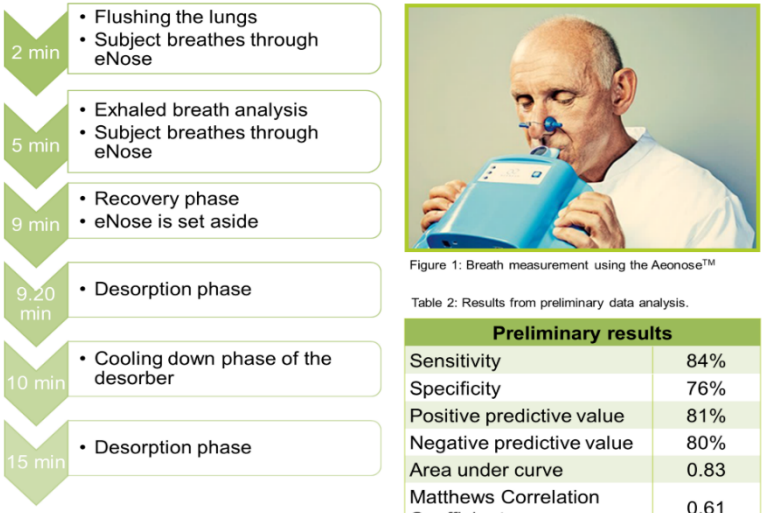


Figure 2: Schematic view of the eNose measurement. During the first five minutes, subjects breathe through the eNose. After five minutes, the eNose is set aside and continues with the measurement for another 10 minutes. Total measurement time is 15 minutes.

Figure 1: Breath measurement using the Aeonose™

Table 2: Results from preliminary data analysis.

Preliminary results	
Sensitivity	84%
Specificity	76%
Positive predictive value	81%
Negative predictive value	80%
Area under curve	0.83
Matthews Correlation Coefficient	0.61
Accuracy	0.81

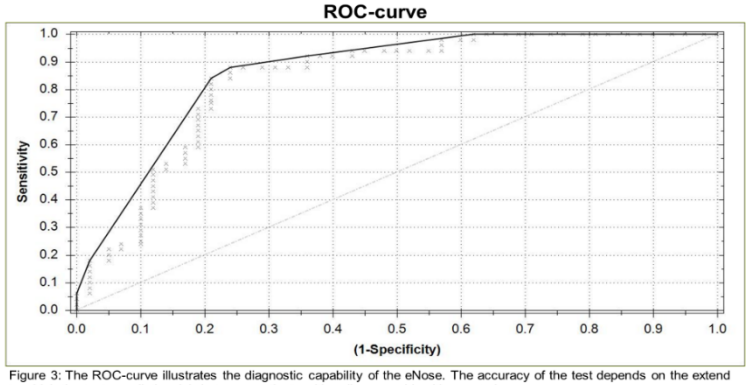


Figure 3: The ROC-curve illustrates the diagnostic capability of the eNose. The accuracy of the test depends on the extend to which the test can distinguish between patients with epilepsy and healthy controls.

Table 1: Characteristics of analysed subjects

Subjects	Male	Female	Age ± SD (y)	Duration of epilepsy ± SD (y)	Number of AED's
Epilepsy patients	30	21	47 ± 17	26 ± 18	2
Healthy controls	14	28	43 ± 16	-	-
Epilepsy patients without AEDs	2	4	35 ± 14	19 ± 11	0-1
Patients with AED's, without epilepsy	2	2	52 ± 25	-	1

Preliminary results

A total of 62 patients with epilepsy were included and 44 control subjects. Additionally, 6 patients with epilepsy, but with reduction of AEDs, and 4 patients using AEDs but without the diagnosis epilepsy were included (Table 1). However, the latter groups were too small to be taken into account for the analysis.

Breathing for five minutes through the eNose is generally well tolerated by the participants, although approximately 5% of them complained about discomfort due to the equipment or shortness of breath. For that reason, 11 epilepsy patients and 2 controls had to be excluded.

The data analysis shows that the eNose can make a distinction between epilepsy patients and healthy controls with a sensitivity of 84% and a specificity of 76% (Table 2, and Figure 3).

Conclusions

The Aeonose™ is capable of distinguishing between patients with epilepsy and control subjects based on their exhaled breath prints.

More breath-print measurements are needed before the eNose can be used as a reliable diagnostic tool for patients with epilepsy. Additionally, investigating the influence of AED's is relevant.

1. Srivasta, A., et al., *Role of inflammation and its miRNA based regulation in epilepsy: implications for therapy*. Clinica Chimica Acta, 2016. **452**: p. 1-9.
2. Iori, V., F. Frigerio, and A. Vezzani, *Modulation of neuronal excitability by immune mediators in epilepsy*. Current opinion in pharmacology, 2016. **26**: p. 118-123.
3. Vezzani, A., et al., *Epilepsy and brain inflammation*. Experimental neurology, 2013. **244**: p. 11-21.
4. Li, G., et al., *Cytokines and epilepsy*. Seizure, 2011. **20**: p. 249-256.
5. The eNose Company. *eNose Technology*. 2016; available from: http://www.enose.nl/rd/technology/.

Kempenhaeghe

UNIVERSITY OF TWENTE.

The eNose company