FACULTY OF MEDICINE AND HEALTH SCIENCES

RESEARCH ON A NONINVASIVE BIOMARKER FOR RESPONDERS TO VAGUS NERVE STIMULATION IN PATIENTS WITH REFRACTORY EPILEPSY

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Charlotte Bouckaert
Student number: 01203156

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A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in the Biomedical Sciences

Academic year: 2016 – 2017

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Preface

In the second bachelor year at Ghent University, I was selected for the Honours Programme in Life Sciences. This gave me the opportunity to get for the first time in touch with research. As I participated for two years in a research project that investigated the efficacy of vagus nerve stimulation as treatment for depression, I was convinced that I wanted to pursue Neuroscience research. With my Master thesis I had the chance to extend my experience from preclinical to clinical research.

In the first place I want to thank my promotor, **Prof. Dr. Kristl Vonck**. Thank you for the chance and the confidence to conduct this research autonomously from early on. I am thankful for the permission to critically review the existing protocol and identify the related problems and to adapt the protocol to let the study run more smoothly. Furthermore, thank you for all other opportunities to attend to meetings, workshops,… and in that way, the opportunities to learn a lot.

I also want to thank my supervisor, **Stefanie Gadeyne**. Thank you for all the feedback you gave on my Master thesis to help me to improve it. During these two years, you were always open to all my questions.

Prof. Dr. Robrecht Raedt, thank you for the confidence, the support and the opportunities you gave me during these two years.

A special thank you to my parents, **Inge** and **Jean-Christophe,** and my brother, **Cedric,** for their support to write this Master thesis and to read and check my work for errors (even if it was difficult to understand the content and the meaning of the scientific terminology ☺).

Furthermore I want to thank my grandfather, **dr. Jan Bouckaert**, for being so interested in all my research. You are always looking to help me and inspire me with your passion for medicine and science. Thanks to you, I was able to visit the Van Drongelen epilepsy lab in Chicago this summer and it was a really interesting experience.

Finally, I want to thank my boyfriend, **Sam**, for all the support and for always believing in me. You are always listening when I am telling enthusiastic about how my experiments went and about things I learnt.

Table of Contents

Samenvatting

Achtergrond: Nervus vagus stimulatie (NVS) is een behandeling voor patiënten met refractaire epilepsie die niet in aanmerking komen voor epilepsiechirurgie. De helft van de patiënten vertoont een goede respons op NVS (≥50% aanvalsreductie), maar een derde zijn non-responders en factoren die de respons kunnen voorspellen ontbreken. Er is evidentie dat de locus coeruleus – belangrijkste bron van noradrenaline – noodzakelijk is voor de anticonvulsieve effecten van NVS. **Methoden:** In deze prospectieve studie bij patiënten met refractaire epilepsie die recent geïmplanteerd werden met een NVS toestel, werden de P3b component van *event-related potentials* en de pupilgrootte onderzocht als potentiële niet-invasieve biomerkers voor vrijstelling van noradrenaline in de hersenen. Dezelfde metingen werden uitgevoerd tijdens transcutane NVS (tNVS) om na te gaan of dit leidt tot gelijkaardige effecten. Afhankelijk van het percentage aanvalsreductie na een jaar werden patiënten opgedeeld in responders (≥50%) en non-responders (<50%). **Resultaten:** Er werden significante effecten gevonden op groepsniveau voor wat betreft P3b amplitude ($F=5,116;p=0,045$), reactietijd ($F=13,708;p=0,003$) en accuraatheid ($F=4,695;p=0,049$) tijdens alle condities (OFF-ON-tNVS). Een model om reactietijd te voorspellen op basis van P3b amplitude kon berekend worden: $RT=633,79–6,95*$ P3b_amplitude ($p=0,037$). Een groep x conditie (ON-OFF) interactie effect op pupilgrootte na een jaar benaderde significantie ($F=4,750;p=0,061$) en modelering wijst erop dat betere outcomes gerelateerd zijn aan een pupildilatatie tijdens NVS. **Besluiten:** De resultaten tonen aan dat de groepen verschillend waren van bij begin. Door de beperkte studiepopulatie en moeilijkheden in het bepalen van de aanvalsreductie moeten de resultaten kritisch geïnterpreteerd worden en is verder onderzoek noodzakelijk.

Summary

Background: Vagus nerve stimulation (VNS) is a therapy for patients with medically refractory epilepsy who are unsuitable candidates for epilepsy surgery. Half of the patients are responders to VNS (≥50% seizure reduction), but one third are non-responders and factors determining a patient's response to treatment are missing. Evidence suggests that the locus coeruleus (LC) – main source of norepinephrine (NE) – is mandatory for the anticonvulsive effect of VNS. **Methods:** In this prospective study in patients with refractory epilepsy recently implanted with a VNS device, the P3b component of event-related potentials (ERPs) and pupil size were investigated as potential noninvasive biomarkers for the release of NE in the brain. The same measurements were performed during transcutaneous VNS (tVNS) to assess whether similar effects are obtained. Depending on the percentage seizure reduction after one year of VNS treatment, patients were subdivided into responders (≥50%) and non-responders (<50%). **Results:** Significant group effects on P3b amplitude (F=5,116;p=0,045), reaction time (F=13,708;p=0,003) and accuracy (F=4,695;p=0,049) during all conditions (OFF-ON-tVNS) were found. A model to predict reaction time based on P3b amplitude was computed: RT=633,79–6,95* P3b_amplitude (p=0,037). A group x condition (ON-OFF) interaction effect on pupil size after one year approximated significance (F=4,750;p=0,061) and modeling indicates that better outcomes are related to a pupil dilatation during VNS. **Conclusions:** Our results suggest that groups were different at the beginning. Furthermore, due to a very limited sample size and difficulty to determine seizure outcome, results must be interpreted with caution and further research is needed.

1. INTRODUCTION

1.1 Epilepsy: What is it?

Epilepsy is a chronic neurological disorder characterized by recurrent – two or more - epileptic seizures. Epilepsy is one of the most common neurological diseases globally, with approximately 50 million people worldwide suffering from this disorder (1). Epileptic seizures are brief episodes of involuntary movements or abnormal behavior resulting from excessive electrical discharges in the brain cortex. The occurrence of one seizure does not necessarily mean that an individual suffers from epilepsy since this seizure may have been provoked by various factors including brain injury or abnormality; central nervous system infections; high fever; contrast agents; medication; metabolic disorders or alcohol/drug use and withdrawal. The individual may never experience a second seizure (2, 3). Some patients have psychogenic seizures (also called pseudoseizures). These seizures are episodes of altered movement; emotion; sensation or experience, similar to epileptic seizures, but have purely emotional causes. For this reason, these episodes are often misdiagnosed as epileptic seizures (4).
The incidence of epilepsy is the highest in children and elderly (See **Figure 1**).

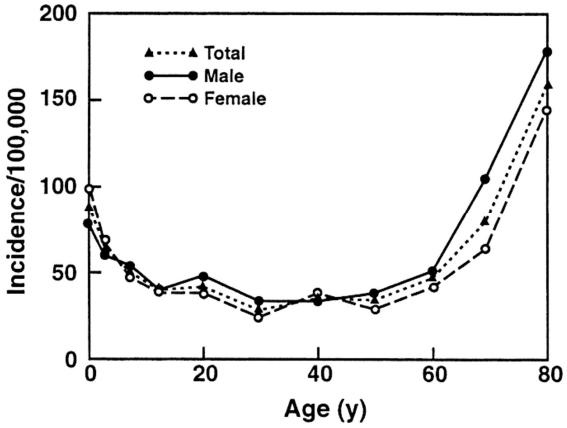

Figure 1: Age-related incidence of epilepsy (5). This figure shows that the incidence of epilepsy is the highest just after birth and decreases until the age of five. From the age of 60 the incidence of epilepsy starts to rise again. y : years.

1.2 Epilepsy: Seizure types

Based on ictal semiology and on electroencephalographic (EEG) characteristics, epileptic seizures can be classified into two categories: 1) focal epileptic seizures originating from networks limited to one hemisphere, either discretely localized or more widely distributed; 2) generalized epileptic seizures that occur in, and rapidly engage bilaterally distributed networks (6). *Focal* or *partial seizures* are further subdivided into simple partial seizures and complex partial seizures. These partial (simple or complex) seizures can sometimes become generalized, and are called secondary generalized seizures, because the electrical activity in a limited area continues to spread throughout the brain. *Generalized seizure types* are caused by abnormal paroxysmal discharges of the whole cerebral cortex and include absence -, myoclonic -, atonic -, tonic -, clonic - and tonic-clonic seizures (7) (see **Table 1**).

Absences cause lapses in consciousness, sometimes with staring. They are often seen in children, start abruptly and only last for a few seconds. **Myoclonic seizures** are brief, shock-like jerks of a muscle or a group of muscles. In **atonic seizures**, muscles suddenly loose strength, which often results in falling of the patient. **Tonic seizures** are seizures in which the tone in body, arm and/or legs is greatly increased, causing the patients to make sudden stiffening movements. **Clonic seizures** consist of rapidly alternating contraction and relaxation of muscles, i.e. repeated jerking. In generalized **tonic-clonic seizures** a tonic phase is followed by a clonic phase. These seizures usually last a few seconds to a minute. A seizure that lasts more than 10 minutes, or three seizures without a normal period in between, indicate a dangerous condition called **status epilepticus** (SE) (8).

Table 1: Overview of the different seizures types and related consciousness.

Seizure categories	Seizure types	Consciousness
Focal epileptic seizures	Simple partial seizures	Conscious
	Complex partial seizures	Altered consciousness
	Secondary generalized seizures	Altered consciousness
Generalized epileptic seizures	Absence seizures	Lapses in consciousness
	Myoclonic seizures	Conscious
	Atonic seizures	Conscious
	Tonic seizures	Altered consciousness
	Clonic seizures	Altered consciousness
	Tonic-clonic seizures	Altered consciousness

Recently a new classification of seizure types based on three key features (i.e. where seizures begin; level of awareness and other features of seizures) has been introduced officially by the International League Against Epilepsy (ILAE).

Epileptic seizures can also be subdivided based on the underlying cause (also called etiology), which will be discussed in the next paragraph.

1.3 Epilepsy: Etiology

In the normal brain, the balance between excitation and inhibition is maintained. In an epileptic brain, there is a disruption of mechanisms that normally create this balance. Disrupting the mechanisms that inhibit firing or promoting the mechanisms that facilitate excitation can lead to epileptic seizures (2).

“Epileptogenesis is a term used to describe the complex plastic changes in the brain that, after a precipitating event, convert a normal brain into a brain debilitated by recurrent seizures. Epileptogenesis is triggered by a diverse range of precipitating factors such as brain injury, stroke, infection or prolonged seizures.” (9)

Based on the underlying cause, epilepsies of known etiology (symptomatic or “secondary” epilepsies), were separated from idiopathic (“primary”) and cryptogenic epilepsies by the ILAE

in 1989 (10). **Symptomatic epilepsy** is caused by structural or metabolic abnormalities in the brain, which can either be acquired (e.g. brain trauma) or genetic (e.g. tuberous sclerosis) in origin (3, 6). **Idiopathic epilepsies** occurring without detectable brain lesions and/or metabolic abnormalities are believed to have a strong underlying genetic basis (3, 7). **Cryptogenic epilepsies** do not meet the criteria for an idiopathic epilepsy and no significant underlying neurologic abnormality or condition is identified, so the etiology of these epilepsies is unknown (11). Focal - and generalized idiopathic epilepsy are generally considered to have an excellent prognosis, whereas cryptogenic and symptomatic generalized epilepsies are generally recognized as having a poor prognosis in children (11). In 2010, the ILAE has proposed a new categorization in which 'genetic', 'structural–metabolic', and 'unknown' represent modified concepts to replace 'idiopathic', 'symptomatic', and 'cryptogenic' respectively (6). This new classification is not yet implemented in the work field.

1.4 Epilepsy: Treatment

The goal of an antiepileptic treatment is to render patients seizure-free without clinically significant adverse effects. Two-thirds of seizures in patients can be fully controlled by antiepileptic drugs (AEDs). Nonetheless, a third of the patients will still experience recurrent seizures despite the best medical treatment, and suffer from so-called medically refractory epilepsy (12). In these patients, epilepsy surgery can provide significant seizure reduction or even complete seizure control (13). Unfortunately, not all patients are suitable candidates for surgery. Electrical stimulation of the brain is a group of rapidly evolving therapies for patients with uncontrolled seizures (14).

1.4.1 Antiepileptic drugs

AEDs are the standard first-line treatment for epilepsy. Different AEDs have different mechanisms of action (MOAs). Possible MOAs are: blockade of voltage-dependent sodium channels; stimulation of the inhibitory GABA system; blockade of T-type calcium channels in thalamic neurons and/or inhibition of the excitatory glutamatergic system. Most AEDs have more than one MOA. Furthermore knowledge of the MOA of various AEDs has limited value in predicting the therapeutic - and adverse effects of these drugs in the clinic (15).

Over half of the patients respond to their first AED prescribed, while less than 20 % respond to subsequent drug trials. Patients with a suboptimal response to their first AED, have a 41 % – 55 % chance of becoming seizure-free with a subsequent drug trial if treatment failure was due to poor drug tolerability or idiosyncratic reactions. Patients who failed their first AED due to the drug being ineffective only have a chance of 11 % of becoming seizure-free in a next trial. Thus, the likelihood of successful treatment by adding other anti-epileptic drugs seems to

diminish with each AED failure. The condition of medically refractory epilepsy, also referred as intractable -; pharmacoresistant -; drug-resistant - or medically intractable epilepsy is defined as the failure of two appropriate and tolerated AED schedules (as monotherapies or in combination) (16).

As AEDs are systemic drugs, they are responsible for a lot of adverse effects. Dizziness; drowsiness and mental slowing are the most commonly encountered side effects. Other adverse effects include changes in weight; inhibition of carbonic anhydrase, which could lead to metabolic acidosis, nephrolithiasis, hypohidrosis and heat intolerance; enzyme induction; visual side effects; dermatological adverse reactions; and others such as hepatotoxicity, nephrotoxicity and colitis; induction of Systemic Lupus Erythematosus; myeloma; movement disorders; behavioral disorders and development of pruritus (See **Table 2**) (17).

Table 2: Adverse effects related to AEDs and their meaning.

Side effects	**Meaning**
Metabolic acidosis	A condition that occurs when the body produces excessive quantities of acid or when kidneys are not removing enough acid from the body (Wiki)
Nephrolithiasis	Calculi (stones) in the kidney
Hypohidrosis	Diminished sweating in response to appropriate stimuli
Hepatotoxicity	Toxicity in the liver
Nephrotoxicity	Toxicity in the kidney
Colitis	Inflammation of the colon
Systemic Lupus Erythematosus	An autoimmune disease in which healthy tissue in many parts of the body are mistakenly attacked by the immune system (Wiki)
Myeloma	A cancer of plasma cells (a type of white blood cell normally responsible for producing antibodies) (Wiki)
Pruritus	Itch

1.4.2 Epilepsy surgery

Patients with medically refractory epilepsy may be suitable candidates for epilepsy surgery. This can either be resective surgery or disconnective surgery. **Resective epilepsy surgery** (e.g. hemispherectomy, temporal lobe resection and extratemporal lobe resection) consists of the complete resection of the epileptogenic zone (i.e. brain tissue responsible for provoking seizures in an individual patient). **Disconnective epilepsy surgery** (e.g. multiple subpial transection (MST) and functional hemispherectomy) consists of the interruption of nerve fibers through which abnormal epileptic activity spreads to the adjacent tissue (18). Up to 80% of the patients becomes seizure-free after epilepsy surgery depending on the localization of the seizure focus, type of surgery and age of patients (19).

1.4.3 Neurostimulation

Despite the development of new pharmacological treatments and surgical treatments with high success rates, a substantial number of epileptic patients does not become seizure-free or

experiences major adverse events. Neurostimulation-based treatments have gained noticeable curiosity in the last decade. In neurostimulation, electrical pulses are administered directly or indirectly (via cranial nerves) to the brain tissue in order to manipulate a pathological substrate and to achieve a symptomatic or even curative therapeutic effect. Several types of neurostimulation exist. These neurostimulation techniques differ with regards to the part of the nervous system affected and the way the stimulation is administered (20).

1.4.3.1 Vagus nerve stimulation

Vagus nerve stimulation (VNS) is a well-established therapeutic add-on treatment in patients with medically and surgically refractory epilepsy (21). It is a safe and effective adjunctive long-term treatment for refractory epilepsy, including partial epilepsy (22) as well as generalized epilepsy. Therefore, VNS can be considered as a broad-spectrum treatment for refractory epilepsy (21). In VNS, the left vagus nerve is stimulated at the level of the neck by a helical cuff electrode connected to a subcutaneously-implanted pulse generator (under the clavicle) (See **Figure 2**). The left vagus nerve is chosen to minimize the effect on heart rhythm because most of the cardiovagal fibers course through the right vagus nerve (23). Since its Food and Drug Administration (FDA) approval for epilepsy in 1997, more than 85,000 people worldwide have been implanted with a VNS device to help control their seizures (24).

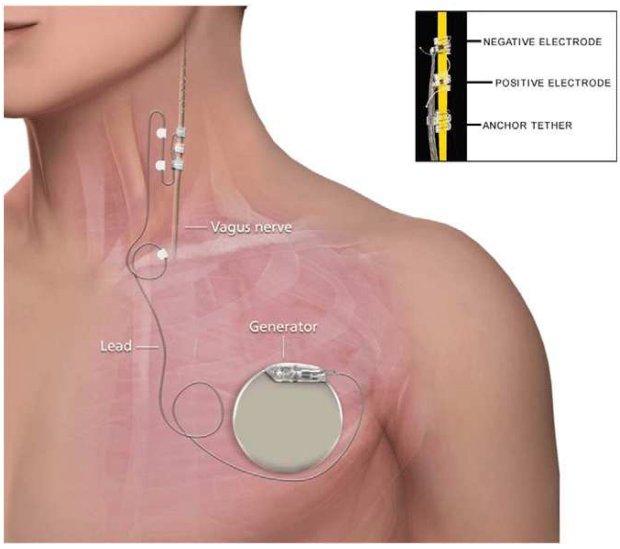

Figure 2 (25): Location of the cuff electrode and the pulse generator. The bipolar cuff electrode is wrapped around the left vagus nerve at the level of the neck with the negative electrode (stimulates) placed rostrally and the positive electrode (blocks) caudally. This electrode is connected through a wire to a subclavicularily-implanted pulse generator.

1.4.3.1.1 Anatomy of the vagus nerve

The vagus nerve is a mixed nerve that consists of 20% efferent and 80% afferent fibers. The *afferent fibers* originate in the end organs and the largest number of these fibers project to the nucleus tractus solitarius (NTS) in the brainstem. From the NTS, axons project throughout the brain to different pontine nuclei, the cerebellum, the mesencephalon, the cortex, the thalamus and the amygdala (23, 26). The vagus nerve also projects directly to the locus coeruleus (LC)

and the dorsal raphe nucleus (DRN), major sources of noradrenergic (NE) and serotonergic neurons respectively (26-28) (see **Figure 3**).

The cervical vagus nerve (CVN) is composed of numerous fascicles. Some fascicles mainly contain thick myelinated axons (efferent Aα- and afferent Aβ), while other fascicles are composed for the most part of small myelinated (Aδ) and unmyelinated axons (C) (29).

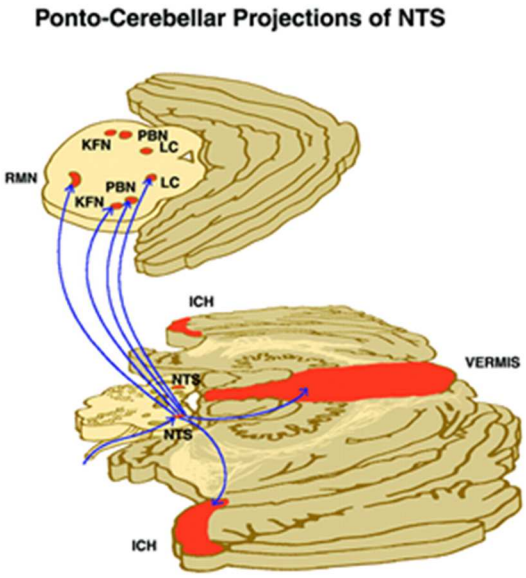

Figure 3: Scheme of the bulbo-cerebellar polysynaptic projections of the nucleus of the tractus solitarius. *Afferent fibers* of the left vagus nerve project densely to the NTS bilaterally, and the NTS projects to inferior and medial cerebellar regions, and to multiple pontine and mesencephalic nuclei. NTS : nucleus tractus solitarius; ICH : inferior cerebellar hemisphere; KFN : Kölliker-Fuse nucleus; LC : locus coeruleus; RMN : raphe magnus nucleus; PBN : parabrachial nucleus (27).

1.4.3.1.2 Mechanism of action

As discussed in the previous section, *vagal afferents* have numerous projections within the central nervous system. An initial hypothesis of the mechanism of action (MOA) of VNS was based on these anatomical connections and the potential of action potentials generated in vagal afferents to affect the entire organism (30). To date, the precise MOA of VNS and how it suppresses seizures is not fully understood. The mechanisms triggered by VNS are more complex than initially thought, and therefore the MOA of VNS is still under intense study.

The vagus nerve is a mixed nerve with both afferent and efferent fibers. Zabara *et al* (31) showed that lesioning of the vagus nerve below the VNS electrode does not result in a loss of efficacy, indicating that afferent stimulation is the route for the antiseizure effects of VNS. As epilepsy is considered a disease with cortical origin and efferent stimulation may cause adverse effects, unidirectional activation of the vagus nerve (i.e. activating only afferent vagal fibers) is often desirable (32), but effective VNS will under all circumstances result in bidirectional activation of both afferent and efferent fibers. In order to induce an anodal block (i.e. conduction block due to hyperpolarization of the nerve) of efferently conducted action potentials during VNS, the anode of the VNS electrode has been placed caudally (see **Figure 2**). In this way, some of the side effects associated with stimulation of *vagal efferents* will be

blocked. Measurements of laryngeal muscle-evoked potentials (LMEPs) in rats showed that when the initial negative phase of the VNS pulse is given at the distal electrode contact instead of at the proximal electrode contact, this reversed stimulation polarity results in unchanged shape and polarity of recorded LMEPs and thus the same activation of efferent fibers (33). Therefore, anodal block seems to be merely a theoretical phenomenon.

Examination of VNS intensities used in anesthetized dogs, showed that the mean recruitment thresholds were 0.37 mA ± 0.18 mA; 1.6 mA ± 0.36 mA; 3.8 mA ± 0.84 mA and 17 mA ± 7.6 mA for afferent A-fibers; fast B-fibers; slow B-fibers and C-fibers respectively. In addition to the stimulation parameters typically used in humans (stimulation amplitude: typically 1.5 mA), this finding suggests that reported therapeutic effects of VNS are associated with the electrical activation of type-A and type-B fibers and are not dependent on the activation of unmyelinated C-fibers (34). This supports the study results of Krahl *et al (35)* who showed that unmyelinated C-fibers do not contribute to the antiepileptic effect of VNS since VNS had the same efficacy in preventing seizures, even though C-fibers in rat vagal nerves were destroyed with capsaicin.

Vagal afferents terminate predominantly in the NTS. The NTS has multiple and complex projections (see Paragraph 1.4.3.1.1) of which direct and indirect projections to the locus coeruleus (LC) (36). Acute electrical stimulation of the vagus nerve in anaesthetized rats induces an increase in discharge rate of single NE neurons of the LC (37). Additionally, basal firing rates in both LC and dorsal raphe nucleus (DRN) significantly increase after long-term treatment of rats with VNS. LC firing rates increase earlier than DRN basal firing rates and because the LC has an excitatory influence on the DRN, the increased DRN firing rate is possibly secondary to an initial increased LC firing rate from VNS (38). VNS induces an increased c-fos and DeltaFosB staining of the LC (39) and increased extracellular concentrations of NE in both hippocampus and prefrontal cortex (40, 41). Moreover, selective lesioning of noradrenergic LC neurons by administration of the neurotoxin DSP-4 was found to suppress acute anticonvulsant effects of VNS in rats (42). Together with the reversal of the seizure-suppressing effect of VNS by intrahippocampal administration of an α2-adrenoceptor antagonist in a hippocampal seizure model (43), these findings provide evidence for a causal role of NE in the anticonvulsant effects of VNS. These findings also support the hypothesis that the degree of NE release in the brain can be a useful biomarker for the therapeutic efficacy of VNS in epileptic patients (44).

Additionally, a potential role for cholinergic modulation in the MOA of VNS on cortical excitability and synchrony (45); a decrease in concentrations of the excitatory neurotransmitters glutamate and aspartate and a VNS-induced simultaneous increase in concentration of the inhibitory neurotransmitter GABA in the cerebrospinal fluid of epileptic patients (46) have been shown.

Using single photon emission computed tomography (SPECT) (47) and positron emission tomography (PET) superimposed on magnetic resonance imaging (MRI) (48) for anatomic localization, cerebral blood flow was found to be increased in both thalami and the cortex in epileptic patients during VNS. Furthermore, Koo *et al* (49) showed that VNS induces desynchronization of EEG rhythms.

1.4.3.1.3 Stimulation parameters

Stimulation of the vagus nerve usually starts one to two weeks after implantation of the VNS system. These two weeks allow the surgical wounds to heal, particularly around the vagus nerve, thereby alleviating irritating sensation of the neck. The stimulation is characterized by different parameters: output current, frequency, pulse width and duty cycle (See **Table 3** and **Figure 4**). There are no clear rules for programming the VNS. Low settings are set at the beginning of the VNS therapy (typically 0.25 mA or 0,5 mA) and adjustments to higher settings (output current (in steps of 0.125, 0.25 or 0.5 mA) or duty cycle) are usually performed on a monthly basis. The frequency and the pulse width of the stimulation are typically set at 20-30 Hz and 250-500 µs respectively. The standard duty cycle is 30 s ON/5 min OFF.

Table 3: Explanation of the different stimulation parameters.

Parameter	**Meaning**
Output current	The current delivered by the vagus nerve stimulator, i.e. the intensity of stimulation
Frequency	The number of electrical stimulation pulses delivered per second
Pulse width	Duration of the electrical stimulation pulse, usually in microseconds (µs)
Duty cycle	ON and OFF time

Long-term titration of stimulation parameters and adjustments of AEDs might help maximize the effectiveness of VNS over time (50). The stimulation parameters used in clinical practice are based on known safety and tolerability and are rather empirically determined than evidence-based. A better clinical outcome can only be achieved by optimizing this stimulation paradigm and therefore, further research is needed.

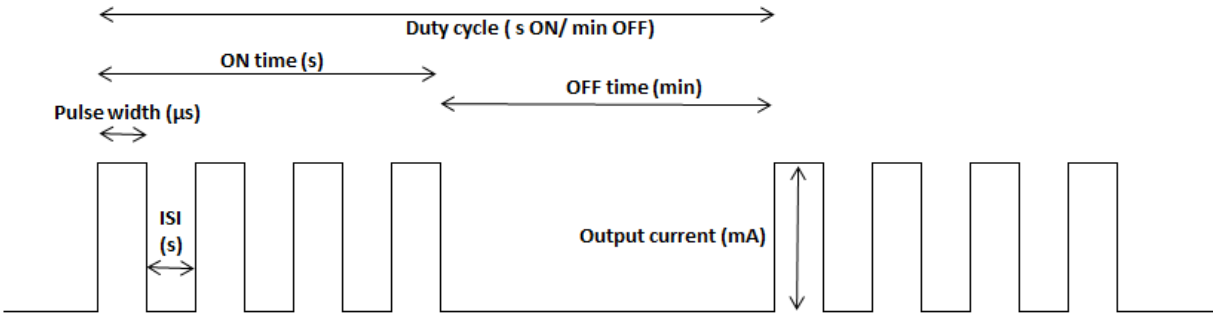

Figure 4: Stimulation parameters. The duty cycle consists of a certain ON time, followed by an OFF time. Frequency = 1/ISI. ISI : Interstimulus interval; Output current : stimulation intensity.

1.4.3.1.4 Efficacy

One third of the patients treated with VNS show a seizure frequency reduction of more than 50 % (these patients are called 'responders'), one third experiences a seizure frequency reduction between 30 % and 50 % ('partial responders') and another third shows no response ('non-responders'). Seizure control seems to improve with increasing duration of the VNS therapy and two retrospective reviews of Elliott *et al (51, 52)* shows that more than 60 % of the patients with refractory epilepsy experienced a seizure reduction of at least 50 %, when used in conjunction with a multidisciplinary and multimodality treatment regimen including aggressive antiepileptic drug regimens and if possible epilepsy surgery. Nevertheless, still 25 - 30% of the patients remain non-responders.

It is unclear why the effects of VNS are limited and despite the growing application, it is still not possible to predict which patients will be responders to VNS therapy. If this responsiveness could be predicted, potential responders might immediately receive an effective VNS system, while the implantation of an expensive VNS system could be avoided in patients with only a low likelihood of response. As a consequence, it is important to determine the success of VNS in order to counsel patients regarding treatment options and inform them about the expected seizure reduction (53).

There are still a lot of uncertainties about VNS responsiveness. Firstly, a correlation between age and success of VNS has been investigated. Labar *et al (54)* found VNS responsiveness to be associated with both older age and longer epilepsy duration, whereas Tecoma *et al (55)* determined it to be independent of epilepsy duration and Ghaemi *et al (56)* revealed it to be associated with younger age. Secondly, a link between VNS duration as well as stimulation settings and success of VNS has been studied. Scherrmann *et al (57)* concluded that seizure outcome was positively correlated with VNS duration and Handforth *et al (58)* observed that seizure reduction was positively correlated with high stimulation settings. Thirdly, the success was found to be negatively related to tonic seizures (59). Finally, the positive effects of VNS

were shown to be related to the absence of bilateral interictal epileptic discharges in the EEG (60).

1.4.3.1.5. Adverse effects

VNS side effects are usually related to stimulation and often improve over time. In a review of Ben-Menachem *et al* (61), almost 98% of the common adverse events were reported as mild to moderate and these could in most cases be resolved by a reduction in stimulation parameters.

The most common side effects of VNS include voice alternation, hoarseness and cough during the active period of stimulation. Incidence and intensity of these side effects are related to the intensity of the output current. Serious adverse events are limited to respiratory difficulties and severe hoarseness. A trend towards diminishing adverse effects can be observed over the years of stimulation (61-63). Other possible side effects include pain; dyspnea; nausea; tingling sensation; nausea; headache; ataxia; dizziness; paresthesias ; fatigue and somnolence (24).

Adverse effects of VNS can also be related to the risk of surgical intervention. Local infections or hemorrhage at the incision site; lower facial weakness; vocal cord paresis and in a few cases bradycardia and asystole belong to this subgroup (21, 24, 61).

1.4.3.1.6 Other indications

VNS is FDA-approved for the treatment of drug resistant epilepsy and depression in both Europe and the United States. The use of VNS is currently being investigated for several other applications such as pain disorders (e.g. migraine (64, 65) and cluster headache (65, 66)); chronic inflammatory disorders (e.g. rheumatoid arthritis (67)); Alzheimer's disease (cognitive-enhancing effects of VNS) (68) and heart failure (right-sided VNS) (69).

1.4.3.1.7 Secondary effects by VNS

The effect of VNS on depressive mood in epilepsy patients has been evaluated because mood improvements were noticed in patients treated with VNS (70, 71).

Hoppe *et al* (70) reported significant improvements in scales that address unspecific aspects of anxiety, whereas more complex and more stable emotional states including cognitive and behavioral aspects such as depression (assessed by Beck-Depression Inventory (BDI)) and health-related quality of life (assessed by QOLIE) appeared unchanged.

In contrast to an unchanged depression state reported by Hoppe *et al*, a study of Harden *et al (72)* revealed a significant decrease over time for all mood scales including BDI (less depressive mood). Positive mood changes were also shown in non-responders, being independent of effects on seizure activity (71). The MOA of VNS with regards to the effect on

seizure frequency as well as on mood is not yet fully understood. Therefore, further research is needed (50).

Furthermore, several other studies were conducted to evaluate the health-related quality of life. Cramer *et al* (73) demonstrated significant improvements regarding energy level; memory difficulties; social aspects; mental effects and fear of seizures in patients with refractory epilepsy, which implies that VNS therapy is associated with a persistent and positive improvement in subjective quality of life (QOLIE) (50). As mentioned before, Hoppe *et al* (70) reported this health-related quality of life to be unchanged. Discrepancies may be attributed to differences in sample size (136 (73) vs 28 (70) patients) and follow-up (3(73) vs 6 (70) months).

1.4.3.2 Transcutaneous vagus nerve stimulation

Transcutaneous vagus nerve stimulation (tVNS) is a relatively new noninvasive method of VNS aiming to achieve similar VNS effects by activating vagal nerve fibers in the ear. The innervation of the external auricle is characterized by a great deal of overlap between multiple nerves: the auriculotemporal nerve; the auricular branch of the vagus nerve (ABVN); the lesser occipital nerve and the greater auricular nerve (see **Figure 5**). The ABVN mainly supplies the auricular concha (74). In tVNS, surface electrodes are applied to this specific region (see **Figure 6**).

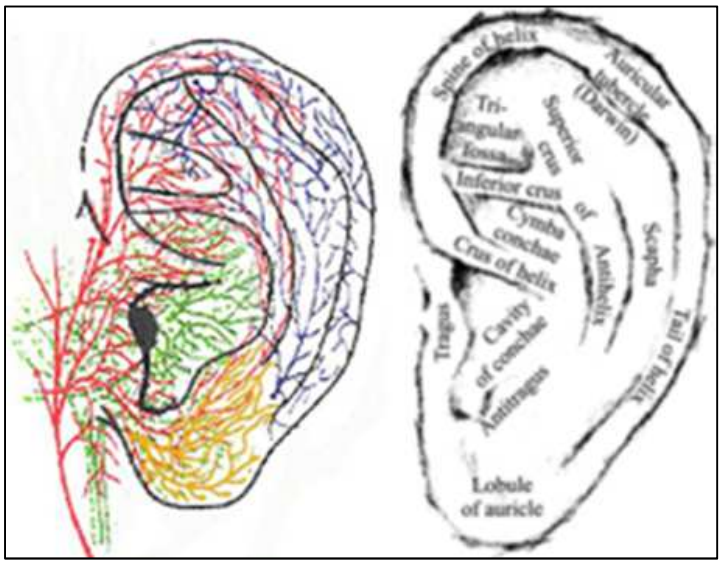

Figure 5: Innervation of the external auricle (Left) (74): Green: Innervations of the auricular branch of the vagus nerve. This peripheral branch of the vagus nerve supplies the auricular concha and most of the area around the auditory meatus. Red: Innervations of the auriculotemporal nerve (a mandibular branch of the trigeminal nerve). Blue: Innervations of the lesser occipital nerve. Yellow: The innervations of the greater auricular nerve. **Left auricle, lateral aspect (right) (75).**

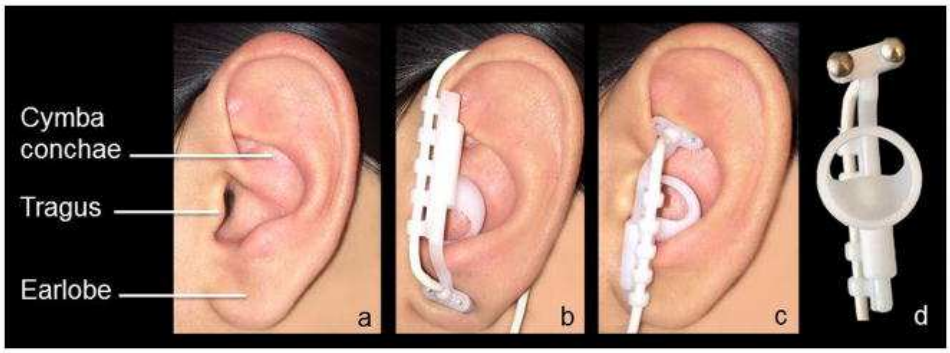

Figure 6: Surface electrodes in tVNS (76): In *panel b* surface electrodes are applied to the earlobe. This is often done to create a control group ("sham stimulation"), because the earlobe does not contain auricular branches of the vagus nerve. *Panel c* shows real tVNS. In tVNS, surface electrodes are applied to the cymba conchae. *Panel d* is a detail of the earpiece and the pair of titanium electrodes used in a commercial tVNS device.

With the amplitudes used in both invasive and transcutaneous VNS, it can be presumed that Aβ-axons are involved. The human ABVN contains these thick myelinated axons of the Aβ-class, which are only five to six times less numerous than those in the cervical vagus nerve (29). A study in cats using the transganglionic horseradish peroxidase (HRP) method showed that, similar to the vagus nerve, the ABVN mainly projects to the NTS in the brainstem (77). Noninvasive stimulation of the cymba conchae in humans produces a significant activation of the ipsilateral NTS, the first central relay of vagal afferents. Taken together, these findings provide evidence that the central projections of ABVN afferents are consistent with the central projections from the cervical vagus nerve (29).

1.5 The P3 component of event-related potentials

There is evidence suggesting that the P3b component of scalp recorded event-related potentials (ERPs) indexes the phasic activity - brief, rapid increases in firing rate - of the neuromodulatory LC/NE system (78, 79). ERPs are EEG changes (i.e. very small voltages generated in the brain structures) thought to reflect the summed activity of postsynaptic potentials produced by synchronous firing of a large number of similarly oriented cortical pyramidal neurons (thousands to millions) during information processing. These ERPs can be elicited by and are time-locked to specific events or stimuli (sensory, motor or cognitive events). They provide a safe and noninvasive approach to study psychophysiological correlates of mental processes. ERPs in humans can be divided into two categories. The early waves peaking roughly within the first 100 ms after the stimulus ('sensory' or 'exogenous' ERPs) and ERPs generated later than 100 ms after the stimulus ('cognitive' or 'endogenous' ERPs). Cognitive ERPs reflect the manner in which the subject evaluates the stimulus. ERP components are described according to their latency, amplitude and topography. The P3b component belongs to the second category, so-called cognitive ERPs (80). The P3b (or P3 or

P300) is a large, broad, positive component in the ERP that typically peaks 300 ms or more after onset of a rare target stimulus. The P3b has a centro-parietal scalp distribution that is maximal at the parietal midline electrode (Pz). A rare, but not task-relevant event (distractor stimulus) may also elicit a positive-going ERP component: the P3a. This P3a can be distinguished from the P3b by use of a three-stimulus oddball paradigm based on an earlier peak latency (250–300 ms) and a midline fronto-central scalp distribution maximum (Cz) (81). To measure the P3b component, an auditory oddball paradigm is performed. This task is a simple and well-established paradigm for the investigation of arousal effects on cognitive performance and has been shown to reliably evoke robust P3 components (79). In this task, three tones are provided: 1) a standard stimulus (low sound), 2) a distractor stimulus (white noise) and 3) a target stimulus (high sound). Patients are instructed to respond only to target tones by pressing the predefined button on the keyboard. Low-probability target and distractor tones, and high-probability standard tones are presented in a randomized order. P3b components are evoked when target stimuli are presented. The distractor stimulus, on the other hand, evokes P3a components, and thus, this stimulus is needed to clearly discriminate P3b from P3a components (see **Figure 7**) (44, 82).

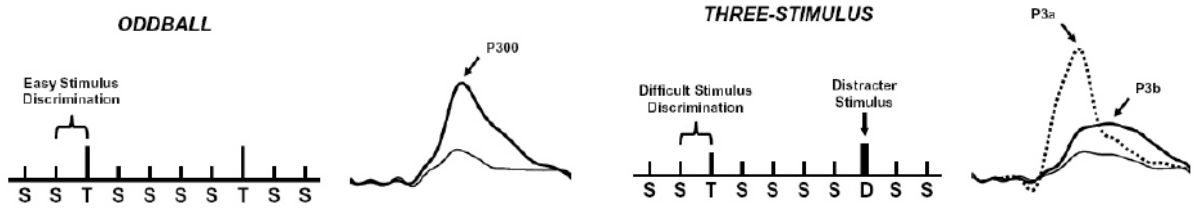

Figure 7: The P300 response (82). Left: The classical oddball paradigm consists of two types of stimuli: frequent standard stimuli (S) and rare target stimuli (T). Early stimulus discrimination evokes a P300 component. Right: A three-stimulus oddball paradigm (used in this thesis) is used to identify the two subcomponents of the P300 response: P3a and P3b. This task includes frequent standard stimuli (S) and infrequent target (T) and distractor (D) stimuli. The attended target stimulus elicits a P3b (P300) potential, whereas the unattended distractor stimulus elicits a P3a response.

1.6 Pupillary measurements

Measurement of pupil diameter is another possibility to index LC activity (79). Pupillary measurements are an easy and noninvasive method to evaluate autonomic functioning. The pupil diameter is under dual autonomic innervation:

1) Parasympathetic (PS) control: fibers project from the Edinger-Westphal nuclei to the ciliary ganglion, to end in the sphincter pupillae muscle, responsible for constriction of the pupil.
2) Sympathetic (S) control: fibers project from the posterior hypothalamus to the spinal cord to the superior cervical ganglion and finally to the dilator muscle, contributing to dilatation of the pupil.

The LC can modulate both PS and S outflow to the pupil (see **Figure 8**) (83). A large pupil diameter appears to be associated with a high tonic LC activity (79).

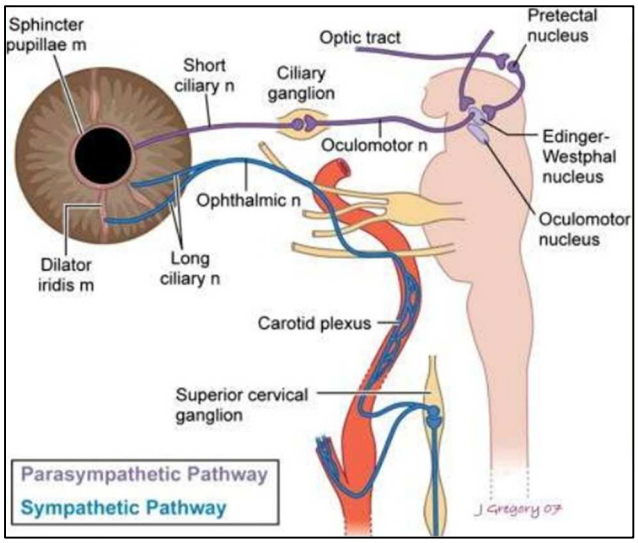

Figure 8: Parasympathetic and sympathetic pathways to the pupil (84). *Parasympathetic fibers* project from the Edinger-Westphal nuclei to the ciliary ganglion, to end via the short ciliary nerve in the sphincter pupillae muscle, responsible for constriction of the pupil. *Sympathetic fibers* project from the posterior hypothalamus to the spinal cord (not shown) to the superior cervical ganglion and finally to the dilator iridis muscle, contributing to dilatation of the pupil.

1.7 Laryngeal Motor-Evoked Potentials

The recurrent laryngeal nerve is a branch of the vagus nerve at the level of the aortic arch, which ascends next to the trachea and carries low-threshold vagal Aα-motor fibers to the larynx, the pharynx and the vocal cords. Therefore, laryngeal motor-evoked potentials (LMEPs) noninvasively measure the activation of motor fibers and can thus be used for objectivation of cervical vagus nerve activation. Effective VNS at the cervical level induces these LMEPs through the co-activation of the recurrent laryngeal nerve and subsequent contractions of the laryngeal muscles and vocal cords. An LMEP is defined as the initial peak that is reproducibly recorded after each stimulation artifact. Non-response caused by lead failure, poor nerve-electrode contact due to gliotic tissue and nerve damage such as demyelination can be ruled out by these measurements (33).

1.8 Hypothesis

The MOA of VNS in epilepsy is not fully understood and one third of patients are non-responders. As biomarkers for the response to VNS are lacking, the aim of this study was to determine factors that predict the clinical response to VNS. Evidence suggests that VNS activates the LC/NE system and that its activation and subsequent increase in extracellular NE levels mediate the anticonvulsive effects of VNS (26, 42, 43). As previously described, the P3b component could be a noninvasive marker for the increase in NE in the human brain. In a first clinical trial, the P3b was significantly different between responders and non-responders in VNS patients who were already treated long-term with VNS. VNS induced a significant

increase in the P3b amplitude at the parietal midline electrode Pz in VNS responders only. Furthermore, logistic regression analysis showed that the increase in P3b amplitude could potentially be used as a noninvasive indicator for VNS responders after at least one year of treatment (44). Additionally, a study of Desbeaumes Jodoin *et al* (83) investigating the effect of VNS on pupil size showed a significant increase in pupil size in VNS ON conditions compared to VNS OFF conditions. However, no distinction between responders and non-responders was made.

This study was based on the first clinical trial using P3b components to distinguish between responders and non-responders, but was conducted in a prospective way. Pupillometry was investigated as an additional potential biomarker for response to VNS. The effect of VNS on both measurements – P3b and pupil size – was investigated because an increase in extracellular NE levels in the brain is believed to be mandatory for the anticonvulsive effects of VNS. By means of P3b components and pupil size we aimed to visualize this NE increase noninvasively.

P3b measurements and pupillometry were performed twice in patients with refractory epilepsy: one time two weeks after VNS implantation when VNS therapy is initiated, and a second time after one year of treatment. In addition, we also investigated in a prospective way whether tVNS, the noninvasive analogue of VNS, is able to affect the P3b measurements and pupillometry in a similar manner in these patients.

As the P3b component and its amplitude are influenced by the NE levels in the brain and considering the evidence for activation of LC/NE system by VNS and subsequent increase in extracellular NE levels that mediate anticonvulsive effects of VNS, we expect the P3b amplitude to be increased in the VNS ON and tVNS conditions as compared to the VNS OFF conditions, with tVNS lying in between VNS OFF and VNS ON. The P3b amplitude during tVNS is expected to be intermediate due to the fact that tVNS only stimulates a side branch (the auricular branch) of the vagus nerve. Furthermore, we expect to observe a dilated pupil in the VNS ON and tVNS condition, because of the increase in NE following activation of the LC/NE system by VNS that is involved in the autonomic control of the pupil. Evidence in rats suggests that acute VNS affects resting pupil size mainly through parasympathetic inhibition, which inhibits constriction of the pupil, thus resulting in pupil dilatation (85).

2. METHODOLOGY

2.1 Study population

This study included patients with refractory epilepsy treated with VNS and took place during the video-electroencephalogram (EEG) monitoring sessions in the Epilepsy Monitoring Unit at Ghent University Hospital, Ghent, Belgium. Patients were tested twice: 1) two weeks after implantation of VNS, and 2) after one year of VNS treatment (see **Figure 9**).

Inclusion criteria were: 1) older than 18 years; 2) full-scale IQ score ≥ 70; 3) agreement with VNS as a treatment method. Approval by the ethics committee of Ghent University Hospital was obtained. Patients received a full description of the procedure before the start of the study, as well as a face-to-face explanation. Afterwards patients decided whether or not to participate in the study by means of a written informed consent.

Figure 9: Schematic timeline of the VNS study. Red dots represent test-moments. d0 : day 0; VNS : vagus nerve stimulation.

For analysis purposes, patients who were also tested after one year were subdivided into two groups depending on their reduction in mean monthly seizure frequency: responders (R; ≥ 50 % reduction) and non-responders (NR; < 50 % reduction). Mean monthly seizure frequency was defined as the mean seizure frequency during the three consecutive months before implantation (pre-VNS) or before testing (post-VNS) (44). Reduction in mean monthly seizure frequency was calculated as follows:

$$Seizure\ reduction\ (\%) = \frac{frequency\ pre{-}VNS - frequency\ post{-}VNS}{frequency\ pre{-}VNS} * 100.$$

2.2 Auditory oddball paradigm

To measure P3b components, an auditory oddball paradigm was performed. As described earlier, this task is a simple and well-established paradigm for the investigation of arousal effects on cognitive performance and has been shown to reliably evoke robust P3 components (79). During a three-stimulus oddball paradigm, three tones (a standard stimulus; a target stimulus and a distractor stimulus) were provided in a randomized order. Standard tones were presented frequently (high-probability; 75%), whereas distractor and target stimuli were rare (low-probability; 12,5% each). To reduce ocular artifacts, patients were instructed to focus on a fixation cross presented on the laptop screen. Patients had to respond only to the target

tones by pressing the predefined button on the keyboard. A practice session allowed the patients to become familiar with the different tones. Stimulus presentation as well as response time and accuracy of response to target tones were controlled using E-prime software 2.0 (Psychology Software Tools, Pittsburgh, PA, USA) on a Dell laptop (Round Rock, TX, USA). Patients performed this task during three stimulation conditions (OFF, ON and tVNS) in a randomized, counterbalanced order. During the ON condition the duty cycle was 7 s ON/ 18 s OFF. Other stimulation parameters during the ON condition were patient-specific (see **Table 4** in **Appendix A**). A 20-minute baseline (OFF, ON or tVNS) was taken before each oddball task to obtain a stable state before initiation of the task. After the oddball tasks during VNS ON or tVNS, a break of 20 minutes (OFF) was introduced in order to eliminate residual stimulation effects.

2.3 Electrophysiological recordings

For the video-EEG monitoring, 27 EEG electrodes were placed on the patient's scalp according to the 10-20 system of electrode positions (see **Figure 10)** and two electrocardiogram (ECG) electrodes above the heart. The EEG was recorded with a Micromed System Plus (Micromed, Mogliano, Italy). The online reference electrode was placed on the right mastoid (G2) and the ground electrode on the left mastoid (G1). To monitor the VNS artifact, two additional electrodes were placed in the neck cranial and caudal to vagal nerve electrode. The EEG, ECG and VNS signals were digitized online with a sampling frequency rate of 1024 Hz, antialiasing filter of 250 Hz, gain of 50 dB, and a resolution of 16 bits. EEG-electrode impedance was maintained below 10 kΩ.

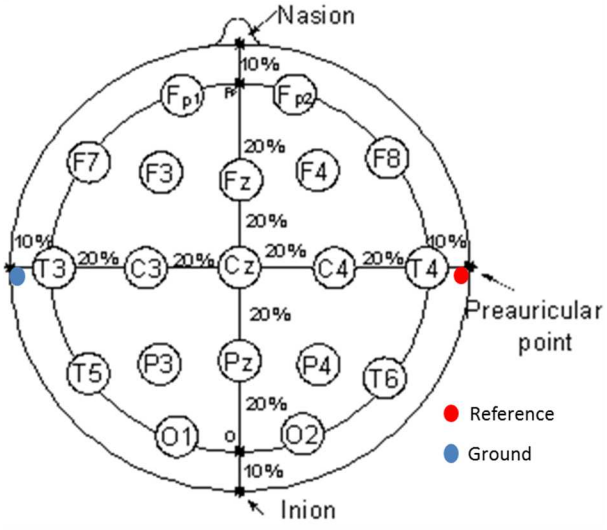

Figure 10: 10/20 scheme for electrode placement (86). Three distances are measured: 1. The distance between the nasion (nasal bridge) and the inion (occipital bone mount); 2. The distance between the two preauricular points; and 3. The circumference between the last two points of the scalp. These distances are divided in proportion of 10-20-20-20-20-10% in both orthogonal axes and in circumference, and a net of imaging quadrates is built on the head surface. The electrodes are placed in a quadrate's angels. The reference electrode is placed on the right mastoid (red dot) and the ground electrode is placed on the left mastoid (blue dot).

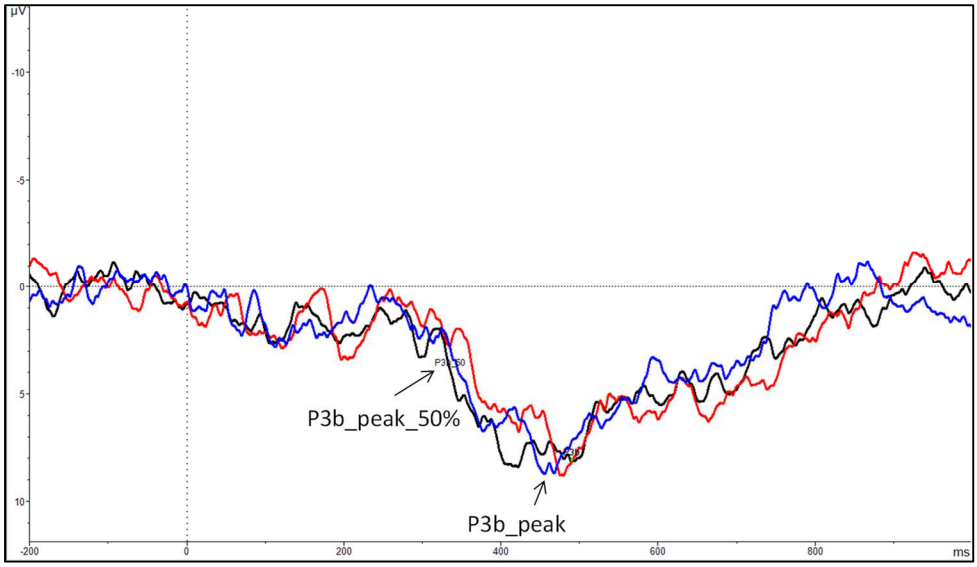

Figure 11: Example of computed P3b components in R04 after one year of VNS therapy. P3b components were recorded on the parietal midline electrode Pz. In this example, the black, red and blue line represent the P3b components measured during OFF, ON and tVNS conditions respectively. P3b_peak indicates the detected peak of P3b components used to calculate mean amplitudes (mean values of 100 ms around these peaks). P3b_peak_50% indicates the point where 50 % of the P3b peak is reached and latencies are measured at this time point. Horizontal axis: time after target presentation in milliseconds (ms); vertical axis: amplitude of the P3b component in microvolts (µV).

The ERP component of interest - the P3b component - was computed in Brain Vision Analyzer 2.0 using a specific analysis sequence (44) (see **Figure 11**):

1. Data filtering (50 Hz notch filter)
2. Independent component analysis (ICA) and inverse ICA: artifact components (vertical and horizontal eye movements, blinks, heartbeat and VNS artifacts) were subtracted from each electrode
3. Re-referencing of the EEG to the average of all recorded channels
4. Data filtering (half-power band-pass filter: 0.1-30 Hz, slope: 12 dB/octave)
5. Segmentation based on a marker position into epochs from -200 ms to 1000 ms relative to the onset of trigger 1.
6. Baseline correction (from -200 ms to 0 ms)
7. Artifact rejection for all scalp EEG electrodes: epochs (± 200 ms) with a voltage exceeding ± 75 µV were excluded
8. Averaging: “average standard”
9. Steps 5 to 8 were repeated for trigger 2: “average target”
10. Steps 5 to 8 were repeated for trigger 3: “average distractor”
11. Difference waves by comparing datasets:
    - target-standard difference waveform
    - distractor-standard difference waveform

12. Peak detection:
    - Difference wave target-standard: P3b peak detection (250-900 ms, positive polarity and marked at Pz)
    - Difference wave distractor-standard: P3a peak detection (200-700ms, positive polarity and marked at Cz)
13. 50% peak detection using MatLab
14. Export peak information
15. Open export files in excel

The amplitudes exported in Excel are the mean values of 100 ms around the detected peak. To minimize the impact of nuisance factors, the percentage difference in P3b amplitude of the VNS ON condition relative to the OFF condition was calculated with the following formula (44):

P3b amplitude (ON–OFF)/OFF

The same was done for the tVNS condition:

P3b amplitude (tVNS-OFF)/OFF

In addition, absolute differences in P3b amplitude between the ON or tVNS condition and the OFF condition were calculated using the following formulas:

P3b amplitude (ON-OFF)

P3b amplitude (tVNS-OFF)

In averaged waveforms, the absolute onset latency will reflect the trials with the earliest onsets rather than the average of the single trial onset latencies. Therefore, the latency of the P3b was measured as the time point at which the voltage reached 50% of the peak amplitude. The peak latency at 50% of the peak amplitude is a much more accurate and sensitive to measure the relative onset time of an ERP component (44).

2.4 VNS-induced LMEP recordings

LMEP recordings were only measured in five patients because these were only added to the protocol in a later stage. For these recordings, six Ag/AgCl recording electrodes were placed in the neck according to three perpendicular axes around the larynx. Electrode pairs 1A-1B, 2A-2B and 3A-3B were placed according to the sagittal, horizontal and vertical axes respectively (see **Figure 12)**. Electrode Fpz on the forehead was used as ground (G1). Furthermore a common reference electrode (rb) was placed onto the sternum (G2). The electromyogram (EMG) was recorded using Micromed System Plus (Micromed, Mogliano, Italy).

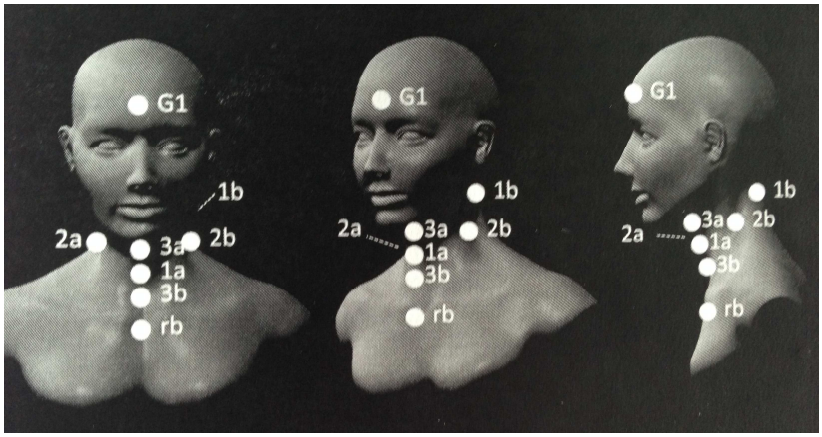

Figure 12: Schematic representation of the electrode placement (87).

For the duration of the measurements, the frequency of the VNS and the duty cycle were set at 30 Hz and 7s ON / 18s OFF respectively in all patients to assure reproducibility and to limit the time of the procedure respectively. A frequency of 30 Hz was chosen because no overlap of stimulation artifacts and electrophysiological responses was observed and because this frequency is most often used in clinical practice. The measurements were repeated three times for different pulse widths (130, 250 and 500 µs). For each pulse width, the output current was ramped-up in steps of 0.25 mA (or 0.125 mA if possible) until the threshold for the patient was reached for newly implanted patients or until the stimulation output of the patient was reached if the patient was tested after one year of stimulation (87). Four trains of stimulation were recorded at each output current. One train of stimulation was defined as 7s ON-18s OFF. The signals were digitized online with a sampling frequency rate of 1024 Hz, an antialiasing filter of 250 Hz, a gain of 50 dB and a resolution of 16 bits.

LMEPs were then computed offline in Brain Vision Analyzer 2.0 using a specific analysis sequence (see **Figure 13**):

1. Edit channels (1A, 1B, 2A, 2B, 3A and 3B)
2. Difference waves by comparing channels: 1A-1B, 2A-2B, 3A-3B
3. Stimulation interval was marked by start and end markers
4. Segmentation based on these markers
5. Data filtering (low cut-off filter: 100-200 Hz, slope 12/24/48 dB/octave, trial-and-error)
6. Level trigger (stimulation peaks marked)
7. Import markers from “6” in non-filtered data (step 4)
8. Segmentation based on a marker position (VNS_peak) and based on time:
   -10 ms before the peak and end 20 ms after the peak.
9. Baseline correction (between -6 and -2 ms)
10. Averaging for the full range plus creating a data set for standard deviation

LMEP amplitudes (µV) were plotted against VNS output currents (mA) using Sigmaplot 13. The best fitting curves were calculated using the following formula: $y = \frac{a}{1+e^{-\frac{(x-x0)}{b}}}$

with y = LMEP amplitude in µV; a = maximum LMEP amplitude in µV (plateau phase); x = VNS output current in mA; x0 = VNS output current in mA resulting in an LMEP of half-maximal amplitude; b = slope. In this way, LMEP amplitude vs VNS intensity curves were created for all measured patients (see **Figure 14** in **Appendix B** and **Figure 15** in **Appendix C**).

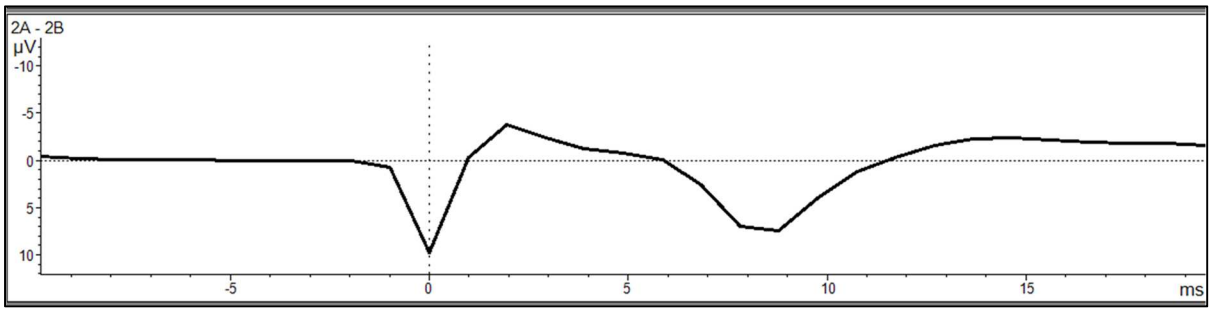

Figure 13: Example of an LMEP computed with Brain Vision Analyzer 2.0. This figure shows the LMEP measured on the horizontal axis (2A-2B). The first peak at 0 ms represents the stimulation artifact and the second peak represents the LMEP evoked by stimulation of the vagus nerve. In this example, LMEPs were recorded after one year of stimulation and recorded at a frequency of 30 Hz, an intensity of 1.0 mA and a pulse width of 250µs. Horizontal axis: time relative to stimulation in milliseconds (ms); vertical axis: amplitude in microvolts (µV).

2.5 Questionnaires

Two weeks after implantation of VNS, patients were asked to complete five questionnaires including Quality Of Life In Epilepsy (QOLIE-31), Beck Depression Inventory (BDI-II), State-Trait Anxiety Inventory (STAI) version DY1 (state anxiety) and DY2 (trait anxiety), and tVNS-sensation questionnaires. The collection of the same questionnaires was repeated after one year of VNS treatment, with exception of the tVNS sensation questionnaires, which were only meant to visualize how patients experience tVNS. Questionnaires were taken during breaks between auditory oddball tasks in the different conditions. Afterwards, total scores of every questionnaire (except tVNS-sensation) were calculated using standard protocols.

The QOLIE-31 questionnaire (88) contains seven multi-scale items: seizure worry; overall quality of life; emotional well-being; energy/fatigue; cognitive; medication effects and social function. For each category a score on a 100-point scale was calculated, with higher scores reflecting better quality of life. These scores were converted into an overall score by use of the following formula:

$$\text{Overall score} = \text{seizure worry} * 0{,}08 + \text{cognitive score} * 0{,}27 + \text{social function score} * 0{,}21 + \text{overall quality of life score} * 0{,}14 + \text{emotional wellbeing score} * 0{,}15 + \text{energy/fatigue score} * 0{,}12 + \text{medication effects score} * 0{,}03$$

For each individual domain score and for the overall score, T-scores were determined. These T-scores represent linear transformations of the scores that produce a mean of 50 and standard deviation of 10 for a cohort of 304 adults with epilepsy. Thus, a person with a T-score of 50 has a score equal to that of the mean for the epilepsy cohort. Higher T-scores reflect a more favorable quality of life (88). T-scores of each QOLIE-31 domain and of the overall score were used for statistical analysis.

The BDI-II questionnaire consists of 21 items that measure the severity of depressive symptoms. Each item is rated on an intensity score of 0-3, with a maximum of 63 (89). Depression severity varies from no or minimal (0-9) over border (10-14); mild (15-20); moderate (21-30) and severe (31-40) to very severe (41-63). Thus, in this questionnaire higher scores represent a more depressive state.

STAI version DY consist of two questionnaires of 20 items each. Scoring should be reversed for anxiety-absent items. The DY1 tests state anxiety and scores on this questionnaire were calculated by reverse-scoring items 1; 2; 5; 8; 10; 11; 15; 16; 19 and 20, followed by calculating the total of all 20 items. The DY2 tests trait anxiety and in this questionnaire items 1; 3; 6; 7; 10; 13; 14; 15; 16 and 19 were reverse-scored, followed by totaling all 20 items. Higher scores indicate greater anxiety.

2.6 Pupillary measurements

The pupil width and height were recorded using an Eyestart Eye-Tracker (ASL, Bedford, MA) with a sampling frequency identical to the EEG recordings (1024 Hz). Using a digital-to-analog converter card (Arrington Research), pupil width and height values were converted to millivolts and provided as input to two DC channels of the EEG system (90). The room was darkened by closing down the rolldown shutters, dimming the main lights and putting the light bulbs on. On the laptop screen, a gray background with a white square was presented in the middle of the patient's visual field. Patients were asked to fixate on this square in order to minimize eye movements and make it possible to visualize the pupil with the camera. The camera was fine-tuned to the right position focusing on the pupil of the left eye (see **Figure 16**). Pupillometry measurements were performed twice, once for VNS and once for tVNS. The randomization order of the three stimulation conditions during auditory oddball paradigms, especially the order of VNS ON and tVNS, determined whether pupillometry for VNS or tVNS was conducted first. Pupillometry was started when the VNS was switched OFF for three minutes. After three minutes VNS or tVNS was switched on and another three minutes of pupillometry was recorded in the VNS ON or tVNS situation (see **Figure 17**). After each session a calibration using a series of artificial pupils with varying diameters (2; 3; 4; 5; 6; 7 and 8 mm) was performed in order to be able to convert the DC channel voltages for width and height back to

millimeters by use of calibration curves (90). These measurements were used to calculate the pupil size: $\frac{Height\ (mm)}{2} * \frac{Width\ (mm)}{2} * \pi$.

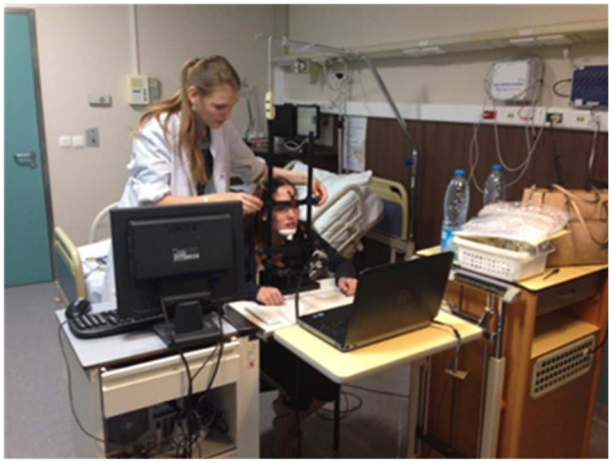

Figure 16: Setup for pupillometry measurements.

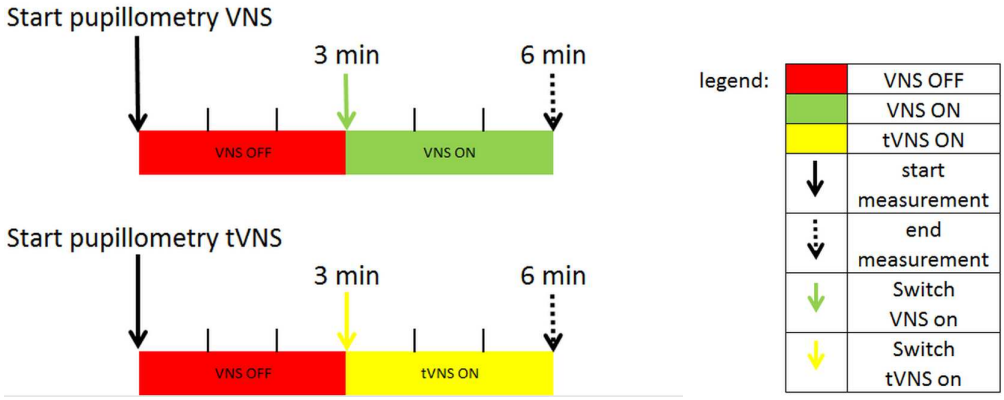

Figure 17: Schematic representation of the pupillometry: Pupillometry measurement was started when the VNS was switched OFF for three minutes. After three minutes, the VNS or the tVNS was switched on, and another three minutes of pupillometry were recorded in the VNS ON or tVNS situation.

The pupillary measurements were analyzed using Brain Vision Analyzer 2.0. A specific analysis sequence was used:

1. Data filtering (50 Hz notch filter for all scalp EEG channels and the ECG channel, low cut-off filter for the VNS channel and no filter for the dc-channels)
2. Whole blink intervals were marked: “blink”
3. Blinks were interpolated using MatLab to correct for missing data
4. 3 min OFF (“start OFF” and “end OFF”) and 3 min ON intervals (“start ON” and “end ON”) were marked (or 3 min tVNS intervals (“start tVNS” and “end tVNS”)
5. Segmentation by these start and end markers:“3min_VNS_OFF” and “3min_VNS_ON” (or “3min_VNS_OFF” and “3min_tVNS”)
6. Area information was exported and export files were opened in excel

In order to avoid interference caused by distractions (movement, talking) when turning on the VNS or tVNS device, ten seconds of data before and after programming the VNS or tVNS device ON were left out for the analysis. A total of 140 seconds of data was analyzed for each condition (OFF-ON and OFF-tVNS) in each patient. Mean pupil sizes were calculated for OFF and ON or OFF and tVNS conditions. These means were used to calculate relative and absolute differences using the following formulas:

Relative: $\boxed{ON/OFF}$ and $\boxed{tVNS/OFF}$

Absolute: $\boxed{ON-OFF}$ and $\boxed{tVNS-OFF}$

2.7 Transcutaneous vagus nerve stimulation

tVNS was performed using a transcutaneous vagus nerve stimulator NEMOS (Cerbomed GmbH, Erlangen, Germany) which consists of a stimulation unit and a dedicated ear electrode (see **Figure 18**). The individual output current for each patient was determined by ramping-up the stimulation output until the detection threshold was reached without sensation of pain (see **Table 4** in **Appendix A**).

Figure 18: tVNS device. NEMOS, Cerbomed GmbH, Erlangen. (http://www.cerbomed.com/)

2.8 Statistical analysis

All statistical analyses were performed in Statistical Package for the Social Sciences (SPSS) version 24 (SPSS, Chicago, IL, USA) with the level of statistical significance set at 0,05.
Electrophysiological, behavioral, pupil and questionnaire results were analyzed using a repeated measures analysis of variance (ANOVA) with group (responders (R) vs non-responders (NR)) as between-subject factor and condition (electrophysiological and behavioral results: OFF, ON and tVNS; pupil results: ON vs OFF and tVNS vs OFF; questionnaire results: one year vs start) as within-subject factor. Two-tailed paired t-tests were computed as post-hoc analyses. Independent-samples t-tests were applied to confirm significant changes in parameters between responders and non-responders.
Correlation between reaction time during auditory oddball tasks and measured P3b amplitudes were tested using Pearson's correlation coefficient. In addition, linear mixed model analysis

with patients as random variable, reaction times as outcome and P3b amplitude as predictor was used to examine whether a model was able to predict reaction time from P3b amplitude. Correlation between pupil size and P3b amplitudes were also tested using Pearson's correlation coefficient.

Since the aim of this thesis was finding a biomarker to predict VNS response, correlation between seizure reduction (%) and effect of VNS and tVNS on P3b amplitude and pupil size were tested using Pearson's correlation coefficient. Models were computed using linear regression analysis.

3. RESULTS

3.1 Patients

24 patients were included in this study of which eleven were categorized as responders, six as non-responders. Seven patients that could not be categorized: one passed away after the first test-moment (NC01), one was lost in follow-up (NC02) and five patients were not yet stimulated for one year. Patient characteristics including sex; age; age at implantation; percentage seizure reduction and VNS parameters for each patient are summarized in **Table 4** in **Appendix A**.

3.2 Electrophysiological results: P3b amplitude and P3b latency

Consistent with the retrospective study of De Taeye *et al* (44), the P3b component of the auditory ERP was recorded at the parietal midline electrode Pz. The generation of this well-characterized P3b component was associated with processing of aberrant target stimuli during the auditory oddball task. P3b amplitudes for each individual patient in the responder and non-responder group, as well as for uncategorized patients, are presented in **Table 5** in **Appendix D**. 24 patients completed the first test session. Two patients (R07 and NR03) were excluded for analysis of electrophysiological results because there was no clear visualization of the P3b component. Of the remaining 22 patients (11 men and 11 women, mean age 42 years), five were categorized as non-responders, ten as responders and seven patients could not yet be categorized. One responder (R11) and one non-responder (NR06) did not receive tVNS. Only ten patients fully completed the VNS study, i.e. they had a test session at the start of their VNS therapy and a second test session after one year of therapy. Of these patients two were categorized as non-responders and eight as responders. P3b components were further analyzed using SPSS. The electrophysiological results of the auditory oddball paradigm for responders and non-responders at start and after one year of VNS therapy are summarized in **Table 6 and 7** in **Appendix E and F** respectively: mean P3b amplitude and mean latency at 50% of the P3b peak, along with F- and p-values of statistical analyses, are shown.

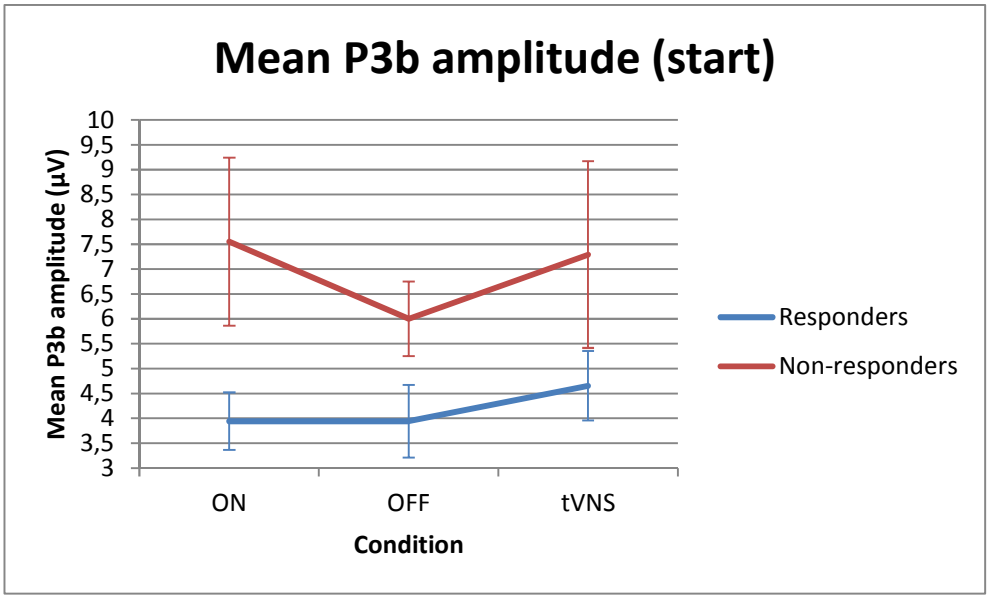

Figure 19: Plot of the mean P3b amplitudes per condition (OFF, ON and tVNS at start) for responders (n = 9) and non-responders (n = 4). Repeated measures ANOVA with condition (OFF, ON and tVNS) as within-subject-factor and group (R vs NR) as between-subject factor revealed a significant effect of group (F = 5.116; p = 0.045) at start of VNS therapy. In this figure we can see higher P3b amplitudes in non-responders as compared to responders during all three condition stimulations (OFF, ON and tVNS). NR: OFF (M = 6.00 µV; SEM = 0.75 µV), ON (M = 7.55 µV; SEM = 1.69 µV) and tVNS (M = 7.29 µV; SEM = 1.88 µV) ; R: OFF (M = 3.94 µV; SEM = 0.73 µV), ON (M = 3.94 µV; SEM = 0.58 µV) and tVNS (M = 4.65 µV; SEM = 0.70 µV).

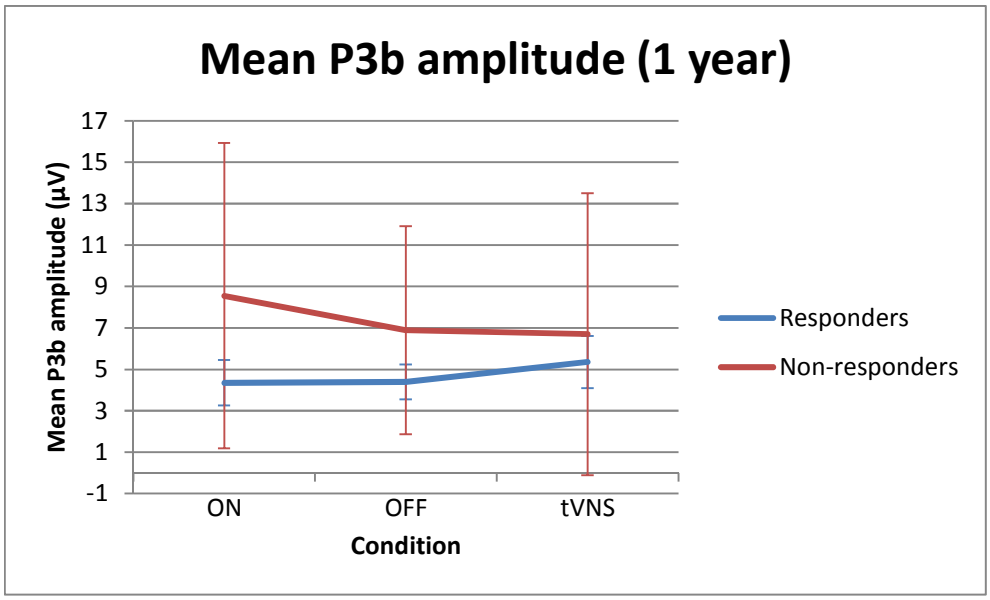

Figure 20: Plot of the mean P3b amplitudes per condition (OFF, ON and tVNS after one year) for responders (n = 7) and non-responders (n = 2). Repeated measures ANOVA with condition (OFF, ON and tVNS) as within-subject-factor and group (R vs NR) as between-subject factor revealed no significant effects of condition (F = 0.503; p = 0.615) , group (F = 0.635; p = 0.452) or group x condition interaction (F = 1.574; p = 0.242) after one year of VNS therapy. In this figure we can see that mean P3b amplitudes are still higher in non-responders as compared to responders during all three condition stimulations (OFF, ON and tVNS), but differences between responders and non-responders are not significant. NR: OFF (M = 6.89 µV; SEM = 5.02 µV), ON (M = 8.55 µV; SEM = 7.37 µV) and tVNS (M = 6.71 µV; SEM = 6.80 µV) ; R: OFF (M = 4.39 µV; SEM = 0.84 µV), ON (M = 4.35 µV; SEM = 1.10 µV) and tVNS (M = 5.35 µV; SEM = 1.26 µV).

Repeated measures ANOVA of the P3b amplitude with condition (OFF, ON and tVNS) as within-subject factor and group (R vs NR) as between-subject factor revealed a significant effect of group (F = 5.116; p = 0.045) at start of VNS therapy (see **Figure 19**), but no significant effects were observed after one year (see **Figure 20**).

If this repeated measures ANOVA was repeated with only two conditions (OFF vs ON) as within-subject factor, the effect of group at start was even more pronounced (F = 10.332; p = 0.007) (see **Figure 21**), but still no group x condition interaction effect was observed (F = 2.308; p = 0.153). No significant differences in latencies could be determined.

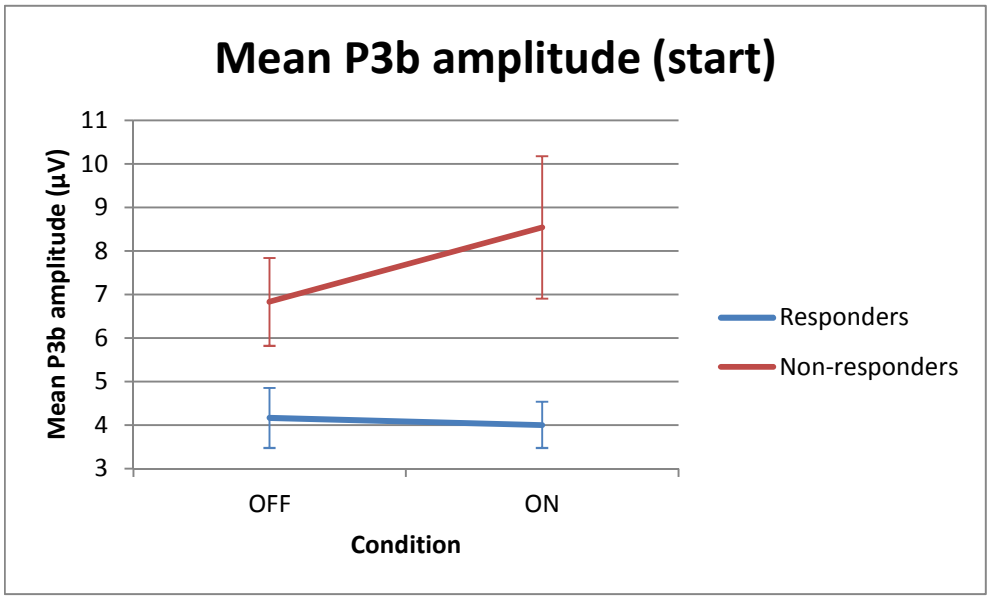

Figure 21: Plot of the mean P3b amplitudes per condition (OFF and ON at start) for responders (n = 10) and non-responders (n = 5). Repeated measures ANOVA with group as between-subject factor (R vs NR) and condition (ON vs OFF) as within-subject factor revealed a significant effect of group (F = 10.332; p = 0.007) at start of stimulation. Higher amplitudes are observed in non-responders during OFF (M = 6.83 µV; SEM = 1.01 µV) and ON (M = 8.54 µV; SEM = 1.64 µV) conditions as compared to responders (OFF: M = 4.16 µV; SEM = 0.69 µV and ON: M = 4.00 µV; SEM = 0.53 µV).

3.3 Behavioral results: accuracy and reaction time

For the analysis of behavioral results, accuracy and reaction times of the same patients were analyzed. The behavioral results for responders and non-responders are summarized in **Table 6 and 7** in **Appendix E and F** respectively: mean accuracy and mean reaction times, along with F- and p-values of statistical analyses, are shown. The high performance (i.e. accuracy) observed during the auditory oddball tasks (mean accuracy: 97.6 ± 2.84 % correct) confirms that patients were paying attention to the stimuli and could easily discriminate between target and non-target stimuli. No significant differences in accuracy were observed between different stimulation conditions at start neither after one year. Repeated measures ANOVA of accuracy with condition (OFF, ON and tVNS) as within-subject factor and group (R vs NR) as between-subject factor revealed a significant effect of group at start (F = 4.695; p = 0.049) (see **Figure 22**), but not after one year of stimulation (F = 0.785; p = 0.405).

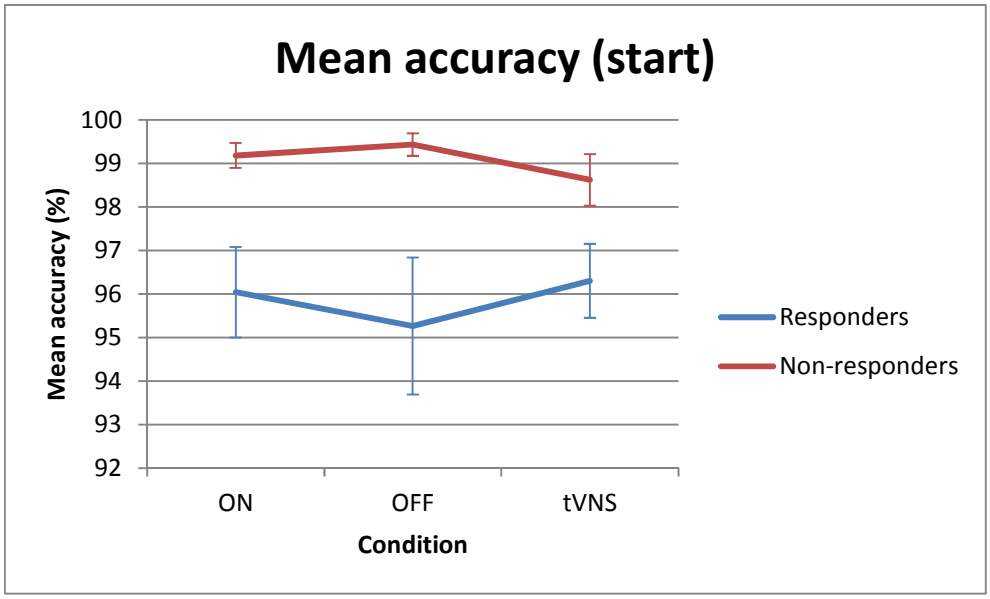

Figure 22: Plot of the mean accuracies per condition (OFF, ON and tVNS at start) for responders (n = 10) and non-responders (n = 5). Repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (OFF, ON and tVNS) as within-subject factor revealed a significant effect of group (F = 4.695; p = 0.049) at start. This graph shows that accuracies are higher for non-responders (OFF: M = 99.43%; SEM = 0.26% - ON: M = 99.18%; SEM = 0.29% - tVNS: M = 98.62%; SEM = 0.59%) as compared to responders (OFF: M = 95.26%; SEM = 1.57% - ON: M = 96.04%; SEM = 1.04% - tVNS: M = 96.30%; SEM = 0.85%) during all three conditions (OFF , ON and tVNS). The difference between responders and non-responders is the highest during OFF conditions (SEM = 4.08 %; SEM = 1.44 %; t(10.431) = 2.843; p = 0.017).

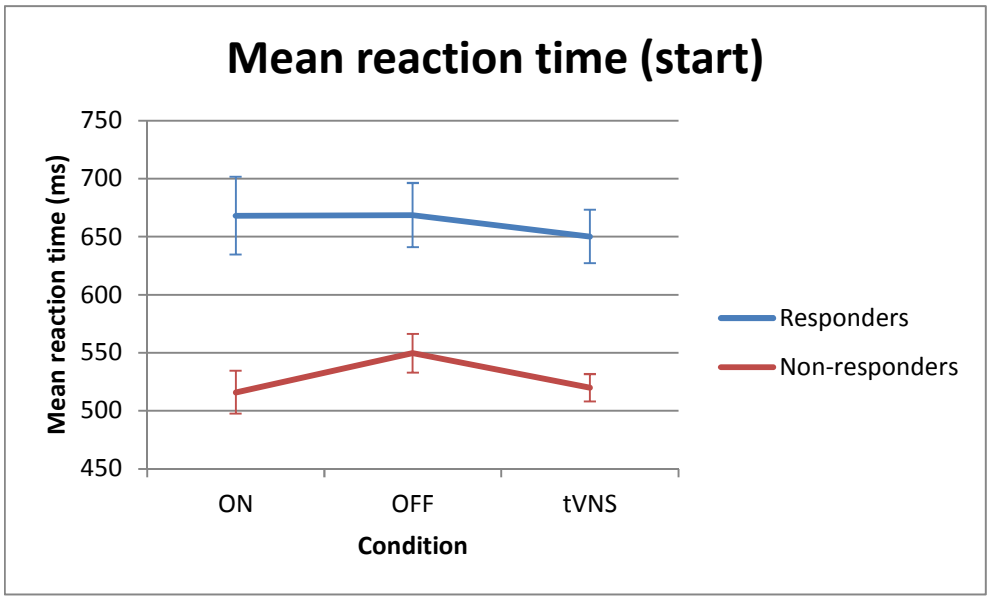

Figure 23: Plot of the mean reaction times per condition (OFF, ON and tVNS at start) for responders (n = 10) and non-responders (n = 5). Repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (OFF, ON and tVNS) as within-subject factor revealed significant effects of group (F= 13.708; p = 0.003) at start. Reaction times of responders (OFF (M = 668.75 ms; SEM = 27.69 ms), ON (M = 668.16 ms; SEM = 33.53 ms) and tVNS (M = 650.11 ms; SEM = 22.95 ms)) were slower as compared to non-responders (OFF (M = 549.57 ms; SEM = 16.87 ms), ON (M = 515.90 ms; SEM = 18.50 ms) and tVNS (M = 519.76 ms; SEM = 11.78 ms)) during all conditions, with the highest difference observed during ON conditions (M = -153.94 ms; SEM = 42.26 ms; t(14.993) = -3.643; p = 0.002).

Analysis of reaction times also revealed no significant differences between different stimulation conditions. Repeated measures ANOVA of reaction time with condition (OFF, ON and tVNS) as within-subject factor and group (R vs NR) as between-subject factor revealed a significant effect of group for reaction times at start (F = 13.708; p = 0.003) (see **Figure 23**), and a significant group x condition interaction effect after one year of stimulation (F = 3.986; p = 0.043) (see **Figure 24**).

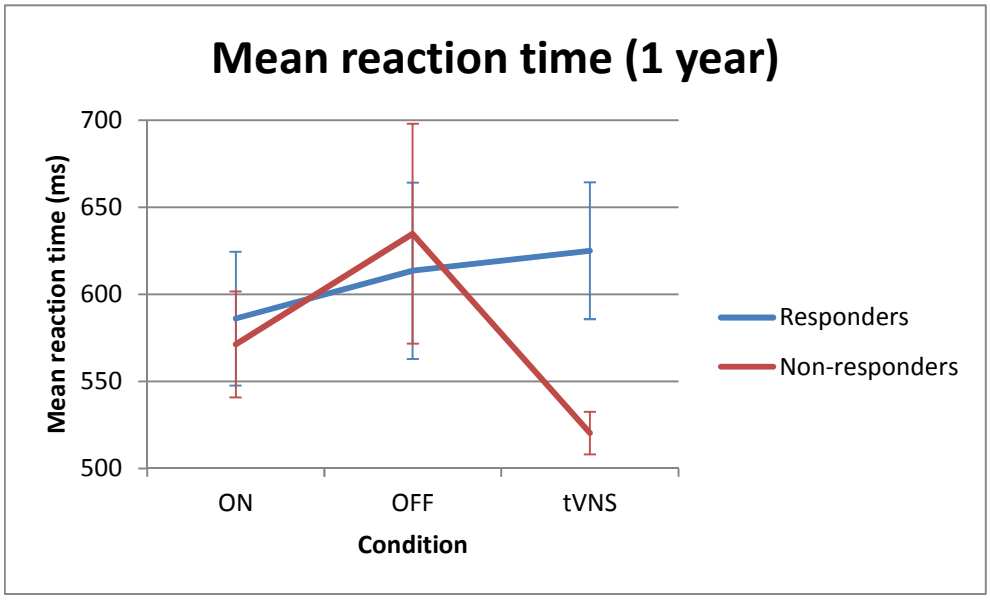

Figure 24: Plot of the mean reaction times per condition (OFF, ON and tVNS after 1 year) for responders (n = 6) and non-responders (n = 3). Repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (OFF, ON and tVNS) as within-subject factor revealed a significant effect of group x condition interaction (F = 3.986; p = 0.043) after one year of stimulation. Reaction times of non-responders decrease during ON conditions and even more during tVNS conditions, while in responders there is only a slight decrease in reaction times during ON conditions. R: OFF (M = 613.40 ms; SEM = 50.68 ms), ON (M = 586,00 ms; SEM = 38.44 ms) and tVNS (M = 624.93 ms; SEM = 39.30 ms) and NR: OFF (M = 634.71 ms; SEM = 63.10 ms), ON (M = 571.19 ms; SEM = 30.44 ms) and tVNS (M = 520.29 ms; SEM = 12.21 ms)

3.4 Correlation between P3b amplitude and reaction time

Correlation analyses using a two-tailed Pearson's coefficients were used to examine whether the effect of VNS and tVNS on P3b amplitude may be related to reaction times. There was a significant correlation between P3b amplitude and reaction times (r = -0.233; p = 0.028).

Using linear mixed model analysis with patient as random variable, reaction time as outcome and P3b amplitude as predictor, a model to predict reaction time by means of P3b amplitude could be computed: RT = 633.79 – 6.95 * P3b_amplitude (p = 0.037) (see **Figure 25**).

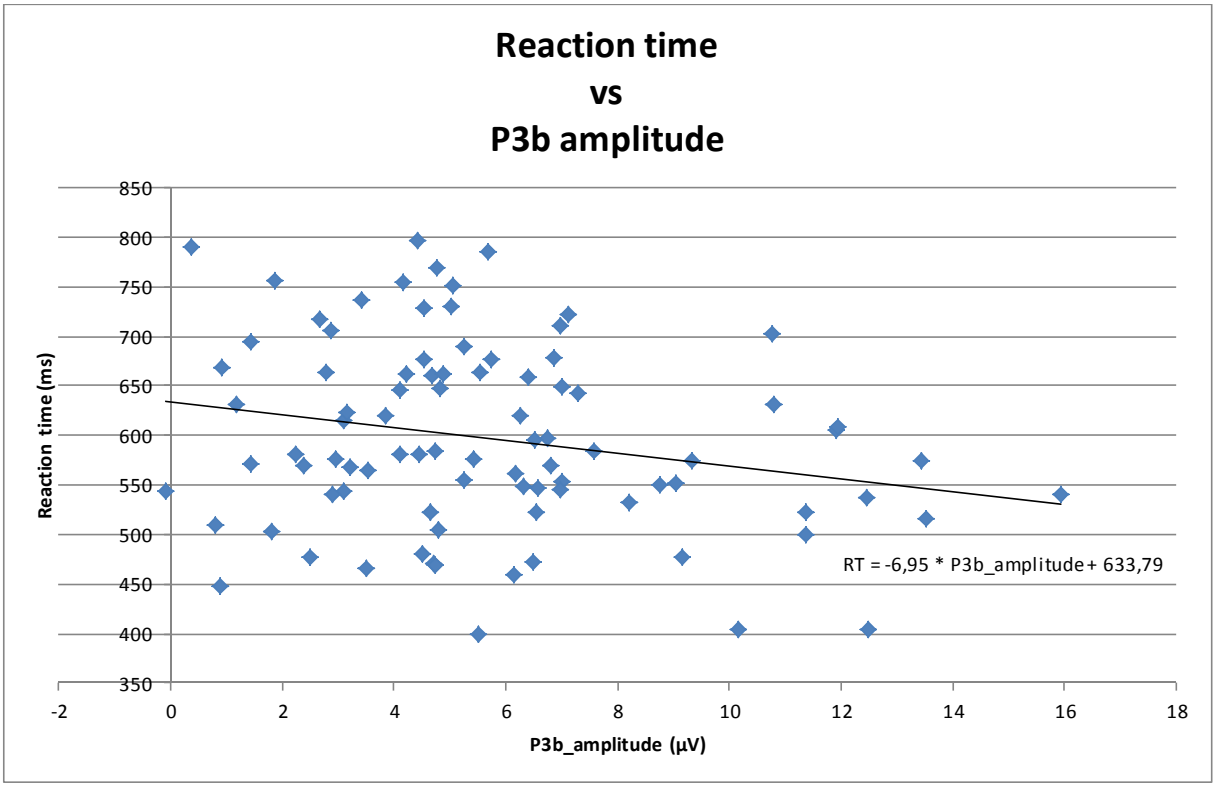

Figure 25: Plot of reaction times versus P3b amplitudes. Each dot in this graph represents a P3b amplitude – reaction time pair. No separation based on group (responders and non-responders), stimulation conditions (OFF, ON and tVNS) or time (start and one year) was made. Using linear mixed model analysis a model to predict reaction time by means of P3b amplitude could be computed: RT = 633.79 – 6.95 * P3b_amplitude (p = 0.037). RT : reaction time.

3.5 Pupil size

For the analyses of pupil size, data of 22 patients (12 men and 10 women, mean age 44 years) were analyzed. Pupil sizes for every patient are shown in **Table 8** in **Appendix G**. Eleven patients were responders, six non-responders and five were uncategorized. Ten patients of which seven responders and three non-responders fully completed the study after one year of VNS therapy. Pupil data for responders and non-responders are summarized in **Table 9 and 10** in **Appendix H**: mean pupil size, along with F- and p-values of statistical analyses, are shown. Repeated measures ANOVA tests with condition (ON vs OFF or tVNS vs OFF) as within-subject factor and group (R vs NR) as between-subject factor did not detect significant effects at start (see **Figure 26**) or after one year for condition, group or group x condition interaction. However, a group x condition interaction effect on pupil size for VNS ON vs OFF conditions after one year approximated significance (F = 4.750; p = 0.061) (see **Figure 27**). As no significant group x condition interaction was observed, post-hoc analyses also revealed no significant differences between ON and OFF conditions after one year in responders (M = 2.21 mm²; SEM = 1.30 mm²; t(6) = 1,.00; p = 0.140) and non-responders (M = -2.86 mm²; SEM = 1.82 mm²; t(2) = -1.571; p = 0.257).

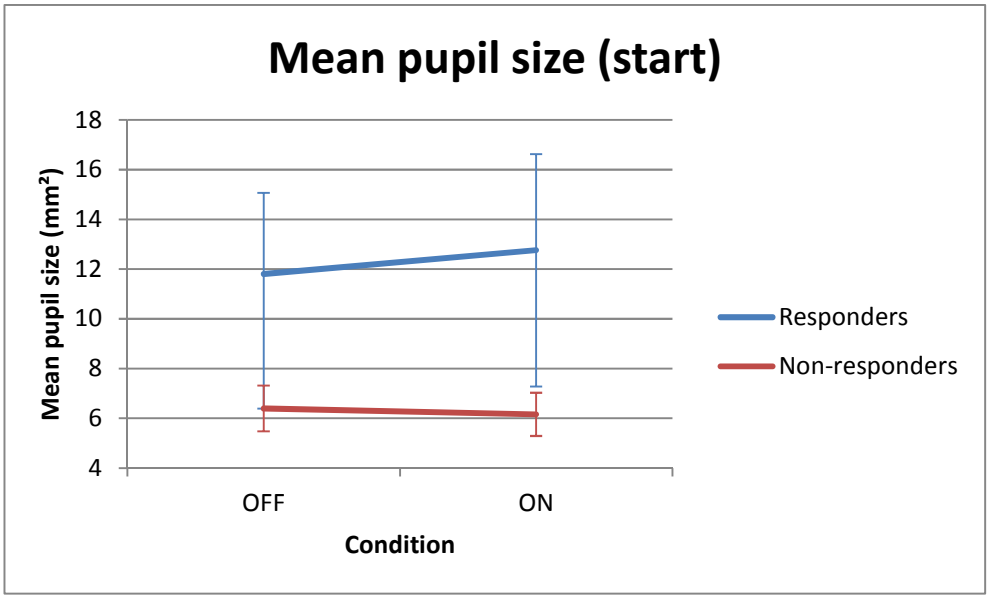

Figure 26 : Plot of the mean pupil sizes per condition (ON vs OFF at start) for responders (n = 10) and non-responders (n = 6). Repeated measures ANOVA with condition (ON vs OFF) as within-subject factor and group (R vs NR) as between-subject factor revealed no significant effects of condition ($F = 0{,}285$; $p = 0{,}602$), group ($F = 1{,}657$; $p = 0{,}219$) or group x condition interaction ($F = 0.796$; $p = 0.387$) on pupil size at start. This graph shows a different trend in responders and non-responders with a pupil constriction during ON conditions in non-responders (OFF ($M = 6.39$ mm²; $SEM = 0.92$ mm²) and ON ($M = 6.15$ mm²; $SEM = 0.87$ mm²)) and a dilatation in responders to VNS (OFF ($M = 11.80$ mm²; $SEM = 3.26$ mm²) and ON ($M = 12.76$ mm²; $SEM = 3.85$ mm²)).

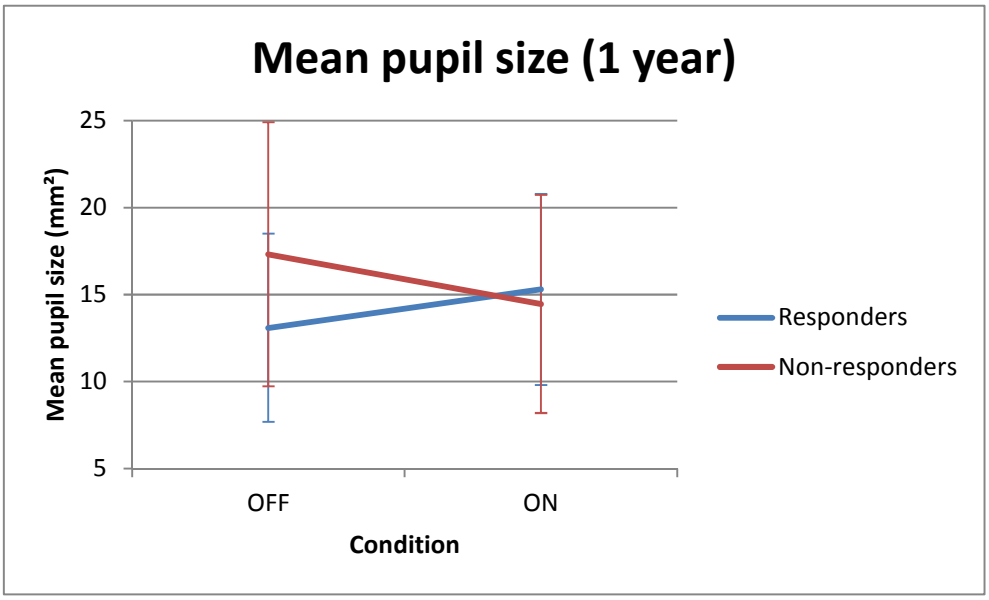

Figure 27: Plot of the mean pupil sizes per condition (ON vs OFF after 1 year) for responders (n = 7) and non-responders (n = 3). Repeated measures ANOVA with condition (ON vs OFF) as within-subject factor and group (R vs NR) as between-subject factor detected a nearly significant group x condition interaction effect on pupil size after one year ($F = 4.750$; $p = 0.061$). This graph shows a pupil constriction during ON conditions in non-responders (OFF ($M = 17.31$ mm²; $SEM = 7.58$ mm²) and ON ($M = 14.45$ mm²; $SEM = 6.27$ mm²)), while a dilatation is observed in responders to VNS (OFF ($M = 13.08$ mm²; $SEM = 5.41$ mm²) and ON ($M = 15.29$ mm²; $SEM = 5.49$ mm²)).

3.6 Pupil size and P3b amplitude as predictors for response to VNS

Two-tailed Pearson's coefficient was used to examine whether the effect of VNS on P3b amplitude or pupil size might be related to seizure outcome. No significant correlations were observed. However, a correlation between absolute difference in pupil size between VNS ON and OFF conditions after one year and the percentage seizure reduction was nearly significant ($r = 0.628$; $p = 0.052$). Based on an R-value of 0.628, 17 patients instead of ten patients would be needed to observe a significant correlation between effect of VNS on pupil size after one year and percentage seizure reduction with a power of 81.3%. Using linear regression analysis with percentage seizure reduction as outcome variable and with the absolute difference in pupil size between VNS ON and OFF conditions after one year as predictor variable, a model to estimate the percentage seizure reduction based on pupil size was computed : %_seizure_reduction = 44.81+ 6.068 * (pupil_size_ON_1y – pupil_size_OFF_1y) ($p = 0.052$) (see **Figure 28**).

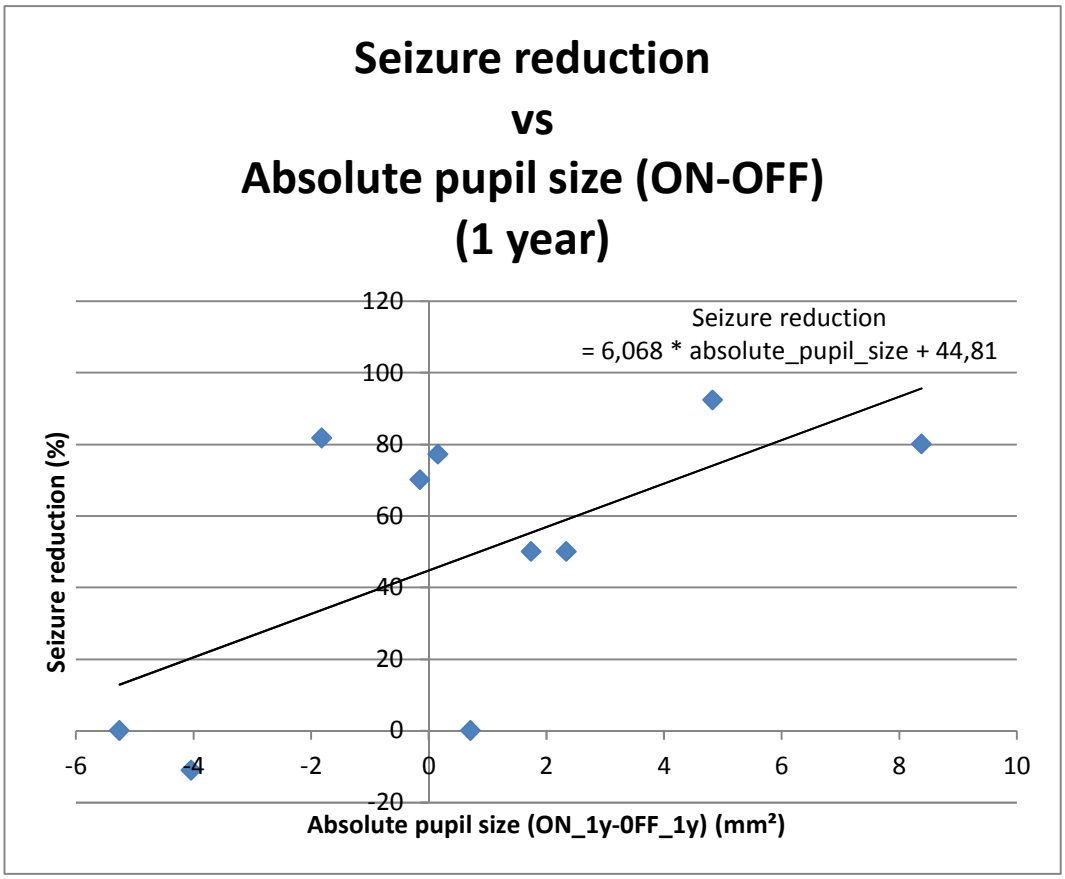

Figure 28: Plot of the percentage seizure reduction versus absolute pupil size (ON-OFF) after one year. Each dot in this graph represents an absolute_pupil_size(ON-OFF) – Seizure_reduction pair. Using linear regression analysis a model to predict seizure reduction (%) by means of the effect of VNS on pupil size after one year could be computed: %_seizure_reduction = 44.81+ 6.068 * absolute_pupil_size(ON-OFF) ($p = 0.052$). From the graph we can see a trend indicating that an increase in pupil size is related to an increase in percentage seizure reduction.

Of special interest in this study was which factors are able to predict the percentage seizure reduction before start of VNS therapy. At start of VNS therapy, no significant correlations between percentage seizure reduction and effect of VNS on P3b amplitude or pupil size (see **Figure 29**) were found. The Pearson's correlation coefficient for a relationship between the absolute difference in P3b amplitude between ON and OFF conditions at start and percentage seizure reduction was the most significant (r = -0.416; p = 0.123). However, 43 patients instead of 15 would be needed to observe a significant correlation between the effect of VNS on P3b amplitude at start and seizure reduction with a chance of 80.9%. The following model was calculated for this correlation: %_seizure_reduction = 47.077 - 6.464 * (P3b_ON_start - P3b_OFF_start) (p = 0.123). To be able to predict which patients will be responders to VNS prior to VNS surgery, a noninvasive analog similar to VNS has to be able to distinguish between responders and non-responders. Therefore, the same analyses were repeated with the effect of tVNS on P3b amplitude or pupil size instead of VNS. No significant correlations and models could be determined.

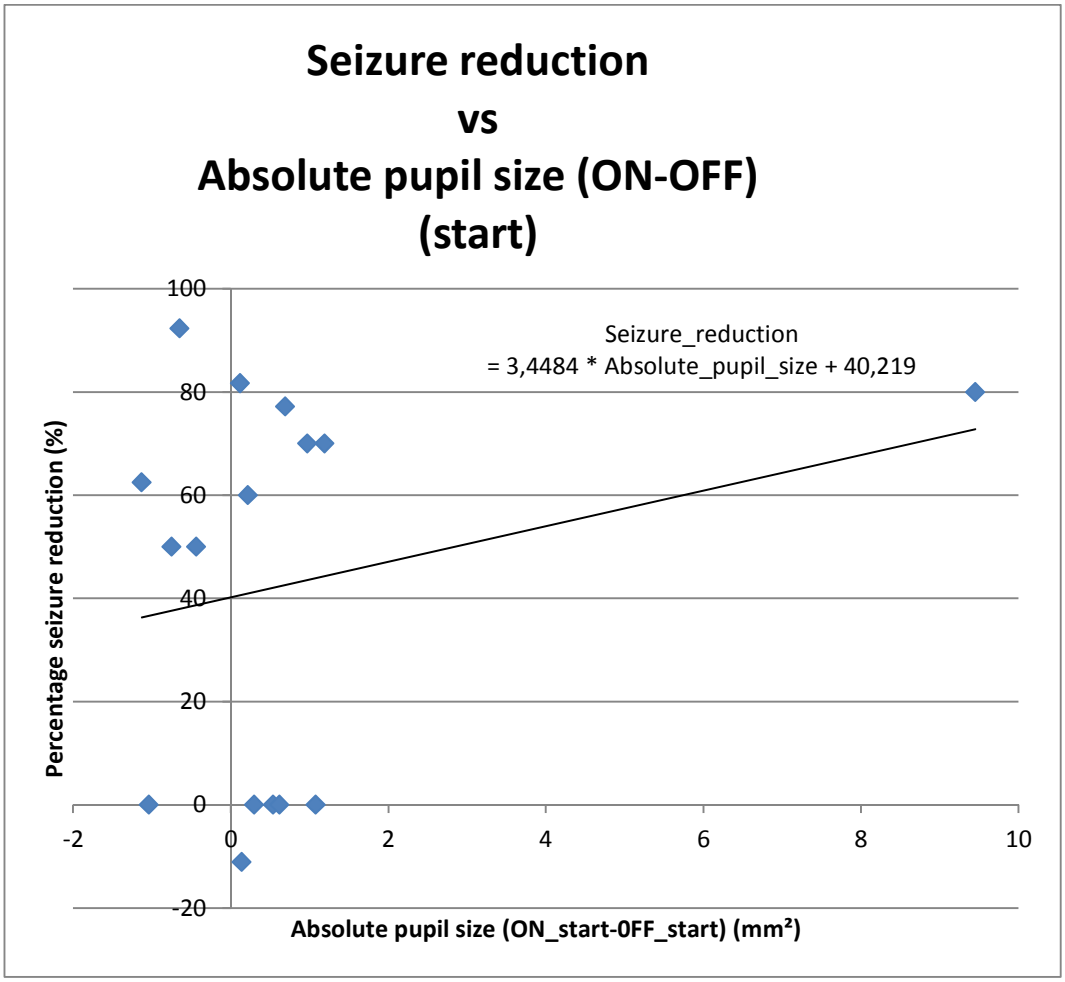

Figure 29: Plot of the percentage seizure reduction versus absolute pupil size (ON-OFF) after one year. Each dot in this graph represents an absolute_pupil_size(ON-OFF) –seizure_reduction pair. Pearson's correlation coefficient showed no significant relationship between effect of VNS on pupil size at start and seizure reduction (r = 0.226; p = 0.400).

3.7 Correlation between P3b amplitude and pupil size

To examine whether the effect of VNS on P3b amplitude might be related to the effect on pupil size, correlation analysis using two-tailed Pearson's coefficients was performed. There was no significant correlation ($r = -0.257$; $p = 0.66$) between absolute pupil sizes ($M = 0.46$ mm²; $SEM = 0.39$ mm²) and absolute P3b amplitudes ($M = 0.50$ µV; $SEM = 0.28$ µV).

3.8 Questionnaires: BDI-II, STAI DY1 and DY2, QOLIE-31

Eleven patients (7 men and 4 women, mean age 45 years) completed BDI-II questionnaires at start and after one year. Comparison of total scores between one year ($M = 17.55$; $SEM = 2.84$) and start ($M = 18.36$; $SEM = 3.13$) revealed no significant differences ($t(10) = -0.258$; $p = 0.802$). Repeated measures ANOVA of BDI scores with group (R vs NR) as between-subject factor and time (one year vs start) as within-subject factor revealed no significant effects of time, group or group x time interaction.

Twelve patients (8 men and 4 women, mean age 46 years) completed STAI questionnaires at start and after one year. For STAI questionnaires, scores of version DY1 and DY2 were calculated separately. Comparison of total scores for DY1 and DY2 revealed no significant differences between one year and start: DY1($M = 3.00$; $SEM = 3.23$; $t(11) = 0.929$; $p = 0.373$) and DY2 ($M = -3.58$; $SEM = 2.65$; $t(11) = -1.354$; $p = 0.203$). For STAI DY1, repeated measures ANOVA with group (R vs NR) as between-subject factor and time (1 year vs start) as within-subject factor revealed a significant group x time interaction effect ($F = 5.448$; $p = 0.043$). This interaction effect could not be observed for STAI DY2 ($F = 0.055$; $p = 0.820$). Post-hoc analysis for STAI DY1 showed significant higher state anxiety scores after one year ($M = 50.38$; $SEM = 2.859$) as compared to start ($M = 42.88$; $SEM = 3.667$) in responders only ($t(7) = 2.792$; $p = 0.027$). Independent-samples t-test showed a significant difference between responders ($M = 7.50$; $SEM = 2.69$) and non-responders ($M = -6.00$; $SEM = 6.38$) in change of DY1 scores between start and after one year ($t(10) = 2.334$, $p = 0.042$), thereby confirming the time x group interaction effect. In seven of the twelve patients, there was at least one missing value, making total scores less reliable. After excluding these patients, five patients were left and scores were analyzed again. The time x group interaction effect for STAI DY1 was no longer observed ($F = 1.914$; $p = 0.261$), but only three non-responders and two responders were included for this analysis. Also no other statistical significant effects were observed after excluding patients with missing values.

Ten patients (7 men and 3 women, mean age 39 years) completed QOLIE-31 at start and after one year. No significant differences in total T-scores between questionnaires after one year ($M = 38.10$; $SEM = 2.41$) and at start ($M = 36.40$; $SEM = 3.24$) could be observed ($t(9) = 1.129$; p

= 0.288). Repeated measures ANOVA of the QOLIE-31 total T-scores revealed no significant effects of time, group or time x group interaction. Next, every domain of the QOLIE-31 (seven in total) was analyzed separately. A significant increase in T-scores for seizure worry over time could be observed: M = 3.91; SEM = 1.63; t(10) = 2.396; p = 0.038. Repeated measures ANOVA tests for T-scores of the seven domains separately with group (R vs NR) as between-subject factor and time (1 year vs start) as within-subject factor revealed a significant effect of time for seizure worry (F = 6.554; p = 0.031) which confirms the outcome of the paired t-tests. Post-hoc tests comparing scores at the two time points showed significant higher T scores for seizure worry after one year (M = 47.50; SEM = 6.59) than at start (M = 41.5; SEM = 6.02) in non-responders only (t(3) = 4.076, p = 0.027). QOLIE-31 questionnaires have a lot of missing values among patients. Two questions are concerned about driving a car (category social function). Because most epileptic patients are not allowed to drive a car, a lot of them left these questions blank. Therefore, patients with more than two missing values in total were excluded for analysis of total T-scores. Still no significant differences in total scores of the remaining eight patients between start and one year were observed. Per category, scores of patients with missing values were excluded. Using paired-samples t-tests, a significant increase in social function scores over time was observed (M = 2.17; SEM = 0.60; t(5) = 3.606; p = 0.015). Repeated measures ANOVA tests were repeated and revealed time effects for seizure worry and social function that approximated significance (F = 4.928; p = 0.068 and F = 5.444; p = 0.080 respectively). A post-hoc test comparing scores after one year with scores at start showed a significant increase in social function scores over time in responders only (M = 2.2; SEM = 0.73; t(4) = 2.994; p = 0.040). It is important to remark that the non-responder group included only one patient for social function in this test.

3.9 Laryngeal Motor-Evoked Potentials

LMEPs were used to noninvasively measure the activation of motor fibers and so to confirm whether there was effective stimulation of the cervical vagus nerve. These LMEPs were only measured in five patients: R08 (1 year), R10 (1 year), R11 (start and 1 year), NR05 (1 year) and NC06 (start). In all patients LMEPs could be recorded at a certain stimulation intensity which verified that every patient's vagus nerve was stimulated effectively. By means of LMEP measurements, whether or not the plateau in the LMEP amplitude vs VNS intensity curve was already reached could be visualized. Our preliminary results indicate that with VNS parameters used at start of VNS therapy (0.25 or 0.5 mA) the plateau level of stimulation was not yet reached. For example, R11 (N.B. therapeutic pulse width used was 500 µs, while LMEPs were only recorded at a pulse width of 250 µs) and NC06 were stimulated at an intensity of 0.5 mA at the start and we can see in the curves that the plateau phase of stimulation is not yet reached

(See **Figure 14 c and e** respectively in **Appendix B**). In contrast, after one year of VNS therapy, the settings of the VNS device were higher in all patients. With these higher stimulation parameters and especially with higher output currents LMEP amplitudes were situated at the plateau level in the LMEP amplitude vs VNS intensity curve in all patients. After one year we can see that for R10 – stimulated at an intensity of 2.0 mA – and, NR05 and R11 – both stimulated at an intensity of 2.75 mA – the plateau of stimulation is already reached (See **Figure 14 b, d and f** respectively in **Appendix B**). R08 was only stimulated at an intensity of 0.75 mA (N.B. therapeutic pulse width used was 500 µs, while LMEPs were only recorded at a pulse width of 250 µs), but even at this lower stimulation intensity the plateau phase seems to be reached. (See **Figure 14 a** in **Appendix B**). In all patients, LMEPs were already recorded at low VNS output currents (0.25 – 0.625 mA) and increased until reaching a plateau at an output current of 0.5 – 0.75 mA (except for NC06 where no plateau was reached yet and for R10 where only around 1.5 mA a plateau was reached) (see **Figure 15** in **Appendix C**).

4. DISCUSSION

This study was unable to find a reliable noninvasive biomarker for response to VNS in patients with refractory epilepsy. Due to unequal group sizes (more responders than non-responders) and due to the small sample size in general, results need to be interpreted with caution.

As evidence suggests that **the P3b component of scalp recorded ERPs** indexes the neuromodulatory LC/NE system (78, 79), this component was measured during auditory oddball tasks. Results of the previously conducted retrospective study (44) which indicated that the increase in P3b amplitude during VNS ON could be a noninvasive biomarker for response to VNS could not be confirmed. No significant increase in P3b amplitude at Pz during VNS ON or tVNS was detected in a subgroup of VNS responders. ERP studies of Hammond *et al* (91) and Brázdil *et al (92)* in patients with refractory epilepsy also found no effect of VNS on P3b amplitude, but both studies did not distinguish between responders and non-responders and had – similar to our study – relative small sample sizes. In contrast to our results, ERP studies of De Taeye *et al (44)* and Neuhaus *et al* (93) showed an increase in P3b amplitude in VNS responders only. It is difficult to compare the results of the latter study with our results, as this study investigated patients treated for depression. As different clinical entities were treated, different cortical mechanisms may contribute to P3b properties post-VNS. Furthermore, effects of VNS on the P3b component were investigated by comparing measurements for OFF and ON conditions at different time points: prior to implantation and ten weeks after standard cycling VNS respectively. In addition, significant increases in amplitude were observed at midline electrodes Fz and Cz, whereas in our study P3b amplitude was investigated at midline electrode Pz (93). In our study, P3b amplitudes in non-responders

were higher compared to responders. A similar trend could be observed in the retrospective study of De Taeye *et al* (44). This difference in P3b amplitude between both groups at start suggests that responders and non-responders were different from the beginning. A plausible explanation for this difference may be the effect of AEDs (or other confounding factors, see third limitation of the study) that might suppress P3b amplitude in responders or increase P3b amplitude in non-responders. Higher P3b amplitudes observed in non-responders at start during all conditions might indicate that even during OFF conditions already higher NE concentrations are released by the LC during task processing, leading to less effective modulation of the LC during VNS ON and/or tVNS conditions in non-responders. However, in the present study no significant group x condition interaction effects on P3b amplitude could be observed neither at start nor after one year. The increase in P3b amplitude during VNS ON and tVNS conditions at start seemed to be more pronounced in non-responders, while this increase was absent during ON conditions in responders (see **Table 6** in **Appendix E**). At start, five non-responders were included of which one (NR06) did not receive tVNS. As in NR01 a very significant increase in P3b amplitude during VNS ON and tVNS conditions was observed, this may have had a major influence on the observed trend. After one year, an increase in amplitude during VNS ON conditions was observed in non-responders, whereas in responders an increase was observed during tVNS conditions (see **Table 7** in **Appendix F**). After one year, only two non-responders were included, making these results unreliable. Furthermore a model to predict seizure outcome based on absolute P3b amplitude at start was calculated: %_seizure_reduction = 47,077 - 6,464 * (P3b_ON_start - P3b_OFF_start) (p = 0,123). Based on an R-value of -0.416, 43 patients instead of 15 would be needed to observe a significant correlation between the effect of VNS on P3b amplitude at start and seizure reduction with a chance of 80.9%. Unless not significant, this model illustrates that higher increases in P3b amplitudes during VNS result in a less effective seizure reduction. This result is contradictory to results obtained in the retrospective study of De Taeye *et al (44)* in which significant increases in P3b amplitudes were found in responders only. Based on the hypothesis that activation of the LC and the concurrent increase in NE in the brain is mandatory for the anticonvulsive effects of VNS, it seems unlikely that higher increases in P3b amplitudes during VNS – reflecting a more efficient release of NE – would result in a less efficient suppression of seizures.

Only for reaction time a significant group x condition interaction effect could be established after one year of VNS therapy (F = 3.986; p = 0.043) with a decrease in reaction time (i.e. faster reaction times) in non-responders during VNS ON conditions and even more during tVNS conditions, whereas only a slight decrease in reaction time during ON conditions and a slight increase during tVNS conditions was observed in responders. However, the observed

decreases in reaction times during ON and tVNS conditions in non-responders did not reach significance. Because a correlation between P3b amplitude and reaction times was found (r = -0.233; p = 0.028), a model to predict reaction times based on P3b amplitudes was computed: RT = 633.79 – 6.95 * P3b_amplitude (p = 0.037). Similar to findings of Murphy *et al* (79), this model indicates that higher P3b amplitudes result in faster reaction times. As P3b amplitudes are believed to reflect phasic responses of the LC and as NE released in the cortex would promote rapid processing of sensory stimuli (94), this is a logic finding supporting the hypothesis that the P3b component can be used as a noninvasive marker to index the neuromodulatory LC/NE system. The significantly greater accuracy and reaction times during VNS ON conditions than during OFF conditions reported by De Taeye *et al* (44) could not be confirmed by our measurements.

As repeated measures ANOVA tests with group (R vs NR) as between-subject factor and condition (OFF, ON and tVNS) as within-subject factor only revealed significant effects of group on P3b amplitude (higher in non-responders), accuracy (higher in non-responders) and reaction time (faster in non-responders) at start, responders and non-responders were probably different before start of the VNS therapy. Based on lower performances of responders during auditory oddball tasks, it could be assumed that responders may have a lower IQ than non-responders. However, no significant differences in IQ between responders and non-responders were established (t(14) = 0.349; p = 0.732). Another explanation for the lower task performances in responders could be the age of the patients. It is possible that older patients may perform less good during auditory oddball tasks than younger patients. Comparing the age between the responder (M = 50 years; SEM = 5 years) and non-responder (M = 37 years; SEM = 5 years) group showed no significant differences (t(15) = 1.715; p = 0.107). We can see a trend of higher ages in the responder group, but with the low number of patients in both groups (6 NR and 11 R) it is difficult to suggest that ages among both groups are different. So, significantly higher P3b amplitudes, greater accuracy and faster reaction times were observed in non-responders at start of VNS therapy. Hence, various potential confounding factors may have contributed to these significant differences between responders and non-responders (see further).

For **pupil size** a group x condition interaction effect almost reached significance (F = 4.750; p = 0.061) after one year of VNS therapy indicating a pupil dilatation in responders and a constriction in non-responders during VNS ON conditions. In addition, a model to predict the percentage seizure reduction based on the effect of VNS on pupil size after one year was computed: %_seizure_reduction = 44.81+ 6.068 * (pupil_size_ON_1y – pupil_size_OFF_1y) (p = 0.052). Based on an R-value of 0.628, 17 patients instead of 10 need to be tested in order to observe a significant correlation between effect of VNS on pupil size after one year and

seizure reduction with a power of 81.3%. From the model we can conclude that after one year of VNS therapy pupil dilatations during VNS ON tend to be related to better outcomes (i.e. higher percentage seizure reduction) which supports the hypothesis that a dilated pupil would be observed in the ON condition as result of a shift in autonomic balance due to VNS (83). However, this relation between seizure reduction and pupil size was not seen at start of VNS therapy and also none of these results were obtained during tVNS conditions. Many factors including medication; luminance; stress and coffee can influence pupil diameter. During pupillary measurements, we controlled as much as possible for potential confounding factors: the room was darkened with only low ambient lighting and pupil size in the VNS ON or tVNS condition was measured immediately after the OFF recordings. No control of medication was possible and therefore, some types (e.g. medication that interacts with the NE system) could decrease the difference in pupil size between OFF and ON or tVNS conditions. In 2015, a research group (83) investigated the effect of VNS on pupil diameter in 14 patients with epilepsy and 7 patients with major depression and found a significant increase in pupil diameter during VNS ON compared to OFF. In this study, potential confounding factors that could affect pupillary measurements were more controlled compared to our study: Patients who took medications that could affect pupil diameters were excluded; patients were asked to refrain from smoking and refrain from drinking coffee three hours before testing. A possible explanation why no significant differences in pupil size between OFF and VNS ON conditions could be obtained in our study is the small sample size (only ten patients after one year of VNS therapy as compared to 21 patients in the study of Desbeaumes Jodoin *et al (83))*. A limitation of the latter study is the lack of categorization of patients as responders and non-responders and the absence of the criteria to only include patients with a similar duration of VNS therapy. Although we were not able to detect significant differences between the stimulation conditions, our results indicated a relation between the percentage of seizure reduction and pupil size. Desbeaumes Jodoin *et al* (83) were unable to find such a relation. Furthermore, in our study rapid cycling was used for VNS whereas in the other study a continuous cyclic pattern of 30 s ON and 3-5 min OFF was used.

In this study, there was **no correlation between pupil size and P3b amplitude** ($r = -0.257$; $p = 0.66$). The LC has two types of firing modes: tonic and phasic. The P3b may index the phasic LC mode, while a large pupil diameter appears to be associated with a high tonic LC activity (79). Depending on which mode of LC activity is facilitated the most by VNS, P3b or pupillary measurements will be affected more. This can explain the fact that no correlation between P3b amplitude and pupil size could be established. As the retrospective study of De Taeye *et al (44)* showed a significant increase in P3b amplitude during ON conditions in responders only, VNS might facilitate for the most part a phasic LC response.

In this study, no significant changes in **BDI-II** and total **QOLIE-31** scores over time could be established. These results are in line with findings of Hoppe *et al* (70) who reported that more complex and stable emotional states including depression (assessed by BDI-II) and health-related quality of life (assessed by QOLIE) appeared unchanged. By contrast, Harden *et al* (72) reported a significant decrease in depression severity over time and Cramer *et al* (73) demonstrated by use of QOLIE-10 questionnaires significant improvements regarding energy level; memory difficulties; social aspects; mental effects and fear of seizures in patients with refractory epilepsy. Our study only reproduced the significant improvement regarding fear of seizures ('seizure worry') ($M = 3.91$; $SEM = 1.63$; $t(10) = 2.396$; $p = 0.038$). After exclusion of patients with missing values per domain, a significant improvement regarding social aspects could be observed over time ($M = 2.17$; $SEM = 0.60$; $t(5) = 3.606$; $p = 0.015$). In the studies of Harden *et al* (72) and Cramer *et al* (73), more patients (20 and 136 patients respectively) were included as compared to our study (11 for BDI-II and 10 for QOLIE-31) and questionnaires were taken at baseline and after three months of VNS therapy. However, improvement was only observed three months after initiation of VNS. Due to this short period between the two questionnaires, it is possible that patients (including non-responders) still had more expectations regarding good outcomes of their VNS therapy. Therefore these studies cannot exclude that mood improvements were only temporary because of initiation of a new therapy and no information about durability of these improvements could be provided. **STAI-DY** questionnaires consist of two subscales: version DY1 measuring state anxiety and version DY2 measuring trait anxiety. No significant changes over time could be established for both questionnaires. However, a significant group x time interaction effect was established for state anxiety scales (DY1) ($F = 5.448$: $p = 0.043$). Only in responders a significant increase in scores over time was found ($t(7) = 2.792$; $p = 0.027$), while the decrease over time in non-responders did not reach significance. As higher scores indicate more anxiety, this is an unexpected finding. On the other hand, state scores (DY1) explore how patients feel at a certain moment in time (in the current study when patients were hospitalized for video-EEG monitoring two weeks after surgery and a second time after one year). A possible explanation could be that responders see no need to come to the hospital and therefore state anxiety is higher. Because of missing values in STAI-DY and QOLIE-31 questionnaires, results before exclusion of patients with missing values need to be interpreted with caution.

A major limitation of this study is the size of the study population. Only ten patients fully completed the study. Their data were analyzed comparing the effect of VNS or tVNS with OFF conditions on P3b components and pupil size for responders and non-responders in both acute and chronic situations. When only changes in P3b amplitude or pupil size during VNS ON and tVNS conditions at start were investigated, 22 patients could be included for the analyses. It

could however be possible that stimulation parameters during acute conditions were too low to observe any effect (see further). This study is still ongoing and new patients implanted with a VNS device in addition to patients who still have to come back for the second test-moment after one year of VNS treatment will be tested. This will increase sample size and improve the power of the analyses. Because of the limited sample size at this moment, results of this study must be interpreted with caution.

A second major limitation of this study is the determination of percentage seizure reduction. Unless patients were asked to write episodes down in a seizure calendar, a lot of them had not done this accurately. Furthermore, evaluation reports by the treating physician in the electronic patient file did not always contain the number of episodes a patient experienced. For many patients it is difficult to keep track of their seizures, for example because they do not realize that they had a seizure or because they forgot to write down that an episode had passed. Due to this fact, no exact determination of seizure reduction rates was possible, but only an estimation. This made it difficult to determine the effect of VNS on seizure frequency and to conclude whether a patient was a responder to VNS or not. In the future, it is important to underline the importance of noting down their seizures as accurately as possible and inform relatives of the patients to help them doing this.

A third limitation and possible explanation for failure to differentiate between responders and non-responders based on P3b characteristics or pupil size can be found in the huge variation in patient population and various potential confounding factors such as AEDs; age of onset; type of epilepsy; type and frequency of seizures; duration of epilepsy; brain lesions; side and localization of the hypothesized epileptogenic zone. In contrast, in the retrospective study of De Taeye *et al (44)* there was a very stringent selection of patients. Responders and non-responders were compared while controlling as much as possible for potential confounding factors stemming from the heterogeneous clinical parameters and the AEDs: both groups took a comparable range of AEDs, none of the patients' AEDs were tapered after they became VNS responders and no reliable modulation of the latency of the P3b component (which could be a consequence of different AEDs) was found depending on the experimental condition. In our study there was no control of AEDs and these AEDs could differ between the test-moment at start and after one year of VNS treatment. Effects on P3b and pupil could be misinterpreted as related to VNS therapy while in fact they were caused by a change in AEDs. Furthermore, in the study of De Taeye *et al* (44) only patients who received VNS therapy for at least 18 months were included. This chronic stimulation presumably led to long-term changes in the neuronal networks and neurotransmitter systems (44, 95). It is possible that these P3b effects cannot be observed in epilepsy patients at start of VNS therapy or after only one year of stimulation. In the future, it may be useful to test more patients and separate these patients in different

groups for analyses depending on certain confounding factors in order to create as homogeneous groups as possible with both responders and non-responders (e.g. groups with the same epilepsy type; groups with a similar age of onset or a similar duration of their epilepsy and who take a similar range of AEDs). It is possible that P3b components and/or pupil size can only be used as biomarkers in certain subgroups of patients.

A fourth possible explanation for the fact that no effect on electrophysiological, behavioral and pupil size measurements were observed in the acute phase of stimulation (start) between OFF and VNS ON conditions could be that the patients were stimulated too low. By means of LMEP measurements, whether or not the plateau in the amplitude vs intensity curve was already reached could be visualized. Because this plateau was not reached in two tested patients at start, these patients were probably stimulated too low at start of their VNS therapy to observe any effects of VNS on reaction times and accuracy during auditory oddball tasks, amplitude and latency of P3b components or pupil size. Although a plateau was reached in all four tested patients after one year of stimulation, still no significant effects of VNS on electrophysiological, behavioral and pupil size measurements were observed. Similar to a clinical pilot trial of Grimonprez *et al (87)*, LMEPs (reflecting Aα-fiber activation) were already recorded at low VNS output currents (0.25 and 0.625 mA) and increased until reaching a plateau at a VNS intensity of 0.5 – 1.5 mA. The VNS intensity required to achieve a therapeutic effect may be underestimated by LMEP recordings as these record the activity of low threshold Aα-fibers, while therapeutic effects of VNS are believed to be mediated by higher threshold A- and B-fibers (87). In future, LMEPs will be measured in more patients at start and after one year of stimulation. These LMEPs need to be studied and analyzed in more detail to find out if and how they can be used in order to optimize individual stimulation parameters. As adequate activation is also observed in non-responders (NR05), other explanations than ineffective VNS, such as heterogeneity in underlying pathophysiological mechanisms or variability in characteristics of more central structures in neural pathways involved – for example, genetic differences in neurotransmitter systems - need to be further investigated (87).

A fifth limitation is that the P3b component of ERPs and pupil size are only indirect measures of LC/NE activity, and other confounding factors could influence these measurements. Therefore, similar to the retrospective study of De Taeye *et al* (44) OFF, ON and tVNS conditions were compared within the same patient and absolute and relative amplitude or pupil size differences were calculated in order to control as much as possible for these factors.

As VNS is a chronic treatment and response to VNS increases with longer duration of the treatment, activation of brainstem structures might outlast the stimulation train. Therefore it is possible that when switching the VNS OFF 20 minutes before each measurement a residual

effect of stimulation was still present and may have reduced some of the differences between the ON, tVNS and OFF conditions (83).

A final limitation of the study is the MOA of VNS itself. The vagus nerve has widespread projections to nuclei in the brainstem and to all cortical regions, as well as to the thalamus, hippocampus and amygdala, modulating activity of various target cells and networks. Furthermore, strong reciprocal connections between different neuromodulatory systems (noradrenaline; dopamine; serotonin and acetylcholine) make it difficult to delineate the role of one single system. As a result, the LC/NE system does not act alone. Other modulatory neurotransmitter pathways interact with this neuromodulatory system and could thus play a contributive role in the antiepileptic effect of VNS (44).

Murphy *et al* (79) showed that P3 components significantly decrease with time-on-task which is consistent with findings in monkeys that phasic LC responses diminish with prolonged task performance (96). Because P3 components were measured during three conditions in our study, patients were on the task for a long time. We tried to control for these time-on-task effects on P3b amplitude as much as possible by use of six randomization arms with the three stimulation conditions following in different orders (see Randomization order in **Table 4** in **Appendix A**).

5. CONCLUSION

Our results were not able to indicate P3b amplitude or pupil size modulations as noninvasive biomarkers to predict response to VNS in patients with refractory epilepsy as no significant modulations during VNS ON conditions were observed at the start of VNS therapy. However, findings on pupil size after one year, indicating a correlation between pupil dilatations and better seizure outcome, will possibly reach more significance when more patients are included for analysis. Because a trend toward pupil dilatations was observed only in responders both at start and after one year, these results are in favor of the hypothesis that VNS induced activation of the LC/NE system is associated with the therapeutic response to VNS in patients with refractory epilepsy. Pupillary measurements may be faster and more easy to perform than P3b measurements. Therefore, these measurements may be used in the future as a potential biomarker to optimize stimulation parameters in patients already treated with VNS if these measurements cannot be used before implantation of a VNS device to predict a patient's response to VNS. After one year of VNS therapy, still no significant modulation of the P3b amplitude was found. Thus, results of the retrospective study on ERPs (44) showing an increase in P3b amplitude during VNS ON in responders to VNS only, could not be reproduced. Even if a different modulation during VNS ON conditions compared to OFF conditions had been observed, these modulations would only have significance as a potential biomarker if a

noninvasive analog of VNS, such as tVNS, is able to modulate these measurements in a similar way. Finding a biomarker for efficacy of VNS is important in order to avoid unnecessary costs of implantation of a VNS device and risks of unnecessary surgery in non-responders (53). The major limitation of this study was the small size of the study population. Therefore, further research including more patients to increase sample size and improve the power of the analyses is needed to investigate the utility of P3b amplitude and pupil size as prospective measurements to predict the therapeutic efficacy of VNS.

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Appendix A

Table 4: Patient characteristics.

Patient	Sex	Age (years)	Age (years) at implantation	% seizure reduction	Randomization order		VNS parameters			tVNS output (mA)
							output (mA)	frequency (Hz)	pulse width (µs)	
Not categorized										
NC01	F	56	52	/	ON-tVNS-OFF	Start	0,25	30	500	0,6
NC02	F	25	24	/	tVNS-ON-OFF	Start	0,25	30	500	1,6
NC03	M	32	31	/	OFF-ON-tVNS	Start	0,25	30	250	0,1
NC04	M	42	41	/	tVNS-OFF-ON	Start	0,5	30	250	0,3
NC05	F	29	28	/	ON-OFF-tVNS	Start	0,25	30	250	0,6
NC06	F	59	59	/	tVNS-OFF-ON	Start	0,5	30	500	1
NC07	M	33	33	/	OFF-ON-tVNS	Start	0,25	30	500	0,5
Non-responders										
NR01	M	22	18	0	tVNS-ON-OFF	Start 1 year	0,25 2,25	30 30	500 500	1,4 0,4
NR02	M	28	24	0	ON-OFF-tVNS	Start	0,50	30	500	0,6
NR03	M	43	40	0	tVNS-OFF-ON	Start 1 year	0,25 2,25	30 30	500 500	0,4 0,4
NR04	F	58	54	0	OFF-ON-tVNS	Start	0,25	30	500	0,3
NR05	M	38	36	0	ON-tVNS-OFF	Start 1 year	0,25 2,75	30 30	500 500	0,3 0,4
NR06	M	30	28	0	ON-OFF-tVNS	Start	0,25	30	500	/
Responders										
R01	M	55	51	50,00	OFF-tVNS-ON	Start 1 year	0,25 1,75	30 25	500 500	0,4 0,3
R02	F	58	54	60,00	OFF-ON-tVNS	Start	0,25	30	500	0,4
R03	F	61	57	70,00	ON-tVNS-OFF	Start 1 year	0,5 2,25	30 25	500 500	0,5 0,3
R04	F	62	59	77,14	tVNS-OFF-ON	Start 1 year	0,25 1,25	30 15	500 250	0,9 0,5
R05	M	31	29	92,31	ON-OFF-tVNS	Start 1 year	0,25 1,75	30 30	500 300	0,3 0,8
R06	F	21	19	81,67	tVNS-ON-OFF	Start 1 year	0,25 1,5	30 20	500 500	0,3 0,4
R07	F	65	63	62,50	OFF-tVNS-ON	Start 1 year	0,25 0,75	30 30	500 500	0,4 0,5
R08	M	48	46	70,00	OFF-ON-tVNS	Start 1 year	0,25 0,75	30 20	500 500	0,3 /
R09	F	47	45	50,00	ON-OFF-tVNS	Start	0,25	30	500	0,9
R10	M	70	69	50,00	ON-tVNS-OFF	Start 1 year	0,5 2	30 30	500 500	0,5 0,3
R11	F	29	28	80,00	OFF-ON-tVNS	Start 1 year	0,5 2,75	30 30	500 500	0,4 0,4

This table summarizes the main clinical characteristics of the patients, as well as VNS parameters and randomization order used during auditory oddball tasks. VNS : vagus nerve stimulation; tVNS : transcutaneous vagus nerve stimulation; NC : Not Categorized; R : responder; NR : non-responder; M : male; F : female.

Appendix B

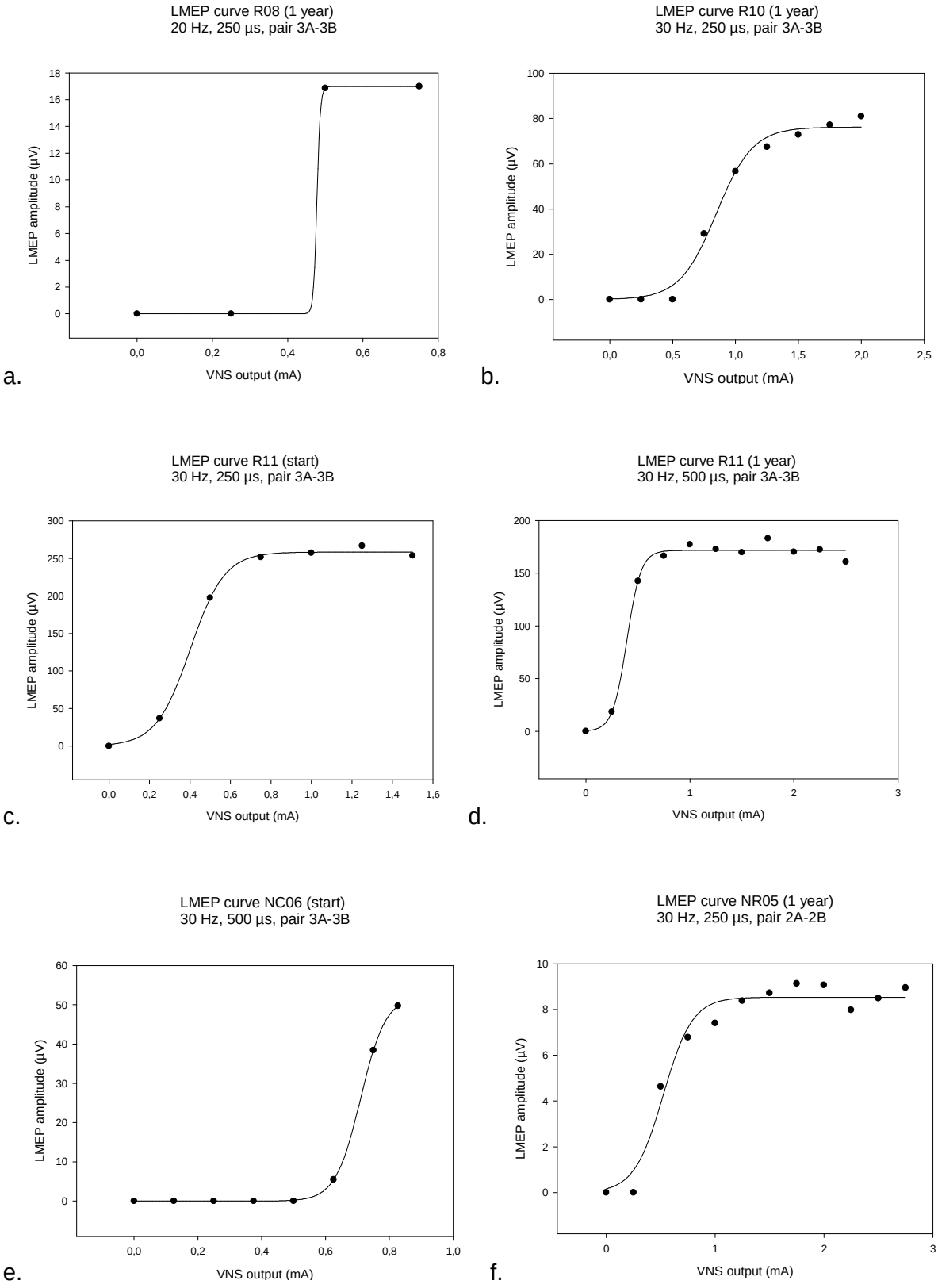

Figure 14: Amplitude vs intensity curves. LMEP amplitudes (µV) are plotted against VNS output currents (mA).

a. LMEP amplitude vs VNS intensity curve for R08 measured after one year (frequency: 20 Hz, pulse width: 250 µs, rapid cycling: 7 s ON / 18 s OFF): $y = \frac{16{,}9800}{1+e^{-\frac{(x-0{,}4779)}{0{,}0045}}}$.

b. LMEP amplitude vs VNS intensity curve for R10 measured after one year (frequency: 30 Hz, pulse width: 250 µs, rapid cycling: 7 s ON / 18 s OFF): $y = \frac{76{,}1981}{1+e^{-\frac{(x-0{,}8474)}{0{,}1445}}}$.

c. LMEP amplitude vs VNS intensity curve for R11 measured at start (frequency: 30 Hz, pulse width: 250 µs, rapid cycling: 7 s ON / 18 s OFF): $y = \frac{258{,}3833}{1+e^{-\frac{(x-0{,}4016)}{0{,}0844}}}$.

d. LMEP amplitude vs VNS intensity curve for R11 measured after one year (frequency: 30 Hz, pulse width: 500 µs, rapid cycling: 7 s ON / 18 s OFF): $y = \frac{171{,}7135}{1+e^{-\frac{(x-0{,}3930)}{0{,}0679}}}$.

e. LMEP amplitude vs VNS intensity curve for NC06 measured after at start (frequency: 30 Hz, pulse width: 500 µs, rapid cycling: 7 s ON / 18 s OFF): $y = \frac{52{,}1834}{1+e^{-\frac{(x-0{,}7096)}{0{,}0393}}}$.

f. LMEP amplitude vs VNS intensity curve for NR05 measured after one year (frequency: 30 Hz, pulse width: 250 µs, rapid cycling: 7 s ON / 18 s OFF): $y = \frac{8{,}5352}{1+e^{-\frac{(x-0{,}5236)}{0{,}1337}}}$.

Appendix C

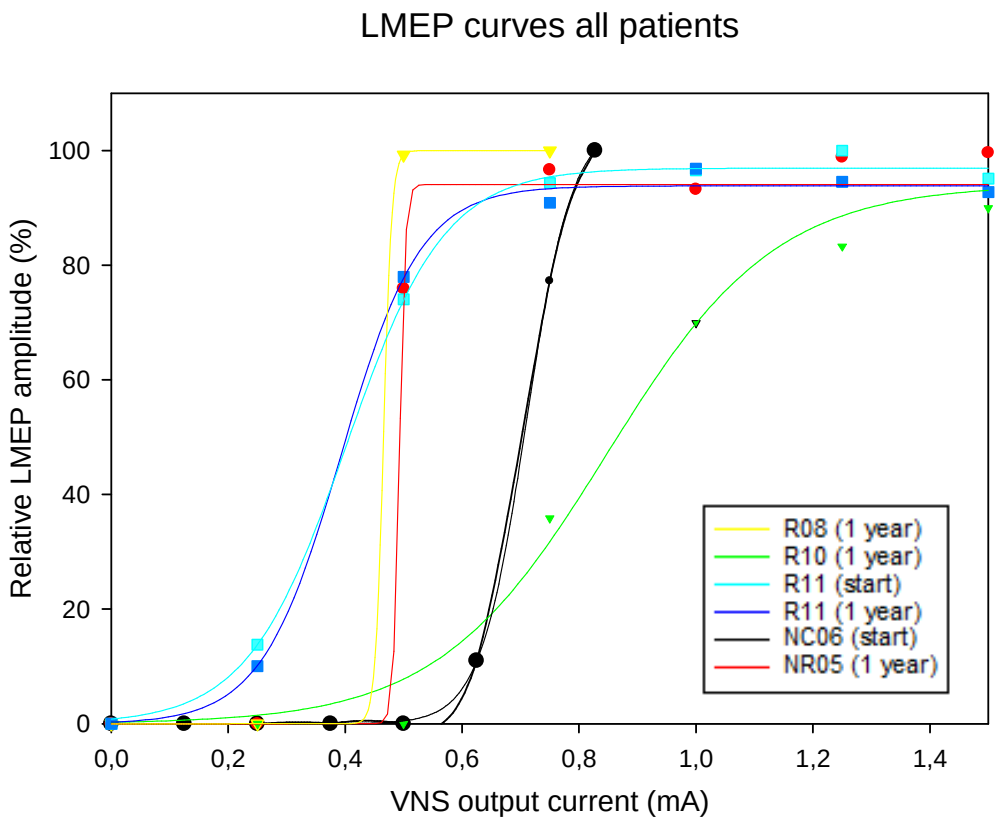

Figure 15: LMEP amplitude vs VNS intensity curves for all patients. Relative LMEP amplitudes (%) are plotted against VNS output currents (mA) for all patients. Relative LMEP amplitudes are calculated by dividing LMEP amplitudes (µV) measured for each VNS output current by the maximal LMEP amplitude (µV) for each individual patient.

Table 5: P3b amplitudes per patient at start and after one year of VNS therapy.

Patient	P3b amplitude start (µV)			Absolute difference in P3b amplitude start (µV)		P3b amplitude 1 year (µV)			Absolute difference in P3b amplitude 1 year (µV)	
	OFF	ON	tVNS	ON-OFF	tVNS-OFF	OFF	ON	tVNS	ON-OFF	tVNS-OFF
Not categorized										
NC01	6,80	6,04	6,74	-0,76	-0,06	/	/	/	/	/
NC02	3,85	6,17	6,31	2,32	2,46	/	/	/	/	/
NC03	6,26	6,86	4,67	0,60	-1,59	/	/	/	/	/
NC04	2,66	4,88	5,25	2,21	2,58	/	/	/	/	/
NC05	10,79	13,44	10,76	2,65	-0,03	/	/	/	/	/
NC06	11,37	9,16	8,21	-2,21	-3,16	/	/	/	/	/
NC07	4,46	5,25	2,39	0,79	-2,07	/	/	/	/	/
Non-responders										
NR01	4,65	12,45	11,37	7,79	6,71	11,91	15,92	13,51	4,01	1,60
NR02	4,79	4,71	2,50	-0,08	-2,29	/	/	/	/	/
NR04	7,58	6,56	8,74	-1,02	1,16	/	/	/	/	/
NR05	6,97	6,49	6,54	-0,48	-0,43	1,87	1,18	-0,10	-0,69	-1,96
NR06	10,16	12,49	/	2,33	/	/	/	/	/	/
Responders										
R01	4,11	3,42	4,81	-0,69	0,71	2,77	2,23	3,09	-0,55	0,32
R02	5,74	5,67	5,06	-0,07	-0,68	/	/	/	/	/
R03	1,43	3,10	3,17	1,66	1,73	3,68	3,09	4,23	-0,59	0,54
R04	6,41	4,77	5,53	-1,64	-0,88	7,11	6,97	7,28	-0,14	0,17
R05	2,96	2,89	3,20	-0,07	0,24	0,89	3,50	1,82	2,61	0,93
R06	6,53	7,02	9,03	0,49	2,50	7,00	9,33	11,93	2,33	4,93
R08	2,86	4,15	4,21	1,28	1,35	5,51	4,11	/	-1,39	/
R09	5,02	3,53	5,41	-1,49	0,39	/	/	/	/	/
R10	0,36	0,91	1,43	0,55	1,07	4,52	4,52	4,41	0,00	-0,11
R11	6,14	4,51	/	-1,62	/	4,72	0,79	4,73	-3,93	0,01

This table shows the P3b amplitudes in µV for each individual patient in the different categories (NC : not categorized; NR : non-responders and R : responders) during the different stimulation conditions (OFF; ON and tVNS). In addition, absolute differences between P3b amplitudes during ON and OFF or during tVNS and OFF conditions are shown.

Table 6: Mean values for P3b amplitude, P3b latency, accuracy and reaction time at start, including statistics.

START									
Dependent variable	Responders			Non-responders			Statistics		
	OFF	ON	tVNS	OFF	ON	tVNS	Group df=1	Condition df=2	Group*condition df=2
Amplitude (µV)	3,94 ± 2,19	3,94 ± 1,75	4,65 ± 2,11	6,00 ± 1,50	7,55 ± 3,37	7,29 ± 3,75	F=5,116 p=0,045	F=1,503 p=0,245	F=0,836 p=0,447
Latency (ms)	416,67 ± 112,94	450,20 ± 144,64	437,17 ± 130,08	395,02 ± 53,87	369,63 ± 23,94	359,38 ± 32,40	F=1,136 p=0,309	F=0,066 p=0,936	F=0,523 p=0,600
Accuracy (%)	95,26 ± 4,96	96,04 ± 3,29	96,30 ± 2,68	99,43 ± 0,57	99,18 ± 0,65	98,62 ± 1,32	F=4,695 p=0,049	F=0,057 p=0,945	F=0,681 p=0,515
Reaction time (ms)	668,75 ± 87,57	668,16 ± 106,04	650 ± 72,58	549 ± 37,73	515,90 ± 41,36	519,76 ± 26,34	F=13,708 p=0,003	F=0,856 p=0,436	F=0,391 p=0,681

This table summarizes the mean values and standard deviations of P3b amplitude, P3b latency, accuracy and reaction time during the different stimulation conditions in responders and non-responders at start. Furthermore, F- and p-values of repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (OFF, ON and tVNS) as within-subject factor are shown. Significant effects are marked in green.

Table 7: Mean values for P3b amplitude, P3b latency, accuracy and reaction time after one year, including statistics.

1 YEAR									
Dependent variable	Responders			Non-responders			Statistics		
	OFF	ON	tVNS	OFF	ON	tVNS	Group df=1	Condition df=2	Group*condition df=2
Amplitude (µV)	4,39 ± 2,22	4,35 ± 2,92	5,35 ± 3,35	6,89 ± 7,10	8,55 ± 10,43	6,71 ± 9,62	F=0,635 p=0,452	F=0,503 p=0,615	F=1,574 p=0,242
Latency (ms)	504,74 ± 215,15	425,64 ± 109,66	486,47 ± 205,14	390,14 ± 44,88	363,28 ± 2,76	359,86 ± 20,03	F=0,657 p=0,444	F=0,642 p=0,541	F=0,266 p=0,770
Accuracy (%)	96,73 ± 4,91	98,07 ± 2,00	97,84 ± 1,95	99,43 ± 0,0	98,44 ± 1,81	99,20 ± 0,39	F=0,785 p=0,405	F=0,114 p=0,893	F=0,767 p=0,483
Reaction time (ms)	613,40 ± 124,14	586,00 ± 94,17	624,93 ± 96,27	634,71 ± 109,29	571,19 ± 52,73	520,29 ± 21,15	F=0,267 p=0,621	F=3,000 p=0,082	F=3,986 p=0,043

This table summarizes the mean values and standard deviations of P3b amplitude, P3b latency, accuracy and reaction time during the different stimulation conditions in responders and non-responders after one year. Furthermore, F- and p-values of repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (OFF, ON and tVNS) as within-subject factor are shown. Significant effects are marked in green.

Table 8: Pupil sizes.

Patient	Pupil size start (mm^2)				Absolute difference in pupil size start (mm^2)		Pupil size 1 year (mm^2)				Absolute difference in pupil size 1 year (mm^2)	
	OFF	ON	tOFF	tVNS	ON-OFF	tVNS-tOFF	OFF	ON	tOFF	tVNS	ON-OFF	tVNS-tOFF
Not categorized												
NC02	/	/	16,52	17,04	/	0,52	/	/	/	/	/	/
NC03	21,51	29,75	20,56	20,14	8,24	-0,42	/	/	/	/	/	/
NC04	21,77	21,39	28,18	21,95	-0,38	-6,22	/	/	/	/	/	/
NC06	11,70	8,28	25,63	25,84	-3,42	0,21	/	/	/	/	/	/
NC07	/	/	12,55	17,93	/	5,38	/	/	/	/	/	/
Non-responders												
NR01	7,09	4,69	4,36	4,32	0,14	-0,04	30,13	26,09	14,43	8,80	-4,04	-5,63
NR02	8,69	7,50	9,21	6,26	-1,04	-2,95	/	/	/	/	/	/
NR03	3,44	3,58	2,21	1,91	0,54	-0,29	3,88	4,60	3,63	2,98	0,71	-0,65
NR04	8,86	9,48	8,49	9,53	0,62	1,05	/	/	/	/	/	/
NR05	4,17	5,26	9,54	11,10	1,08	1,56	17,92	12,67	16,63	18,23	-5,26	1,60
NR06	6,08	6,38	/	/	0,30	/	/	/	/	/	/	/
Responders												
R01	1,98	1,20	1,63	1,60	-0,44	-0,03	5,12	7,46	1,26	1,75	2,34	0,50
R02	26,97	27,43	23,03	23,87	0,22	0,84	/	/	/	/		
R03	6,74	7,92	8,46	7,73	1,19	-0,73	2,89	2,74	4,85	4,48	-0,15	-0,37
R04	5,21	5,90	3,25	3,44	0,69	0,18	1,93	2,08	3,81	4,14	0,16	0,33
R05	3,99	3,34	4,64	2,95	-0,65	-1,69	7,31	12,15	12,51	10,09	4,83	-2,41
R06	10,69	10,81	11,61	11,76	0,12	0,14	41,18	39,36	30,85	29,44	-1,82	-1,40
R07	3,30	2,17	3,07	2,96	-1,13	-0,12	/	/	31,08	38,91	/	7,83
R08	7,15	8,12	6,59	6,46	0,97	-0,13	/	/	/	/	/	/
R09	25,24	24,48	25,64	28,59	-0,75	2,95	/	/	/	/	/	/
R10	/	/	/	/	/	/	9,85	11,60	6,45	5,54	1,74	-0,90
R11	26,74	36,19	27,36	30,57	9,45	3,20	23,27	31,64	20,14	23,05	8,38	2,91

This table shows measured pupil sizes per patient during different stimulation conditions (OFF, ON, tOFF and tVNS) in responders and non-responders at start and after one year. Furthermore, absolute differences in pupil sizes between ON and OFF or between tVNS and OFF are shown. tVNS : transcutaneous vagus nerve stimulation; tOFF : OFF condition measured during tVNS pupillometry; NC : not categorized; R : responder; NR : non-responder

Appendix H

Table 9: Summary of mean values for pupil size measurements at start, including statistics.

START							
Dependent variable	Responders		Non-responders		Statistics		
	OFF	ON	OFF	ON	Group df=1	Condition df=1	Group*condition df=1
Pupil size (mm²)	11,80 ± 10,30	12,76 ± 12,16	6,39 ± 2,26	6,15 ± 2,12	F=1,657 p=0,219	F=0,285 p=0,602	F=0,796 p=0,387
	tOFF	tVNS	tOFF	tVNS			
Pupil size (mm²)	11,53 ± 10,01	11,99 ± 11,33	6,76 ± 3,29	6,63 ± 3,75	F=1,040 p=0,326	F=0,139 p=0,715	F=0,465 p=0,507

This table summarizes the mean values and standard deviations of pupil size during the different stimulation conditions in responders and non-responders at start. Furthermore, F- and p-values of repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (ON vs OFF and tVNS vs tOFF) as within-subject factor are shown. tOFF : OFF condition measured during tVNS pupillometry.

Table 10: Summary of mean values for pupil sizes measurements after one year, including statistics.

1 YEAR							
Dependent variable	Responders		Non-responders		Statistics		
	OFF	ON	OFF	ON	Group df=1	Condition df=1	Group*condition df=1
Pupil size (mm²)	13,08 ± 14,31	15,29 ± 14,51	17,31 ± 13,13	14,45 ± 10,86	F=0,032 p=0,863	F=0,078 p=0,787	F=4,750 p=0,061
	tOFF	tVNS	tOFF	tVNS			
Pupil size (mm²)	13,87 ± 12,09	14,68 ± 13,95	11,56 ± 6,96	10,01 ± 7,70	F=0,187 p=0,675	F=0,109 p=0,748	F=1,090 p=0,324

This table summarizes the mean values and standard deviations of pupil size during the different stimulation conditions in responders and non-responders after one year. Furthermore, F- and p-values of repeated measures ANOVA with group (R vs NR) as between-subject factor and condition (ON vs OFF and tVNS vs tOFF) as within-subject factor are shown. A group x condition interaction effect on pupil size after one year during VNS pupillometry approximated significance and is marked in red. tOFF : OFF condition measured during tVNS pupillometry.