Moving beyond belief:

A narrative review of potential biomarkers for transcutaneous vagus nerve stimulation

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Abstract

Transcutaneous vagus nerve stimulation or tVNS is a non-invasive neurostimulation technique that is currently being tested as a potential treatment for a myriad of neurological and psychiatric disorders. However, the working mechanisms underlying tVNS are poorly understood, and it remains unclear whether stimulation activates the vagus nerve for every participant. Finding a biological marker of tVNS is imperative, as it can help guide research on clinical applications, and can inform researchers on optimal stimulation sites and parameters to further optimize treatment efficacy. In this narrative review, we discuss five potential biomarkers for tVNS, and review currently available evidence for these markers for both invasive and transcutaneous VNS. While some of these biomarkers hold promise from a theoretical perspective, none of the potential biomarkers provide clear and definitive indications that tVNS increases vagal activity or augments activity in the locus coeruleus-noradrenaline network. We conclude the review by providing several recommendations for how to tackle the challenges and opportunities when researching potential biomarkers for the effects of tVNS.

KEYWORDS: transcutaneous vagus nerve stimulation; biomarker; heart rate variability; vagal somatosensory evoked potential; pupil size; P300; salivary alpha-amylase; noradrenaline; locus coeruleus

1. Introduction

Vagus nerve stimulation (VNS) is a neurostimulation technique that has received renewed attention in recent years. During invasive VNS, the vagus nerve is electrically stimulated by two electrodes that have been surgically implanted and sutured around the vagus nerve at the level of the neck. This technique was first described in humans by Penry and Dean (Penry & Dean, 1990) and has mainly been used to treat treatment-resistant epilepsy and treatment-resistant depression, although potential broader cognitive effects of this technique have also garnered attention (Vonck et al., 2014). Due to the invasiveness of the procedure as well as its accompanied health risks and medical costs, VNS is not a commonly used intervention. However, with the invention of non-invasive devices that stimulate areas of the skin which are either directly innervated by the vagus nerve (e.g. the ear) or that lie in close proximity to it (e.g. the neck), transcutaneous vagus nerve stimulation (tVNS) is now being studied and marketed for the treatment of depression, anxiety, headache, epilepsy, diabetes, pain, and heart failure (Groves & Brown, 2005). It is still unclear how tVNS could achieve such a myriad of beneficial effects. Although for some conditions the proposed working mechanism is the increased vagal activity itself (Gidron, Deschepper, De Couck, Thayer, & Velkeniers, 2018), alternative potential working mechanisms that have been suggested include modulation of the locus coeruleus-noradrenaline (LC-NA) network, increased neuronal plasticity and neurogenesis, and increase of GABAergic activity (Grimonprez, Raedt, Baeken, Boon, & Vonck, 2015). Crucially however, and contrary to traditional invasive VNS, is that it remains unclear whether tVNS is successful at activating the vagus nerve specifically (rather than other nerves that lie in close proximity of the stimulation area). Thus, there is a clear need for a biological marker that can be used as an indication of whether the vagus nerve is actually being stimulated.

Ideally, a biological marker for tVNS can provide information on the extent to which the vagus nerve is actually being stimulated, or could provide indications on whether the stimulation affected biological processes related to the presumed working mechanisms underlying the effects of tVNS. In

such a way, a biological marker would greatly elucidate at least some of the processes that are now shrouded within the 'vagal black box'. Currently, tVNS research is predominantly focused on documenting potentially beneficial effects of stimulating the vagus nerve on specific aspects of behavior, cognition or emotion. Without a reliable biomarker, results from these studies will be significantly less informative. When these studies do not find an effect of tVNS, it is unclear whether this is due to a failure to activate the vagus nerve, or whether successful stimulation of the vagus nerve simply does not affect the process under scrutiny (relative to an active control condition). Similarly, when studies do find an effect of tVNS, the absence of a biomarker that may inform researchers about the underlying working mechanisms hampers conclusions and follow-up research. In this review we will provide readers with an overview of possible biomarkers and the evidence base for their use.

1.1 The rationale for non-invasive vagus nerve stimulation

The vagus nerve is the tenth cranial nerve and is the largest autonomic nerve of the body, innervating most of the peripheral organs. The vagus nerve is mainly known for its parasympathetic control over the heart, lungs and its role in the cholinergic anti-inflammatory pathway. However, approximately 80% of the fibers of the vagus nerve are afferent fibers that transfer sensory information from peripheral organs to the brainstem, a process which is neither parasympathetic nor sympathetic (Yuan & Silberstein, 2016). It is this afferent pathway of the vagus that is being targeted during non-invasive vagus nerve stimulation

The auricular branch of the vagus nerve (ABVN) – the target for most tVNS interventions – appears to be a phylogenic remnant of nerves that supply the lateral line organs in amphibians and fish to sense vibrations and movements in the surrounding water (Engel, 1979; Gupta, Verma, & Vishwakarma, 1986). In mammals, the ABVN is an exclusively afferent, sensory nerve that innervates part of the skin of the outer ear, as well as the external acoustic meatus (Peuker & Filler, 2002;

Sherrington, 1897). The afferent fibers of the ABVN terminate in the nucleus of the solitary tract – similarly to the thoracic vagus nerve – as well as the spinal trigeminal nucleus (He et al., 2013; Nomura & Mizuno, 1984). The vagus nerve can be described as consisting of different fibers that can be broadly categorized into A-, B- and C fibers, ranging from large, highly myelinated, fast-conducting A fibers to smaller and lightly myelinated B fibers, and small, unmyelinated, slow-conducting C fibers. The ABVN seems to be innervated predominantly by the large, myelinated A-fibers (Safi, Ellrich, & Neuhuber, 2016). Studies conducted in anesthetized dogs revealed different stimulation thresholds for these fiber types; 0.4mA for A-fiber, 3.8mA for B-fiber and 17mA for C-fibers (Yoo et al., 2013). Given the similarities in fiber thickness between dog and human vagal nerves, it seems likely that human vagus nerves follow the same pattern (Yoo et al., 2013). As stimulation intensities of VNS and tVNS typically vary between 0.3-3mA, it seems likely that these techniques almost exclusively recruit the A-fibers of the vagus nerve, and not the smaller B- or C-fibers (Helmers et al., 2012). It currently remains unclear whether recruiting the smaller, unmyelinated fibers of the vagus nerve would boost the therapeutic effectiveness of vagus nerve stimulation, although the destruction of peripheral C-fibers of the vagus nerve in rats did not affect the suppression of seizures as a result of cervical VNS (Krahl, Senanayake, & Handforth, 2001).

There is a paucity of research on the specific anatomical distribution of the vagus nerve in the outer ear. Sherrington, in an anatomical study performed on macaques in 1897, noted that the ABVN innervates the cavum and cymba conchae and the antitragus, and also part of the tragus and the antihelix. In 1927, a case study was published concerning a patient suffering from severe pain in the ear and throat. In an initial procedure, Fay performed a subtotal resection of the trigeminal nerve supplying the area of the tongue and throat. This initial surgical procedure resulted in complete analgesia of the tragus, indicating that although the ABVN may also innervate this part of the ear, it is not the only nerve to provide sensory feedback from this part of the ear. In a subsequent procedure, the ABVN was sectioned, resulting in complete anesthesia in the cymba concha, and to a lesser degree the antihelix and antitragus (Fay, 1927). Finally, a study performed on human cadavers showed that

the ABVN is the only nerve to stimulate the cymba concha (Peuker & Filler, 2002). Additionally, the ABVN may innervate the tragus, antihelix and cavum concha, although the study reports contradictory innervation percentages in the main text and the corresponding table, making it impossible to assess whether and to what extent the ABVN innervates these areas of the outer ear (A. Burger & Verkuil, 2018).

During tVNS, electrodes are placed on the skin of either the ear or the neck. Electrical stimulation is then applied to the skin via the electrode. Given a sufficiently high stimulation intensity, the electrical stimulation will permeate the skin and trigger action potentials in nerve cells underneath the stimulated area. tVNS researchers have mainly used devices that stimulate the cymba concha of the ear, given the anatomical evidence that the ABVN innervates this part of the ear. Alternatively, researchers have opted to stimulate the skin at the area of the neck (Goadsby et al., 2017), or the tragus of the ear (Jacobs, Riphagen, Razat, Wiese, & Sack, 2015). An important limitation of stimulating the ABVN in both latter areas is that they are not exclusively innervated by the vagus, and thus any effects of the stimulation may also be attributable to the activation of other nerves. Moreover, it seems unclear whether the tragus is actually innervated by the vagus nerve at all (A. Burger & Verkuil, 2018).

fMRI studies have shown widespread activation patterns in participants receiving tVNS compared to sham stimulation of the earlobe, indicative of successful activation of the afferent fibers of the vagus nerve (Frangos, Ellrich, & Komisaruk, 2014; Yakunina, Kim, & Nam, 2016). However, activation of the nucleus of the solitary tract - the terminal site of the vagus nerve in the brain stem - was only observed in studies where the cymba concha had been stimulated (Badran, Dowdle, et al., 2018a). The lack of effects on the nucleus of the solitary tract found in studies where the tragus or ear canal was stimulated cast some doubts on whether neuronal effects found in these studies were truly due to activation of the vagus nerve, or were simply random fluctuations in activation patterns. The large differences between studies in neuronal effects of tVNS strongly limit the reliability of any of these measurements. Possible reasons for these discrepancies include differences in stimulation

parameters including stimulation sites, different regions of interest, different task set-ups, and different scanners. There is a clear need for more direct replications to be conducted, to assess the reliability and validity of any of these results.

Given the lack of research on the anatomical distribution of the ABVN in the human ear and the lack of knowledge on optimal stimulation parameters, there is a clear need for a biomarker to assess vagal activation. In this review, we aim to provide a short background to different measurements that could potentially serve as either a direct marker of vagal activity or as an indirect marker by measuring one of the presumed pathways through which VNS is hypothesized to affect cognition. Specifically, we will discuss five potential biomarkers that have been suggested as indices for either efferent vagus nerve activity (i.e. heart rate variability), afferent vagus nerve activity (vagus somatosensory evoked potentials), or markers of the proposed working mechanism of tVNS (LC-NA activity; indexed via pupil diameter, P300 amplitude, and salivary alpha-amylase). In our review of each potential biomarker, we will discuss how this marker is related to the vagus nerve, and provide an overview of current experimental studies that have studied the effects of either invasive or transcutaneous VNS on these biomarkers. We would like to emphasize that research on tVNS is still in its infancy, and many of the proposed potential biomarkers will be discussed more from a theoretical point of view than based on any direct evidence that these markers are actually affected by tVNS.

2. Markers of vagal activity

2.1 Heart Rate Variability

The vagus nerve provides the primary parasympathetic innervation of the heart. Efferent cardiac B-fibers of the vagus nerve, originating from the nucleus ambiguous and the dorsal motor nucleus, project to the sinoatrial node, atrioventricular node, and atrial cardiac muscle. Efferent activity in these

cardiac B-fibers triggers acetylcholine release in the vagus nerve's terminals. Acetylcholine, in turn, binds to the muscarinic receptors in the heart, thereby decreasing the depolarization rates in the sinoatrial and atrioventricular nodes, which subsequently reduces heart rate (Shaffer, McCraty, & Zerr, 2014). Both the left and the right vagus nerves innervate both the sinoatrial and the atrioventricular node, although this innervation is not symmetrical. Specifically, the left vagus nerve preferentially innervates the atrioventricular node, whereas the right vagus nerve innervation the sinoatrial node more strongly (Ardell & Randall, 1986; Randall, Ardell, & Becker, 1985).

Even though activity of efferent cardiac fibers of the vagus nerve promotes bradycardia, differences in heart rate between or within individuals can be a result of parasympathetic activity, sympathetic activity, or a combination of the two. Therefore, heart rate should therefore not be interpreted as a marker of vagal activity (Task Force of The European Society of Cardiology & The North American Society of Pacing and Electrophysiology, 1996). By contrast, certain components of heart rate variability (HRV) have been proposed as reliable indices of cardiac vagal control. Indeed, due to differences in response times to either sympathetic or parasympathetic input – vagal impulses have an effect on heart rate within one second, whereas there is a delay of up to 5 seconds after increased sympathetic activity (Hainsworth, 1995) –, indices of HRV that specifically capture rapid beat-to-beat changes, typically at respiratory rhythms, are thought to provide reliable estimates of parasympathetic vagal modulation of heart rate (Chapleau & Sabharwal, 2011; Task Force of The European Society of Cardiology & The North American Society of Pacing and Electrophysiology, 1996). Specifically, HRV indices that are thought to capture vagal activity include (1) the root mean square of successive R-R interval differences (RMSSD), (2) the percentage of consecutive R-R intervals differing by >*x* ms (pNNx, %), (3) the respiratory sinus arrhythmia (RSA), and (4) the high frequency (HF) spectral component of HRV (Laborde, Mosley, & Thayer, 2017). These indices can be extracted from ECG data of both humans and animals, although the specific parameters need to be adjusted based on what animal is being studied (e.g., due to the higher HR of mice, HF HRV typically corresponds to 1.5-5 Hz instead of 0.15 Hz fluctuations; Thireau, Zhang, Poisson, & Babuty, 2008). Other parameters of HRV potentially

represent some combination of sympathetic and vagal activity, and are therefore less suitable as biomarkers for vagal activity (examples of such parameters include Low Frequency (LF) HRV, LF/HF ratio, or SDANN). A comparison of these and other HRV parameters and how they relate to vagal activity is beyond the scope of this review, although we would like to refer interested readers to several sources where this is discussed in detail (Berntson et al., 1997; Berntson, Quigley, & & Lozano, 2007; Chapleau & Sabharwal, 2011; Laborde et al., 2017; Penttila, Helminen, Jartti, & Kuusela, 2001; Task Force of The European Society of Cardiology & The North American Society of Pacing and Electrophysiology, 1996). In table 1, we provide a brief overview of HRV indices that have been used in tVNS and VNS research.

Table 1. Heart rate variability indices used in (t)VNS studies.

Time Domain	**RMSSD**	**Root mean square of successive R-R interval differences**
	pNNx	**Percentage of consecutive R-R intervals differing by >*x* ms**
	RSA	**Respiratory sinus arrhythmia**
	SDNN	Standard deviation of all N-N intervals
	SDANN	Standard deviation of average N-N intervals for 5 min segments within a 24h HRV recording
	SDSD	Standard deviation of succesive N-N differences
Frequency Domain	**HF**	**High frequency (> 0.15 Hz in humans) component of HRV**
	LF	Low frequency (0.04 – 0.15 Hz) component of HRV
	LF/HF ratio	Ratio between low and high frequency components of HRV

Note. Indices that have been used in HRV research. Indices that are thought to reflect cardiac vagal tone are presented in bold.

Despite large differences between studies in the stimulation parameters that are used, invasive right-sided VNS consistently increases HRV in animals (Huang, Wang, Jiang, Zhou, & Huang,

2010; Kozasa et al., 2018; Lee, Anderson, et al., 2018; Lee, Kulkarni, et al., 2018; Yoshida et al., 2018; Zhang et al., 2009). By contrast, studies that focused on stimulating the left vagus nerve have yielded mixed results: some report effects on HF HRV (Jin et al., 2017; Samniang et al., 2016; Sun et al., 2013), while others were unable to find such an effect (Li & Wang, 2013; Martlé et al., 2014). These discrepant results suggest that the effects of left cervical VNS on heart rate is less pronounced compared to right cervical VNS. Indeed, two studies that compared left and right cervical VNS showed that although both types of stimulation successfully reduced HR, stimulation of the right vagus nerve produced more pronounced effects on HR (Ng, Brack, & Coote, 2001; Yoo et al., 2016), whereas left vagus nerve stimulation led to a stronger prolongation of atrioventricular conduction (i.e. a larger delay between atrial activity and ventricular activity) (Ng et al., 2001). These findings are thus in line with anatomical evidence showing that the right vagus nerve innervates the sinoatrial node more diffusely, whereas the left vagus nerve contributes more strongly to the distribution of post-ganglionic vagal fibers to the atrioventricular node (Ardell & Randall, 1986).

In humans, effects of cervical VNS on HRV seem less clear. When stimulating the left cervical vagus, the majority of studies did not find effects of VNS on any index of HRV (Barone et al., 2007; Galli et al., 2003; Garamendi et al., 2017; Jansen et al., 2011; Liu et al., 2018; Pruvost et al., 2006; Sperling & Reulbach, 2010). Curiously, left cervical VNS was found to increase rather than decrease LF/HF ratio in children suffering from epilepsy (Jansen et al., 2011). In a large scale trial to assess whether right cervical VNS would improve cardiac function in 87 heart failure patients, right cervical VNS did not affect RMSSD, SDNN, or HR, but showed a modest increase in SDANN compared to when VNS was switched off (Zannad et al., 2015). Similarly, in 28 epilepsy patients, right cervical VNS did not affect HR, HF HRV, or LF/HF ratio (Verrier, Nearing, Olin, Boon, & Schachter, 2016).

Effects of left and right cervical VNS on HR and HRV were directly compared in the ANTHEM-HF study, an open-label study that aimed to test the efficacy of VNS on myocardial function in heart failure patients). During the initial titration period in this study, both left- and right-sided VNS

significantly reduced HR, although this reduction was stronger for right VNS. Additionally, the decrease in heart rate was correlated with stimulation strength, with stronger associations for right-sided VNS (r = 0.88) than for left-sided VNS (r = 0.49) (Nearing, Libbus, Amurthur, Kenknight, & Verrier, 2016). After 6 months of VNS, a 24-hour ECG recording (during which VNS followed its standard duty cycle) indicated that vagally mediated HRV as indexed by SDNN had increased significantly compared to pre-stimulation baseline, with minimal differences between participants who received left or right sided VNS (Premchand et al., 2014). Finally, during a 12-month follow-up, another 24-hour recording indicated that both left- and right-sided VNS significantly increased RMSSD and HF HRV compared to pre-stimulation baseline, although effects on SDNN were no longer significant (Libbus, Nearing, Amurthur, KenKnight, & Verrier, 2016).

Finally, several studies did not specify whether the left or right cervical vagus nerve had been stimulated. These studies provided mixed evidence for the effects of VNS on HRV. In epilepsy patients, acute cervical VNS significantly decreased LF and LF/HF ratio one week after surgery, although HF HRV was unaffected (Schomer, Nearing, Schachter, & Verrier, 2014). In a different study, long-term application of VNS (i.e. application for over one year) was found to increase some time domain parameters of HRV (RMSSD, SDSD, pNN50, but not SDNN), but did not affect power-spectrum parameters (LF, HF, LF/HF) (Cadeddu et al., 2010). In children suffering from epilepsy, VNS was found to increase all time-domain and frequency-domain HRV parameters after 6 months of stimulation compared to baseline, although this effect was no longer present after 12 months (Hirfanoglu et al., 2018).

In sum, the effects of cervical VNS on HRV are less clear in humans than in animals. Although it is difficult to determine the cause of this discrepancy, it is interesting to notice differences between animal and human literature in the stimulation intensity that is typically applied. Studies in animals do differ from each other in stimulation parameters, but mostly utilize identical stimulation parameters within a study. By contrast, human studies typically follow a titration procedure, where stimulation

intensity is progressively increased until either a clinical response is found (oftentimes this clinical response is not directly related to cardiac activity, as in the case of epilepsy symptoms) or until tolerability is exceeded. As a result, the stimulation intensity provided to some patients may not have been sufficient to activate the efferent cervical B-fibers.

Contrary to the direct stimulation of efferent cardiac branches of the vagus nerve during invasive VNS, auricular tVNS is thought to stimulate an exclusively afferent branch of the vagus nerve. Therefore, any potential effects of tVNS on HRV are indirect consequences of afferent activation. Specifically, as proposed by Murray and colleagues (Murray, Atkinson, Mahadi, Deuchars, & Deuchars, 2016), tVNS may increase input to the nucleus tractus solitarii (NTS), thereby increasing activity of NTS neurons projecting to the two vagal efferent nuclei: the dorsal motor nucleus and the nucleus ambiguous. Increased activation in these nuclei may in turn increase vagal control of cardiac activity.

Similarly to the effects of invasive VNS, it is challenging to review the effects of transcutaneous VNS on vagally mediated indices of HRV due to the large differences in design characteristics. Not only do studies differ in the specific indices of HRV that were assessed, studies also differ on what part of the ear was stimulated (concha or tragus), whether the left or right ear was stimulated, what stimulation parameters were used, and what control group was used. In the section below, we discuss the most important findings from these studies, while differentiating between what part of either the left or right ear had been stimulated (see table 2 for an overview of all included studies).

Studies that have stimulated the tragus of the left ear have found preliminary, albeit mixed, indications that tVNS may affect certain indices of HRV. For tragus stimulation on the left ear, studies have reported significant increases in RSA (Lamb, Porges, Lewis, & Williamson, 2017), reductions in LF/HF ratio (Antonino et al., 2017; Tran et al., 2018) and increases in HF HRV (Tran et al., 2018). By contrast, other studies did not find any effects of left tragus stimulation on HF HRV (Antonino et al., 2017; Weise et al., 2015) or LF/HF ratio (Weise et al., 2015). One parametric study found that higher pulse width and stimulation frequency (i.e. 200 and 500μs pulse width, 10 and 25Hz) were associated

with more pronounced cardiac deceleration, although no assessments of HRV were included (Badran, Mithoefer, et al., 2018).

The effects of tragus stimulation of the right ear have only been described in one study so far, which showed no significant effects on HF HRV, but a significant reduction in LF/HF ratio compared to a no-stimulation baseline (Weise et al., 2015). Two other studies did not explicitly mention whether the left or right ear was being stimulated. The first of these studies found that stimulation of the tragus significantly reduced LF/HF ratio compared to a 'stimulation off' condition, but did not significantly affect the HF or LF components (Clancy et al., 2014). The second study described three separate experiments that tested the effects of tragus stimulation on various indices of HRV (Bretherton et al., 2019). The first experiment describes a comparison between tVNS and sham stimulation, but does not report a direct comparison between conditions. In the second experiment, active tVNS (compared to a pre-stimulation baseline) was associated with significantly increased LF HRV, RMSSD, pNN50, and non-linear indices of HRV (SD1 and SD2), while not affecting HF HRV, LF/HF ratio, RR intervals, or SDNN. The third experiment tested the effects of tVNS compared to pre-stimulation baseline before and after a two-week period of daily tVNS. Here, the authors found significantly higher LF, SDNN, RR intervals, and non-linear indices of HRV during tVNS compared to pre-stimulation baseline. This experiment also showed some preliminary evidence for a cumulative effect of long-term tVNS, as some indices of HRV (RMSSD, SDNN, and SD1) were significantly increased after two weeks of daily tVNS, although others remained unaffected.

The results of stimulation of the tragus should be interpreted with caution, however: Most studies compared active stimulation to a 'stimulation off' sham condition (Clancy et al., 2014; Lamb et al., 2017) or a pre-stimulation baseline (Weise et al., 2015). Out of the two studies that did include an active sham stimulation condition (Antonino et al., 2017; Tran et al., 2018), one study did not report a comparison between tVNS and sham stimulation, and only compared tVNS to pre-stimulation baseline (Antonino et al., 2017).

Studies on the effects of electrical stimulation of the cymba concha on HRV have also provided inconsistent findings. In a two-part study, De Couck and colleagues found significant effects of both left and right sided cymba concha stimulation compared to a pre-stimulation baseline on SDNN, although these effects were not replicated in RMSSD, HF, LF, or LF/HF measures (De Couck et al., 2017). In a second study, the authors found that one hour of right ear cymba concha stimulation did not affect any HRV parameter after controlling for confounders (De Couck et al., 2017). Similarly, in 10 healthy individuals, stimulation of the left cymba concha did not significantly affect HF HRV (Gancheva et al., 2018). During a fear conditioning procedure, stimulation of the left cymba concha did not significantly affect RMSSD throughout generalization and extinction learning (A. Burger, Van Diest, et al., 2019). Finally, in a sample of high-trait worriers, stimulation of the left cymba concha did not significantly affect RMSSD compared to earlobe sham stimulation (A. Burger, Van der Does, Thayer, Brosschot, & Verkuil, 2019).

In sum, similar to invasive VNS, tVNS in humans has produced mixed results on HRV. There are no clear differences in cardiac effects between left- and right-sided tVNS, although direct comparisons are scarce.

Perhaps because there is a clear anatomical link between the vagus nerve and the heart, and certain indices of HRV offer a reliable index of *efferent* vagal activity, quite some studies have investigated the effects of vagus nerve stimulation on HRV. Animal studies have provided clear evidence that both left and right cervical vagus nerve stimulation affect HR and vagally mediated HRV. These effects are more pronounced when stimulating the right relative to the left vagus, likely because the primary pace maker of the heart (sinoatrial node) is predominantly innervated by post-ganglionic fibers of the right vagus nerve. In humans, the influence of cervical VNS on cardiac activity is far less clear. Both left and right cervical VNS produce inconsistent effects on HR and HRV, which is likely due to several factors. Firstly, most studies have used relatively small sample sizes, meaning that they may have been statistically underpowered to detect potentially meaningful effects as significantly different

from zero. Secondly, there is large heterogeneity between studies in the tested samples, ranging from healthy adults to children to heart failure patients. Thirdly, stimulation intensities vary considerably between studies: whereas the sensory threshold for stimulation of the cymba concha lies somewhere between 0.5 and 1.5mA (De Couck et al., 2017; Gancheva et al., 2018), studies that stimulate at the tragus vary anywhere from roughly 4 mA (Badran, Dowdle, et al., 2018b; Jacobs et al., 2015) up to 50 (Antonino et al., 2017; Clancy et al., 2014). It seems unlikely that the sensory threshold of the tragus lies a hundred times higher than that of the cymba concha, indicating that certain settings besides the frequency, stimulation wavelength and target current may differ between these devices. Fourthly, the large variation of HRV parameters and the inclusion of parameters that are not clear indices of vagal activity (such as LF-HF ratio) decrease the comparability between studies, increase researcher degrees of freedom and thereby can inflate type I error rates. A better research practice may be to preregister (prior to data collection) which HRV parameters will be assessed, thereby favoring a limited number of parameters that are recognized to specifically reflect cardiac vagal efferent activity (i.e. RMSSD, pNNx, RSA, or HF HRV). Additionally, we would argue that until the effects of VNS on respiratory activity are elucidated, it may be more informative to focus on time domain parameters, as these seem to be less affected by changes in respiratory frequency compared to HF HRV (Hill, Siebenbrock, Sollers, & Thayer, 2009; Penttila et al., 2001). Finally, different types of 'control' conditions are being used in different types of studies. With the exception of two studies (Badran, Mithoefer, et al., 2018; Tran et al., 2018), all studies with tragus stimulation compared active stimulation with conditions in which the tVNS device was switched off. By contrast, the more convincing sham stimulation placebo condition (stimulation of the earlobe) was used predominantly in studies stimulating the cymba concha. This difference in the predominant control condition may potentially explain why studies that stimulate the tragus seem to yield positive findings more consistently than studies targeting the cymba concha.

An important limitation of HRV as a biomarker for vagal nerve stimulation is that this index represents a marker for *efferent* vagal activation, whereas most research on tVNS focus on *afferent* effects of on cognitive, emotional, or neurological functioning. Although changes in HRV may be a

sufficient requirement for demonstrating vagal activation, it is unlikely to be a *necessary* requirement for the activation of *afferent* vagal fibers.

Table 2. Overview of effects of tVNS on HRV.

First author (year of publication)	tVNS stimulation location	Sham control condition	N *	Stimulation duty cycle (on/off)	Stimulation parameters (intensity, pulse width, frequency)	HRV parameters	Effect of tVNS on HRV?	
Clancy et al (2014)	Tragus, unclear whether left or right ear	Tragus with electrode switched off after tVNS intensity calibration	48 (34 tVNS, 14 sham)	Continuous	10-50 mA 200 µs 30Hz	LF, HF, LF/HF	Significant decrease in LF/HF ratio, no significant effects on HF or LF components.	?
Weise et al (2015)	tragus & outer ventral edge of external auditory meatus of left and right ear	Pre-stimulation baseline	28**	Not reported	8mA 100 µs 0.5Hz	LF, HF, LF/HF, HRV index	Significant decrease in LF/HF ratio during right but not left tVNS. No effects on LF, HF, or HRV index.	?
Antonino (2017)	Left tragus	Left earlobe	13	Continuous	10-50mA 200µs 30Hz	LF, HF, LF/HF	Significant reduction of HR and LF/HF ratio compared to baseline during tVNS, but not during sham. No comparison tVNS vs sham.	?
Lamb (2017)	Left tragus	Tragus with electrode switched off after tVNS intensity calibration	22	Not reported	Mean intensity 5.6 mA (3–11.3 mA) 20 Hz	RSA	Increased RSA during tVNS compared to sham.	+
De Couck (2017) Experiment 1	Left and Right cymba concha	Left cymba concha, not active stimulation	30	30s on, 30s off	Mean intensity 0.7mA 250 µs 25Hz	RMSSD, SDNN, HF, LF/HF	Right-sided tVNS significantly increased SDNN compared to baseline, but no effects on RMSSD, HF or LF/HF. Also no significant differences compared to sham or compared to left-sided tVNS.	?
De Couck (2017) Experiment 2	Right cymba concha	Pre-stimulation baseline	30	30s on, 30s off	Mean intensity 1.0mA 250 µs 25Hz	RMSSD, SDNN, HF, LF/HF	No effects of one hour of tVNS on HRV in total sample. Significant time*gender effect revealed significant increase in SDNN over time in women, but not in men. This effect was not replicated in other HRV parameters.	?

Badran (2018) Experiment 1	Left tragus stimulation	Left earlobe stimulation	15	60s on	Mean intensity 3-9.3mA, depending on pulse width 100µs-250µs-500µs 1Hz-10Hz-25Hz	HR***	tVNS led to a significantly stronger drop in HR compared to sham in all conditions with pulse width of 200 and 500µs. Higher pulse width combined with higher stimulation frequency also decreased HR rebound after stimulation offset.	
Badran (2018) Experiment 2	Left tragus stimulation	Left earlobe stimulation	20	60s on	Mean intensity 2.1 (SD = 1.0) mA 500µs 10Hz-25Hz	HR***	tVNS led to a significantly stronger drop in HR compared to sham in 10Hz condition, but not in 25Hz condition.	
Gancheva (2018)	left cymba concha	Left earlobe	14 (7 tvns, 7 sham)	Continuous	0.6-1.4mA 250 µs 25Hz	LF, HF, LF/HF	No effects of tVNS on HRV.	0
Burger (2019)	Left cymba concha	Left earlobe	85 (43 tVNS, 42 sham)	30s on, 30s off	0.5 mA 250 µs 25Hz	RMSSD	No effects of tVNS on HRV.	0
Burger (2019)	left cymba concha	Left earlobe	97 (48 tVNS, 49 sham)	30s on, 30s off	0.5 mA 250 µs 25Hz	RMSSD	No effects of tVNS on HRV.	0
Bretherton (2019) Experiment 2	Tragus, unclear whether left or right ear	Pre-stimulation baseline	51	Continuous	2-4 mA 200 µs 30Hz	LF, HF, LF/HF, RR interval, SDNN, RMSSD, pNN50, non-linear indices of HRV (SD1, SD2)	tVNS led to a significant increase in LF, RMSSD, pNN50, and SD1, SD2, compared to pre-stimulation baseline. No effects reported on the other indices.	?
Bretherton (2019) Experiment 3	Tragus, unclear whether left or right ear	Pre-stimulation baseline	26	Continuous	2-4 mA 200 µs 30Hz	LF, HF, LF/HF, RR interval, SDNN, RMSSD, pNN50, non-linear indices of HRV (SD1, SD2)	tVNS led to a significant increase in LF, SDNN, SD2, and mean RR interval. No effects reported on the other indices.	?

Note.*Total number of participants in study. For studies utilizing a between-subjects design, participants per experimental condition are reported between brackets. ** Although the methods section describe a sample of 100 participants, the reported test statistics suggest that the statistical analysis of HRV data was based on data of only 28 participants. ***Although technically the studies by Badran and colleagues did not report HRV, we believe their systematic approach towards testing the effects of various stimulation parameters of tVNS on heart rate still deserves a mention here.

2.2 Somatosensory Evoked Potentials

Another possible indication of vagus nerve activity may be obtained by recording electrical activity of the brain at the scalp in response to tVNS, obtaining so called somatosensory evoked - or far field - potentials. Fallgatter proposed to use tVNS in combination with recordings of evoked potentials to test for the neurological integrity of the vagus brainstem nuclei (Fallgatter et al., 2003), which could theoretically aid the early detection of neurodegenerative diseases such as Alzheimer's. That is, after a single short stimulation pulse of the ABVN, electrical signals (evoked potentials) at the scalp are recorded bipolarly from the electrode positions (C3–F3, C4–F4, Fz–F3, Fz–F4). Repeated delivery of these single stimulation pulses allow a reliable estimate of the evoked potentials that occur within the first 10 msec after the stimulation. Both amplitude and latencies of the different components of the evoked potentials can be examined. According to Fallgatter and colleagues, the occurrence of such far field potentials can be interpreted as indicative for the activity of the vagus nerve nuclei in the brainstem.

Fallgatter and colleagues initially tested this procedure on Fallgatter himself in five different sessions, as well as 5 other healthy subjects in single sessions. Only stimulation at the inner side of the tragus of the left and right ears (but not at the lobulus, the scapha, the crus antihelix superior and top of the helix) yielded evoked potentials within the first 6 msec: a positive deflection (P1) followed by a negative deflection (N1) and another positive deflection (P2), together termed vagus sensory evoked potentials (VSEPs). The occurrence of VSEPs has been replicated in several studies and has also been observed after non-invasive cervical VNS in a small sample of 12 healthy participants (Nonis, D'Ostilio, Schoenen, & Magis, 2017). Yet, VSEPs cannot be observed in each single participant. That is, in a first replication study, Fallgatter could not demonstrate the VSEPs in 2 of 22 young participants and 4 of 43 elderly participants (Fallgatter, Ehlis, Ringel, & Herrmann, 2005).

Subsequent work focused on VSEP latencies, as prolonged latencies may be indicative of a reduced myelination of the vagus nerve and a concomitant reduced conduction velocity of the nerve fibers. Prolonged latencies of the VSEPs after tVNS have been documented in elderly (Fallgatter et al., 2005) and people suffering from mild cognitive impairments and Alzheimer's disease (Polak et al., 2014, 2007), but not in people with vascular dementia (Polak et al., 2009), Parkinson's disease (Weise et al., 2015) or major depression (Polak et al., 2014). Yet, it is not always the full spectrum of P1-N1-P2 potentials that is delayed after tVNS. Only P1 latencies were also found to be prolonged in people suffering from multiple sclerosis, especially in those patients with known neurodegeneration at the brainstem (Polak, Zeller, Fallgatter, & Metzger, 2013). Furthermore, in people reporting to have

experienced a worsening of their memory performance, only those who worried about this showed a prolonged P2 component (Hagen et al., 2015). In addition, no P1 differences could be observed when comparing people with Alzheimer's disease to healthy controls and people with mild cognitive impairments, possibly due to the heterogeneity in the Alzheimer's disease group (Metzger et al., 2012).

Although VSEPs are typically observed after tVNS, the vagal origin of these evoked potentials is debatable: administration of a muscle relaxant completely removed the late P2-N2 complex of the evoked potential, although early P1-N1 complex remained unchanged (Usami, Kawai, Sonoo, & Saito, 2013). These results indicate that the early components of the VSEP could theoretically reflect vagal activity, while later P2-N2 potentials can be attributed to neuromuscular activity. In a similar paradigm, Leutzow and colleagues measured VSEPs in response to auricular tVNS before and during general anesthesia in 14 patients (Leutzow et al., 2013). They observed that under general anesthesia and after the muscle relaxant agent cisatracurium had been administered, neither the early nor the late components of the VSEPs could be observed anymore. Additionally, they noted that in the original paper by Fallgatter, the VSEP was only found ipsilateral and not contralateral to the stimulation, and that the N2-P2 pattern was actually reversed contra laterally. Given that evoked potentials that indicate brainstem activity are typically observed both ipsilateral and contralateral (after auditory stimulation for example), these findings question the vagal-brainstem origin of the VSEP reported in earlier tVNS studies (Leutzow, Nowak, & Usichenko, 2014).

As a muscular origin of at least some components of the tVNS evoked potentials cannot be ruled out, a more thorough examination of the vagal origins of the VSEPs seems warranted before considering VSEPs as reliable markers of afferent vagal activity during vagal nerve stimulation.

3. Noradrenergic markers of tVNS

Increased levels of noradrenaline (NA) as a biomarker of vagus nerve stimulation may seem paradoxical, given that the vagus is often described as part of the parasympathetic nervous system, whereas NA is a neurotransmitter that is strongly related to its peripheral sympathetic counterpart, adrenaline. However, since adrenaline cannot cross the blood brain barrier, it instead binds on the beta-adrenergic receptors of afferent peripheral nerves such as the vagus nerve. Indeed, peripheral injections of adrenaline lead to increased firing of the vagus nerve, which in turn increases central NA

levels (Chen & Williams, 2012; Miyashita & Williams, 2006). Specifically, the vagus nerve terminates on the NTS, where it projects onto the LC, the main hub of NA in the cortex.

Studies on the effects of VNS in rats have repeatedly and consistently found effects of VNS compared to sham stimulation on firing rates of LC neurons. These effects were found both acutely after initial stimulation onset (Chen & Williams, 2012; Dorr & Debonnel, 2006; Groves, Bowman, & Brown, 2005; Hulsey et al., 2017; Manta, El Mansari, Debonnel, & Blier, 2013) and after a long term stimulation period (over a period of 90 days, Dorr & Debonnel, 2006; after 14 and 90 days, Manta, Dong, Debonnel, & Blier, 2009). Similarly, acute effects of VNS on NA levels in the hippocampus (Raedt et al., 2011; Roosevelt, Smith, Clough, Jensen, & Browning, 2006), basolateral amygdala (Hassert, Miyashita, & Williams, 2004) and medial prefrontal cortex (Follesa et al., 2007) were found.

Although the effects of VNS on LC-NAactivity is well established in animals, studies on the noradrenergic effects of (t)VNS in humans are lacking. Direct measurement of NA requires an invasive procedure and suffers from poor reliability and sensitivity (Grassi & Esler, 1999). Instead, activity of the LC-NA network can be derived from indirect markers, including pupil diameter, ERPP300 amplitude, and salivary alpha amylase. In the sections below, we will discuss how each of these markers may relate to LC-NA activity, and we will review currently available evidence for effects of invasive and transcutaneous VNS on these markers.

3.1 Pupil Dilation

Two opposing muscles control pupil size: the pupillary dilator muscle and the pupillary sphincter muscle, which promote pupil dilation and constriction respectively (Eckstein, Guerra-Carrillo, Miller Singley, & Bunge, 2017). Activity in the LC-NA system affects activity in both muscles: first, excitatory projections of the LC increase activity of the dilator muscle, whereas inhibitory pathways decrease activity of the Edinger-Westphal Nucleus (EWN), thereby inhibiting activity in the sphincter muscle (for a more complete overview of these pathways, see Samuels & Szabadi, 2008).

Several measures can be derived from pupillometry that measure distinct aspects of LC-NA activity. First, measures of pupil diameter at rest give an estimate of tonic levels of LC-NA activity, indicative of baseline arousal (Breton-Provencher & Sur, 2019; Reimer et al., 2016). Corresponding to the anatomical link between the LC and the pupillary dilator and constrictor muscles, intracranial recordings performed in monkeys have shown correlations between LC neuron activity and pupil

diameter (Joshi, Li, Kalwani, & Gold, 2016; Rajkowski, Kubiak, & Aston-Jones, 1993). Unfortunately, the correlation between tonic LC activity and pupil diameter is only small, which can be partly explained by cholinergic and GABAergic neurons that concurrently affect pupil diameter (Breton-Provencher & Sur, 2019; Reimer et al., 2016). Additionally, studies performed in humans have shown that administration of α2-adrenoreceptor agonists leads to a constriction of the pupil, whereas α2-adrenoreceptor antagonists lead to a dilation of the pupil (Hou, Freeman, Langley, Szabadi, & Bradshaw, 2005; Hou, Langley, Szabadi, & Bradshaw, 2007; Phillips, Szabadi, & Bradshaw, 2000). Second, apart from measuring pupil size at rest to assess tonic LC-NA activity, pupillometry can also be used to assess phasic, task-related changes in LC-NA activity (Aston-Jones & Cohen, 2005; de Gee et al., 2017; Gilzenrat, Nieuwenhuis, Jepma, & Cohen, 2010; Jepma & Nieuwenhuis, 2011; Murphy, Robertson, Balsters, & O'connell, 2011). Pupillary responses to cognitive demand have a relatively short response latency of approximately 1.5s (Eckstein et al., 2017), and temporal resolution can be enhanced further using data deconvolution methods (Wierda, van Rijn, Taatgen, & Martens, 2012). Finally, activity of the LC-NA system can also be indirectly derived from the amplitude and latency of the pupillary light reflex, which is a response to a change in light intensity reaching the retina. Specifically, increases in luminosity increase activity in the parasympathetically mediated constrictor muscle, and noradrenergic inhibition of activity in the EWN reduces the size of the pupillary light reflex. Indeed, pharmacological studies have shown that noradrenergic receptor agonists decrease the amplitude and increase the latency of the pupillary light reflex, whereas noradrenergic receptor antagonists have the opposite effect (for a review, see Samuels & Szabadi, 2008).

Only four studies have been published so far that have reported effects of VNS on pupil dilation. In rats, resting pupil diameter was significantly increased after VNS compared to a pre-stimulation baseline (Bianca & Komisaruk, 2007). –In a group of patients suffering from refractory epilepsy, Jodoin and colleagues (2015) observed larger resting pupil diameters during periods where VNS was switched on compared to when it was switched off. However, pupillary light response amplitude and magnitude were unaffected by VNS. In a different sample of patients suffering from refractory epilepsy, Schevernels and colleagues (2016a) found no significant differences in resting pupil diameter between periods where VNS was switched on compared to when it was switched off. The authors noted, however, that the non-significant difference found in their study may have been due to the limited sample size which hampered their statistical power. Indeed, although this difference was not statistically significant, participants' pupil sizes were larger during active stimulation than when VNS was switched off, both during baseline and during a stop-signal task. Finally, a recent preprint demonstrates a VNS parameter-dependent modulation of pupil diameter in rats, where increased stimulation charge (μC) substantially increased pupillary response to VNS (Mridha et al., 2019). The

parameter-specific modulation of LC activity (Hulsey et al., 2017) as well as pupil diameter (Mridha et al., 2019) clearly demonstrates that careful selection of stimulation parameters is of the utmost importance for measuring pupil diameter as a biomarker for invasive or transcutaneous VNS. Only three studies have been published that assessed the effects of tVNS on pupil size. In a within-subject cross-over study comparing stimulation of the left cymba concha to stimulation of the earlobe, tVNS did not increase resting pupil diameter in a sample of 16 healthy participants (Warren et al., 2019). In a second study, tVNS did not affect resting pupil diameter, nor did it affect phasic pupil dilation during an auditory oddball task (Keute, Demirezen, Graf, Mueller, & Zaehle, 2019). Finally, in a series of three experiments, there was no effect of tVNS compared to sham stimulation on resting pupil diameter or phasic pupil dilation during an attentional blink task (Burger, Van der Does, Brosschot, & Verkuil, 2020). Two more studies that are currently in preparation have tested the effects of tVNS on pupil diameter. Firstly, authors MdA, AB, IVD, and colleagues found no effects of tVNS on resting pupil dilation. 0, two experiments tested the effects of tVNS at an individually calibrated stimulation intensity (Sharon, Fahoum & Nir, poster presented at Society of Neuroscience conference). In the first experiment, the authors found no significant effects of tVNS on tonic pupil diameter. In the second experiment, the authors utilized a 3s on, 30s off duty cycle and found that tVNS significantly increased phasic pupil dilation compared to sham stimulation.

To conclude, in studies that aim to assess the effects of tVNS on attentional or memory processes, including tonic or phasic measurements of pupil dilation would be a worthwhile consideration to test a possible mediating effect of LC-NA activity. Unfortunately, published reports on the effects of VNS on pupil dilation have focused on the effects of invasive VNS, and results found on both phasic and tonic components of pupil dilation have been inconsistent. As of yet unpublished results primarily found null results of tVNS on pupil dilation when compared to sham stimulation. It should be noted, however, that with the possible exception of Keute and colleagues (2019) and Sharon and colleagues (unpublished), all studies that have assessed the effects of tVNS on pupil diameter have utilized similar stimulation parameters. Given the parameter-dependent effects of VNS on pupil dilation found in animals (Mridha et al., 2019), future research may want to systematically test the effects of various stimulation parameters on pupil diameter in humans.

Table 3. Overview of studies on the effects of tVNS on pupil diameter.

First author (year of publication)	tVNS stimulation location	Sham control condition	N *	Stimulation duty cycle (on/off)	Stimulation parameters (intensity, pulse width, frequency)	Pupil Diameter Measurements	Effect of tVNS on Pupil Diameter	
Keute (2019)	left cymba conchae	Left ear lobe	31	continuous	3.0 mA 200 µs 25 Hz	Tonic pupil diameter, phasic pupil dilation	No effects of tVNS on tonic pupil diameter or phasic pupil dilation	
Warren (2019) experiment 2	left cymba conchae	Left ear lobe	16	30s /30 s	0.5 mA 200–300 µs 25 Hz	Tonic pupil diameter	No effects of tVNS on tonic pupil diameter.	-
Burger (2020) Experiment 1	left cymba conchae	Left ear lobe	97 (48 tVNS, 49 sham)	30s /30 s	0.5 mA 200–300 µs 25 Hz	Tonic pupil diameter	No effects of tVNS on tonic pupil diameter.	-
Burger (2020) Experiment 2	left cymba conchae	Left ear lobe	30	30s /30 s	0.5 mA 200–300 µs 25 Hz	Tonic pupil diameter, phasic pupil dilation	No effects of tVNS on tonic pupil diameter or phasic pupil dilation.	-
Burger (2020) Experiment 3	left cymba conchae	Left ear lobe	80 (40 tVNS-40 sham)	30s /30 s	0.5 mA 200–300 µs 25 Hz	Tonic pupil diameter, phasic pupil dilation, pupillary light reflex	No effects of tVNS on tonic pupil diameter or phasic pupil dilation.	-

D'Agonisti (unpublished)	left cymba conchae	Left ear lobe	67 (32 tVNS, 35 sham)	30s /30 s	0.5 mA 200–300 µs 25 Hz	Tonic pupil diameter	No effects of tVNS on tonic pupil diameter.	-
Sharon (Unpublished) Experiment 1	left cymba conchae	Left ear lobe	23	30s /30 s	Mean intensity 2.2 mA 200–300 µs 25Hz	Tonic pupil diameter	No effects of tVNS on tonic pupil diameter.	-
Sharon (Unpublished) Experiment 2	left cymba conchae	Left ear lobe	24	3s / 30 s	Mean intensity 2.2 mA 200–300 µs 25Hz	Phasic pupil dilation	Significantly stronger pupil dilation after tVNS compared to sham.	+

Note.*Total number of participants in study. For studies utilizing a between-subjects design, participants per experimental condition are reported between brackets.

3.2 P300

The P300 (or P3) refers to a positive deflection in the scalp-recorded event related potential (ERP) that starts from around 300ms after the onset of a task-relevant or rare stimulus and is maximal over the midline electrodes (Fz, Cz, Pz). A reliable and established way to evoke the P300 is the oddball paradigm. In the classical oddball task, one has to respond to infrequent target stimuli that are interspersed with frequent non-target stimuli not requiring a response. The novelty oddball task also includes infrequent non-target 'distractors' that do not require a response and which are added as a third stimulus category (Duncan et al., 2009). Typically, P300 is increased both to infrequent targets and distractors. A distributed network of neuroinhibitory processes is presumed to promote efficacious processing of (potentially) relevant stimuli, as reflected in an enhanced P300 (Polich, 2007).

Some authors distinguish between two types of P300 that are thought to depend on different brain areas as well as neurotransmitter systems (Polich, 2007). More frontally, the P300 would depend on dopaminergic activity and relate to a novelty-driven orienting response to distractors (sometimes called P3a). At more temporal-parietal recording sites, the P300 would relate also to memory and decision-making (P3b) and would be driven by phasic noradrenergic activity (Nieuwenhuis, Aston-Jones, & Cohen, 2005; Polich, 2007). Typically, the P3b amplitude is larger for target stimuli than for distractors, which is in line with animal studies showing increased phasic activity in LC neurons in response to unpredicted stimuli that demand an immediate behavioral response (Aston-Jones, Rajkowski, Kubiak, & Alexinsky, 1994). Important in the present context is that the P3b and its underlying phasic LC-NA activity are thought to depend on background tonic levels of LC-NA activity in an inverted U-shape manner, with maximal phasic reactivity and larger P3bs at intermediate levels of tonic LC-NA activity (Aston-Jones & Cohen, 2005; Murphy et al., 2011). Despite differences in neural origins of the P3a and P3b subcategories, most studies do not include infrequent non-target 'distractor' trials in their oddball paradigm, and therefore are unable to differentiate between both components, opting instead to focus on the P300 ERP in general. In the following sections, we will specifically indicate whether results refer to the P300 in general, of one of its subcomponents specifically.

As LC-NA activity seems involved in the anti-epileptic and antidepressive potential of VNS, researchers have sought to study the relation between patients' P300 and their clinical response to VNS. The initial studies compared patients' P300 in an oddball task before and after long-term treatment with VNS. Using an auditory oddball task in epileptic patients, the P300 did not change in latency or amplitude from prior to surgery to following chronic VNS treatment (Brázdil et al., 2001; Hammond, Uthman, Reid, & Wilder, 1992). In contrast, an increased P300 amplitude following chronic

VNS was found with a visual oddball task, and this effect was more pronounced in patients who clinically benefited from VNS (less seizures). In a similar vein, Neuhaus et al. (Neuhaus et al., 2007) reported an enhanced auditory P300 in a subgroup of 5 (out of the 13) depressed patients who responded well to VNS (reduction of depressive symptoms). It is unclear whether the increased P300 may have been secondary to a general clinical improvement, as both depression and epilepsy are associated with widespread cognitive impairments and altered P300 responses (Kemp et al., 2010; Sowndhararajan, Kim, Deepa, Park, & Kim, 2018). As such, the observed increased P300 after VNS treatment in patients who clinically benefit from VNS does not necessarily relate directly and specifically to a VNS-induced increased phasic LC-NA activity. Interesting in this respect is a more recent study that has investigated P300 in epileptic patients performing a standard auditory oddball and a stop-signal task during periods in which the VNS was either ON or OFF (De Taeye et al., 2014; Schevernels et al., 2016b; Wostyn et al., 2016). A first paper on the findings with the oddball task reported that P300 was increased during the ON period at the parietal midline electrode (Pz) in VNS responders only (De Taeye et al., 2014). In a more recent report, all 60 electrodes were incorporated in the analyses (Wostyn et al., 2016). The mean amplitude of 6 EEG channels at temporoparietal sites (CP2, C2, C4, Pz, C6, CP4) differentiated responders from nonresponders in relation to whether the VNS was ON or OFF. When VNS was OFF, responders had a reduced P300 amplitude in these channels compared to nonresponders. The amplitude of responders increased when VNS was ON compared to off, whereas the reverse was true for nonresponders. The same group of patients also performed a stop-signal task. Findings with this response inhibition task showed that the P300 amplitude to the auditory stop signal was enhanced when VNS was ON compared to OFF, though this effect was not modulated by the clinical improvement associated with VNS (Schevernels et al., 2016b).

In summary, some studies with invasive VNS have generated indirect findings that are compatible with the idea that an increased P3b may be a marker for VNS-induced phasic LC-NA activity. However, they should be considered preliminary at best, as all reported findings are based on (very) small samples, have not been replicated thus far, and lack a control condition with sham stimulation. Furthermore, three of the 6 mentioned publications draw on the same study investigating a sample of epileptic patients.

Table 4 summarizes findings from recent studies using a within-subject cross-over design to investigate the effects of tVNS versus sham stimulation on P300 in healthy subjects. Five studies looked at P300 during an auditory or visual oddball task and one study during a response conflict task (Simon task). Overall, findings from these studies yield an inconsistent pattern of findings. For the oddball paradigm, two out of 5 studies report on an enhancement of P300 / P3b for tVNS compared to sham

stimulation (Lewine, Paulson, Bangera, & Simon, 2018; Rufener, Geyer, Janitzky, Heinze, & Zaehle, 2018). One additional study found a similar positive effect, but only in the context of an exploratory post-hoc analysis (Ventura-Bort et al., 2018). Consistent with the idea that especially P3b but not P3a draws upon noradrenergic activity, no effect of tVNS was present for P3a (Lewine et al., 2018; Ventura-Bort et al., 2018), supporting the idea that tVNS-induced enhancement of central NA levels is reflected in an increased P3b in the oddball task. However, two other studies with the oddball task could not confirm an effect of tVNS on P3b (experiments 1A and 1B from Warren et al., 2019). In a similar vein, no effect of tVNS on P300 was found in the study using the Simon task (Fischer, Ventura-Bort, Hamm, & Weymar, 2018). None of the studies in Table 4 found a reversed effect (an attenuated P300 for tVNS compared to sham stimulation).

It is clear from Table 4 that the reported studies differ on various factors that may mediate or moderate a potential effect of tVNS on P300, including variations in the oddball task, tVNS stimulation characteristics, duration of stimulation, presence of stimulation during the task, the presence of stimulation already prior to the task, stimulation location, and the operationalization of the sham stimulation. With respect to the latter, it is notable that the two studies that observed a clear positive effect of tVNS on P300 had a sham condition that differed from the most commonly used sham, which is stimulation at the left ear lobe.

Together, the currently available evidence cannot confirm that P300 or P3b constitutes a reliable marker of (t)VNS-related enhancement of phasic noradrenergic activity. Evidence is preliminary at best and further studies are needed to disentangle under which conditions the effect shows up or not.

Table 4. Overview of studies on the effects of tVNS on P300.

First author (year of publication)	tVNS stimulation location	Sham control condition	N*	Stimulation duty cycle (on/off)	Stimulation parameters (intensity, pulse width, frequency)	Task**	total stimulation time	effect of tVNS on P300?	
Ventura-Bort (2018)	left cymba conchae	left ear lobe	20	Continuous	1.3 mA (0.4–3.3 mA) 200–300 µs 25 Hz	visual novelty oddball 70/15/15%	28 min	P3b enhanced only for EASY targets in a post-hoc exploratory analysis	?
Fisher (2018)	left cymba conchae	left ear lobe	20	Continuous	1.3 mA (0.4–3.3 mA) 200–300 µs 25 Hz	Simon task	35 min	no effects of tVNS on P300	0
Rufener (2018)	left cymba conchae	tVNS at left cymba conchae turned off after 10 s	20	30s/30 s	0.5 mA 200–300 µs 25 Hz	auditory classical oddball 80/20%	100,5 min	P3 amplitude increased; P3latency decreased	+
Lewine (2018)	neck (left carotid sheath)	neck (muscles, most posterior aspect of the jaw)	8	2 min/1min	12-20 V 1000 µs 25 Hz	auditory multistimulus oddball 70/20/10%	2x 120 sec prior to task	P3b enhanced at 15 and 120 min after stimulation	+
Warren (2019) experiment 1A	left cymba conchae	Left ear lobe (within subject)	24	30s/30s	0.5 mA 200–300 µs 25 Hz	4 oddball tasks: auditory/visual classical/novelty classical: 88/12% novelty: 76/12/12%	80 min	no effects	0
Warren (2019) experiment 1B	left cymba conchae	Left ear lobe	20	30s/30 s	0.5 mA 200–300 µs 25 Hz	visual, classical oddball task 88/12%	80 min	no effects	0

Note. *Total number of participants in study. All studies utilized a within-subjects comparison of tVNS and sham stimulation. ** Percentages denote the percentage distractor/target or distractor/target/novelty trials within a task.

3.3 Salivary alpha amylase

Salivary alpha-amylase (sAA) is a salivary protein involved in the digestion of starch in the oral cavity (Baum, 1993). It has been proposed as a promising marker of sympathetic nervous system activity, as it is strongly affected by local sympathetic nerves, and pharmacological studies have shown involvement of noradrenergic activity in sAA secretion (Ehlert, Erni, Hebisch, & Nater, 2006; Warren, van den Brink, Nieuwenhuis, & Bosch, 2017). Although a clear neuroanatomical description of how central NA mediates protein secretion is missing, one possible path entails the dense projections from the locus coeruleus onto the pre-ganglionic sympathetic nuclei in the spinal cord (Samuels & Szabadi, 2008; Warren et al., 2017). Such sympathetic nuclei innervate the sympathetic ganglia (e.g., superior cervical ganglion), which in turn increases sAA secretion (Proctor, 2016). It should be noted, however, that sAA secretion cannot be interpreted as an exclusively sympathetic marker, as sAA release is co-determined by activity in the parasympathetic nervous system. Specifically, activity in the parasympathetic nervous system affects sAA activity in three ways: (1) through stimulation of glands that are parasympathetically innervated, (2) through synergistic sympathetic-parasympathetic effects on sAA secretion, and (3) through an increase in salivary flow rate (i.e., saliva fluid output per minute – ml/min, Bosch, Veerman, de Geus, & Proctor, 2011).

For tVNS researchers interested in the effects of tVNS on NA activity, sAA secretion can only be interpreted as a somewhat reliable marker of NA if tVNS does not increase the parasympathetically driven salivary flow rate. Only when a change in parasympathetic modulation of sAA secretion is ruled out as a mediator of changes in sAA secretion, can sAA secretion be interpreted as reflecting a change in noradrenergic activity with some level of confidence (Bosch et al., 2011). As such, researchers should measure sAA secretion (i.e., net salivary alpha amylase per minute - U/min) as a product of both concentration (i.e., net salivary alpha amylase per milliliter of fluid - U/ml) and salivary flow rate (i.e., saliva fluid output per minute – ml/min).

To our knowledge, there are no published reports describing the effects of invasive VNS on sAA. However, the effects of tVNS on sAA have been tested in three independent studies. Ventura-Bort and colleagues (2018) tested how sAA concentration was affected by tVNS compared to sham stimulation in a within-subjects design. The authors found that sAA concentration increased over time for participants after both tVNS and sham stimulation, with no significant differences between experimental conditions. However, a post-hoc analysis performed on sham and tVNS conditions separately revealed that SAA concentration increased significantly after tVNS but not after sham stimulation (Ventura-Bort et al., 2018). In a very recent, other study, D'Agostini and colleagues

(unpublished data) collected samples of saliva at the start of the experiment (t0), before the stimulation started (t1) and roughly around the last minutes of the stimulation (t2). The authors found that sAA concentration was unaffected by tVNS.

The findings of both studies should be interpreted with caution since both Ventura-Bort et al. (2018) and D'Agostini et al. collected stimulated saliva using cotton sponges. When chewing the sponges, the parotid glands (which are very rich in sAA) can increase their contribution to saliva secretion independently of central regulation. Under such conditions, sAA is likely a less reliable index of central NA activity (Bosch et al., 2011). Moreover, the authors measured sAA concentration instead of sAA secretion, ignoring parasympathetic influences on salivary flow rate. Thus, it is not possible to rule out parasympathetic effects on sAA secretion following tVNS.

In a third study, Warren et al. (2019) overcame these limitations by employing the spitting method, thereby ensuring collection of unstimulated saliva (Bosch et al., 2011). The authors pooled together sAA data from two studies, both employing a tVNS versus sham within-subject design and an identical stimulation protocol. sAA was assessed at 3 points in time: 5 min before stimulation, 45 and 75 min after stimulation began. Post-hoc analysis for the tVNS and sham conditions separately revealed that sAA secretion significantly increased following tVNS only. Importantly, there was no main effect of stimulation on flow rate, which may indicate that sAA enhancement by tVNS is predominantly sympathetically mediated.

Although preliminary results point to sAA as a potentially interesting marker of NA-enhancement modulated by tVNS (Ventura-Bort et al., 2018; Warren et al., 2019), some limitations and recommendations deserve attention. First, the lack of a clear neuroanatomical description of how central NA mediates sAA secretion is still lacking, which complicates a full understanding of the mechanisms implicated in sAA secretion and the valid interpretation of sAA as an index of NA activity. Second, experimenters in future trials would benefit from collecting unstimulated saliva (e.g., spitting method or passive drooling) and may want to investigate the effects of VNS on both sAA secretion and flow rate. Third, the timing of sAA collection needs some consideration. Ventura-Bort et al. (2018) assessed sAA levels following stimulation, whereas D'Agostini et al. and Warren et al. (2019) did so during stimulation. It is difficult at this stage to establish how fast sAA levels decrease after the stimulation stops. While animal research has shown central NA release drops immediately after iVNS is turned off (Follesa et al., 2007), the temporal dynamics of how tVNS affects sAA in humans is currently unclear. Future studies should assess sAA levels during active stimulation to avoid measuring the effects of tVNS at moments when the effects has already tapered off.

Table 5. Overview of studies on the effects of tVNS on sAA.

First author (year of publication)	tVNS stimulation location	Sham control condition	N*	Stimulation duty cycle (on/off)	Stimulation parameters (intensity, pulse width, frequency)	Saliva collection method	Total stimulation time	Effect of tVNS on sAA	
Ventura-Bort (2018)	Left cymba conchae	Left ear lobe	20	Continuous	1.3 mA (0.4–3.3 mA), 200–300 µs 25 Hz	Salivettes (60 s)	35 min	sAA increased only in tVNS in a post-hoc analysis	?
D'Agostini (unpublished)	Left cymba conchae	Left ear lobe	67 (32 tVNS, 35 sham)	30s /30 s	0.5 mA 200–300 µs 25 Hz	Salivettes (60 s)	40 min	No effect of tVNS on sAA.	0
Warren (2019) experiment 1A&2	Left cymba conchae	Left ear lobe	25	30s /30 s	0.5 mA 200–300 µs 25 Hz	Spitting method (3 mins)	80 min	sAA increased only in tVNS in a post-hoc analysis	?

Note.*Total number of participants in study. For studies utilizing a between-subjects design, participants per experimental condition are reported between brackets.

4. General Discussion

The stimulation of the vagus has received renewed attention in recent years, due to recent technological advances and commercializing of devices allowing for transcutaneous VNS. Indeed, a myriad of promising potential effects of tVNS can be found in recent literature, and tVNS has been proposed as a potential treatment for a broad spectrum of neurological and psychiatric disorders, including but not limited to depression, anxiety, epilepsy, pain, chronic cluster headache, coronary artery disease and atrial fibrillation (Groves & Brown, 2005; Stavrakis et al., 2015). These promising tentative findings are in stark contrast with the lack of evidence showing that tVNS actually increases vagus nerve activity or augments activity in the LC-NA network. Finding a biomarker is imperative for tVNS research going forward, as it can help guide research on clinical applications, and can inform researchers on optimal stimulation sites and parameters to further optimize treatment efficacy.

As this review clearly demonstrates, none of the potential biomarkers that have been discussed here provide clear and definitive indications that tVNS can increase vagal activation or augments central activity in the LC-NA network (see figure 1 for a graphical overview). We would argue that this may be due at least partly to suboptimal experimental assessment standards and large heterogeneity in experimental designs. In the following section, we would like to discuss several recommendations to tackle some of the challenges and opportunities for future research on the topic of biomarkers for tVNS.

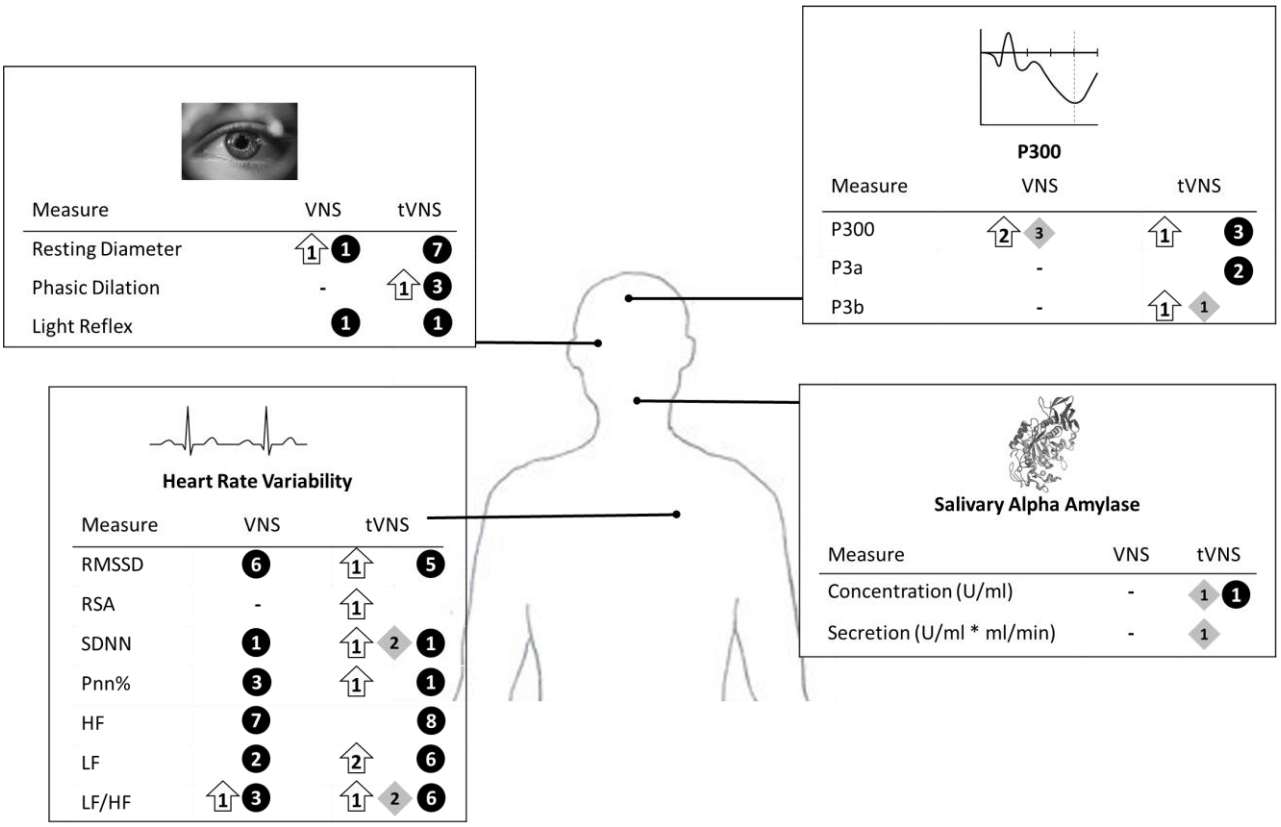

Figure 1. Schematic overview of results in humans.

: Number of studies that reported significant effects of stimulation on biomarker.

: Number of studies that reported mixed or post-hoc findings of stimulation on biomarker.

: Number of studies that reported no significant effects of stimulation on biomarker.

4.1. Recommendations and challenges for future research

1. Many of the reviewed studies seem statistically underpowered, which is an important source for the large number of mixed effects found in studies on tVNS. **It is imperative that researchers perform power analyses to guide their decisions on required sample sizes for their studies, and that they justify their sample size based on these power analyses**. Although recruiting large samples is costly in terms of both time and money, studies that include small sample sizes run a real risk of being "scientifically useless, and hence unethical in its use of subjects and other resources" (Moher, 1994). Specifically, statistical analyses of studies with small samples sizes are oftentimes underpowered, thereby increasing the risk of false negative findings. In the case of the studies that were discussed in this review, the median sample size was *n* = 20 for within-subjects designs, and *n* = 33 per condition for between-subjects designs. In a hypothetical scenario where tVNS would be compared using a t-

test, and given an alpha of .05 and a desired power of .80, this would allow researchers to reliably detect effect sizes of roughly Cohen's *d* = 0.7 in either design. True effect sizes of this magnitude are very rare in experimental clinical research (Schäfer & Schwarz, 2019), and from a theoretical and clinical point of view researchers should also be interested in detecting smaller effects. What's more, given that this reflects the reliably detectable effect size for the median sample size, this indicates that half of the experiments that were included in this review were powered to only detect effect sizes that were even larger than *d* = 0.7, making null results in these studies relatively meaningless. We urge researchers to determine beforehand what effect sizes are theoretically or clinically meaningful, and subsequently perform power analyses to determine the sample size needed to reliably detect these effects.

2. **To improve transparency in sample calculations and research questions, we urge researchers to adopt more open science practices in their research.** First and foremost, preregistering studies is an important step in study preparation that we believe should become common practice within psychological science. Not only does it increase research transparency and reduces researcher degrees of freedom, it also helps researchers initiate and foster collaborations with other researchers in their field. Moreover, sharing materials, analysis code and experimental data can help other researchers interpret your results, and can invite cross-lab attempts at exact replication projects. This is especially pertinent for biomarkers such as the P3 and pupil diameter, for which there are no standard preprocessing guidelines. To help researchers get started with their open science initiatives, we have created a general folder on the Open Science Framework, where researchers can report on their projects. To visit the 'tVNS biomarkers' folder and contribute your own preregistrations or materials, please go to https://osf.io/sn7wt/.

3. Whereas some studies did not include any control condition and compared active stimulation to a pre-stimulation baseline, other studies included either a passive 'stimulation OFF' control, or an active sham condition (e.g. stimulation of the earlobe). **To account for possible placebo effects of tVNS,**

researchers should include an active and credible control condition. We would argue that both pre-stimulation baseline and passive control conditions suffer from serious limitations. Active tVNS elicits clear sensations in the ear, and thus participants are not blind to when the stimulation is on or off. As such, active tVNS can be expected to elicit stronger placebo effects than passive control conditions or pre-stimulation baselines. Indeed, similar transcutaneous stimulation protocols using TENS devices have been shown to elicit pronounced placebo effects (Thorsteinsson, Stonnington, Stillwell, & Elveback, 1978). These placebo effects cannot be disentangled from actual effects of vagal activation without an active placebo condition that elicits similar physiological sensations.

Even though active sham stimulation is preferable to pre-stimulation baseline or passive control conditions, the optimal use of active control conditions is still under debate (Keute, Ruhnau, & Zaehle, 2018; Rangon, 2018). Active sham stimulation in tVNS studies is usually applied to the earlobe or the scapha, both of which are innervated only by the great auricular nerve, and not by the ABVN (Peuker & Filler, 2002). Neuroimaging studies showed that the brain activation pattern after earlobe stimulation is somewhat comparable to stimulation of the ABVN (e.g. deactivation of limbic areas, including the hippocampus and the poster cingulate gyrus), which could suggest that stimulation of the great auricular nerve is not solely a placebo condition but may actually have therapeutic properties. It should be noted, however, that brain areas proposed to be central to the working mechanisms of tVNS, including the NTS and the LC, are not affected by earlobe stimulation (Frangos et al., 2014; Yakunina et al., 2016). Further research is necessary to disentangle whether the central effects of earlobe stimulation are due to stimulation of the great auricular nerve or are simply due to a placebo effect. Additionally, further research is warranted to study whether stimulation of the scapha, or possibly a location further away from the auricle, can be a more suitable sham stimulation condition than earlobe stimulation.

4. It was remarkable that when measuring the biomarkers, not all studies relied on state-of-the-art assessments or indices. **We believe tVNS research would benefit from studies that adhere to**

assessment standards for the respective biomarkers. For example, a limitation in the sAA limitation is the assessments of alpha-amylase using a cotton swab that participants have to chew. This assessment of 'stimulated saliva' has been demonstrated to increase noise, as inter- and intra-individual differences in the mastication process directly affect the involvement of the parotid glands in determining the levels of sAA in saliva, independent of central noradrenergic involvement. Several guideline papers have actively argued against the use of the assessment of stimulated saliva for the assessment of sAA as a noradrenergic marker (e.g. Bosch et al., 2011).

More so than biomarkers of central noradrenergic activity discussed above, comparison of HRV findings is thwarted by the large variety of HRV indices that can be derived from the raw ECG signal. Not every index of HRV is a good representation of efferent vagal activity, and we would urge researchers to focus their attention specifically on those indices of HRV that have been recommended by the psychophysiological guidelines on ECG as reflections of cardiac vagal tone (i.e. RMSSD, pNNx, RSA, or HF HRV; Laborde et al., 2017; Task Force of The European Society of Cardiology & The North American Society of Pacing and Electrophysiology, 1996). Other indices, including the LF/HF ratio which was found to be influenced by tragus stimulation in some studies (Antonino et al., 2017; Clancy et al., 2014; Weise et al., 2015) but not in others (Couck et al., 2017; Gancheva et al., 2018), are influenced by other factors than vagal activity, and thus should not be interpreted as a definite sign that the vagus nerve is activated.

5. Apart from the methodological quality of individual research papers, we would also like to advocate the need for clear reporting guidelines. Although stimulation amplitude and frequency are commonly reported, other stimulation parameters are often left out. **We would like to propose that future studies adhere to reporting on at least some elementary components of the electrical stimulation that is applied, namely the location and laterality, applied current or voltage, frequency, pulse width, duty cycle, and the skin cleaning (see table 6).** This will increase the replicability of our research, and facilitate comparisons between studies.

Table 6. Reporting checklist for stimulation parameters.

1.	Target Site and Stimulation Device[a]
2.	Stimulation Current or Voltage[b]
3.	Frequency
4.	Pulse width
5.	Duty cycle
6.	Skin cleaning & electrode attachment

Note.

[a]: depending on whether a constant current or constant voltage device was used.

[b]: In case of custom-made electrodes, also report their size.

Example report:

"Auricular electrical stimulation was conducted using a bipolar constant current stimulator (DS5 stimulator, Welwyn Garden City, UK) connected to two titan electrodes (NEMOS®, Cerbomed, Erlangen, Germany). During active tVNS, the electrodes were attached to the cymba concha of the left ear. During sham stimulation, the electrodes are connected to the center of the earlobe instead (1). Stimulation intensity was individually calibrated to be 0.1mA below pain threshold (2). The cymba concha was stimulated at 25Hz (3) with a pulse width of 250µs (4). A 30s ON, 30s OFF duty cycle of tVNS was applied (5). Prior to attaching the electrodes, the surface of the stimulation area was cleaned for 15 seconds with an alcohol wipe. The electrode was put in place and secured with a piece of medical tape (6)."

6. Studies that assessed potential biomarkers of tVNS have predominantly focused on healthy individuals. We would argue that establishing the validity of certain biomarkers in healthy persons prior to incorporating these measurements in clinical studies is sensible, as it avoids placing an unnecessary burden on vulnerable populations. **However, if a reliable biomarker of tVNS is found in the future, incorporating it in clinical tVNS studies could provide important information on the extent to which this marker relates to clinical outcomes**. As an example, invasive VNS was found to increase the P300 magnitude only in epilepsy patients who responded favorably to VNS, and did not affect the P300 magnitude in non-responders (De Taeye et al., 2014; Wostyn et al., 2016). It remains unknown whether the P300 can be used to identify patients who will respond to VNS. If biomarkers could be used to prospectively identify potential responders to tVNS, this would greatly increase the clinical applicability of tVNS.

4.2 Summary

In contrast to the animal literature, in which the effects of invasive VNS on both a central (in the form of increased LC activation and higher NA concentrations) and cardiac (increased vagally mediated HRV) level have been clearly demonstrated, neither invasive nor transcutaneous VNS in humans has so far produced robust effects on any of the reviewed biomarkers. The mixed findings that pervade throughout the human VNS literature highlight the necessity for larger sample sizes, more stringent methodology, and the formulation of and adherence to clear reporting guidelines in these future projects. We have attempted to provide future researchers with recommendations on how to handle these potential challenges and pitfalls when studying potential biomarkers of tVNS. Given the promising preliminary effects of tVNS that have been reported on a variety of clinical and cognitive processes, we would argue that it is worthwhile to continue searching for a biomarker for tVNS.

5. Declaration of interest

None.

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