RESEARCH PROTOCOL

Exploring the effect of Transcutaneous Auricular Vagus Nerve Stimulation (taVNS) on the acute stress responses

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PROTOCOL TITLE 'Exploring the effect of Transcutaneous Auricular Vagus Nerve Stimulation (taVNS) on the acute stress responses'

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)
AE	Adverse Event
ANS	Autonomic Nervous System
AR	Adverse Reaction
BFI	Big Five Inventory
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CPT	Cold Pressor Test
CRF	Corticotropin Releasing Factor
CRU	Clinical Research Unit
CTCM	Clinical Trial Centre Maastricht
CV	Curriculum Vitae
DMP	Data Management Plan
DMNV	Dorsal Motor Nucleus of Vagus nerve
DSMB	Data Safety Monitoring Board
EU	European Union
EudraCT	European drug regulatory affairs Clinical Trials
GAD-7	Generalized Anxiety Disorder 7-Item Scale
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)
HPA	Hypothalamus-Pituitary-Adrenal axis
HRV	Heart Rate Variability
IB	Investigator’s Brochure
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
IC	Informed Consent
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier

I-PANAS-SF	International Positive and Negative Affect Short Form
MAST	Maastricht Acute Stress Task
METC	Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)
NTS	Nucleus of the Solitary Tract
PFC	Prefrontal Cortex
PHQ-9	Patient Health Questionnaire
PNS	Parasympathetic Nervous System
(S)AE	(Serious) Adverse Event
SAM	Sympatho-Adrenal-Medullary axis
SNS	Sympathetic Nervous System
SPC	Summary of Product Characteristics; in Dutch: officiële productinformatie IB1-tekst
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
TaVNS	Transcutaneus Auricular Vagus Nerve Stimulation
UAVG	Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG
VAS	Visual Analogue Scale
WMO	Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Dysregulation of the autonomic nervous system (ANS) has been shown to be associated with various diseases. Stress is a significant factor capable of inducing such sympatico-vagal imbalance by favouring sympathetic responses. Restoring normal vagal tone is a key objective in treating these conditions. Transcutaneous auricular vagus nerve stimulation (taVNS) offers a non-invasive approach to modulate the ANS, given the unique access point of the external ear to the vagus nerve. This modulation can influence numerous physiological processes and bodily states associated with information transfer between the brain and the body. While previous studies show promising results, the precise physiological impact of taVNS on vagal or autonomic function remains unclear. A better understanding of the mechanisms of action for taVNS is essential for implementing taVNS-based treatment strategies in everyday practice.

Objective: The primary aim of this study is to assess the efficacy of taVNS in mitigating the acute stress response induced by the Maastricht Acute Stress Task (MAST) among healthy subjects, measured by cortisol levels in saliva samples. It is hypothesized that taVNS will significantly reduce the cortisol response to the MAST by 35%. Since prior research showed that 85% of healthy participants exhibited a measurable cortisol response ((i.e. cortisol increase ≥ 2.5 nmol/l) to the MAST, a responder rate of 85% is expected in the sham group compared to 50% in the taVNS group (1).

Secondary objectives include evaluating taVNS's potential to counteract stress-induced sympathetic activation, thereby reducing negative affect as measured by the International Positive and Negative Affect Schedule Short Form (I-PANAS-SF), as well as feelings of stress, pain, and unpleasantness, on 0-100 Visual Analog Scales (VAS). Additionally, the study will assess taVNS's impact on autonomic outflow parameters and examine the relationship between stress responses and affective symptoms and personality trait, using the Generalized Anxiety Disorder 7-Item Scale (GAD-7), Patient Health Questionnaire (PHQ-9), and the Big Five Inventory (BFI).

Study design: This study concerns a single-centre, prospective, double-blind, randomized, placebo-controlled interventional trial with a (1:1) parallel design, with all measurements conducted at Maastricht University.

Study population: This study aims to enrol 60 healthy volunteers aged 18-65 years.

Intervention: Participants will be randomly assigned to either the taVNS or sham stimulation group, administered 30 minutes before the MAST.

Main study parameters/endpoints: The primary endpoint is a significant reduction in the neuroendocrine stress response, measured by saliva cortisol samples, in the taVNS group compared to the sham treatment group following the MAST.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: This study, involving 60 healthy volunteers aged 18-65 years, is a low-risk, single-center, double-blind, randomized, placebo-controlled trial conducted at Maastricht University. Participants will undergo two short visits: an initial visit for obtaining written informed consent and completing (digital) questionnaires, and a test day lasting 2-3 hours. During the test day, participants will receive either 30 minutes of taVNS or sham stimulation, followed by the 15-minute MAST. Participants will rate feelings of negative affect using the I-PANAS-SF and assess their perceived stress, pain, and unpleasantness using 0-100 Visual Analogue Scales (VASs). Saliva cortisol samples will be taken at eight fixed time points, up to 55 minutes after the MAST. The study does not involve incapacitated or minority groups. While participants will not directly benefit, risks are minor and proportional to scientific value. TaVNS is non-invasive, with no reported serious adverse events. The MAST is a well-established research tool and is not expected to induce negative effects beyond mild discomfort and emotional responses. Additional procedures include administering questionnaires, collecting cortisol saliva samples, and measuring autonomic parameters using a blood pressure monitor, a FitBit smartwatch, and the Shimmer3 GSR sensor. All these procedures carry minimal risks.

1. INTRODUCTION AND RATIONALE

1.1.The autonomic nervous system (ANS)

The autonomic nervous system (ANS) plays a pivotal role in coordinating bodily functions that generally are entirely subconscious. The ANS comprises two major components: the sympathetic nervous system (SNS), responsible for the body's 'fight-or-flight' reaction, and the parasympathetic nervous system (PNS), characterized by 'rest-and-digest'. An equilibrium between the excitatory SNS and inhibitory PNS is required to adaptively respond to external stressors, and therefore plays a crucial role in maintaining homeostatic control (2). The primary component of the parasympathetic nervous system is the vagus nerve, providing a mutual connection between the brain and major body structures, with innervation from the neck to the transverse colon (3). The vagus nerve is a mixed nerve, with its main function being afferent, conveying information from internal organs such as the heart, lungs, gut, and liver to the brain (4).

1.2.Role of stress in autonomic dysfunction

Dysregulation of the ANS has been implicated in the pathogenesis of various diseases, including cardiovascular diseases (5) and chronic pain disorders, such as fibromyalgia (6) and chronic low back pain (7), supported by evidence of an increased resting heart rate and reduced heart rate variability (HRV) (2). Stress is one of the significant factors capable of inducing such sympatico-vagal imbalance. Stressors encompass both acute and chronic events, varying from daily nuisances to life-threatening situations, which prompt the "fight or flight" response (8). During acute stress, the sympathico-vagal balance is heavily tilted towards the sympathetic autonomous responses, facilitated by autonomic-related projection neurons from the hypothalamus to the dorsal motor nucleus of the vagus (DMNV) in the brainstem, and sympathetic pre-ganglionic neurons in the spinal cord (9, 10). Subsequently, the release of central corticotropin releasing factor (CRF) inhibits vagal output and stimulates sympathetic activity. This in turn stimulates the release of catecholamines (e.g. epinephrine and norepinephrine) via adrenal sympathetic nerve activity (sympatho-adrenal-medullary (SAM) axis), and the release of glucocorticoids (hypothalamus-pituitary-adrenal (HPA) axis). That in turn leads to increases in heart rate, blood pressure, and respiration frequency (1, 9). Over time, chronic or recurrent stress imposes greater demands on physiologic systems, including, among others, the HPA axis and the ANS, termed "allostatic load", triggering enduring behavioural patterns and physiologic reactions that may contribute to the onset of diseases in predisposed individuals, as evidenced in conditions such as irritable bowel syndrome (IBS) (11, 12).

1.3.Rationale for taVNS as therapeutic entity

Transcutaneous auricular vagus nerve stimulation (taVNS) is an emerging technology in the field of bioelectronic medicine, prompting interest as a non-pharmaceutical treatment option for various diseases (3). TaVNS allows for non-invasive stimulation of the vagus nerve, eliminating the need for surgical procedures (4). The auricular vagus nerve serves as the target of taVNS. This provides a unique access point to the ANS as the external ear is the only place on the body where the vagus nerve sends its only peripheral branch. The modulation of this solely afferent vagus nerve serves as a 'gateway' for influencing a number of physiological processes and bodily states associated with information transfer between the brain and the body (3). Furthermore, the auricular branch allows for easy external access for electrical stimulation, connecting the applied stimuli directly to the brainstem, with the nucleus of the solitary tract (NTS) as the primary relay station for sensory vagal afferents, which then project directly or indirectly to a range of higher brain regions (13). Thus, offering an affordable and non-invasive method for manipulating the central nervous system (3). In addition, the extensive array of projections from the vagus nerve to both the brain and peripheral regions, along with their associated functions, indicates a wide spectrum of disorders potentially suitable for taVNS therapy.

Previous studies investigating the efficacy of taVNS have demonstrated promising results in various chronic pain conditions, such as migraine and fibromyalgia (3, 14). However, despite the increasing number of randomized controlled trials on taVNS, many of them remain preliminary case studies. Furthermore, the precise physiological impact of taVNS on vagal or autonomic function in humans is yet to be fully understood, primarily relying on assumptions derived from animal studies or invasive VNS procedures (3). The lack of conclusive evidence regarding why taVNS elicits therapeutic effects further complicates regulatory approval (15). Therefore, understanding the mechanisms of action for taVNS is essential for implementing taVNS-based treatment strategies in everyday practice.

1.4 The presumed influence of taVNS on autonomic disbalance

Stress, whether interoceptive or exteroceptive, activates the sympathetic nervous system while concurrently suppressing the activity of the vagus nerve (16, 17). Consequently, prolonged exposure to either chronic or recurrent stress may lead to a reduction in vagal tone. This autonomic disbalance has been linked to a reduced HRV, elevated fasting glucose levels, heightened overnight urinary cortisol, and increased levels of pro-inflammatory cytokines and acute-phase proteins (18). Together, these factors collectively contribute to increased allostatic load and diminished health (19). Furthermore, the activity of the prefrontal cortex (PFC) and the amygdala is strongly influenced by stress, leading to an imbalance between these areas, and consequently, an imbalance between the ANS and the

HPA axis (19). This is supported by a suggested inverse relationship between vagal function and cortisol levels observed during acute stress (20). A decrease in vagal tone, assessed through heart rate variability as an indicator of sympatho-vagal balance, as well as an imbalance between the ANS and HPA axis have been observed in various conditions such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (21, 22). Restoring a normal vagal tone is a key objective in treatment of such diseases (19). Therefore, considering this autonomic disbalance, stimulation of vagal afferents through taVNS may be a potent therapy, with the expected mechanism being the activation of parasympathetic efferents and the inhibition of sympathetic activity, similar to the baroreflex mechanism (23). Thus, taVNS stimulation may provide negative inhibitory feedback to counteract otherwise forward-propagating sympathetic activation and restore homeostasis (3).

1.5. Maastricht Acute Stress Task (MAST)

The potential of taVNS to counteract stress-induced sympathetic activation can be assessed by exposing healthy individuals to acute stress using the previously validated Maastricht Acute Stress Task (MAST) (1). The MAST, a brief and simple stress protocol, comprises a cold-water hand immersion test alternated with a mental arithmetic task, eliciting strong autonomic and glucocorticoid stress responses. This is supported by the understanding that physical stressors promptly activate the autonomic nervous system and HPA axis through mechanisms involving the hypothalamus and brainstem, whereas psychosocial stressors elicit responses through frontal lobes and limbic structures that are linked to the hypothalamus (24). To evaluate the effect of taVNS on vagal function, subjective, cardiovascular, and neuroendocrine stress responses can be measured. Firstly, salivary cortisol levels can be used to assess to the activity of the stress-responsive HPA-axis. In addition, vagal tone can be examined through cardiac vagal tone, measured by heart rate variability (HRV), along with other autonomic parameters such as blood pressure and respiratory rate. Lastly, multiple subjective stress ratings can be measured using a Visual Analogue Scale (VAS), providing inside into the perceived stress, pain, and discomfort experienced during the stress procedure (1).

1.6.Aim of study

The primary objective of the present project is to explore whether taVNS has the potential to mitigate the acute stress response in healthy individuals by comparing stress-related outcomes between the taVNS and the sham group. This will help to gain a deeper understanding of the specific physiological effects of taVNS on vagal and autonomic function, elucidating the mechanisms underlying its therapeutic effects across various diseases.

2. OBJECTIVES

2.1 Primary Objective

To investigate the efficacy of taVNS in attenuating the acute stress response induced by the validated Maastricht Acute Stress Task (MAST) among healthy subjects, as measured by cortisol levels in saliva samples.

Hypothesis

We hypothesize that taVNS can significantly mitigate the cortisol response to the MAST by 35%, which we assume to be a biologically relevant difference. Specifically, we anticipate a cortisol increase of 85% from baseline in the sham group compared to an 50% increase from baseline in the taVNS group. This hypothesis builds upon prior research, where 85% of participants exhibited a measurable cortisol response (i.e. cortisol increase ≥ 2.5 nmol/l from baseline) to the MAST, indicating an evident and quantifiable response to the acute stress task (1).

2.2 Secondary Objectives

1. To evaluate whether taVNS has the potential to counteract stress-induced sympathetic activation and thereby alleviate stress-related effects, including negative affect, as measured using the I-PANAS-SF questionnaire, and feelings of stress, pain, and unpleasantness, as measured with 0-100 Visual Analog Scales (VAS).
   Hypothesis: We hypothesize that the I-PANAS-SF negative affect scores and VAS scores for stress, pain, and unpleasant feelings will decrease to a greater degree in the taVNS group compared to the sham group.

2. To evaluate the effect of taVNS on parameters related to autonomic outflow, using a blood pressure monitor for blood pressure, a FitBit smartwatch for heart rate variability, and a Shimmer3 GSR sensor for heart rate variability and skin conductance.
   Hypothesis*:* We hypothesize that treatment with taVNS will lead to significant changes in the autonomic outflow reflective of an increase in parasympathetic activity.

3. To evaluate stress responses in relation to affective symptoms and personality traits, utilizing the GAD-7, PHQ-9, and BFI.
   Hypothesis: We hypothesize that there will be a significant association between affective symptoms, personality traits, and the degree of stress responses.

3. STUDY DESIGN

The proposed project concerns a monocentric, prospective, double-blind, randomized, placebo-controlled interventional trial with a (1:1) parallel design. It is anticipated to span a total duration of 2 years, encompassing recruitment, intervention, and singular measurements conducted at the NUTRIM Clinical Research Unit (CRU) at Maastricht University.

Recruitment procedures are outlined in paragraph 11.2. Interested study participants will receive a comprehensive electronic information letter and verbal explanation of the study by phone. At least 7 days later, they will be invited to attend an inclusion visit at Maastricht University. During this visit, participants will undergo eligibility screening and will receive detailed instructions about the study procedures. When eligible for inclusion, subjects will be asked to provide written informed consent to participate in the study. Following this, participants will be asked to complete several (digital) questionnaires, including the baseline questionnaire, GAD-7, PHQ-9, and the BFI. All information obtained during this screening visit, including participant data and study-related details, will be securely recorded using Castor, an electronic data capture system. After signing the informed consent form, participants will be randomized in a 1:1 parallel fashion by an investigator to receive either taVNS or sham stimulation on the second test day, with appropriate blinding procedures in place.

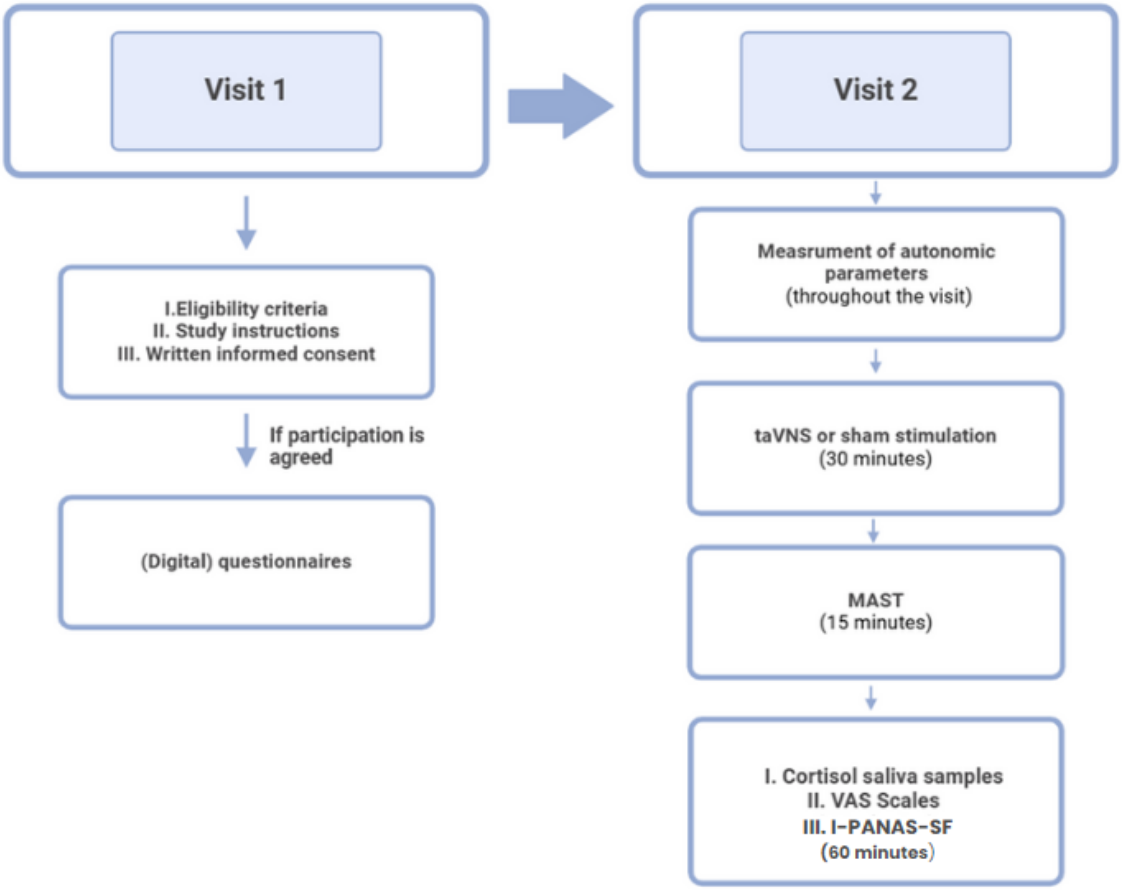
A second visit, which is the test day, will be scheduled. During this second visit participants will receive either taVNS or sham stimulation, 30 minutes prior to the MAST, depending on their allocation. This timeframe was chosen based on the following. TaVNS is assumed to act via the activation of the nucleus of the solitary tract (NTS) in the brainstem. The NTS is the primary relay station of all vagal input. Previously, Frangos, et al. (25) showed a gradual decrease in NTS activity that peaked *after* cessation of a 7-min cymba conchae stimulation. Other regions also showed a gradual increase in activity during stimulation. Among all brain regions, the right amygdala showed the greatest activation during stimulation, reaching a peak during the post-stimulation period. Nearly all regions became maximally active during the post-stimulation period; their activity declined gradually, persisting throughout the 11 min. Hence, we expect maximum effect direct after the cessation of taVNS, which is to coincide with the stress stimulus delivered through the MAST. This is hypothesized to create the most-optimal setting to maximize the counterbalancing impact of taVNS on the acute stress response.

Both taVNS treatment and sham stimulation will be administered using a tVNS device that provides stimulation to the cymba concha of the right ear. The right side is chosen based on the innervation pattern of the right vagus nerve and to ensure comparability with previous studies, such as the RESILIENCE study (NL84720.068.23 / METC 23-032). In addition, our ultimate clinical interest is related to conditions such as irritable bowel syndrome, which is considered to originate from the distal bowel. This part of the intestine is predominantly innervated by the right vagus nerve. The stimulation parameters comprise a biphasic rectangular pulse wave with an impulse frequency of 25Hz, impulse duration of 30 seconds, impulse pause of 30 seconds, and pulse width of 450ms. These parameters have been identified as the most optimal treatment frequencies for taVNS based on recent systematic reviews (26, 27). Prior to the experiment, the stimulation current will be adjusted according to the subject's sensitivity to ensure it remains below the pain threshold, with current intensities ranging between 0.25 and 10 mA. Active stimulation will be programmed in a way that it only delivers subthreshold stimuli. The device will blink when the stimulation is delivered. In the sham (control) group, the same location of the electrode will be used but the electrode will be non-conducting. The device will still blink to maintain blinding (27). Sham stimulation at a different anatomical location without vagal innervation (such as the earlobe) is considered at risk for deblinding given the information available in lay media. Nonetheless, the electrodes used in the tVNS and sham groups differ slightly. Consequently, the investigator placing the electrodes will be aware of the treatment allocation. To ensure proper blinding, two researchers will be involved on the test day. One researcher will manage the part of the tVNS stimulation and will be responsible for programming the devices, while the other researcher will conduct the second part of the test day, the Maastricht Acute Stress Task (MAST). This division ensures that the researcher conducting the MAST and responsible for data analysis is blinded to the treatment allocation.

After 30 minutes of taVNS or sham stimulation, participants will undergo the Maastricht Acute Stress Task (MAST) (1). The MAST protocol (as was previously used in the DISCOvERIE study, NL75159.068.20) includes a cold-water hand immersion test and an arithmetic task (counting backward from 2043 in steps of 17 as rapidly as possible, restarting upon error) to induce acute stress. The acute stress phase lasts for 10 minutes, preceded by a 5-min preparation phase. Cortisol levels will be measured using saliva samples collected before the start of either taVNS or sham stimulation (baseline), 5 minutes before the stress induction period (t-pre-stress), and at six subsequent intervals afterward, up to 55 minutes post-MAST. In addition, participants will be asked to rate negative affect using the I-PANAS-SF and to assess perceived stress, pain, and unpleasantness of the procedure at four time points using 0-100 Visual Analog Scales (VAS). Autonomic parameters will be recorded from before the

start of either taVNS or sham stimulation until 60 minutes after the acute stress task using a blood pressure monitor, FitBit smartwatch, and Shimmer3 GSR sensor (see 8.3.2). Blood pressure will be monitored at four different time points. Finally, after completing all measurements, participants will fill out the reimbursement form.

Figure 1: Schematic overview of the study design and the main procedures.
TaVNS, transcutaneous auricular vagus nerve stimulation; MAST, Maastricht Acute Stress Task; VAS scales, Visual Analogue Scales.



4. STUDY POPULATION

4.1 Population

To investigate whether taVNS has the potential to mitigate the acute response to stress, a total of 60 healthy volunteers will be enrolled. Participants will be recruited through various channels, including public advertisement and local newspapers. Both males and females aged between 18-65 years are eligible for inclusion.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all the following criteria:

- Healthy participants (defined as those without a pre-existing medical comorbidity);
- Aged between 18-65 years;
- Ability to understand and speak the Dutch language.

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Medical history or condition affecting the cardiovascular, respiratory, urogenital, gastrointestinal/hepatic, haematologic/immunologic, HEENT (head, ears, eyes, nose, throat), dermatological/connective tissue, musculoskeletal, metabolic/nutritional, endocrine, neurological/psychiatric systems, as well as prior major surgeries or ongoing laboratory abnormalities that could potentially limit participation or completion of the study protocol;
- Any use of medication, especially those that may influence the autonomic nervous system or the hypothalamus-pituitary-adrenal axis (e.g., beta-agonists or corticosteroids), with the exception of contraceptives and paracetamol;
- Current or lifetime psychopathology (including PHQ-9 and GHD-7 scores $\geq$ 10);
- Substance abuse (including excessive alcohol consumption);
- Smoking;
- Pregnancy, lactation, or intention to become pregnant during the study period;
- Use of devices (e.g., cochlear implants) or other reasons (e.g. wounds, permanent ear-piercing) which complicate the use of the tVNS device;
- Participation in another clinical study in which the MAST was used;

- Administration of investigational drugs or participation in any scientific intervention study that might interfere with this study (to be determined by the principal investigator) within 180 days preceding the commencement of the study;
- Students and employees of Maastricht University are not precluded from participation, unless they have a direct personal, professional or hierarchical position with regards to any of the study team members or their department.

4.4 Sample size calculation

There are no directly comparable studies available for an accurate sample size calculation, as previous research investigating the effect of tVNS on stress used different stimuli, making comparison challenging. However, previous research on the Maastricht Acute Stress Task (MAST) indicated that 85% of participants demonstrate a noticeable and quantifiable cortisol response (i.e., cortisol increase ≥ 2.5nmol/l from baseline) to the acute stress task (1). Anticipating that tVNS is expected to counteract the cortisol response to the MAST by 35%, resulting in an estimated 85% cortisol increase from baseline in the sham group vs. a 50% increase from baseline in the tVNS group, a sample size of 54 participants is required to detect this difference (b=0.8, a=0.05). This estimate is consistent with other somewhat similar studies on tVNS. Based on previous experience with studies involving healthy volunteers, an additional six participants (10%) will be recruited to account for potential dropouts. This approach is deemed appropriate and feasible given the short an non-invasive nature of the intervention.

5. TREATMENT OF SUBJECTS

This study aims to investigate the efficacy of taVNS in attenuating the acute stress response by comparing stress-related outcomes between the active and sham groups.

5.1 Investigational product/treatment

The intervention of interest is taVNS, with sham stimulation employed as the appropriate control.

5.1.1. taVNS

Electrical stimulation will be administered to the auricular branch of the vagus nerve, specifically at the cymba conchae of the right ear, using a custom-made device called tVNS R (tVNS Health GmbH, Grünwald, Germany). This device comprises a stimulation unit and an ear electrode. This electrode is connected as an anode to the stimulation device by a cable and is worn similarly to an earphone. The stimulation unit sends electrical impulses through the electrode, which stimulates the auricular branch of the vagus nerve. On the test day, subjects assigned to the taVNS group will receive 30 minutes of active stimulation before the MAST procedure. According to recent systematic analyses determining the most optimal treatment frequencies for taVNS, the stimulation parameters will consists of biphasic rectangular pulse trains with an impulse frequency of 25Hz, impulse duration of 30 seconds, followed by an impulse pause of 30 seconds, and an impulse width of 450ms (26, 27). Each participant will undergo sensory testing prior to the commencement of the treatment period during which the current will be adjusted to suprathreshold stimulation intensity, with current intensities ranging between 0.25 and 10 mA. The device will indicate stimulation delivery by blinking. Concurrently with taVNS stimulation autonomic parameters, using a blood pressure monitor, FitBit smartwatch, and a Shimmer3 GSR sensor, will be collected.

5.1.2. Sham stimulation

In the control group, sham stimulation will be administered to the same location of the electrodes (right cymba conchae) using an identical device as that used for taVNS. The electrode will be non-conducting, ensuring that there is no stimulation of the vagus nerve. However, as the device is identical for both tVNS and sham stimulation, the electrodes used in the tVNS and sham groups are slightly different, resulting in the investigator placing the electrodes being unblinded to the treatment allocation. Nevertheless, participants will not be aware of the treatment received since stimulation is provided to the same location and the device will still blink to maintain blinding (27).

5.2 Use of co-intervention: MAST

The Maastricht Acute Stress Task (MAST) (1) will be implemented as the co-intervention following either 30 minutes of taVNS or sham stimulation. The MAST (as was previously used in the DISCOvERIE study, NL75159.068.20) will be utilized to investigate the effects of taVNS on the acute stress response. This validated tool to induce acute stress comprises a brief and simple stress protocol involving a cold-water hand immersion test and an arithmetic task (counting backward from 2043 in steps of 17 as rapidly as possible, restarting upon error) to induce acute stress. The acute stress phase lasts for 10 minutes and is preceded by a 5-min preparation phase. For a detailed description of the steps undertaken in this task, please see paragraph 8.3.

5.3 Escape medication

Not applicable.

6. INVESTIGATIONAL PRODUCT

In this clinical investigation we will use tVNS R. tVNS L already has a declaration of conformity. However, tVNS R and tVNS L are completely equivalent concerning all electrical as well as mechanical aspects. The only difference is the software of the products. Where in the tVNS L device the stimulation parameters are fixed, these can be changed in the tVNS R for research purposes. See annex
'D2_Declaration_of_conformity_tVNS_L_signed_Versie1.0_dd 31-05-2021' and D4_Confirmation_of_equivalence_tVNS_Versie1.0_dd 22-10-2021'. Because the tVNS device will be used outside the intended use of the declaration of conformity this investigation falls under article 82 of the MDR.

6.1 Name and description of investigational product(s)

The tVNS R is a vagus nerve stimulation device comprising a stimulation unit and an ear electrode that is worn like an earphone. The stimulation unit delivers electrical impulses through the electrode, stimulating the auricular branch of the vagus nerve transcutaneously (through the skin). Further details are provided in paragraph 5.1.

6.2 Summary of findings from non-clinical studies

Animal studies have demonstrated that transcutaneous vagus nerve stimulation in rats experiencing transient focal ischemia revealed activation in the locus coeruleus and a reduction in the volume of the infarct zone (28). Furthermore, another study revealed that transcutaneous vagus nerve stimulation can decrease systemic tumor necrosis factor levels and enhance survival in mice with lethal endotoxemia (sepsis) (29).
In healthy humans, transcutaneous vagus nerve stimulation has been demonstrated as a safe and well-tolerated procedure, particularly in patients without a history of cardiac disease (15, 27, 30). Notably, heart rate and blood pressure remain unaffected.
Transcutaneous vagus nerve stimulation likely operates through a mechanism similar to invasive VNS, involving the activation of the locus coeruleus (and noradrenaline) and the elevation of GABA levels (31). Healthy participants undergoing transcutaneous vagus nerve stimulation reported decreased pain levels compared to the sham condition (32).

6.3 Summary of findings from clinical studies

Transcutaneous vagus nerve stimulation has been effectively utilized in a controlled trial with patients diagnosed with epilepsy, resulting in a reduction in seizure frequency and improvements in EEG measures, depressive symptoms, anxiety, and quality of life (33-35). In addition, a pilot study indicated that taVNS enhances mood and reduces handicap scores in patients with tinnitus (36). Furthermore, a recent systematic review and meta-

analysis highlighted that taVNS shows promise in reducing the number of migraine days and headache intensity (37). However, a systematic review examining taVNS in cardiac, pain, and other conditions revealed conflicting results regarding heart rate variability (HRV) as an indicator of sympathovagal balance (27). In studies that reported changes in heart rate, a modest decrease of 2-3 beats per minute was observed in the active group. Most trails, however, found no significant difference in heart rate between the control and active stimulation groups. Nevertheless, the calculation of HRV varied inconsistently across the studies, making it challenging to draw definitive conclusions from the aggregated data. Furthermore, one study investigating the impact of taVNS on markers of noradrenergic activity in healthy individuals found a significant decrease in cortisol levels over time, using saliva samples, following taVNS treatment. However, compared to sham, there was no significant difference between taVNS and sham stimulation in cortisol levels overall or as a function of time (38). Furthermore, post-hoc analysis in two studies showed that cortisol levels significantly declined throughout the experiment following sham stimulation, but not after taVNS treatment (39, 40).

6.4 Summary of known and potential risks and benefits

Transcutaneous vagus nerve stimulation is a non-invasive treatment that is well-tolerated and safe in patients with no pre-existing cardiac pathology (41, 42). Thus far, no serious risks have been mentioned in the literature. Some cases have reported dizziness or daytime drowsiness in long-term stimulation, which alleviated upon reducing the stimulation intensity (34). In another pilot study, taVNS exhibited no serious or long-lasting side effects, and no significant heart rate changes were observed during stimulation. A few individuals reported fatigue, concentration problems, or a tingling sensation at the ear, which subsided after 90-minutes (43). Furthermore, in a randomized controlled trial conducted with adolescents using a more invasive method involving percutaneous electrical nerve field stimulation, eight individuals discontinued treatment due to aesthetic reasons, discomfort from the device's fit, needle phobia, or anxiety, as well as adhesive allergy (44). However, in this current study, ear electrodes are utilized, fitting perfectly without the need for plasters and capable of stimulating the vagus nerve in a non-invasive, transcutaneous manner, rendering the aforementioned reasons less relevant. In addition, patients may experience slight hearing impairment on the side where the electrode is placed. A recent systematic review and meta-analysis demonstrated no severe side effects. Mild instances of dizziness, headache, and slight redness around the stimulation area, which dissipated shortly after discontinuing the intervention, were reported (45). Furthermore, taVNS can be provided safely both left and right-sided, as was illustrated by a review article (46).

6.5 Description and justification of route of administration and dosage

Not applicable.

6.6 Dosages, dosage modifications and method of administration

Not applicable.

6.7 Preparation and labelling of Investigational Medicinal Product

Not applicable.

6.8 Drug accountability

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

Not applicable.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

- A significant reduction in the neuroendocrine stress response triggered by the MAST following taVNS or sham treatment, assessed through saliva cortisol samples, with a defined threshold of a 35% decrease.

8.1.2 Secondary study parameters/endpoints

- Subjective stress responses to the MAST following taVNS or sham treatment, assessed through scores on the I-PANAS-SF and 0-100 Visual Analog Scales (VAS);
- Autonomic response to stress following taVNS or sham treatment and the MAST, assessed using a combination of a blood pressure monitoring, FitBit smartwatch, and the Shimmer3 GSR sensor;
- Stress responses in relation to potential affective symptoms and personality traits, using the GAD-7, PHQ-9, and BFI;
- Number and severity of adverse events.

8.1.3 Other study parameters

- Patient characteristics: sex, age, lifestyle factors, medical history, and mental health.

8.2 Randomisation, blinding and treatment allocation

After confirming suitability for inclusion and obtaining written informed consent during the initial visit, participants will be scheduled for a second visit (referred to as the test day). Prior to this test day, an independent investigator will randomise the subjects in a 1:1 parallel fashion to receive either taVNS or sham stimulation. The randomisation of participants will be conducted using an online environment provided by the CTCM (TENALEA) software. The concealment list will be generated by the CTCM and provided to a co-worker in the same department as the coordinating investigator. For emergencies, drs. Sweerts will be provided with the concealment list and will be able to de-blind. The concealment list provides information on which participant will get active taVNS and who will get sham stimulation. This information will not be given to the investigators, and the concealment list will be sealed until after the initial analysis.

In this study, blinding procedures will be implemented. The tVNS device used will be identical for both the active and sham groups, and the ear electrodes will be placed at the same anatomical location. Furthermore, the device will blink in both groups to ensure that participants remain blinded to their treatment allocation. The only differences are that the sham group will receive a device with a non-conducting electrode, and that the electrodes for the taVNS and sham groups will be slightly different. Consequently, the investigator placing the electrodes will be aware of the treatment allocation. To maintain proper blinding, two researchers will be involved on the test day. One researcher will manage the tVNS stimulation and will be responsible for programming the devices, while the other researcher will conduct the second part of the test day, the Maastricht Acute Stress Task (MAST). This division ensures that the researcher conducting the MAST and responsible for data analysis is blinded to the treatment allocation.

8.3 Study procedures

8.3.1 Visit 1

The screening visit will take place at the NUTRIM Clinical Research Unit (CRU) of Maastricht University. At this first visit, all participants will be asked to confirm that they have read the information letter, received oral information, and have no further questions regarding the study. The investigator will ensure the participant understands all the information through an interview. Afterward, the eligibility criteria will be checked, and if suitable, the participant will be asked to complete written informed consent prior to any study procedures. Following inclusion, participants will be requested to complete several questionnaires, including a baseline questionnaire (F1.1_Baseline_Characteristics_Questionnaire_Versie1.0_dd 06-06-2024), GAD-7 (F1.2_GAD7_Versie1.0_dd 06-06-2024), PHQ-9 (F1.3_PHQ9_Versie1.0_dd 06-06-2024), and BFI (F1.4_BFI_Versie1.0_dd 06-06-2024), with the support of Castor EDC. Given that current or lifetime psychopathology serves as an exclusion criterion and the GAD-7 and PHQ-9 assess for the presence of anxiety and depression disorders, respectively, participants scoring 10 or above on either questionnaire will be excluded from the study (47).

8.3.2 Visit 2

The test day will take place at the NUTRIM Clinical Research Unit (CRU) of Maastricht University. Upon arrival at the CRU, all subjects will complete a checklist to verify compliance with the test day regulations. These regulations include not eating or drinking anything except water, brushing their teeth, using chewing gum, or smoking one hour prior to the start of the test session (until the last saliva sample

has been taken). Furthermore, participants must not consume alcohol on the day before the test day, nor consume caffeine or engage in physical exertion on the day of the test session.
Once compliance is confirmed, the investigator, responsible for the first part of the test day, will have the participant complete the I-PANAS-SF questionnaire and the VAS scales. Blood pressure will then be measured, and the FitBit smartwatch and the Shimmer3 GSR sensor will be applied to allow for continuous measurement of autonomic parameters. Afterward, the sensory threshold of the tVNS device will be calculated. The tVNS device will be programmed to deliver only subthreshold stimuli to assure blinding. Subjects will then receive 30 minutes of either active taVNS or sham stimulation, followed by the MAST.

Maastricht Acute Test Task (MAST)

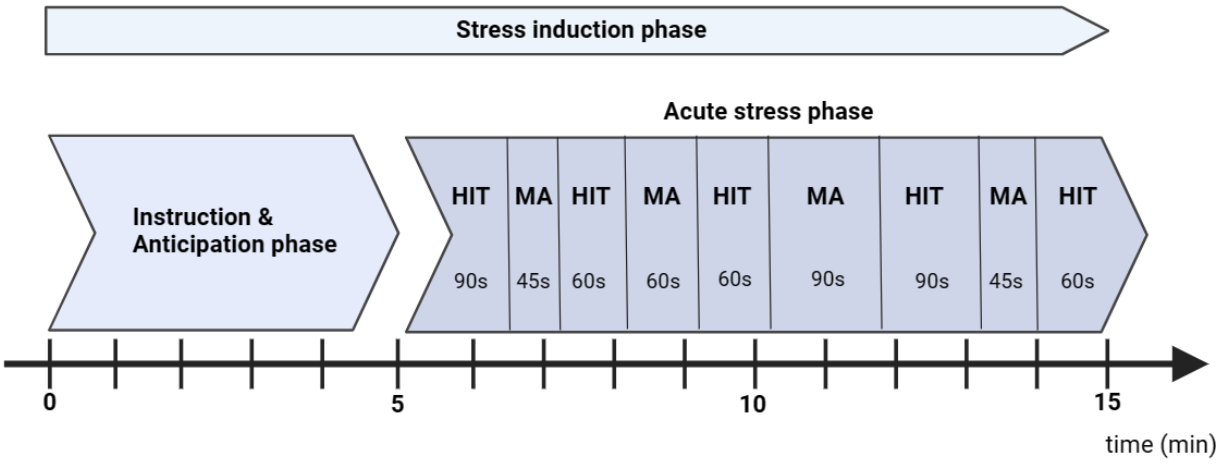
To investigate the effects of taVNS on the acute stress response, the MAST will be administered after 30 minutes of taVNS stimulation by the second investigator. The MAST is a validated tool for inducing acute stress (1). It comprises a 5-minute preparation phase followed by a 10-minute acute stress phase, which includes a cold-water hand immersion test and a mental arithmetic task (Figure 2).
During the 5-minute preparation period, participants will be seated in front of a computer screen and provided instructions about the upcoming task through a PowerPoint presentation (see F4.2_MAST_Powerpoint_Versie1.0_dd 06-06-2024). Participants will be informed about the multiple trials, including both the cold-water hand immersion test and the arithmetic task. Furthermore, participants will be notified that they will be monitored by the experimenter as well as videotaped, for later facial expression analysis. Participants will also be informed about the requirement to provide written informed consent for the videotaping (see E1-E2b_Informatiebrief_en_toestemmingsformulier_Versie2.0_dd 18-07-2024) and their right to withdraw from the task at any time. However, in reality, subjects will be informed that they will be videotaped to elevate their stress levels, but no videos will be recorded, and no facial expression analysis will be conducted.

During the acute stress phase, participants will be required to immerse their hand multiple times in cold (4 ◦C) water. Subjects will be informed that the duration of these trials will be randomly determined by the computer but will never exceed 90 seconds. Between the hand immersion trials, subjects will be instructed to put their arm on a towel beside the water bath and immediately engage in the mental arithmetic test. This test involves counting backward from a defined number (e.g.

2043) in steps of 17 as quickly and accurate as possible. If a mistake is made, the subject will receive negative feedback and must restart from 2043. Participants will be instructed to continue with the mental arithmetic task until the computer signals the start of the next hand immersion trial, which will be told to last at least 45 seconds. In reality, the duration of the hand immersion trials alternated with the mental arithmetic task will be predetermined and consistent for all participants (see Figure 2). This task has been validated and is effective in eliciting a strong subjective and physiological stress response (1, 48). See F4.1_MAST_Protocol_Versie1.1_dd 02-01-2025 for a detailed protocol of the MAST.

Following the stress phase, participants are promptly briefed on the nature and objectives of the task and the video recording. The subjects are informed that no video recording occurred during the test, and that the written consent for video recording was not genuine. It will be clarified that this was staged to heighten stress levels as part of the test and not for facial expression analysis. Before the participants go home, efforts will be made to ensure that the participants are calm and have no remaining questions.

Figure 2: Schematic timeline of MAST



HIT = Hand Immersion Test
MA = Mental Arithmetic

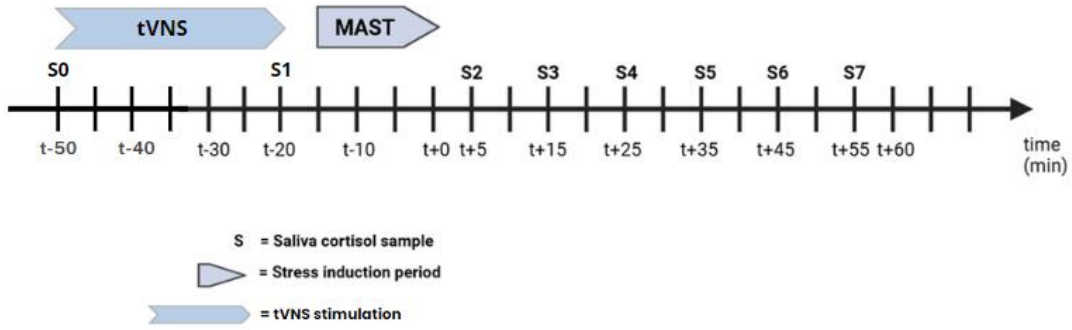
During the test day subjective, cardiovascular, and neuroendocrine stress responses will be measured:

- *Neuroendocrine stress response*: Cortisol levels will be quantified in response to the stress induction procedures as an indicator of the activity of the stress-

responsive HPA axis. Saliva samples will be collected using synthetic Salivette devices before the start of either taVNS or sham stimulation (baseline), 5 minutes before (i.e. t-pre-stress) and 6 times afterwards (i.e. t+05, t+15, t+25, t+35, t+45, t+55) relative to the end of the stressor (thus, a total of 8 samples will be collected: see Figure 3).

- *Cardiovascular stress response:* Autonomic parameters will be continuously recorded from the start (i.e. prior to taVNS or sham stimulation) until 60 minutes after the acute stress task. Heart rate variability and skin conductance will be assessed using a FitBit smartwatch and the Shimmer3 GSR sensor. Blood pressure will be measured at four fixed time points: before the taVNS or sham stimulation, prior to the MAST, immediately after the MAST, and one hour following the MAST.
- *Subjective stress response:* Participants will be asked to evaluate the extent of negative affect using the I-PANAS-SF, and to rate feelings of stress, pain, and discomfort experienced before and after the procedure by marking four Visual Analog Scales (VASs) ranging from 0 to 100 (anchors: 0 = ‘not at all’; 100 = ‘extremely’) (see F1.5_VAS_Scales_Versie1.1_dd 02-01-2025).

Figure 3: Overview of timing of saliva sample collecting following the MAST



Shimmer3 GSR sensor

The Shimmer3 GSR sensor will be used to collect data on heart rate variability and skin conductance. This CE-certified device (see K6.1_Shimmer_GSR_Declaration_of_conformity_Versie1.0_dd 04-09-2014), designed and manufactured in conformity with the essential requirements and provisions of the European Council Directive 2014/35/EU, provides real-time Galvanic Skin Response (GSR) biofeedback by measuring the electrical conductance of the skin. It supports one channel of GSR data acquisition (Electrodermal Resistance Measurement – EDR/Electrodermal Activity – EDA) and captures an Optical

Pulse/PPG (photoplethysmogram) signal to estimate heart rate using the Shimmer ear clip or optical pulse probe. The sensor monitors skin conductivity between two reusable electrodes attached to two fingers on one hand. In this study, the electrodes will be attached to the index and middle fingers that do not come in contact with water during the hand immersion trial. When the sweat glands become more active due to a stimulus, moisture on the skin increases, enhancing current flow and thus increasing skin conductance. The Shimmer3 GSR sensor allows simultaneous, real-time measurement of all signals, with data streamable for live visualization via Consensys software and raw data loggable to an SD card for later access (49, 50).

FitBit Smartwatch

The FitBit wearable device will be used to collect patient data concerning heart rate variability. Participants will wear the FitBit device from the start of the test day until 60 minutes after the acute stress task. The device will be connected to the coordinating investigator's smartphone via Bluetooth and the Fitbit app. Heart rate data will then be automatically collected by the FitBit.

8.3.3 Biological samples

Saliva cortisol samples: During the study visit, saliva cortisol samples will be collected using synthetic Salivette devices: before the start of either taVNS or sham stimulation (baseline), 5 minutes before (t-pre-stress) and 6 times afterward (t+05, t+15, t+25, t+35, t+45, t+55) the MAST (thus, a total of 8 samples will be collected; see Figure 3). Samples will be processed as soon as possible and will be promptly stored at -20 ◦C until further analysis. The analysis of the samples will be conducted at the end of the study period in the laboratory of Maastricht University using immunoassay (ELISA SLV-2930). See F4.4_MAST_SOP_Speekselsamples_Versie1.1_dd 02-01-2025 for a detailed description of the saliva cortisol sample collection process and subsequent processing.

8.3.4 Questionnaires

All questionnaires will be completed in a secure electronic environment, named Castor, accessible via the Internet through a telephone or computer. Castor was developed by the Maastricht Center and Information and Data Management (MEMIC), affiliated with Maastricht University. Castor utilizes a NEN7510 and ISO 27001:2005 certified server called “True”. Our research group has previously used Castor in the FORTITUTE study (NL67607.068.18/METC 18-037).

Baseline characteristics questionnaire

‘F1.1_Baseline_Characteristics_Questionnaire_ Versie1.0_dd 06-06-2024’

At visit 1, subjects will complete this questionnaire concerning baseline characteristics, demographics, and lifestyle.

Generalized Anxiety Disorder 7-Item Scale (GAD-7)

‘F1.2_GAD7_Versie1.0_dd 06-06-2024’

The GAD-7 is a validated 7-item questionnaire, which has been demonstrated to be an efficient tool for screening generalized anxiety disorder and assessing its severity in clinical practice and research (51). It includes seven anxiety symptoms, with cut-off levels for mild, moderate, and severe anxiety. Subjects will be asked to complete the GAD-7 during the first visit to screen for anxiety.

Patient Health Questionnaire (PHQ-9)

‘F1.3_PHQ9_Versie1.0_dd 06-06-2024’

The PHQ-9 is a validated instrument utilized for screening, diagnosis, and measuring the severity of depression. It evaluates each of the nine DSM-IV criteria for depression along with key symptoms of major depressive disorder, condensing them into a concise self-report questionnaire. The questionnaire establishes cut-off points for mild, moderate, moderately severe, and severe depression (52). During the initial visit, participants will complete the PHQ-9 to assess for the presence of depression.

The Big Five Inventory (BFI)

‘F1.4_BFI_Versie1.0_dd 06-06-2024’

The BFI is an instrument designed to assess the personality of individuals aged 18 and above. It comprises 44 items divided into five sub-scales, each representing

one of the five personality factors: extraversion (8 items), neuroticism (8 items), conscientiousness (9 items), agreeableness (9 items), and openness to experience (10 items). Participants are required to rate the extent to which they agree with the statement using a 5-point Likert scale ranging from 1 (Disagree strongly) to 5 (Agree strongly) (53). During the initial visit, participants will complete the BFI to evaluate their personality traits.

0-100 Visual Analog Scales (VASs)

'F1.5_VAS_Scales_Versie1.1_dd 02-01-2025'

Participants will be requested to assess the degree of stress, pain, and discomfort encountered before and during the procedure by providing a score ranging from 0 to 100 four times, where 0 indicates 'not at all' and 100 indicates 'extremely'. This scoring system is used to measure the subjective stress response.

International Positive and Negative Affect Schedule Short Form (I-PANAS-SF)

'F1.6_PANAS_Versie1.0_dd 02-01-2025'

The I-PANAS-SF is the short version of the 20-item Positive and Negative Affect Schedule (PANAS) and includes two categories: positive and negative affect, each containing 5 items (59). Participants will be asked to rate their feelings of positive and negative affect on a scale from 1 ('never') to 5 ('always'). This scoring system is used to measure the subjective stress response.

8.4 Withdrawal of individual subjects

Participants are free to withdraw from the study at any time, for any reason, without facing any consequences. They are not required to provide a specific reason for their decision. In urgent medical situations, the investigator reserves the right to withdraw a participant from the study.

8.4.1 Specific criteria for withdrawal (if applicable)

We accounted for potential dropouts in our sample size calculation.

8.5 Replacement of individual subjects after withdrawal

If a subject decides to discontinue participation before completing all study procedures, this subject will be considered a dropout and will be excluded from analyses. In case this happens during the recruitment period of the study, researchers may choose to replace

the subject. However, subject IDs will not be reused to prevent data confusion; instead, a new subject ID will be assigned to the “replacing” participant.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

There are no expected reasons for premature termination of the study. However, in the event of urgent medical issues arising during the study (for example SAEs that result in death or are life threatening) the investigator (if necessary, together with the medical committee) can decide to halt the study to check whether it is safe to proceed or if termination is preferred.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to transcutaneous vagal nerve stimulation or the MAST. All adverse events reported spontaneously by the subject or observed by the investigator, or his staff will be recorded. Subjects are promptly asked to report any changes in their health in the 24 hours following the test day via phone or email to the coordinating investigator. These adverse events will then be recorded in Castor.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any adverse event that led to any of the following:

- Death,
- Serious deterioration in the health of the subject, that resulted in any of the following:
    - Life-threatening illness or injury,
    - Permanent impairment of a body structure or a body function,
    - Hospitalisation or prolongation of patient hospitalisation,
    - Medical or surgical intervention to prevent life-threatening illness or injury or permanent impairment to a body structure or a body function,
    - Chronic disease,
- Foetal distress, foetal death or a congenital physical or mental impairment or birth defect.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events. The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable.

9.3 Annual safety report

Not applicable.

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

9.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable.

10. STATISTICAL ANALYSIS

Demographic data and patient-reported data will be registered and saved in Castor by the coordinating investigator. Database cleaning will be carried out by the study coordinator and the local research nurses, using internal consistency checks and identification of database entries outside expected ranges.

Statistical analysis will be performed using IBM SPSS, R, Python, and Excel. Subject characteristics will be presented and analysed using descriptive statistics. Continuous parameters will be presented as means and standard deviations, and assessed between the two groups (taVNS vs. sham) with a two-sample unpaired t-test (for normally distributed data) or a nonparametric Mann-Withney U test (for non-normally distributed data). Categorical variables will be represented as frequencies and analyzed using a Chi-square test or a Fisher's exact test. A p-value < 0.05 will be considered statistically significant.

Data interpretation will involve effect size estimates as appropriate for each statistical test (e.g. Cohen's *d* for t-tests, *f* for Mann-Whitney U test) as well as estimated intervals, such as 95% confidence intervals. Thereby, conclusions will not be drawn from a singular p-value alone. No missing data are expected. Primary and secondary outcomes data will be analysed based on an intention-to-treat approach.

10.1 Primary study parameter(s)

The efficacy of taVNS in attenuating the acute stress response induced by the validated Maastricht Acute Stress Task (MAST) among healthy subjects will be determined based on cortisol levels in saliva samples, using an intention-to-treat principle. A repeated measures ANOVA will be utilized to evaluate both intergroup differences and within-group changes in cortisol levels across the different time points. If the assumption of normality is violated, a nonparametric equivalent will be employed. Multivariable regression analysis will be performed to correct for confounding effects of age, gender, and mental health (i.e. GAD-7, PHQ-9, BFI). Differences in cortisol saliva levels, with corresponding 95% confidence intervals (CIs) and two-sided p-values, will be calculated at a type I error level of 0.05.

10.2 Secondary study parameter(s)

The efficacy of taVNS and sham stimulation in alleviating stress-related effects, such as negative affect and feelings of stress, pain, and unpleasantness, will be determined

based on scores on the I-PANAS-SF and the 0-100 Visual Analog Scales (VASs). Scores will be compared between the two groups using the same statistical approach as that for the primary outcome.

To evaluate the effect of taVNS vs. sham on autonomic parameters, such as heart rate variability and skin conductance, a mixed-effects model will also be used. This model allows for the evaluation of the impact of taVNS on each autonomic parameter while accounting for the repeated measure within subjects.

To assess the effect of taVNS vs. sham on pulse rate variability (PRV), as a measure of vagal tone of the autonomous nervous system, all photoplethysmography data files will be processed using a custom-written MATLAB script (MATLAB R2020a). Systolic peaks will be detected using the peak detection method and considered as the pulse times for calculating pulse intervals and PRV. Root mean square of successive differences (RMSSD) will be calculated first for the 6-minute baseline period and then over fixed 5-minute intervals, following standard practice. For statistical analysis, the RMSSD change from pre-intervention (baseline) will be calculated for each subject under each condition (i.e. taVNS and sham). Marginal linear mixed models (using the R lme4 function) will be used to compare RMSSD change from baseline between conditions, using R Statistical Software version 3.6.3 (2020-02-29). RMSSD (change from baseline) will be the dependent variable, with condition (taVNS versus sham) as the within-subject independent variable. Both the main effects and the two-way interaction will be included in the model. Model fit will be assessed using the Kronecker product of an unstructured and a first-order autoregressive variance-covariance matrix, based on the lowest value of Akaike's Information Criterion. The main effect of condition will test the hypothesis of a significant difference in RMSSD between conditions over the entire time course.

Furthermore, HRV data obtained from the FitBit and Shimmer3 GSR will be compared for accuracy using linear mixed-effects models.

10.3 Other study parameters

Not applicable.

10.4 Interim analysis

Not applicable.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the most recent version of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013), the Medical Research Involving Human Subjects Act (WMO), and adheres to the Medical Device Regulation (MDR, EU no 2017/745).

11.2 Recruitment and consent

Healthy volunteers will be recruited through a variety of channels, including advertisements in local and faculty newspapers, flyers/posters in public spaces, social media, and by reaching out to individuals who have previously participated in research and consented to be contacted for future studies, utilizing advertisement document E3_Wervingsmateriaal_Versie2.0_dd 22-07-2024. This document will outline the study's objectives, inclusion criteria and the timeline of this study. The contact details of the coordinating investigator will be provided at the bottom of the advertisement document, including a link/QR-code, that automatically generates an email, for easy access by potential participants. Upon contacting the study team via telephone, volunteers will receive a detailed oral explanation of the study from a researcher, and the written information brochure and informed consent form (see E1-2b_Informatiebrief_en_toestemmingsformulier_Versie2.0_dd 18-07-2024) will be sent via regular mail or email. For volunteers who contact the study team via email, the written information brochure and informed consent form will be promptly provided. Additionally, a verbal explanation of the study will be given over the phone. After sending the written information brochure and providing the verbal explanation, a minimum period of one week is given for the participant to decide whether they would like to participate. The participant is encouraged to contact the investigator by telephone to ask additional questions and to discuss their decision to participate. If the participant does not initiate contact with the researcher within this timeframe, the researcher will reach out to them once, at the earliest 7 days after sending the written information brochure and the verbal explanation, and only if the participant has given permission to do so during the initial conversation or in the initial email. During this contact, the researcher ensures that the participant understands the provided information and addresses any remaining questions. If the participant expresses a positive decision to participate, the first visit will be scheduled at Maastricht University. During this visit, participants will be explicitly asked to state in their own words what the study involves, including the associated risks and burdens. After this, the written informed consent form will be signed, multiple (digital) questionnaires will be

answered, and a second visit for the measurements will be planned. No study procedures will be performed until the informed consent form is signed.

11.3 Objection by minors or incapacitated subjects

Not applicable.

11.4 Benefits and risks assessment, group relatedness

This study does not involve any incapacitated or minority groups, and is considered a low-risk study. Although volunteers will not benefit directly from participating in this study, the risks associated with participation are minor and proportional to the scientific value of the research. This study can contribute to understanding the putative mechanism of action for taVNS, providing new insights and perspectives for further research into the application of taVNS in pathophysiological states, and potentially leading to new treatment options for various indications.

Subjects will be informed about the risks and burdens associated with the measurements beforehand. However, the burdens and risks of the current study are considered to be minimal to the benefits. The study protocol involves two short visits, with the test day having a maximum expected duration of 2-3 hours. These visits are not overly time-consuming for participants and are not expected to interfere with their regular daily activities. TaVNS stimulation is a non-invasive treatment approach, and to date, no serious adverse events have been reported in literature. For a detailed overview of previously reported risks of taVNS, see paragraph 6.4 ‘Summary of known and potential risks and benefits’. The MAST (as was previously used in the DISCOvERIE study, NL75159.068.20) is not expected to induce negative effects or safety hazards beyond mild discomfort and the emotional responses of stress, fear, and mild pain. Specifically, a previous version focusing on the hand-immersion-in-cold-water aspect of the task, that is the cold pressor test (CPT), indicated that it is acceptable even for use with children. Discomfort from the CPT dissipates quickly after hand removal from the water, adverse events are rare and transient, and the majority of researchers, children, and parents reported positive experiences with the CPT. Participants will be promptly debriefed on the nature and objectives of the task immediately after the procedure, and efforts will be made to ensure that participants are calm and have no remaining questions before leaving. Further study procedures involve the completion of questionnaires and collection of biological materials (i.e. cortisol saliva samples), both of which carry no significant risks. In the event of incidental findings during the study period, the general practitioner

will be contacted. If a subject does not wish to be informed of incidental findings, they will not be allowed to participate in the study.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO. The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.6 Incentives

Subjects who complete the entire study period, including the initial and second visits with taVNS vs. sham stimulation, MAST, collection of cortisol saliva samples, and completion of all questionnaires, will receive a compensation of 25 euros. Furthermore, travel expenses will be compensated, reimbursing €0.23 per kilometre if travelled by car or full reimbursement for public transportation costs. Participants who decide to stop earlier will be reimbursed for travel expenses but will not receive any other compensation.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

All obtained data will be handled confidentially and coded to protect the privacy of the participants, in accordance with EU General Data Protection Regulation (Algemene Verordening Gegevensbescherming, 2016) and the Dutch Act on Implementation of the General Data Protection Regulation (Uitvoeringswet Algemene Verordening Gegevensbescherming, 2020). At study entrance, all subjects are assigned an individual study ID that is not directly traceable to the subject. The code starts with “UM” to indicate the corresponding center, followed by a participant number consisting of three digits in chronological order, starting with “001” for the first subject, and so on (for example: “UM001). The coordinating investigator will keep the key of the code in a locked cabinet, to which only the coordinating and principal investigator have access. In case of inspection, the Inspectie van Gezondheidszorg en Jeugd (IGJ) and the appointed monitor will have access as well.

A Data Management Plan (DMP) will be created in collaboration with an expert from MEMIC. All (coded) data will be stored and analysed at Maastricht University, using a certified database.

The data collection framework will consist of the following elements:
1.Castor EDC for Case report forms (CRF) and questionnaires: Castor EDC is a web-based tool for collecting (clinical) data. Within the electronic CRF, various information will be recorded, including the inclusion and exclusion criteria, the findings of the researcher during the visits, and any adverse events. CRFs will be coded and will not contain personal details of the subjects. Completed validated questionnaires (i.e. GAD-7 questionnaire) will be saved within Castor EDC. Castor Electronic Data Capture, Ciwit BV, http://castoredc.com, Amsterdam, uses “True”, a NEN7510 and ISO 27001:2005 certified server, in line with previous studies performed by our group (PERSUADE and TENDER, NL56000.068.16/METC 162009 and NL62932.068.17/METC 173051 resp.).

2.Shimmer3 GSR sensor: For the Consenys software, an anonymized test subject ID will be used to ensure no traceable data is recorded. The key to this code will be stored securely in a locket cabinet, accessible only to a select part of the research team, specifically the principal investigator (Prof. Dr. D. Keszthelyi) and the coordinating investigator (Drs. F. Veldman). All data storage is managed by end users, with Shimmer having no visibility or access to the data. Shimmer adheres to all EU guidelines and

directives, ensuring data handling complies with European data protection legislation, including the General Data Protection Regulation (GDPR).

3.FitBit wearable

For the FitBit application, an anonymized test subject ID will be used to ensure no traceable data is recorded. The key to this code will be stored securely in a locket cabinet, accessible only to a select part of the research team, specifically the principal investigator (Prof. Dr. D. Keszthelyi) and the coordinating investigator (Drs. F. Veldman). All data on phones, on the server and in-transit are encrypted and pseudo-anonymized using industry-standard encryption techniques. Encrypted data from Fitbit will be stored via servers of Amazon Web Services (United States). As a result, data exchange of the encrypted data will take place outside the European Economic Area. However, all parties concerned state that the data exchange is in accordance with European data protection legislation, including the General Data Protection Regulation (GDPR). In addition, we will inform the subjects about the data exchange outside the European Economic Area and the fact that the level of protection for data transfer to countries outside the European Economic Area is not exactly the same as the level of protection for data transfer to countries inside the European Economic Area. We will ask for explicit consent for the data exchange to a country not part of the European Economic Area in the Informed Consent form. Our group previously used the Fitbit applications for similar purposes (DISCOvERIE, NL75159.068.20/METC 20-076 & RESILIENCE, METC23-032 / NL84720.068.23).

4.Datahub: All data will eventually be stored in Datahub in accordance with the FAIR principles, the EU General Data Protection Regulation (in Dutch: AVG) and the Dutch Act on Implementation of the General Data Protection Regulation (in Dutch: UAVG) . Datahub is a MUMC+ initiative designed to support researchers from MUMC+ and Maastricht University in the field of research data management for both clinical and non-clinical studies. Datahub provides a central infrastructure including an institutional repository for storing metadata and research data. Datasets will be archived in Datahub Maastricht. The datahub infrastructure will ensure that only individuals with the appropriate authorisation can upload new data and access existing data.

All primary documents and data will be stored for 15 years after the end of the study at Maastricht University and will be accessible to the principal investigators (Prof. Dr. D. Keszthelyi), coordinating investigator (Drs. F. Veldman), the Dutch Health Care Inspectorate (Inspectie Gezondheidszorg en Jeugd), and monitors assigned by the

Clinical Trial Center Maastricht (CTCM). Samples taken from the subjects during the study will also be kept for 15 years after the end of the study for possible additional analysis for this study. The label of each sample will contain the individual study ID and the date of collection. The primary investigator (Prof Dr. D. Keszthelyi) and the coordinating investigator (Drs. F. Veldman) have access to the stored samples. In the informed consent form, subjects indicate whether they give consent for storing and keeping personal data and biological samples, which may be used for additional analysis in the line of the current investigation. If subjects deny this consent, personal data and biological samples will only be used for the analysis as described in this protocol. By any means, personal data and biological samples will be securely stored for 15 years within Maastricht University.

12.2 Monitoring and Quality Assurance

A qualified monitor of the CTCM will monitor the conduct of the study. A monitoring plan will be drafted after the first application to the METC.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient’s last visit.
The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.
In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.
Within one year after the end of the study, the investigator/sponsor will submit a final

study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

Publication will occur in accordance with the CCMO-statement on publication policy (CCMO-statement publicatiebeleid, 2002).

13. STRUCTURED RISK ANALYSIS

13.1 Potential issues of concern

The tVNS R is a transcutaneous vagus nerve stimulation device that will be investigated outside the intended use of declaration of conformity (D2_Declaration_of_conformity_tVNS_L_signed_Versie1.0_31-05-2021).

a. Level of knowledge about mechanism of action

The therapeutic potential of taVNS is hypothesized to result from its ability to influence sensory feedback from the body to the brain. The auricular vagus nerve serves as the target of taVNS, providing a unique access point to the ANS since the external ear is the only location where the vagus nerve sends its only peripheral branch. Modulation of this solely vagal afferent leads to activation of the nucleus of the solitary tract (NTS), which serves as the primary relay station for sensory vagal afferents in the brainstem. The NTS has direct or indirect projections to the nuclei that provide noradrenergic, endorphinergic, and serotonergic fibers to various brain regions (3). Efferent outflow is then generated either via the efferent vagus through the vago–vagal reflex loop or via splanchnic nerves to various organs. Brain imaging studies have shown that taVNS can activate the NTS (54). However, the physiological impact of this activation on vagal or autonomic function in humans remains uncertain and is merely based on assumptions derived from animal studies or invasive VNS (3). Hypotheses suggests that taVNS stimulation may restore reduced vagal tone, which is commonly seen in various diseases, and subsequently restore homeostasis. Nevertheless, there is no conclusive evidence explaining why taVNS elicits therapeutic effects (15).

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

Studies have demonstrated the clinical efficacy of VNS in patients with depression (45), migraine (37), epilepsy (33-35), and tinnitus (36), among others. For a more detailed description of these studies, see paragraph 6.3. In addition, a previous study in adolescents with functional abdominal pain has also shown promising results of vagal neuromodulation (44).

c. Can the primary or secondary mechanism be induced in animals and/or in *ex-vivo* human cell material?

TaVNS has been investigated in mice (29) and rats (28). See paragraph 6.2 for further details regarding these studies.

d. Selectivity of the mechanism to target tissue in animals and/or human beings

Not applicable.

e. Analysis of potential effect

The tVNS device is considered safe. Previous studies have shown no serious or long-lasting adverse events (34, 41-43). See paragraph 6.4 ‘summary of known potential risks and benefits’ for previously reported risks.

f. Pharmacokinetic considerations

Not applicable.

g. Study population

All participants are healthy volunteers aged 18-65 years. No risks have been identified for this population (see also paragraph 6.2). Vagus nerve stimulation has been applied to both healthy controls and patient populations for several years (55-58).

h. Interaction with other products

Not applicable.

i. Predictability of effect

Not applicable.

j. Can effects be managed?

In healthy humans, taVNS has been demonstrated to be a safe and well-tolerated procedure, particularly in individuals without a history of cardiac disease (31, 41, 42). Subjects with cardiac pathology or pacemakers will be excluded from the study. However, autonomic parameters will be monitored continuously throughout the entire taVNS stimulation period and after the MAST. No serious complications have been reported in literature. A few subjects have reported dizziness, fatigue, concentration problems and a tingling sensation at the ear, which are typically associated with longer stimulation durations or with higher stimulation intensities (43). Therefore, prior to the experiment, the stimulation current will be adjusted according to each subject’s sensitivity to ensure it remains below the pain threshold. Participants are allowed to withdraw from the study at any time.

13.2 Synthesis

TaVNS is a non-invasive and a safe approach to use in research. The risks associated with participation in this study are minor and proportional to the scientific value of the research. Serious adverse events have not been reported in studies examining the efficacy of the tVNS device. Mild to moderate adverse events, including dizziness, fatigue, concentration problems, and a tingling sensation at the ear, have been reported. However, these symptoms typically and quickly disappear after removing the tVNS device. It is expected that this study will reveal important insights into the physiological impact of taVNS on vagal and autonomic function in healthy individuals.

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