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VINCI-AD: Investigating Transcutaneous Vagus Nerve Stimulation in Amnestic Mild Cognitive Impairment

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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I declare that this thesis is my own work. The Joint Research Ethics Committee of St James's Hospital and Tallaght University Hospital, Dublin, Ireland approved this trial (REC reference 307). This trial was conducted in accordance with the Declaration of Helsinki.

Helena Dolphin

Signed:

Date: __1st May, 2024____

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Abstract

Introduction

New treatments are urgently needed for individuals with Mild Cognitive Impairment (MCI) - in particular for those with amnestic MCI as they are at highest risk for developing dementia. Transcutaneous Vagus Nerve Stimulation (tVNS) is a non-invasive neuro-modulatory treatment which has not been extensively examined in older adults with amnestic MCI. The primary aim of this research programme was to examine the feasibility tolerability and safety of tVNS when administered to persons with MCI. Secondary aims included investigating the effects of a limited dose of tVNS on cognitive performance and serological immune responses.

Methods

A single site, single-blind, randomised three-arm crossover pilot trial of acute (60 minutes) tVNS (baseline, sham or active stimulation) was conducted at a Regional Specialist Memory Service. Forty participants (age 71.7 $\pm$6.9; 22/40 male) with amnestic MCI were recruited. Given the reported association between MCI and neuro-cardiovascular instability, potential adverse effects of active tVNS were assessed using beat-to-beat peripheral (Blood Pressure (BP) and Heart Rate [HR]) and central (via Near Infra-red Spectroscopy) haemodynamic responses to Active Stand (AS). Cognition was assessed between 21.3 ($\pm$4.9) and 60.5 ($\pm$4.4) minutes using a domain-specific cognitive performance battery with results analysed using mixed-effects linear regression.

Results

In older adults with amnestic MCI, tVNS was safe, tolerable and acceptable with 98% of participants stating they would use the device again. There was no significant effect on BP, or HR responses to AS and cerebral oxygenation remained stable during AS. After tVNS stimulation, performance on tests of spatial navigation were significantly improved compared to both baseline (β= -8.76; [-14.91, -2.56]; p=0.01) and sham (β= -4.15; [-7.32, -0.99]; p=0.01) conditions. There was no effect of tVNS on haemodynamic indices during AS. There was no effect of tVNS on inflammatory cytokines or chemokines.

Conclusion

tVNS is a safe and tolerable treatment modality in older adults with MCI. Future studies should explore sustained effects and feasibility of domiciliary use.

Lay Abstract

Introduction

There are many different types of memory impairment. For some people, their memory may become poorer for a while, and then go back to normal. For some people, their memory impairment may get worse and develop into a dementia, whereby they are no longer able to complete some tasks for themselves as well as previously. There are very few treatment options for people with mild memory difficulties (MCI). tVNS is a method of gently electrically stimulating the outer part of the ear to activate a nerve (the vagus nerve) which may positively affect memory.

Methods

A trial was designed in the Memory Service of a University – affiliated hospital. The trial involved 40 patients with MCI, and involved 3 visits to the hospital, 7 -10 days apart. The aim was to investigate whether using the tVNS device to actively simulate the vagus nerve, compared to both baseline (no stimulation) and sham stimulation was tolerable in patient with MCI. Blood pressure, heart rate and brain oxygenation responses to a standing test, memory tests and blood tests looking at inflammation were also undertaken at three study visits.

Results

In this population, the tVNS device was found to be safe, tolerable and acceptable with 98% of participants stating they would use the device again. There was no effect of tVNS on BP, HR or brain cerebral oxygenation during standing. After active tVNS stimulation, performance on certain memory tests (specifically those testing spatial navigation) were significantly improved compared to both baseline and sham stimulation. There was no effect of tVNS on blood tests taken after the memory tests were completed.

Conclusion

tVNS was found to be safe and tolerable in older adults with MCI. Future studies should explore whether the positive effects on memory found during the trial last over many weeks, and should investigate whether the device can be used by people in their own homes.

Summary

The Vagus Nerve (VN) is the longest cranial nerve in the human body, and has intimate connections with nearly all viscera, as the primary mediator of the Parasympathetic Nervous System. Though the VN has well-established roles in moderating cardiovascular reflexes, mediating glandular secretion and peristalsis in the Gastro-Intestinal (GI) tract, mediating broncho-constriction and secretion in the pulmonary system and mediating various pelvic functions, what is less well known is the role central neurotransmitters of the VN play in moderating cognition and attention, and also the role the peripheral VN has in mediating inflammatory responses. Invasively stimulating the VN (via iVNS devices) has been undertaken for decades in cases of refractory epilepsy and depression. However, given the natural limitations of invasively stimulating the VN (involving lateral neck dissection, general anaesthesia, implanted device in a pectoral cavity in the thorax) this has prompted wider investigation of ways to manipulate the VN non-invasively.

Transcutaneous Vagus Nerve Stimulation (tVNS) involves using a light, portable neuromodulation device that, via a gentle current of electricity, stimulates the Auricular Branch of the Vagus Nerve which is postulated to afferently supply a portion of the skin of the external auricle. There are other means of non-invasively stimulating the VN, involving non-invasive stimulation at the cervical VN, however for the purposes of this thesis I will focus on auricular tVNS. Recent metanalysis of published data of the effects of auricular tVNS on cognitive tasks in Cognitively Unimpaired (CU) healthy adults indicates a significant improvement. However to our knowledge only one previous study has investigated the effects of tVNS in a cognitively impaired population, and none to date have simultaneously

investigated the effects of tVNS on Neuro-CardioVascular (NCV) indices in a cognitively impaired population.

Cognitive impairment exists on a spectrum from healthy adults to persons with both cognitive and functional impairment (i.e. dementia). Mild Cognitive Impairment (MCI) is a diagnosis of cognitive impairment without any functional loss, and can evolve to dementia over time. Recent data suggests that Brain Health interventions in this group can stabilise cognitive decline, as a high degree of the population-attributable risk factors for dementia are potential modifiable. Non-invasive neural stimulation is being considered in this context of augmenting cognitive performance and reserve, and studies are urgently needed to establish its safety and potential efficacy in a cognitively impaired population. Persons with MCI are known to have more circulating inflammatory cytokines and chemokines than CU peers, and also have worse NCV instability which may even herald further decline from MCI to dementia. The VN likely plays a fundamental role in mediating both NCV losses and impaired inflammatory responses in this group, and non-invasively modulating the VN may be an exciting new therapeutic avenue in MCI.

This thesis is the cumulative results of a pilot trial undertaken in a tertiary referral University Hospital Memory Assessment Service, which involves a three-arm crossover device trial in patients with amnestic MCI. The primary aim of this study was to establish the tolerability, acceptability, safety (via side effect profile and in-depth analysis of NCV responses to an orthostatic challenge) and independent usability of this device in this population. Pre-defined exploratory secondary aims were to investigate the effect on cognitive and inflammatory outcome measures.

Research Questions:

Primary aim:

1. Is non-invasive stimulation via a tVNS neuromodulation device feasible for those with amnestic MCI?
    i. Is it safe?
    ii. Is it tolerable? Are the side effects acceptable?
    iii. Is it usable?

Pre-defined secondary aims:

2. Does acute tVNS affect domain-specific cognition in amnestic MCI?
    i. Associative memory
    ii. Executive Function
    iii. Spatial Navigation
    iv. Language and Working Memory

3. Does acute tVNS affect Neuro-Cardio Vascular Indices in amnestic MCI?
    i. Systolic BP (SBP), Diastolic BP (DBP), Heart Rate (HR) and Tissue Saturation Index (TSI) responses to Active Stand
    ii. Resting 5-minute Heart Rate Variability (HRV) analysis

4. Does acute tVNS affect circulating whole blood cytokines and chemokines in amnestic MCI?
    i. Battery of cytokines and chemokines known to be associated with MCI: IFN-γ, IL-10, IL12p70, IL-6, TNF- α, Eotaxin, IP-10, MCP-1, MIP-1 β, IL-17A

5. Exploring Blood Bio-Markers (BBMs) of Alzheimer's Disease in this cohort, and associated responses to tVNS
    i. p-tau217, p-tau181, total tau (t-tau), Neurofilament Light (NfL) and Glial Fibrillary Acid Protein (GFAP)

Value of this body of research

This thesis reports on a successfully completed sham- controlled single-blinded three-arm crossover randomised trial of a medical device in older adults with amnestic Mild Cognitive Impairment (MCI). This adds to the body of research that advocates for including older adults in medical research, especially clinical research of medications and devices. The device non-invasively stimulates the vagus nerve, and is termed a Transcutaneous Vagus Nerve Stimulation (tVNS) device.

Frequently as clinicians we extrapolate published data in Randomised Controlled Trials (RCTs) in decision-making with older patients in our care. These RCTs are commonly undertaken in younger adults, free of the complexity of older age. The natural way to meet this challenge is to include older, medically-complex patients in RCTs of devices and medications, to better represent real-world scenarios.

All participants in this trial had a diagnosis of amnestic MCI, the participants were older (average age 71.2), and had relatively high medical complexity, with 23/40 (57.5%) of participants having a Charlson Comorbidity Index ≥ 4 and 12/40 (30%) taking ≥ 6 medications per day. Despite this, 38 of 40 adults completed all 3 study visits to a tertiary referral centre. This speaks to the willingness and graciousness of older patients and their families in selflessly participating in clinical research.

This body of work also develops on the theme of digital self-management, that older adults are both interested in and capable of adapting to new technology. Not only did we

successfully trial a non-invasive neuromodulation device in this population, but the means by which participants were assessed also involved utilising various technology such as a laptop device to undertake the associative memory and executive function tasks, and a tablet device to undertake the spatial navigation cognitive task.

It was noted during literature review of tVNS studies that no studies to date had designed a three-arm crossover trial. Given the anatomy of the afferent supply to the external auricle, active stimulation with tVNS is undertaken at either the cymba conchae or tragus, and sham stimulation is undertaken at the earlobe which is free of vagus afferent innervation. All published work to date has compared either sham or active stimulation in age- and sex-matched groups (parallel design) or both active and sham stimulation in an internal crossover design, nested within participant. These designs do not account for the potential bias of the participant knowing they are being stimulated (sham or active), especially when undertaking cognitive testing. The three-arm crossover design was conceived of, such that any effect would have to be evident comparing active to baseline assessments but also active compared to sham stimulation. Thus the participant acts as their own control, and all statistical analysis is undertaken nested per participant.

It was also noted that despite individual trials and metanalysis suggesting an acute improvement in cognition with tVNS, that only one study published to date has also investigated tVNS in a cognitively impaired population (Wang et al., 2022). This study noted that in a parallel design, over a 24 week period, there was an improvement in a composite cognitive score. Our study adds to this growing body of literature suggesting a beneficial effect on acute cognitive of tVNS, but has many other strengths including our

aforementioned robust trial design, assessment of device tolerability and usability, in-depth haemodynamic assessment during an orthostatic stress, and captures plasma inflammatory cytokine and chemokine data as well.

This trial was designed as a pilot feasibility study, with the primary aim being to ascertain feasibility (safety, tolerability, acceptability and usability) of a tVNS device in participants with MCI. The secondary aims of this study were to assess haemodynamic responses to an orthostatic stress, to assess the effect of tVNS on domain-specific cognition and to assess the effect of tVNS on whole blood cytokine and chemokines.

Assessment of responses to orthostatic stress is important, as patients with MCI often have Orthostatic Hypotension (OH), and stimulating the vagus nerve could theoretically aggravate an OH response. We found no effect on any haemodynamic index (blood pressure, heart rate or tissue saturation – a measure of cerebral oxygenation) during the active stand test. A notable discovery was that during active tVNS stimulation, while undertaking the tablet-based spatial navigation task, there was a statistically significant trend towards quicker accurate time to target (the measure being examined) for two of the more challenging levels. To our knowledge no previous study has specifically researched tVNS' effects on spatial navigation, an area of cognition commonly affected in MCI and dementia due to Alzheimer's disease and it would be interesting to see if these findings are replicated in larger, fully- powered studies.

Future studies of tVNS in MCI should focus on the following areas:

1. A human factors bio-engineering project investigating why participants struggled to place the tVNS device in the correct part of their ear, and how to potentially augment or change the device design to account for this would be useful
2. An adequately-powered study to detect absolute between-condition differences in cognitive outcomes in a population with MCI would be useful to confirm the changes observed in this pilot trial
3. A longer term project designed to investigate the acceptability of daily domiciliary use of a tVNS device over many weeks (e.g. 24 weeks) with interim analysis of cognition and haemodynamic indices as primary outcomes would be useful to ascertain the real-world benefit of this intervention in this population

Publications from this thesis

- Dolphin H, Dyer AH, Dukelow T, Finucane C, Commins S, Kennelly SP. Safety and feasibility of transcutaneous vagus nerve stimulation in mild cognitive impairment: VINCI-AD study protocol. *BMC Neurol*. 2023;23(1):289. Published 2023 Aug 2. doi:10.1186/s12883-023-03320-5

- Dolphin H, Dukelow T, Finucane C, Commins S, McElwaine P, Kennelly SP. "The Wandering Nerve Linking Heart and Mind" - The Complementary Role of Transcutaneous Vagus Nerve Stimulation in Modulating Neuro-Cardiovascular and Cognitive Performance. *Front Neurosci*. 2022;16:897303. Published 2022 Jun 16. doi:10.3389/fnins.2022.897303

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- Morrison LM, Dyer AH, Dolphin H, et al. Circulating IL-17A is Associated with Executive Function in Middle Aged Adults With and Without Type 2 Diabetes. Submitted to Frontiers in Ageing Neuroscience, March 2024

- Connolly E, O'Connor, A, Dolphin H, et al. Projected Annualised Lecanemab Treatment Eligibility in an Irish Specialist Memory Clinic. Submitted to International Journal of Geriatric Psychiatry, April 2024

Chapter 1: The Vagus Nerve and Central Autonomic Function

Anatomy and Physiology of the Vagus Nerve

The longest Cranial Nerve (CN) in the body, the Vagus Nerve (VN) derives its name from the Latin for 'straying' or 'wandering'. It is the tenth CN, often referred to as CN X. The nerve has an extensive course; it arises in the brainstem medulla and through the length of its course it innervates most visceral structures, including the heart, lungs, Gastro-Intestinal (GI) and biliary tracts, pelvic organs and, indirectly, the spleen. It is involved in many homeostatic functions via autonomic reflexes, including mediating complex cardioinhibitory responses via the baroreceptor reflex, smooth muscle tone and glandular secretion in the lungs via the pulmonary plexus, smooth muscle tone (controlling peristalsis) and enzyme secretion in the GI tract as far as the transverse colon, and modulating complex inflammatory pathways indirectly via the splenic system [1,2].

The VN exits the skull base with CN IX, XI and XII lateral to the atlas bone and medial to the styloid process of the skull. One of the first branches of the VN as it exits the skull is the Auricular Branch of the VN (ABVN or Arnold's Nerve) which is described in further detail below. Each VN (right and left) travels with the common carotid artery and jugular vein of its respective side in the carotid sheath to the level of the carotid body, at the carotid bifurcation. Here the VN supplies abundant afferent cells to complete the reflex arc of the baroreceptor reflex (mediated via baroreceptor firing during low peripheral Blood Pressure [BP][3]).

The VN is the primary mediator of parasympathetic activity. The Parasympathetic Nervous System (PNS) is classically associated with “rest and digest” conditions whereas the sympathetic nervous system drives the adrenergic “fight or flight” response in stressful situations.[4] The PNS serves a myriad of purpose, including regulation of visceral functions such as pupil dilation, peristalsis, glandular secretion and urination, is intricately involved in maintaining peripheral and central BP which will be explored in greater detail below**.

There are cervical, intrathoracic, intra-abdominal and pelvic branches of the VN on each side with numerous sub-branches of each supplying efferent neurons to make visceral plexuses and carrying afferent signals [1]. The cervical and thoracic cardiac plexuses are of most relevance to this thesis. Reviews of the function of the intra-abdominal, splenic and pelvic VN distributions is beyond the scope of this thesis but can be found at [5,6].

Fibre Types and Axonal Function

The specific fibre types (Aα, Aβ, Aδ, B and C fibres) of the VN may be classified by conduction velocity [7] and in general, the larger A and B fibres are myelinated, thicker, with quicker conduction velocities than unmyelinated C fibres which mainly carry afferent visceral pain, stretch and nociceptive information [8]. Please see Table 1 for further detail of the VN fibre types.

	Fibre Type				
	Aα	Aβ	Aδ	B	C
Fibre diameter	13-20mm	6-12mm	1-5mm	1-5mm	0.4-2mm
Function	Efferent: Motor innervation of laryngeal muscles	Afferent: Sensation (stretch, pain) of laryngeal and pharyngeal mucosa	Afferent: Visceral mechanoreceptor: baroreceptor, stretch, temperature	Efferent: Parasympathetic autonomic control to viscera	Afferent: Visceral pain, stretch, chemical sensation, temperature
Myelin	+	+	+	+	-
Threshold mA	0.02-0.2mA	0.02-0.2mA	0.02-0.2mA	0.04-0.6mA	0.3-6mA
Conduction velocity m/s	8-120m/s	35-75m/s	3-30m/s	3-15m/s	0.5-2m/s
Purported effect of VNS on EEG	Synchronization	Synchronization	Synchronization	Synchronization	Desynchronization

Table 1 details the constituent fibres of the VN. Adapted from Groves et al., 2005 [8] and Ahmed et al., 2002[9]

CN function can be divided into seven broad categories: General Somatic Afferent (GSA), General Somatic Efferent (GSE), General Visceral Afferent (GVA), General Visceral Efferent (GVE), Special Somatic Afferent (SSA), Special Visceral Afferent (SVA), and Special Visceral Efferent (SVE). The term somatic refers to skin and skeletal muscle innervation, visceral refers to smooth muscle, cardiac muscle, and glandular innervation, afferent refers to sensory function, efferent refers to motor function, special refers to nerve functions possessed by the Central Nervous System (CNS), and general to CN functions that are the same as other spinal nerves [1]. Of these seven functional categories, the VN carries all fibre types except GSE and SSA fibres. Table 2 details the five distinct fibre types of the VN and their function.

Fibre Type	Function	Cell Body Location	Distribution
General Somatic Afferent (GSA)	Cutaneous sensation	Superior ganglion	External auditory meatus, tympanic membrane, and posterior dura fossa mater
General Visceral Afferent (GVA)	Visceral sensation	Inferior ganglion	Cervical, thoracic and abdominal branches of the VN, and carotid and aortic arch bodies
Special Visceral Afferent (SVA)	Taste	Inferior ganglion	Taste receptors around the pharynx and upper oesophagus
General Visceral Efferent (GVE)	Visceral smooth muscle and glands	DMN of VN (brainstem)	Muscles and glands of alimentary canal, cardiac and pulmonary plexuses
Special Visceral Efferent (SVE)	Swallowing and phonation	NA (brainstem)	Pharyngeal, superior laryngeal and recurrent laryngeal nerves

Table 2 details the afferent and efferent fibre types of the VN, adapted from Ottaviani et al., 2022 and Camara et al., 2015 [1,2]

The VN is comprised of approximately 80% afferent and 20% efferent fibres [10,11]. This indicates that a large volume of the VN's modulatory processes are afferent i.e. from peripheral tissues to the CNS. Afferent VN fibres originate in peripheral viscera or other tissues, they carry afferent information to the brainstem nuclei of the VN and from there modulate various CNS functions. Efferent VN fibres originate in the CNS, synapse in the brainstem and from there send post-ganglionic fibres to tissues and viscera to module visceral function.

Brainstem Vagal Nuclei

VN fibres synapse centrally on four specialised vagal brainstem nuclei; the Nucleus of the Solitary Tract (NTS) and Spinal Trigeminal Nucleus (STN) receive VN afferent signalling and from there modulate subcortical and cortical function. The Nucleus Ambiguus (NA) and Dorsal Motor Nucleus of the Vagus (DMN) are responsible for "top-down" signalling and are the site from where VN efferent post-ganglionic axonal fibres arise and from hence modulate peripheral reflexes and visceral function. [12,13]

Afferent

Afferent VN fibres terminate primarily in the NTS [14,15] with a smaller proportion terminating on the STN. The VN synapses bilaterally in the NTS; so VN afferent information is processed bilaterally in the CNS [16]. Second order neurons from the NTS project to the Parabrachial Nucleus of the Pons (PNP) and also densely innervate both noradrenergic (i.e the Locus Coeruleus [LC]) and serotonergic (raphe nuclei) neuromodulatory systems [13,17]. This link between the NTS vagus nucleus and the LC is explored in more detail below, and is thought to have specific importance in the context of the VN and cognition.

From the NTS and STN vagal information is relayed to several mostly subcortical structures, including the hypothalamus, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the intralaminar thalamic nucleus. Vagal afferent information is also relayed to the anterior insular cortex which communicates with more rostral regions of the cortex (orbital and ventrolateral prefrontal cortex) and indirectly with the medial prefrontal cortex [18,19]. These cortical and subcortical regions will be explored in further detail below, as they form part of the Central Autonomic Network (CAN).

It is noteworthy that afferent VN cell bodies are located in one of two ganglia, the jugular or nodose ganglion [20]. Somatic afferent axons from the ABVN, the internal branch of the superior laryngeal nerve, the recurrent laryngeal nerve and the meningeal branch of the VN (supplying the dura of the posterior cranial fossa) have cell bodies within the jugular ganglion, while GVA fibres from the epiglottis, pharynx, and all visceral organs have cell bodies in the nodose ganglion.[2,21,22]

In the brainstem the jugular ganglion neurons project to the STN, while nodose ganglion neurons mostly project to the NTS in the medulla.[1] Vagal afferents terminate in the NTS in a topographical manner (i.e. cardiovascular afferents synapse in the dorsal subnuclei with GI afferents mostly in the medial and commissural subnuclei) resulting in discrete NTS functional divisions in several subnuclei.[22]

Efferent

The descending VN pathways involve primarily activation of the DMN[23] and the NA which is in the reticular formation of the medulla. The DMN and NA have multiple central connections (NTS, magnocellular paraventricular nuclei and several medullary nuclei[24,25])

however the primarily role of the fibres originating from the DMN is efferent innervation of cardiac, pulmonary and GI organs via parasympathetic ganglia located close to or in the walls of viscera [2]. The efferent fibres from the DMN exert a cardio-inhibitory effect mediated via the sinoatrial and atrioventricular ganglia.[26,27] The right VN mostly innervates the Sino-Atrial (SA) node (involved in the pacemaker function of the heart) whereas the left VN is mostly thought to innervate the Atrio-Ventricular (AV) node (regulating the force of contraction of the cardiac myocytes with less influence over heart rate), which is discussed in further detail in Baroreceptor Reflex below**. It is noteworthy that most of the data describing this anatomical layout have been preclinical studies, and comprehensive human studies confirming this precise delineation are needed.[28,29]

The NA is in the central tegmentum, dorsal to the inferior olivary nucleus and ventromedial to the STN. Similar to the NTS, NA neurons are organised topographically from the viscera they innervate.[2] The NA can be subdivided into an external part which contains preganglionic cardiac parasympathetic neurons and a dorsal somatic division. Axons from the cardiomotor portion of the NA terminate in small ganglia embedded in the heart and exert cardioinhibitory effects whereas the rostral part of the NA contains vagal motoneurons innervating striated muscles of the pharynx and the oesophagus.[30,31] It is noteworthy that both the DMN and NA are involved in the efferent arm of vagal reflexes such as the baroreceptor reflex and chemoreceptor reflex. See Figure 1 for a schematic representation of the VN fibres and central projections.

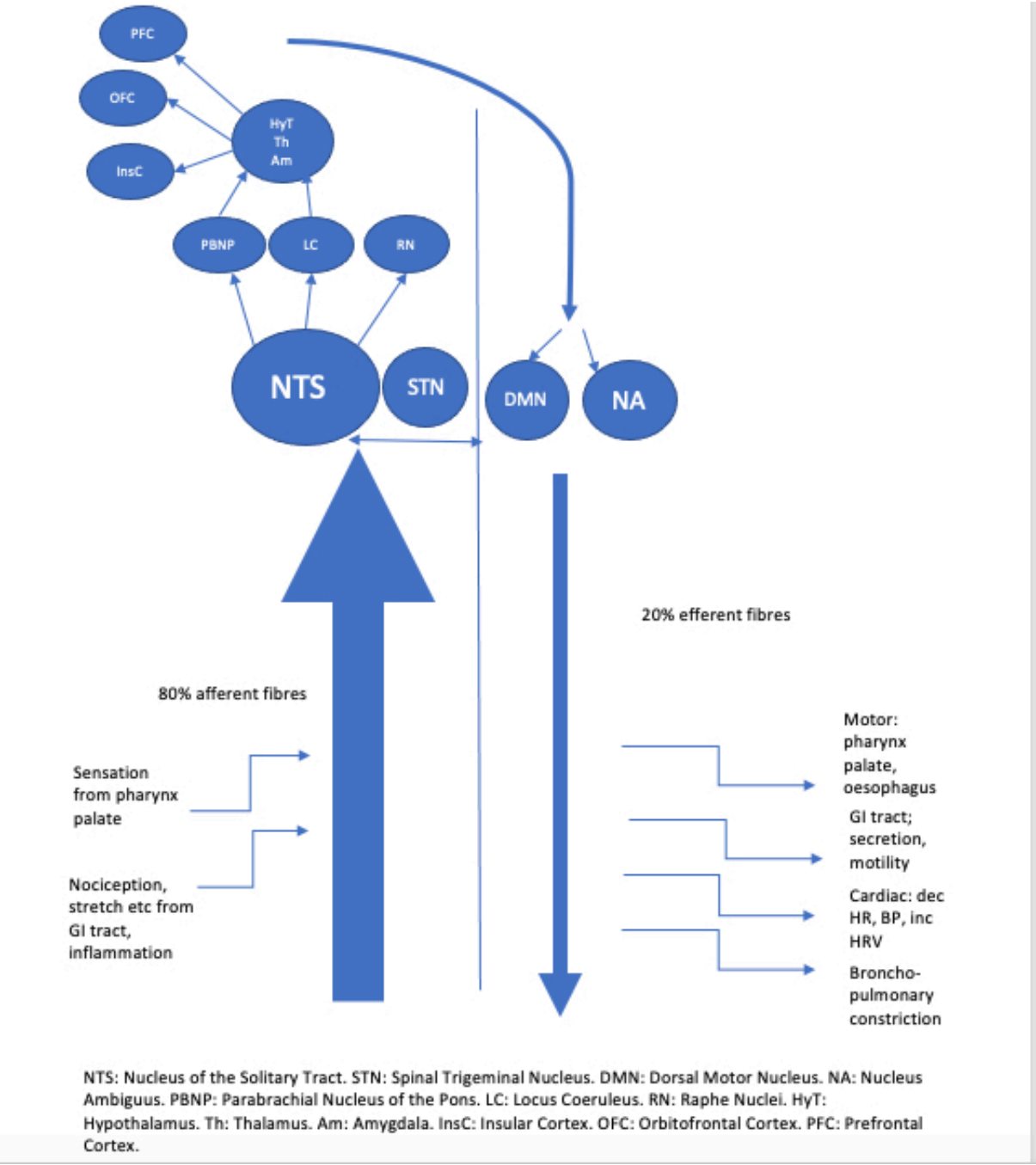


Figure 1: Central and peripheral projections of the VN, afferent and efferent fibres

Neurochemistry

Vagal axons contain a vast array of neurotransmitters and neuropeptides on their effector cells, each with differing time courses of action. While classical neurotransmitters are packaged in the nerve terminals, neuropeptides are synthesized in the nodose and petrosal

ganglia's cell bodies and transported centrally and peripherally to modulate the principal neurotransmitters' actions.[32]

In general, parasympathetic motor axons are cholinergic, releasing Acetylcholine (ACh), but can also release co-transmitters, such as Adenosine Tri-Phosphate (ATP) and other monoamine messengers.[2] Several other amino acid type transmitters have also been identified in the terminals of vagal efferents, including glutamate, γ-aminobutyric acid (GABA), serotonin and precursors for dopamine.[33]

Vagal sensory neurons in the nodose and jugular ganglia project to the brainstem's sensory nuclei via central process. Vagal afferents have mostly glutamatergic terminals and activate second-order brainstem neurons via N-Methyl-D-Aspartate (NMDA) type glutamate receptors producing via the G-protein coupled glutamate receptors long-lasting effects on second-order neurons in the brainstem.[22,34] The NTS neurons contain an extensive array of receptors and neurotransmitters such as GABA, glutamate, catecholamines, and glycine, as well as a vast range of neuropeptides which play a fundamental role in the modulation of neuronal activity.[2]

Innervation of the External Ear and Arnold's Nerve (Auricular Branch of the Vagus Nerve)

The auricle (the visible part of the ear that consists of cartilage and skin and sits outside the cranium) is formed from parts of the first two pharyngeal embryonic arches.[35] The ABVN is thought to be a remnant of a more extensive embryonic nerve which originally supplied the first pharyngeal arch and may be derived evolutionarily from a system of sense organs (lateral line organs) detecting movement, vibration and pressure in surrounding water.[36,37] This is noteworthy as this afferent vagal information regarding safety and potential danger could have an important evolutionary purpose, as the VN can instantaneously modulate pulmonary and cardiovascular reflexes which may be necessary in times of threat.

However the auricle has multiple sources of innervation and each auricle likely demonstrates a high degree of inter-individual variability, as well as cross-innervation by more than one nerve in specific areas. Generally, the ABVN is thought to innervate mostly the concha, cymba concha and tragus of the auricle; the auriculotemporal nerve is a sensory branch of the mandibular division of the facial nerve (V3) which is thought to innervate the helix, scapha and antihelix; the greater auricular nerve innervates the antitragus and lobule and the lesser occipital nerve innervates the cranial portion of the ear and potentially some of the middle helix. The greater auricular nerve and the lesser occipital nerve are superficial branches of the cervical plexus, i.e. are formed by contributions from the C2 and C3 spinal nerves.[38–41] Please see Figure 2 for an illustration of the known innervation patterns of the human auricle.

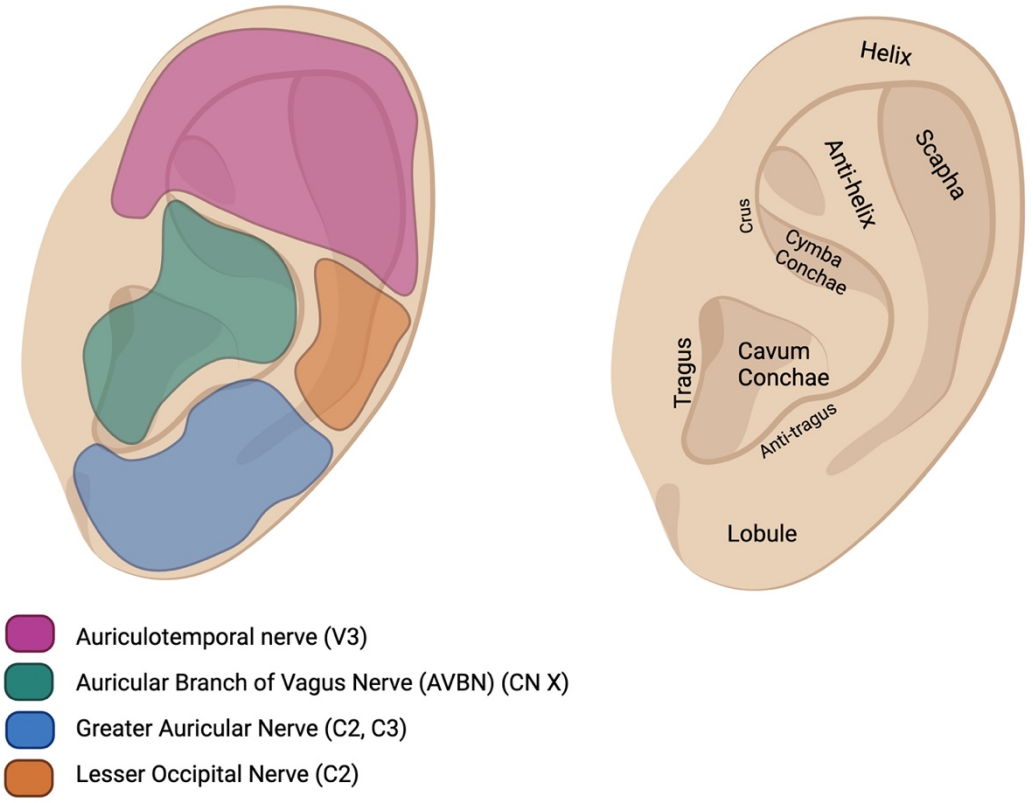


Figure 2 illustrates the known innervation patterns of the human auricle

The cutaneous distribution of the ABVN including the skin it innervates was initially researched by human cadaveric specimens in the late 1800's [42] which although illuminating were small studies with sparse published data. More recent cadaveric dissection studies of human nerve supply of the external ear have been summarised in a recent review.[43] The variability in ABVN pathways (e.g. which ganglion they arise from, whether they share branches with the glossopharyngeal or facial nerve branches) in addition to the relatively small calibre of the nerve itself make reliable gross anatomical data of the nerve difficult to obtain. Regarding fibre type, the ABVN has been shown to contain myelinated Aβ fibres (though with less numerous Aβ axons than in cervical VN dissections) and afferent

unmyelinated C fibres [44]. Electrical stimulation parameters and how this may effect ABVN fibre types is explored in Chapter 2.

Notably recently Functional Magnetic Resonance Imaging (fMRI) data has demonstrated that electrical stimulation of the tragus [45–48] or cymba concha [49,50] of the external ear causes activation of brainstem nuclei and other brain regions known to be associated with the VN which suggests that the previous cadaveric data regarding auricular innervation was accurate. The specifics of VN stimulation, location of electrode placement and sham stimulation will be discussed in detail in Chapter 2.

Since 2022, the National Institute of Health (NIH) in the United States of America (USA) via its Stimulating Peripheral Activity to Relieve Conditions (SPARC) programme has launched over (US) $21 million in funding to research the precise locations of the human VN (both the Reconstructing Vagal Anatomy Initiative (REVA) and Vagus Nerve Stimulation Endpoints from Standardised Parameters (VESPA) study) which may help explain the variability in the anatomical VN literature and aid future neuromodulation research.[51]

The Central Autonomic Network

The CAN is an intricate network of spinal cord, brainstem, subcortical and cortical brain regions that monitor and modulate Autonomic Nervous System (ANS) function, through neurochemical, humoral and endocrine mechanisms. It was historically predominantly studied in preclinical and clinical work via lesional (stroke or traumatic injury) or epileptic subjects and from that body of work, the primary brain regions implicated were the insula,

amygdala, hypothalamus, periacqueductal grey matter, PNP, NTS and Ventral Medulla (VM) [23].

The CAN may be conceptualised in three hierarchies; the first at the spinal cord level which has direct reflex control of ANS functions. The second is at the level of the brainstem and comprises the NTS, VM and PNP which are involved in immediate and reflexive control of circulation, respiration and GI tract function. The forebrain CAN structures include the hypothalamus which integrates autonomic, sleep and thermoregulatory information, the Anterior Cingulate Cortex (ACC), amygdala and insula together integrate peripheral sensations, pain, and the persons' emotional state, and modulate emotional and goal-directed autonomic responses [52,53]. It is also now known due to fMRI studies that parts of the salience network and Default Mode Network (DMN) including the OrbitoFrontal Cortex (OFC), the medial prefrontal cortex, the precuneus, the thalamus and the cerebellum are all involved in the modulation of autonomic function in the CAN [54,55].

It is important to note that the CAN receives afferent information regarding a wide variety of signals, including pain, temperature, bodily sensations, environmental information, threat and perception of oneself in space. There is constant bidirectional feedback between viscera and the CAN monitoring and modulating based off the afferent information. There are strong functional connections between the brainstem nuclei of the CAN, the insula and the hippocampus [56] which is evolutionarily advantageous; one should remember if a situation was dangerous, threatening or hostile so it can be avoided in future.

Figure 3 illustrates the brainstem, subcortical and cortical regions now postulated to be part of the CAN. It is noteworthy that some regions (such as the precuneus and hippocampus) have now been also clearly described as part of the DMN (a functional brain network involved in decision making and emotional processing [57]) however the "core" regions of interest of the CAN are illustrated alongside these.

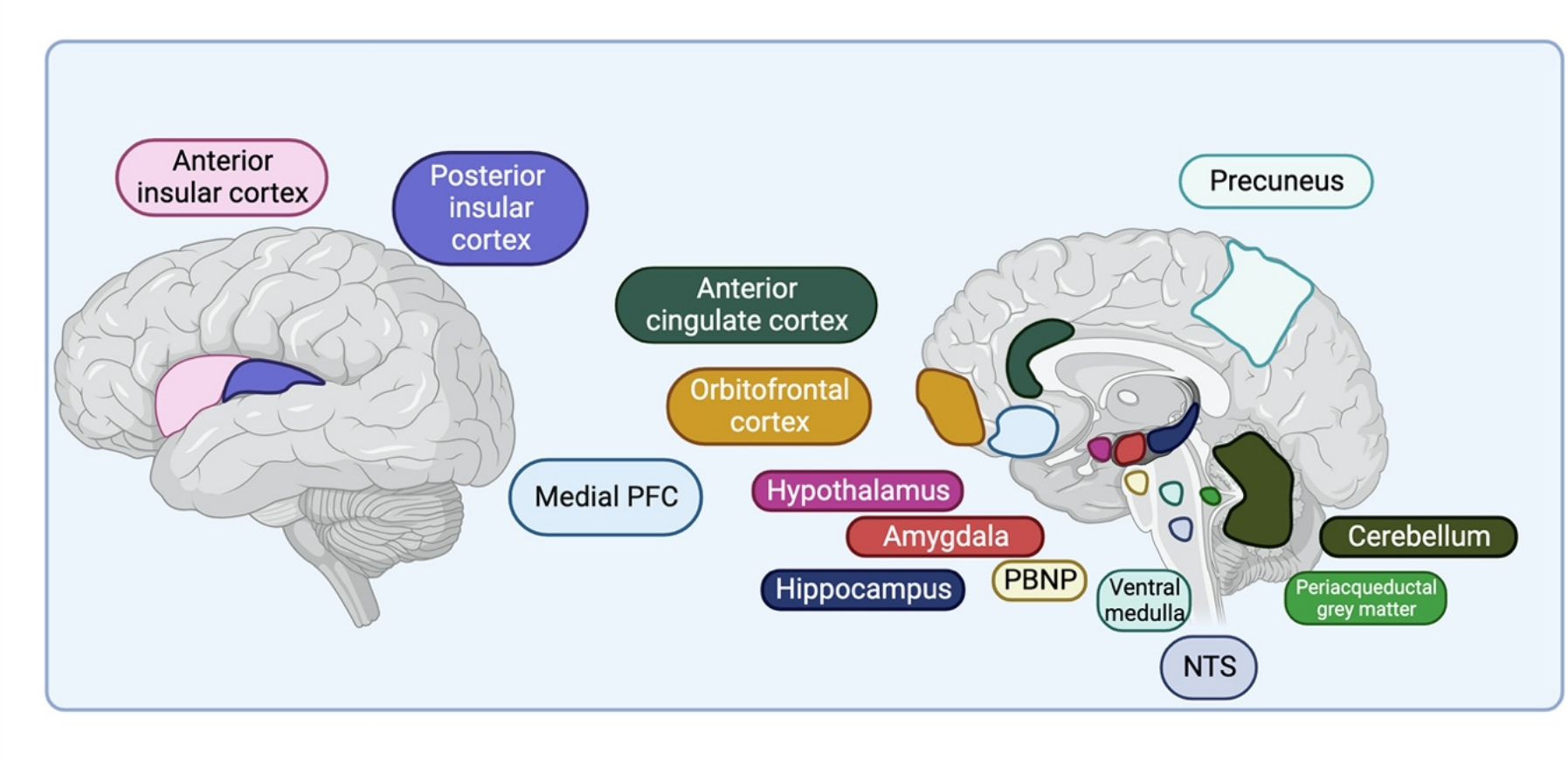


Figure 3 illustrates the various cortical and subcortical structures that form to Central Autonomic Network (CAN)

The CAN receives myriad inputs, monitoring and processing visceral information and instituting appropriate responses. The inputs may be nervous (e.g. baroreceptors in the cardiac arch detect lack of circulating volume), endocrine (the Hypothalamic- Pituitary-Adrenal [HPA] axis may be activated by a stress trigger) or otherwise humoral (immune signalling, glycaemic control etc. [58]). It is noteworthy that the "gut instinct" or sensation of threat or hostility in the gut area is intimately linked to the CAN, similarly lacrimation when experiencing joy or sadness and piloerection or "butterflies" when feeling nervous are all modulated via the CAN [59,60]. Traditionally the CAN was thought to be under only subconscious control and related to ancient evolutionary survival mechanisms [23] however recent advances in the field of neuroimaging and biofeedback (discussed below) suggest it may be, to a certain degree, responsive to conscious control.

Interoception and Biofeedback

Related to the CAN is a concept known as interoception. Interoception refers to a person's ability to monitor their internal biological environment, e.g. awareness of respiratory rate, cardiac cycle and heartbeats, awareness of peristalsis or pelvic organ function [61]. Impaired interoception (i.e. hypo- or hyper-sensitivity to afferent signals) has been postulated to be related to various disease states, as our ability to accurately monitor our internal environment is likely related to our ability to accurately appraise risk [62]. An example of this may be in the case of severe anxiety, afferent signalling to the CAN is heightened, functional connectivity with the hippocampus and DMN is impaired, and the person may maladaptively over-estimate that potential risk of a situation, perpetuating anxiety signalling [63]. Similarly in

obesity, central processing of satiety signalling is likely impaired, leading to inaccurate monitoring of biological needs and ultimately maladaptive behaviours.[64]

Interoception is related to the concept of biofeedback, which postulates that via awareness and monitoring of one's internal autonomic signals that these processes may, to some degree, come under conscious control. Biofeedback measures and provides live feedback to patients on their physiological, autonomic responses with the overall goal being to change those responses. The most common types of measurements are muscle activity, heart rate function and variability, respiration, blood pressure and flow, Electro-Encephalogram (EEG) waveforms, skin temperature, and electrodermal activity.[65] The hypothesis upon which biofeedback is based is that receipt of this feedback increases a person's awareness of their internal physiological processes. Ideally, when this awareness is paired with interventions to change behaviour, thoughts, or emotions, a beneficial change in the physiological process occurs.[66]

Respiratory and cardiac biofeedback processes are similar to the contemporary idea of "mindfulness meditation" a method of monitoring one's respiratory cycle and associated monitoring and acceptance of any thoughts as they arise (for a review of the autonomic effects of Buddhist-style meditation please see.[67] Recent studies have started to explore whether via biofeedback (e.g. heartrate modulation) the outputs of the ANS may be consciously modified, with some interesting results. Studies are sparse and require reproduction but notably have reported that short interventions of biofeedback training improved symptoms of anxiety,[68–71] impulsivity,[72] Raynaud's phenomenon,[73] urinary and faecal incontinence,[74,75] headache disorders, [76] post-traumatic stress disorder, [77]

fibromyalgia[78] and sleep disorders among cancer survivors.[79] These data suggests that the CAN may be consciously manipulated to a degree and may offer an exciting avenue to non-pharmacologically manage certain illnesses in future.

Locus Coeruleus – Norepinephrine System

Second-order neurons in the NTS integrate visceral information and send axonal projections to third-order neurons in many brainstem locations, including the brainstem reticular formation, the periaqueductal gray, the LC and multiple raphe nuclei. [2,80]

The LC is a small blue nucleus in the pons. It is the main source of cortical Norepinephrine (NE) and plays a significant role in regulating brain function. The LC can be loosely considered a cortical analogue of the adrenal glands, influencing arousal and helping optimise cognitive states for varied environmental demands.[81] It provides near- exclusive NE innervation of the cortex and is characterized by diffuse efferent projections to both subcortical and cortical structures.[82] It consists of small unmyelinated fibres and forms a wide antero-posterior branching network to reach the raphe nuclei, the cerebellum, and almost all areas of the midbrain and forebrain.[83]

The LC-NE system is involved in a complex matrix of behavioural and electrophysiological actions that mostly process salient environmental information. The LC-NE system contributes to the initiation and maintenance of certain behaviours, and facilitates the processing of sensory information (i.e. arousal, wakefulness). It then modulates the gathering and management of salient sensory information through a range of cortical and

subcortical systems, primarily involving attention and memory circuits.[84] However, similar to other brainstem nuclei, the LC-NE system modulates the activity of other sensory inputs to target subcortical and forebrain regions.[85]

LC modulates attention

Despite the vast efferent network of the LC, there are two major LC afferent inputs: the nucleus paragigantocellularis (part of the ventrolateral rostral medulla) and the nucleus prepositus hypoglossi (in the dorsomedial rostral medulla).[86] These two pathways modulate the LC via the NTS and are considered disynaptic: there is an excitatory pathway (via the nucleus paragigantocellularis) and an inhibitory pathway (via the nucleus prepositus hypoglossi).[31,87]

There are distinct modes of LC firing that are associated with equally distinct modes of attentional strategy.[88] Projections to the OFC and ACC are thought to drive the LC-NE system into one of these two stable states of activity, a high tonic (sustained) mode or a phasic (bursting) mode accompanied by moderate tonic activity.[88] This switching of attentional state via tonic LC activity is thought to result in a flexible attentional system that allows cycling between behaviours to find and meet task demands in one's environment i.e. the Adaptive Gain Theory.[88]

When one is focused on a singular task the LC-NE system employs high-amplitude transient phasic bursts preceding behavioural responses to task-relevant stimuli, while sustained firing is maintained at a moderate level, and task performance is relatively strong. As task utility wanes, and tonic LC firing increases, phasic firing is reduced. This level of tonic LC activity

allows for a decoupling of attention from the current focus, which allows a re-evaluation of the current environments and the generation of goals of potentially greater value.[89]

Figure 4 illustrates the LC-NE system and associated innervated structures.

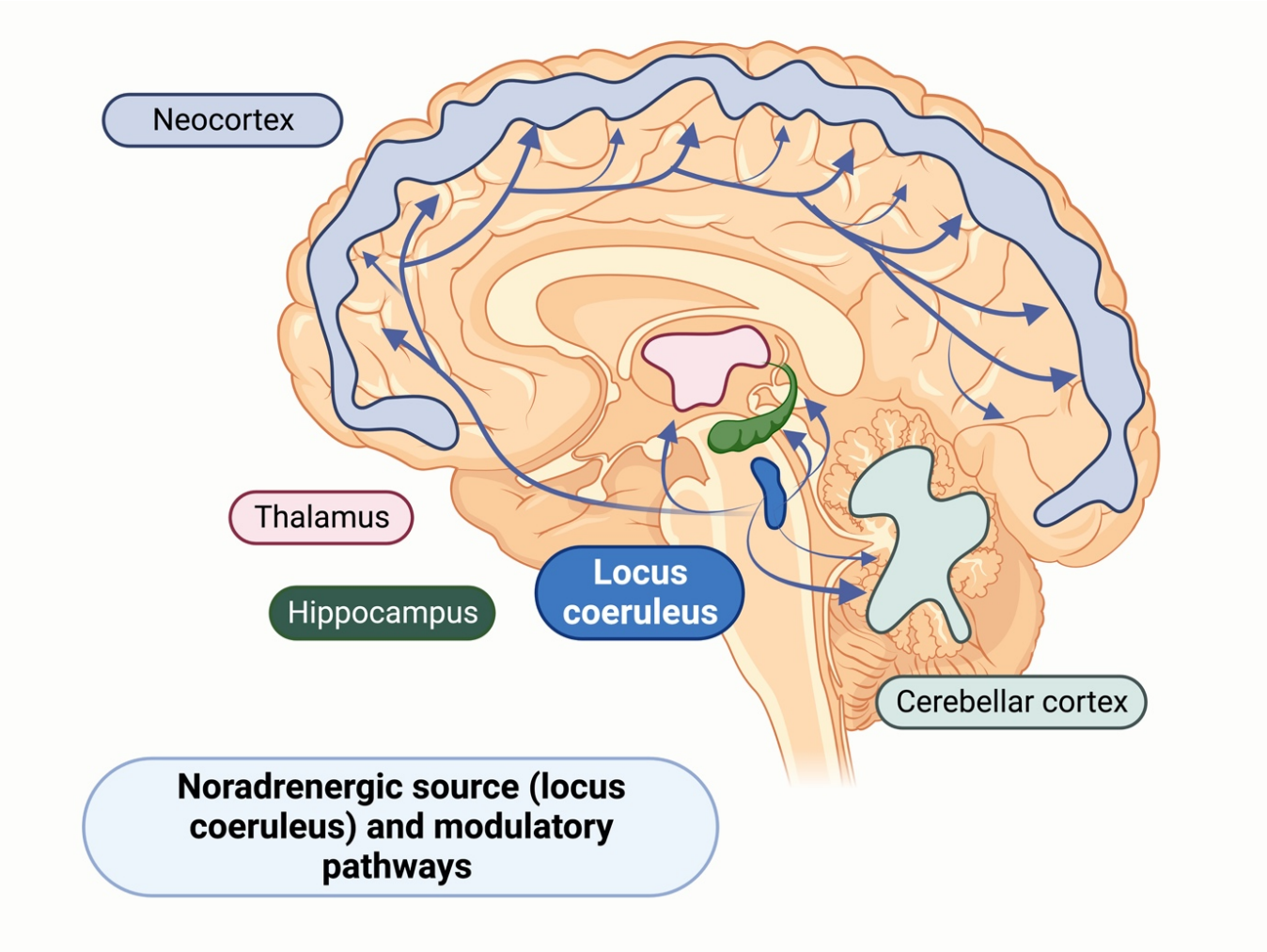


Figure 4: Schematic illustration of the Locus Coeruleus- Norepinephrine (LC-NE) modulatory system and associated structures

Heart Rate Variability

Heart Rate Variability (HRV) analysis is based on extrapolating time intervals between each R wave peak on an Electro-Cardiogram (ECG), excluding any ectopic beats or arrhythmias like Atrial Fibrillation (AF). The most commonly applied methods for HRV determination include time-domain analysis and frequency/spectral analysis. Time-domain analysis produces indices quantifying variance in selected inter-beat intervals, such as the Standard Deviation of Normal beat intervals (SDNN) and the Root Mean Square of Successive Differences between normal beats (RMSSD) [90]. Spectral analysis identifies oscillatory rhythms in specific frequency ranges, including the Very Low Frequency band (VLF, below 0.04 Hz), influenced by thermoregulatory mechanisms and circadian rhythms; the Low-Frequency band (LF, 0.04–0.15 Hz), influenced by baroreflex and sympathetic and parasympathetic modulation; and the High-Frequency band (HF, 0.15–0.4 Hz), a marker of VN modulation influenced by respiratory activity [91–93]. Longer HRV measurement intervals, such as 24 hours, can reduce variability but pose obvious practical challenges and studies suggest that short (5-minute) HRV analysis are reliable [94–96].

The ANS influences cardiac beat-to-beat interval length in response to several factors. The sympathetic and parasympathetic systems are the principal rapidly reacting systems that control heart rate. The two systems have different latency periods with sympathetic effects on heart rate slower than parasympathetic [97–99] i.e. the parasympathetic system has the ability to alter heart rate within 1-2 beats, while sympathetic effects take up to 10 seconds to take effect. Further detailed analysis of HRV is explored in Chapter 2.

Human neuroimaging studies have successfully linked changes in neuronal function in specific brain regions of the CAN to fluctuations in HRV [100]. These highlight key components of the central autonomic network that include the OFC, the ACC, medial prefrontal and insular cortices [101–103] alongside subcortical regions of the CAN including the amygdala, thalamus, hypothalamus, periaqueductal grey, and dorsal pons [104,105]. It has been proposed that via the CAN, vagally-mediated HRV indices may modulate prefrontal cortical function[103,106] which may offer an exciting mechanism to both index neuronal activity of the CAN, or - in a mechanism similar to biofeedback - harness the VN via HRV and modulate cardiac-neuronal coupling.

Low HRV has been associated with poorer prognosis in ischaemic heart disease, cancer, metabolic syndrome, anxiety disorders and Alzheimer's disease, and it has been postulated that there may be related pathophysiological mechanisms to this, namely dysregulated inflammation and sympathetic overactivity.[106–110] Another mechanism by which the VN may indirectly modulate disease states is via oxidative stress, and reduced VN activity has been found to be correlated with oxidative stress in preclinical models [108,111] which may have uses in clinical applications in the future.

The Baroreceptor Reflex

The baroreceptor reflex orchestrates intricate adjustments of the ANS to maintain systemic and cerebral BP within a narrow range. Primarily situated in the carotid sinuses and the aortic arch, these specialized stretch receptors continuously monitor changes in arterial pressure.[112] The afferent arm of the reflex involves baroreceptor activation and subsequent

transmission of afferent information to the NTS in the medulla via the nodose ganglion. This region integrates the sensory input and orchestrates the efferent arm via the NA and DMN. In response to increased blood pressure, the baroreceptors sense stretch in the carotid sinus or aortic arch. This activates the reflex, leading to reduced inotropy (force of contractions) via the AV node and chonotropy (heart rate) via the SA node. As described above, the left cervical VN is thought to innervate the AV node of the heart while the right VN more densely innervates the SA node, hence the right VN is thought to have a more powerful effect on HR and could mediate bradycardia more than the left.[113] For this reason, any therapeutic manipulation of the VN tends to be via the left VN.[114]

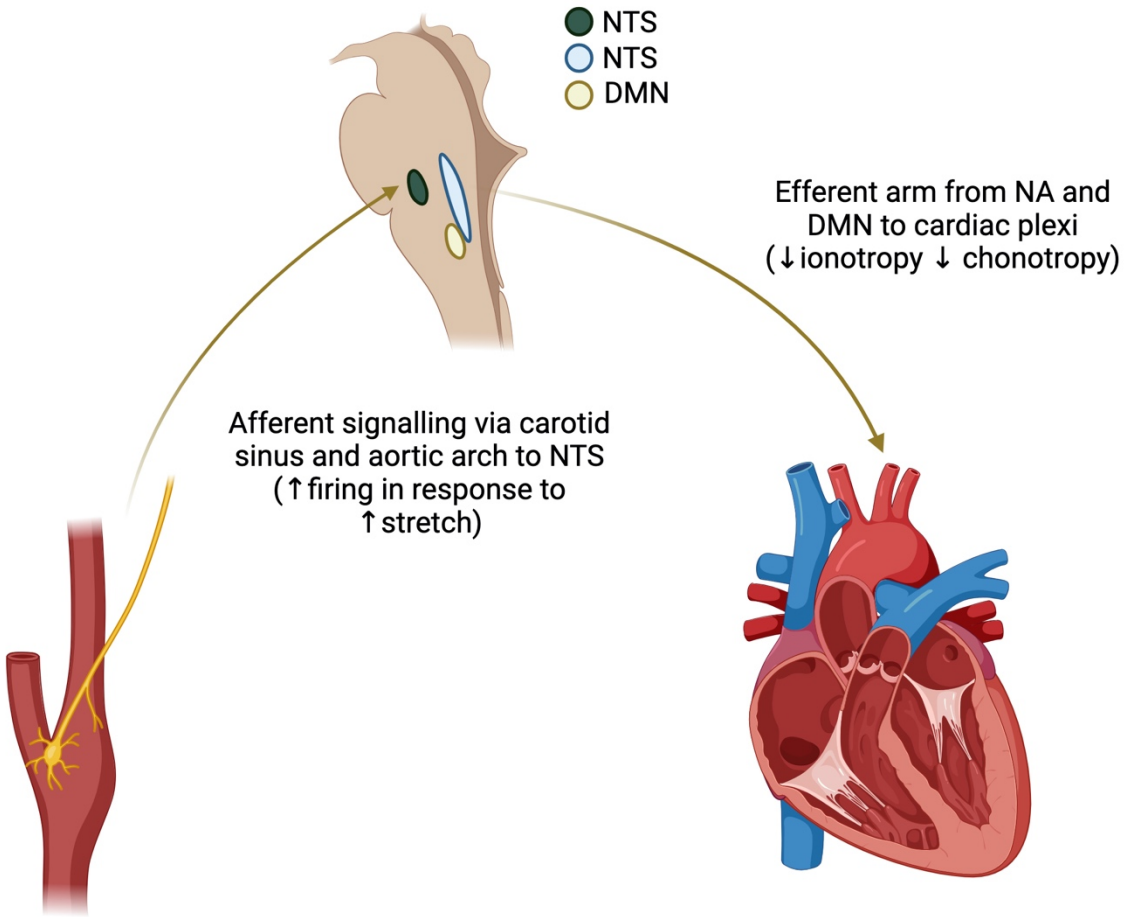


Figure 5 details the pathways involved in the Baroreceptor reflex

Cerebral auto-regulation, crucial for maintaining constant cerebral blood flow despite fluctuations in systemic blood pressure, is heavily influenced by the baroreceptor reflex. The baroreceptor-mediated adjustments in vascular resistance help preserve cerebral perfusion within a narrow range, preventing the brain from experiencing hypoperfusion during systemic hypotension or hyperperfusion during systemic hypertension [115]. This delicate balance is particularly vital given the brain's high metabolic demands and susceptibility to ischemia.

Orthostatic Hypotension (OH), and Postural Orthostatic Tachycardia Syndrome (POTS) represent clinical conditions where the baroreceptor reflex plays a pivotal role [116,117]. Orthostatic hypotension manifests as a drop in BP upon standing leading to symptoms such as dizziness and potentially loss of consciousness. Dysfunction in the baroreceptor reflex, inadequate compensatory vasoconstriction, or impaired autonomic responses contribute to this. POTS, characterized by an excessive heart rate increase upon standing, often involves abnormal baroreceptor responsiveness or hypersensitivity to ANS signals [116]. Neuro-Cardio-Vascular Instability (NCVI) encompasses a spectrum of autonomic dysregulation, with baroreceptor reflex dysfunction being a potential unifying underlying factor [118]. Understanding these intricate interactions is critical for elucidating the pathophysiology of these disorders and devising targeted therapeutic strategies.

The Cholinergic Anti-Inflammatory Pathway

The Cholinergic Anti-Inflammatory Pathway (CAIP) represents a critical link between the CNS and the peripheral immune system, playing a pivotal role in modulating inflammatory responses [119,120]. This pathway relies on the release of ACh via the efferent arm of the VN to interact with alpha-7 nicotinic acetylcholine receptors (α7nAChR) on immune cells. Activation of the CAIP leads to a downregulation of pro-inflammatory cytokine production and a suppression of immune responses, contributing to the maintenance of immune homeostasis [121].

α7nAChR's are expressed on various immune cells, including macrophages, monocytes, lymphocytes, and dendritic cells. The α7nAChR is a ligand-gated ion channel, and its activation can be achieved by the binding of acetylcholine or other cholinergic agonists. Upon activation, α7nAChR signalling inhibits the nuclear factor-kappa B (NF-κB) pathway, a central regulator of pro-inflammatory gene expression. NF-κB is a transcription factor that, when activated, translocates to the cell nucleus and promotes the transcription of pro-inflammatory genes, including those encoding cytokines such as Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 beta (IL-1β), and Interleukin-6 (IL-6) [122].

α7nAChR activation inhibits the NF-κB signalling pathway at multiple levels. It prevents the degradation of inhibitory kappa B (IκB), an inhibitor of NF-κB, and inhibits the nuclear translocation of NF-κB. As a result, the transcription of pro-inflammatory genes is suppressed. This leads to a decrease in the production and release of pro-inflammatory cytokines by immune cells [123].

In addition to inhibiting NF-κB signaling, α7nAChR activation also induces anti-inflammatory pathways. It promotes the activation of the Signal Transducer and Activator of Transcription 3 (STAT3) pathway [124] which contributes to the expression of anti-inflammatory cytokines, such as interleukin-10 (IL-10). IL-10 acts as a potent inhibitor of pro-inflammatory responses, further contributing to the overall anti-inflammatory effect [125].

The activation of α7nAChR is part of the broader inflammatory reflex, a neural circuit that involves the vagus nerve and its communication with the spleen and other organs. Stimulation of the vagus nerve releases acetylcholine, and via the splenic nerve activates α7nAChR on immune cells [126]. This reflex mechanism is crucial for maintaining immune balance and preventing excessive inflammation.

Summary

In summary, the VN is a crucial component of the ANS, playing a central role in regulating various physiological functions. Its anatomy and physiology involve intricate connections to the brainstem, particularly the vagal nuclei, which serve as command centres for autonomic responses. The VN has afferent connections with somatic and visceral areas, including the external ear through the ABVN. This complex network of connections underscores the VN's multifaceted impact on both sensory and motor functions.

The CAN forms the neural infrastructure responsible for coordinating autonomic activities throughout the body. Interoception and biofeedback mechanisms are integral components

of this network, enabling the body to monitor internal states and adjust responses accordingly. The LC-NE system modulates arousal and stress responses, contributing to the regulation of autonomic functions. HRV, a measure of the variation in time between heartbeats, serves as a valuable indicator of ANS activity, reflecting the balance between sympathetic and parasympathetic influences.

The Baroreceptor Reflex and the CAIP represent two critical regulatory mechanisms within the autonomic nervous system. The Baroreceptor Reflex is responsible for maintaining BP stability by adjusting heart rate and vascular resistance. Conversely, the CAIP highlights the VN's anti-inflammatory role, emphasizing the modulation of immune responses through cholinergic signalling. These concepts collectively illustrate the intricate web of interactions within the ANS, showcasing its prevalent influence on physiological homeostasis and health.

Chapter 2: Vagus Nerve Stimulation, Cognition and Neuro-Cardiovascular Effects

Vagus Nerve Stimulation

Vagus Nerve Stimulation (VNS) involves the use of electrical impulses to electrically stimulate the VN. VNS has typically been employed to treat medical conditions that are not adequately managed through interventions such as medications. There are two primary methods of administering VNS: invasive VNS (iVNS) and transcutaneous VNS (tVNS) which we discuss below.

VNS was first conceptualised in the 1870's to modulate nerve function [127]. The first practical application was suggested by James Corning, a neurologist in New York, who created a carotid "electro compressor" which was intended to both compress and electrically stimulate the carotid sheath bilaterally [128,129]. Over the next 100 years preclinical feline and canine studies [130,131] indicated that VNS could both activate central afferent projections and terminate induced seizures [132,133]. This preclinical work led to the first implanted iVNS devices in clinical trials in 1988.

Invasive VNS

iVNS requires a surgical procedure to implant the device directly onto the VN, typically the left cervical VN. This method is typically more targeted and precise but involves a surgical intervention, and has risks associated with it such as the risks of general anaesthesia, a lateral neck dissection to isolate the cervical VN, risk of damage to surrounding structures such as the great vessels of the neck, lead misplacement over time or lead infection and battery pack removal or replacement involving reopening the thoracic subcutaneous pocket

[134]. It has also been noted that hoarseness, throat pain and localised skin paraesthesia are common post-surgery, and up to half of patients experience side effects post iVNS implantation [135].

iVNS received US Food and Drug Administration (FDA) approval for the adjunctive treatment of epilepsy in 1997 and for use in treatment-resistant depression in 2005 [136]. It is noteworthy in these cases, that there has been a phenomenon noted in the literature of "VNS-responders" and "VNS non-responders" with up to 30% of persons with epilepsy for example who receive an iVNS device not responding to the treatment [135]. This will be of importance when discussing the potential other benefits of VNS, such as via modulating inflammation or augmenting cognition.

Given the invasive nature of iVNS and the risks associated with implantation, the concept of non-invasive VNS was proposed in 2000 [137] whereby, drawing on evidence from acupuncture studies, it was hypothesised that transcutaneous VNS could represent a valuable tool in epilepsy treatment. Non-invasive VNS involves using stimulating electrodes on the skin to excite afferent vagal fibres and can be performed via the auricle or the neck (cervical VNS: cVNS).

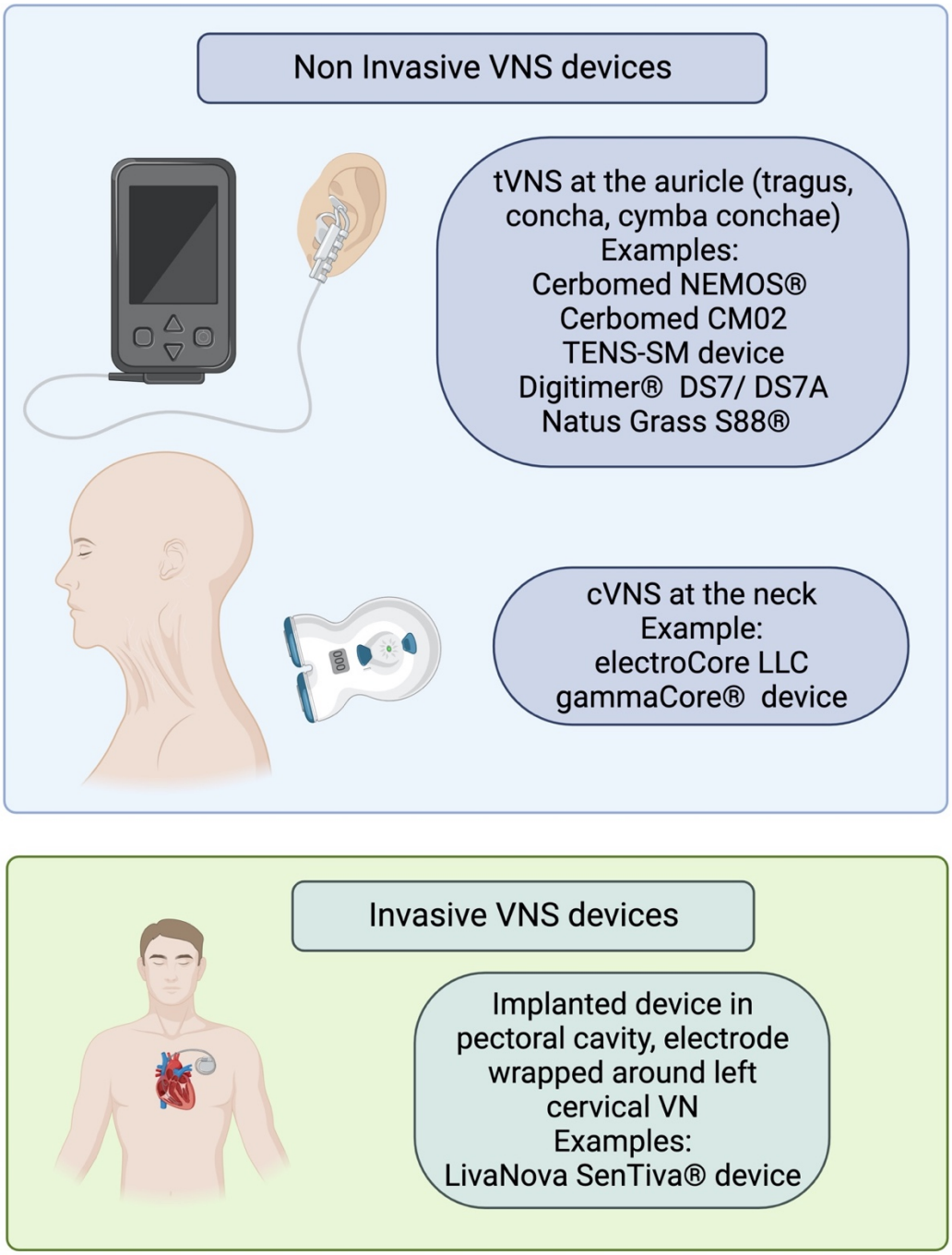


Figure 6 illustrates various VNS devices commercially available and their anatomical location

Non- Invasive VNS

Cervical VNS

cVNS involves electrically stimulating the cervical portion of the VN where it lies medial to the lateral border of the sternocleinomastoid muscle. It is primarily undertaken with a handheld neuromodulation device such as the gammaCore ® device from electroCore LLC [138]. There are active studies investigating the effects of cVNS in modulating migraine [139], cluster headaches [140], GI pain and gastroparesis [141], Sjogren's syndrome [142] and Parkinson's Disease [143,144].

For the purposes of this thesis we will focus on auricular tVNS, as this is the type of device we utilised in our research study, and we wished to investigate the safety, feasibility and tolerability of an auricular tVNS device especially in an older population.

Transcutaneous VNS

tVNS employs non-invasive techniques to modulate the VN through specific sites on the ear, notably the cymba concha and tragus. There have been positive results reported in studies investigating the therapeutic effects of tVNS for the following conditions: pain and sensitization [145–152], migraine [153,154], cluster headache [155], depression [156], anxiety [157,158] Parkinson's Disease [159], schizophrenia [160] impaired glucose tolerance [161], obesity [162,163], tinnitus [164], atrial fibrillation (AF) [165,166], osteoarthirits [167], post operative inflammation [168] and ileus [169], dyspepsia [170], Post-Traumatic Stress Disorder (PTSD) [171], and insomnia [172]. Studies of the effects of tVNS on cognition, memory and neurocardiovascular outcomes will be discussed in detail below. tVNS waveforms can be delivered at a variety of different parameter settings which vary frequency (Hz), amplitude (mA), pulse width (µs-msec) and duration of stimulation [138] The mechanism of action of tVNS is illustrated in Figure 7.

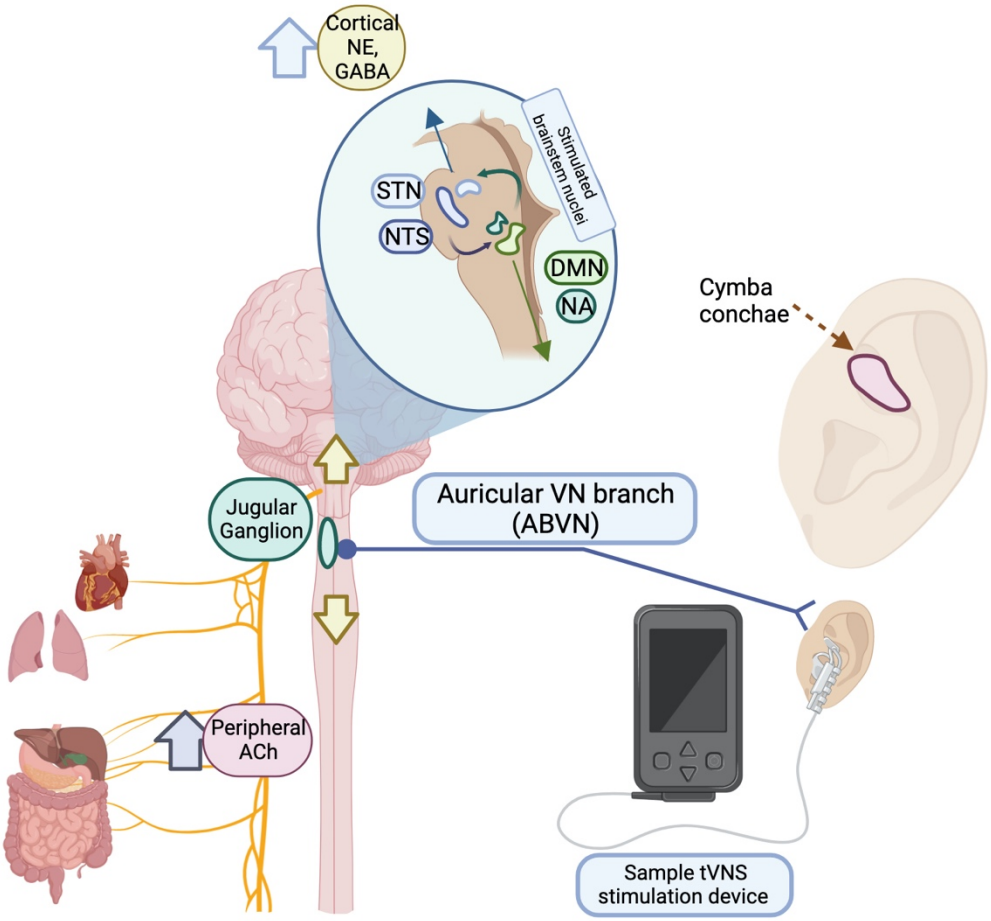


Figure 7 illustrates a schematic representation of the anatomy of the Auricular Branch of the Vagus Nerve (ABVN) and central vagal modulatory structures activated by tVNS

Parameters, Fibres and Settings

As we recall from Chapter 1, the depolarization of a nerve axon is influenced by its diameter and myelination, with larger myelinated requiring lower stimulus intensity to depolarise. This is due to increased current flow which leads to earlier depolarization [173]. The ABVN is

reported to have approximately 20% thick myelinated Aβ fibres with a diameter exceeding 7 μm which is far fewer than the reported Aβ fibre density of the cervical VN [44].

Given that pain-signalling fibres are typically smaller diameter Aδ or C-fibres, determining the appropriate current level for tVNS involves utilizing the individual pain threshold of the study participants. Small diameter Aδ and C-fibres necessitate significantly higher stimulus intensities for depolarization compared to the larger diameter Aα or Aβ fibres with thicker myelination [174].

To prevent unpleasant tingling or pain during tVNS, the stimulus intensity required to activate Aα or Aβ fibres can be determined by increasing the output intensity of the stimulator until the subjective pain threshold is reached, then reducing the stimulus intensity just below the individual pain threshold [138]. This approach ensures successful stimulation of thickly myelinated nerve fibres without activating the small diameter pain-mediating fibres [175].

However, nerves can remain refractory after depolarisation, such that very elevated current or stimulation frequency could cause refractory depolarisation and could delay repolarisation, which has been demonstrated with kHz stimulation of the VN which activated C fibres but blocked myelinated fibres [176]. The precise mechanism underlying this remains incompletely understood, necessitating further future research to elucidate the specific stimulation settings required to definitively activate the ABVN.

Neuroimaging during tVNS

Stimulation of the external ear regions innervated by the ABVN through tVNS results in activation within the medulla region of the brainstem, including the NTS. Furthermore, NTS projections extend to various areas associated with the CAN such as the PBN, substantia nigra, STN, LC, amygdala, insular cortex, NA, paracentral lobule of the cortex, and frontal cortex, as indicated by fMRI studies conducted by [49,50,177–179]. Notably, deactivations in response to this transcutaneous approach have been observed in the hippocampus and the hypothalamus [50,180] which warrants further study.

Biomarkers of VNS

Various biomarkers of VNS have been explored, which provide insights into its physiological effects. One notable biomarker associated with iVNS is the P300 Event-Related Potential (ERP). The P300 ERP is a positive wave in cortical electrical activity, typically occurring around 300 milliseconds after a stimulus [181]. Studies have shown that iVNS can modulate the P300 ERP, suggesting its influence on cognitive processes and attention [182,183]. However studies confirming whether tVNS can also modulate the P300 ERP are lacking, and some have suggested it is not affected by tVNS [184] so further research is needed in this area.

Salivary Alpha-Amylase (SAA) is another biomarker influenced by tVNS. SAA is an enzyme secreted by the salivary glands, and its levels are indicative of ANS activity. Monitoring SAA provides a non-invasive way to assess the impact of tVNS on autonomic function and recent pooled research has demonstrated that tVNS is associated with a significant increase in SAA levels, suggesting tVNS modulates the ANS likely via NE [185].

Pupil dilatation has been investigated as a biomarker of tVNS, with mixed results. Pupil dilation is controlled by the ANS, and alterations in this response can reflect sympathetic and parasympathetic activity. While some studies have demonstrated that tVNS induces pupillary dilation [186–188] others have failed to replicate this [189]. This, plus a specific light-controlled testing environment is needed to reliably test pupillary dilation, make it a less reliable marker of tVNS activity.

HRV is a biomarker of autonomic function, reflecting the variation in time intervals between successive heartbeats. tVNS has been linked to changes in HRV, indicating its impact on cardiac autonomic regulation[190–192]. Increased HRV is generally associated with improved cardiovascular health and parasympathetic dominance. Studying HRV in the context of tVNS provides valuable information about its influence on cardiac autonomic control, contributing to the assessment of its potential therapeutic applications in cardiovascular health and stress management. Analysis of HRV indices in the context of tVNS will be explored in greater detail below.

Cognitive Performance and VNS

In this section we will discuss the noted effects VNS has on cognition. Given that there are two distinct modalities of delivering VNS (iVNS and tVNS) we will first discuss the effects of iVNS on cognition and then the effects of tVNS on cognition, and in what populations this was studied.

Interest in the potential role of VNS as a cognitive enhancer began after the publication of a preclinical study of an inhibitory-avoidance task whereby subjects received a single exposure to a foot shock followed immediately by active VNS or sham. Those undergoing active VNS compared to sham demonstrated significantly enhanced avoidance to the noxious stimulus.[193] Similar preclinical laboratory work has confirmed these findings and found VNS induced cortical changes in areas of the hippocampus compared to sham [194,195]. It is noteworthy that the positive learning effect was modulated by the intensity of the stimulus, with 0.4 mA demonstrating an effect on stimulus avoidance but 0.2 mA and 0.8 mA having no significant effect. This will be of importance as we discuss how various stimulation parameters may activate certain fibres of the VN at discrete thresholds and is discussed in further detail below.

Cognition and iVNS

Following from the preclinical data suggesting a cognitively beneficial effect of VNS, researchers studied the effects of iVNS devices in clinical subjects who had iVNS devices implanted for control of treatment-resistant epilepsy or depression. In this subsection, we will examine the reported cognitive effects of iVNS in this clinical populations and examine the effects of iVNS on executive function and language.

It is worth noting that, except for one interesting study which we detail below, these patients had iVNS devices implanted for control of a refractory medical condition (epilepsy or depression), so any noted cognitive benefits would have been secondary to the primary reason for device implantation. We are also aware that study experiments would not have

been originally powered to detect a change in cognition for participants, so definitively noting cognitive improvements in these populations should be done with caution. It is also worth noting that the micro- and macro- architecture of the brains of patients with treatment resistant epilepsy [196] or depression [197] are different to the general population, and distinct from brains of people with Mild Cognitive Impairment (MCI) or dementia [198,199]. Further analysis of the differences between MCI and dementia will be explored in Chapter 3.

Further potential confounders to this data include the fact that that Anti-Epileptic Drugs (AEDs) have a deleterious effect on cognition [200] and may bias these results, and that controlling frequent seizures of depression itself would moderate cognitive performance [201]. Nevertheless, these studies investigated the potential cognitive effects of iVNS and prepared the field for further scientific investigation.

It is noteworthy and interesting that the beneficial effects of iVNS on cognition in epilepsy may be maximally demonstrated in so-called “VNS responders”. As mentioned above, up to 30% of patients who have an iVNS device implanted for control of treatment-resistant epilepsy do not respond to the therapy and have no appreciable reduction in seizures [135]. In some circumstances, only VNS-responders had demonstrably improved cognition, as measured by improved reaction times and reduced distractor interference [202].

One distinct set of studies investigated iVNS in a population without epilepsy or depression, consisted of a pilot study, with one year follow up, undertaken to investigate the cognitive benefits of iVNS devices implanted in patients with Alzheimer’s Disease (AD) with the

primary aim being to investigate the cognitive benefit of iVNS for AD. [203,204] We discuss these studies below.

iVNS and Executive Function

Executive function refers to a complex set of cognitive skills, encompassing attentional selection, task focusing, working memory and response inhibition. Impaired executive function manifests as rigidity of thought or processing, impaired social cueing, inability to task-shift or turn take, and difficulty with appreciating motivation or reward [205]. Executive function is tested using a variety of tools, mostly testing response times, meta-cognition, set shifting, or response inhibition and examples include the Stroop Test [206], Trail-Making test, Clock-Drawing Test, Digit-span forward and backward [207] and stop-go tasks such as the Eriksen-Flanker test [208] and Sustained Attention to Response Task (SART) [209]. Executive function commonly incorporates the concept of cognitive control i.e. the regulation of behaviour to achieve a goal, which involves suppression of goal-irrelevant stimuli via response and attention-inhibition [210]. Inhibitory control is commonly measured via forced-choice reaction time tasks that require participants to selectively respond to target stimuli whilst ignoring distracting stimuli [206,211].

Working memory refers to a cognitive process of temporary storage and manipulation of information necessary for complex cognitive tasks [212]. It may be conceptualised as a part of executive function. The impact of iVNS on working memory has been appraised in one experimental paradigm, wherein twenty participants with poorly controlled epilepsy were

required to perform a computer-based executive-reaction time test. The participants' ability to memorise and store the orientation of a shape and indicate its position in response to a go signal were assessed whilst VNS was delivered in a cyclic fashion. Active iVNS stimulation was associated with fewer errors in the subtask relying on working memory [213].

The effect of active iVNS on response inhibition was assessed by employing a stop-signal task in participants with refractory epilepsy [214]. Quicker response inhibition was demonstrated during active iVNS stimulation in patients, particularly those who had previously demonstrated a higher therapeutic benefit (i.e. greater seizure control) with iVNS, again suggesting that VNS-responders may reveal greater cognitive benefits.

iVNS has been shown experimentally to have mixed results when examining the subdomain of decision making, specifically on the Iowa Gambling Task (IGT). In one paradigm eleven patients with refractory epilepsy and iVNS devices completed a gambling task involving control and experimental trials with active VNS synchronised to stimulate in the latter. Whilst improved performance was demonstrated in the earlier part of the task, this trend was reversed later in the experimental trial with active stimulation trending towards being detrimental to performance [215]. Technical failure and a cumulative stimulation-dose effect were amongst the potential explanations proposed by the authors to explain this phenomenon.

iVNS and Language

Assessment of language involves both assessing categorical fluency, a semantic memory language task involving the temporal lobe, and word recognition and retrieval which mostly involves the prefrontal cortex and medial temporal structures [216,217] In the first study of its kind, building on previous preclinical research, the impact of iVNS on word retrieval memory was assessed via a sham-controlled experimental protocol with a population of participants who had iVNS devices inserted for the management of treatment-resistant epilepsy. The group who underwent active stimulation after the learning phase, during consolidation, had significantly higher word retrieval than the sham group [193]. The propensity for iVNS to positively impact word retrieval memory in a population of patients being treated with iVNS for intractable epilepsy was highlighted again in 2006 whereby the impact of iVNS on performance in the Hopkins Verbal Learning Test was assessed, demonstrating a significant improvement in word retention when active (amplitude 0.5mA) compared to sham stimulation was applied during memory consolidation [218].

iVNS in patients with AD

In one previous study, iVNS devices were experimentally implanted into 17 patients with AD, diagnosed on the basis of clinical phenotype (i.e. before the era of accurate AD biomarkers) with follow up of cognitive (via the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) and Mini-Mental State Examination [MMSE]), depression and Quality-of-Life (QOL) outcomes one year later[203] The population studied was 63 years old (range 57-81) with 11 female participants. The outcomes were noteworthy: iVNS was well-tolerated, with few side effects reported. The authors noted improvement or lack of decline after one year in 7/17 participants on the ADAS-Cog and 12/17 on the MMSE, without notable changes in

other outcome measures. To our knowledge this experiment has not been replicated globally but offered an interesting insight into the potential effects of iVNS in a cognitively impaired population.

iVNS, Cognition and Stimulation Parameters

Several studies in these areas have noted that there is a "'sweet spot" specifically regarding the amplitude of stimulation to promote cognition. The earliest note of this was in the early 1990's, wherein investigators noted an "inverted U-shaped relationship" regarding stimulus intensity and modulation of cognitive performance, with memory enhancing effects demonstrated only at moderate intensities, namely 0.5mA but not at higher (1mA) or lower (0.1mA) intensities[193]. These results were in part corroborated by a subsequent study which employed higher stimulation intensities (>1mA) and failed to demonstrate enhancement of verbal recognition memory, in fact demonstrating a reversible deterioration in figural memory [219]. It is noteworthy that in the groups with higher mA stimulation settings[220] stimulation mA was set at 1.2mA- 1.3mA with alterations in stimulation frequency (Hz) investigated, but no significant improvements in cognition were noted in either group. As we recall from Chapter 1, myelinated A fibres of the VN are abundant in the cervical portion of the nerve [44] which require lower stimulation thresholds to depolarise than unmyelinated C fibres.

Table 3 highlights the studies investigating the effects of iVNS on various cognitive outcome measures in discrete clinical populations.

COGNITION AND iVNS								
		Stimulation Parameters						
Study	**iVNS/tVNS**	**Hz**	**mA**	**Pulse width**	**Time**	**Population**	**Task**	**Outcome**
Clark et al., 1999	iVNS 2- 24/52 post implantation	30Hz	-0.5mA -0.75-1.5mA	0.5 ms	30 sec	Intractable epilepsy n=10	Word recognition task	Improved word recognition memory only when 0.5mA delivered post reading
Sjogren et al., 2002 [204]	iVNS Assessed at 3 and 6 months	20Hz	0.25mA, increased 0.25mA increments over 2 weeks then fixed	500 µs	30 sec followed by 5 min pause	Probable AD n=10 age 67±7.6 8 women 2 men	Median change in ADAS-cog Median change in MMSE after 3 and 6/12 Depression, behaviour and QOL variables	After 6/12 8 of 10 patients showed improvement from 3/12 ADAS-cog scores After 6/12 7 of 10 patients improved MMSE score by average 2.5 points No change in other variables

Martin et al., 2004 [215]	iVNS	30Hz	0.5mA	500 µs	60 sec	Intractable epilepsy n=11	Iowa Gambling Task	Conflicting results, deleterious at higher doses
Merrill et al., 2006 [203]	iVNS At least one year of VNS treatment	20Hz	0.25mA, increased in 0.25mA increments over 2 weeks then fixed	500 µs	30 sec followed by 5 min pause	Probable AD n=17 (age 63 range 57-81) 11 women 6 men	Median change in ADAS-cog Median change in MMSE after 1 year Depression, behaviour and QOL variables	At one year, 41% had improvement or no decline from baseline on ADAS-cog 70% had improvement or no decline on MMSE No change in other variables
Helmsteadter et al., 2001 [219]	iVNS 5-7/12 post implantation	30Hz	Mean 1.75mA (range 1-2.5)	500 µs	30 sec – 4.5 min	Intractable Epilepsy n=11	Word recognition task Design recognition task	Deterioration in figural recognition memory
Dodrill et al., 2001 [220]	iVNS 12-16/52 after implantation	30 Hz in high stim group 1 Hz in low stim group	Avg 1.3mA in high simulation group Avg 1.2mA in low	500 µs 130 µs	30 seconds on every 5 minutes	Intractable Epilepsy n=160	Wonderlic personell test, Stroop test, Digit cancellation, Symbol Digit Modalities	No significant changes were noted in the cognitive tests in low or high stimulation

			stimulation group		30 seconds on every 3 hours			
Ghacibeh et al., 2006 [218]	iVNS >3/12 post implantation	X	0.5mA	x	30 sec	Intractable Epilepsy n=10	Hopkins verbal learning test	Improved retention index
McGlone et al., 2008 [221]	iVNS 12/12 post implantation	30 Hz	0.5-3mA avg 1.72 +/- 0.53	500 µs	30 sec every 5 min	Intractable epilepsy n=16	Memory Observation Questionnaire	Improved subjective and objective memory scores compared to baseline, but similar to medical management
Schevernels et al. 2016 [214]	iVNS > 18/12 post implantation	Avg 25 (20-30)	Avg 2.3mA (0.75-3.0)	Avg 431 µs (130-500 µs)	7 sec on / 18 sec off	Intractable epilepsy n=20	Stop signal task	VNS responders demonstrated quicker response inhibition
Sun et al., 2017 [213]	iVNS 2-130 months post implantation	30Hz	1.5-1.75mA	250 µs	30 sec on / 48 sec off	Intractable epilepsy n=20	Executive reaction time test (go-no-go task)	Improved working memory (only when 3 participants with cognitive impairment removed)

Bochove et al., 2018 [202]	iVNS	20 or 30 Hz	Avg 2.28mA (0.75-3.0)	250 µs or 500 µs	7 sec on / 18 sec off	Intractable epilepsy n=17	Eriksen Flanker task	VNS responders demonstrated improved reaction times and decreased distraction interference

Table 3 details the studies investigating parameter settings and cognitive outcomes during invasive VNS (iVNS)

Cognition and tVNS

As discussed above, fMRI imaging during tVNS demonstrates activation of vagal projections to various regions of the CAN including NTS, LC, ventral medulla, insular cortex, frontal cortex and cerebellum. However, not all studies have confirmed the same areas of central activation, and interestingly during sham stimulation of the earlobe with tVNS devices, there was still brainstem activation noted.[50]

Possible reasons for this include anatomical variation and lack of parameter optimisation during tVNS stimulation. If we recall from Chapter 1, there are numerous nerves that innervate the skin of the auricle, including the auriculotemporal nerve, the greater auricular nerve and lesser occipital nerve. Precise human cadaveric and functional imaging studies confirming the exact delineation of innervation sites in the ear are lacking [43] and further precise research in this area may help clarify these issues.

Here we discuss the reported cognitive effects of tVNS to various sites on the auricle. The studies mostly involve healthy, young volunteers which, though illuminating for proof of concept for tVNS, are limited in their scope, as cognitive benefits in young healthy adults may be challenging to elicit due to ceiling effects [222]. We note with interest one study of the cognitive effects of tVNS in healthy older adults[223] and one study of tVNS in an older population with MCI [224] which we explore in detail below. Overall, recent metanalysis of the effects of tVNS on cognition demonstrated a weighted effect size of 0.21 for the effect of

tVNS on overall cognitive performance, with significant effects on measures of executive function [225].

tVNS and Executive Function

Post error slowing refers to appropriate slowing after negative feedback or unforeseen errors and is part of the matrix of cognitive control and response inhibition. Enhanced response times, as reflected by participants' ability to stop a process and change to another response simultaneously and sequentially, and increased post error slowing were demonstrated during tVNS [226,227]. As with the above trials, there were fewer false alarms during a more challenging paradigm with tVNS when working memory processes were simultaneously engaged [228] and improved response selection and control performance was demonstrated with tVNS in a serial reaction time test in young volunteers. [229]

In a sequence learning paradigm, the presentation of so-called reversal trials is associated with longer response latencies as compared to non-reversal trials, a result attributable to the 'inhibition of return' type phenomenon. Inhibition of return refers to an inhibitory after-effect of attention whereby, following exogenous orientation of attention to a stimulus, processing of stimuli at this location is first facilitated and then inhibited [230]. Jongkees and colleagues demonstrated that active tVNS, as compared to sham stimulation in the context of a serial reaction time test, reduced reaction time for reversal trials, eliminating the inhibition-of-return effect [229] In a similar experimental set up, increased attention, globally enhanced accuracy and reduced performance costs were demonstrated in a Stop-Change paradigm with tVNS [231].

The majority of recent studies in this area have involved a spatial stimulation and response inhibition multitask, with notable improved results in accuracy with 25 minutes pre-assessment tVNS stimulation [232], improved objective attention, arousal and multitasking ability in sleep-deprived military personnel [233] and initial improved ability to dual task in healthy volunteers [234].

The effect of tVNS on executive function tests in healthy volunteers has not reported uniformly positive results. In a testing paradigm using higher than average amplitude settings there were no improvements in the Stroop test, modified Erisken-Flanker test or a number/letter working memory task with tVNS [235] Similar previous studies failed to show any improvements on a novelty oddball task and the Simon task, while a biomarker of attention and potentially tVNS, the P300 ERP, was noted to be significantly elevated in the intervention group. [236,237] These apparently contradictory results may be due to small sample sizes, and the pilot nature of a lot of these studies that are poorly powered to detect absolute changes. A further study investigating EEG amplitudes affected by tVNS and cognitive control paradigms included one involving an acoustic rather than visual oddball paradigm and again tVNS augmented the P300 ERP [238].

tVNS and Language

Fluency scores in healthy volunteers during a convergent and divergent thinking task were significantly higher during active tVNS at the left conchae, and categorical flexibility (i.e. participants' ability to think of more and varied categories of nouns) was also significantly

improved [239]. However, an experimental design investigating the difference in effect of tVNS on word recognition memory in young compared to older volunteers (average age 22.2 and 55.1) whereby tVNS was delivered for 30 seconds during the consolidation phase of a word recognition memory task showed no improvement in accuracy scores for immediate recall or delayed recognition in both age groups [240]. A recent investigation of word retention, stimulating the left tragus with tVNS at again similar parameters but wider amplitude found improved accuracy in word retention but only in items that rhymed i.e. were phonologically similar [241].

The only published study we are aware of that has investigated the effect of tVNS on cognition in a population with diagnosed MCI noted a significant improvement after 24 weeks of tVNS at the cymba conchae and concha in the an Auditory Verbal Learning Test (AVLT) and the Montreal Cognitive Assessment (MOCA) tool B [224]. It is noteworthy that the MOCA-B is designed for use in illiterate or poorly educated older persons [242] even though the mean years of education in their population was 9 which may have had implications for their findings. tVNS in this population was well-tolerated with few side effects, opening an avenue for future replication studies in this area.

tVNS and Associative Memory

Associative memory is a subcategory of declarative episodic memory and involves the ability to link disparate novel stimuli.[243] tVNS has been tested in a group of healthy older adults to determine the technique's impact on performance in a Face-Name Association Task (FNAT)[223] tVNS was employed in the encoding and consolidation phases of the task with active and sham

stimulation compared in a randomised crossover design. Active tVNS significantly increased the number of accurate "hits" during the FNAT. Stimulation parameters employed differed from those referenced in the broader literature concerning the impact of tVNS on cognitive function. A stimulation intensity of 5.0 mA, a pulse width of 200 μs, and a frequency of 8Hz were utilised, citing previous functional and electrophysiological studies [244]. A stimulation lead in time of 17 minutes was also utilised, which has been theorised to be beneficial for targeted neuronal plasticity [245].

tVNS and Emotion Recognition

Emotion recognition as a subtype of cognition involves areas of the cortex involved in perceiving social information including the medial prefrontal cortex and the orbitofrontal cortex [246] and subcortical structures including the amygdala, hypothalamus, basal ganglia and the periaqueductal grey matter [247].

The ability to recognise different emotions in others was investigated and found to be enhanced by tVNS at the left outer auditory canal in young healthy adults but only for objectively easy, not challenging, items via the Reading the Mind in the Eyes test [248]. Subsequent investigations of fear conditioning and extinction in young volunteers, after previous positive studies, found that tVNS at the left cymba conchae did not infer any difference in physiological or declarative indices of fear or improve fear extinction [249] The effect of tVNS on participants' ability to recognise facial emotions in three experimental paradigms (graded presentation, static images and in a go-no-go task) was also assessed in a group of adolescents diagnosed with Major Depressive Disorder (MDD). In non-depressed

controls tVNS demonstrated enhanced recognition of emotions, but notably led to a significant decrease in the ability of those with MDD to recognise sad emotions. [250]

Summary tVNS, Cognition and Stimulation Parameters

The afferent innervation site of the ABVN is the proposed site for these tVNS effects. One seminal dissection study referenced above but warrants repeating noted that approximately 20-30% of the ABVN on dissection were myelinated Aβ axons [44]. It is presumed that the stimulation of these axons leads to the most precise activation of brainstem nuclei such as the NTS, and that at a participants' pain threshold at the site of stimulation, C-afferent nociceptive fibres are preferentially recruited [251]. It is noteworthy that the negative trials investigating cognitive effects of tVNS had relatively high mA settings [236,252] though not uniformly, as some trials with high mA settings yielded positive results, but with lower frequency settings [223]. This supports again the idea of an inverted U-shaped curve when stimulating a nerve to a desired frequency.

As recently described, to clarify these inconsistencies researchers should adhere to the minimum data set required when publishing tVNS research data [253] and ideally since the ABVN is so small and anatomical innervation sites may vary, a biomarker of activation should be used to prove target engagement during trials seeking to prove efficacy of tVNS.[41] Please see Table 4 for details of studies investigating cognition and tVNS.

COGNITION AND tVNS								
		Stimulation Parameters						
Study	**iVNS/tVNS**	**Hz**	**mA**	**Pulse width**	**Time**	**Population**	**Task**	**Outcome**
Steenbergen et al., 2015 [227]	tVNS	25Hz	0.5mA	200 - 300 µs	30 sec blocks	Healthy young adult volunteers n=30	Stop change paradigm	Enhanced response selection and faster response times when two actions executed in succession
Sellaro et al., 2015 [226]	tVNS left outer auditory canal	25Hz	0.5mA	200 - 300 µs	30 sec blocks	Healthy young adult volunteers n=40	Modified Flanker test	Increased post error slowing during active tVNS
Jacobs et al., 2015 [223]	tVNS left external acoustic meatus	8Hz	5.0mA	200 µs	17 min	Healthy older adults avg age 60.5 n=30	-Face name recognition task -15 word learning test -Digit span forward / backward -Verbal fluency test -Concept shifting task	Higher number of accurate “hits” during tVNS for face name recognition

							-Letter digit subtraction -Stroop colour word test	
Beste et al., 2016 [228]	tVNS left inner ear	25Hz	0.5mA	200-300 µs	30 sec on / 30 sec off	Healthy young volunteers n=51	Inhibitory control (go-no-go task)	Fewer false alarms in the more challenging paradigm i.e. when working memory processes also engaged
Colzato et al., 2017 [248]	tVNS Left outer auditory canal	25Hz	0.5mA	200-300 µs	30 sec on/ 30 sec off	Healthy young volunteers n=38	Emotion recognition Reading the mind in the Eyes test	Enhanced emotion recognition for easy (not challenging) items suggesting it promoted the ability to decode salient social cues
Fischer et al., 2018 [236]	tVNS	25Hz	Avg 1.3mA (0.4-3.3)	200 - 300 µs	Continuous	Healthy adult volunteers n=21	-Adapted response conflict Simon task	No behavioural change noted Down-regulated N2

							-Novelty oddball task	potential EEG reading
Ventura-Bort et al., 2018 [237]	tVNS Left cymba conchae	25 Hz	-1.3mA (0.4–3.3) active -1.49mA (0.6−4.8) sham	200–300 µs	28 mins task 1 7 mins task 2	Healthy young volunteers n=21	-Novelty oddball task -number version of the Simon task	-No difference with tVNS with difficult targets or novel stimuli -Difference between tVNS and sham stimulation (P3 amplitude) in EEG parameters for easy targets associated with larger increase in sAA levels after tVNS
Rufener et al., 2018 [238]	tVNS Left cymba conchae	25Hz	0.5mA	250 µs	30 sec on / 30 sec off Started 90 mins prior to task	Healthy young volunteers n=20 avg age 24.8	-Acoustic oddball paradigm (respond as quickly as possible whenever a target tone was detected)	- tVNS increased EEG parameter P3 amplitude - Random noise stimulation reduced the reaction time
Maharjan et al., 2018 [254]	tVNS at left ear both anterior (cymba	-80Hz -10Hz	10- 15mA	180 µs in square waveform	25-35 min lead in time	Healthy adult males n=18	Two olfactory tests (odour threshold test	High frequency (80Hz) VNS

	conchae) and posterior of ear	-No stim					(OTT) and supra-threshold test (STT)	positively modulated olfactory performance in healthy participants and showed significant increase in NIRS recordings of the right hemispheric orbitofrontal cortex
Colzato et al, 2018 [239]	tVNS left concha n=40 sham left earlobe n=40	25Hz	0.5mA	200 - 300 µs	15 min lead in time	Healthy young volunteers n=80 (50 females, 30 males, mean age 20.96)	Convergent and divergent thinking tasks	-Fluency scores were significantly higher in the active tVNS group (able to generate more answers) -tVNS affected cognitive flexibility i.e. participants could think of more different

								categories than sham
Jongkees et al., 2018 [229]	tVNS left medial acoustic meatus	25Hz	0.5mA	200 - 300 µs	30 sec blocks 15 min lead in time	Healthy young adult volunteers n=40	Serial reaction time test	Enhanced response selection process and action control performance
Burger et al., 2019 [249]	tVNS Left cymba conchae	25Hz	0.5mA	250 µs	30 sec on/ 30 sec off 10 minute lead in time	Healthy young volunteers n=61	Computerised fear conditioning, fear generalization, and fear extinction paradigm	No difference in physiological and declarative indices of fear between tVNS and sham conditions
Mertens et al., 2020 [240]	tVNS cymba conchae	25Hz	0.5mA 0.54-0.57mA	250 µs	30 sec during consolidation	Healthy volunteers -n=41 age avg 22.2 -n=24 age avg 55.1	Word recognition task	No effect on verbal word memory
Giraudier et al., 2020 [255]	tVNS left cymba conchae (active) or left earlobe (sham)	25Hz	Active 1.48 mA +/- 0.59 sham 1.31mA +/- 0.5	200 - 300 µs	30 sec on/ 30 sec off Stimulated for 23 minutes 5min before 13 min during and 5 in after lexical decision task	Healthy volunteers -n=60 -46=female -avg age 23.45	Lexical decision task and recognition memory task of selected German words (either	Overall no effect of tVNS on task performance or word recognition memory –

							emotionally charged or neutral) Also – BP, HR and sAA	however higher recollection based memory performance was observed during tVNS than sham
Borges et al., 2020 [235]	tVNS	25Hz	2.19mA (±0.93)	200 - 300 µs	30 sec on / 30 sec off 4 mins lead in time	Healthy adult volunteers n=35	-Modified Flanker test -Spatial Stroop task -Number / Letter task -Dimensional change card sorting task	Only the DCCS shows improvement with tVNS
Keute et al., 2020 [231]	tVNS left cymba conchae	25Hz	2.37mA (±0.16)	200 µs	30 sec on/ 30 sec off 30 minutes lead in time	Healthy adult volunteers n=22	Stop Change paradigm (go-no-go task)	Globally enhanced accuracy across conditions -Reduced the performance costs of go/change response conflicts -increased attention

Sun et al., 2021 [232] Study 1	tVNS Left cymba conchae Sham: no stimulation	25Hz	Online 0.7 ± 0.36mA Offline 0.69 ± 0.38mA Sham 0.73 ± 0.27mA	500 µs	30 s on and 30 s off 25 min pre task (offline) or 15 min during task (online)	Healthy young volunteer n=46 (25 female, average age 20.39 ± 1.96)	Spatial stimuli task Four blocks with 72 experiment trials in each block	Offline (pre-task stim for 25 min) tVNS significantly increased hits in spatial 3-back task but not rejections or reaction times
Sun et al., 2021 [232] Study 2	tVNS left cymba conchae sham; active stimulation of earlobe	25 Hz	Active: 0.74 mA ± 0.37 Sham: 0.84 mA ± 0.39	500 µs	30 s on and 30 s off Both 25 mins stimulation	Healthy young volunteers n=58 (24 female, average age 19.9 ± 1.49)	Spatial stimuli task Four blocks with 72 experiment trials in each block	Offline (pre-task stimualtion for 25 mins) tVNS improved hits but not correct rejections or reaction time of accurate trials in spatial WM performance
Kaan et al., 2022 [241]	tVNS Left tragus	25Hz	1-6mA	250 µs	Not specified	Healthy young volunteers n=33 control n=29 experiment	-Word retention: – non-rhyming, easily	tVNS was associated with higher accuracy but only when the items are

							separable words -rhyming words	phonologically similar
Koenig et al., 2021 [256]	tVNS Left conchae	1Hz	0.5mA	250 μs	30 sec on/ 30 sec off 15 minute lead in time	Adolescents with major depressive disorder n=33 control group: adolescents with headache n=30	Facial emotional recognition in three tests 1. As a graded presentation 2. As static images 3. in a go – no - go task	-In non-depressed controls tVNS enhances the general ability to recognize emotions -tVNS specifically led to a decrease in the recognition of sad emotions in patients with MDD
McIntire et al., 2021 [233]	Cervical VNS via gammaCore device cVNS vs sham (n=20 both groups)	25Hz	Not available	Not available	2 min cycles	Healthy young military recruits n=40 (M:F 33:7) avg age 28 ± 6 years	34 hours of continuous sleep deprivation Air Force–Multi-Attribute Task Battery (AF-MATB);	cVNS significantly improved objective arousal and multitasking for as long as 24-h post-stimulation

							simultaneously monitor and respond to four separate cognitive process tasks	Subjective ratings of fatigue also improved
Wang et al., 2022 [224]	tVNS (TENS type device) to the concha and cymba conchae Sham stim = scapha of helix Parallel trial design	20 Hz for 10 s and 100 Hz for 50 s in each minute	0.6- 1 mA determined by participant	Not reported	30 mins per session, two sessions daily, 5/7 a week for 24/52	Participants with diagnosed MCI n = 60 age = 67.0 ± 4.36, 43 females	MOCA-B, AVLT and a battery of neuro-psych test	Significantly improved MOCA-B and AVLT after tVNS
Sommer et al., 2023 [234]	tVNS (NEMOS © device) to left cymba conchae Internal crossover (2/7)	25 Hz	1.6 ± 0.8 mA for active stim and 1.5± 0.8mA for sham	250 µs	30 seconds on / 30 seconds off	Healthy volunteers, n=32 completed (19 females; 25.5 years, ± 2.6 years)	Cognitive control in multitasking via dual task assessment	Significantly improved dual task assessment under tVNS with early stimulation- however effect was transient and disappeared after Test-Block 1

Table 4 details studies investigating parameter settings and cognitive outcomes of transcutaneous VNS (tVNS)

VNS and Neuro-Cardio-Vascular Effects

The VN is intimately involved in maintaining cardiovascular homeostasis, predominantly via the baroreceptor reflex (for detail on the physiology of this reflex please see Chapter 1. In general, heightened VN activity can mediate either a drop in peripheral BP (vasodepression) or a drop in pulse rate (cardio-inhibition) and either one of these mechanisms can cause temporary lack of cerebral perfusion and could result in Transient Loss Of Consciousness (TLOC). In clinical practice, and especially in older adults, a combination of both vasodepression and cardio-inhibition often act synergistically to result in reflex mediated syncope [257]. One method employed by syncope experts to unmask VN hypersensitivity is a Carotid Sinus Massage (CSM) which, under controlled conditions, can unmask bradycardia or asystole if cardio -inhibition is the cause of TLOC in a patient, or drop in SBP if the underlying cause is vasodepression [258]. The act of massaging the carotid sinus stimulates the stretch receptors of the baroreceptors and causes activation of the VN. As noted in previous chapters, especially when the right VN is stimulated a more pronounced bradycardic effect may be elucidated due to the anatomic innervation of the cardiac plexi via cervical VN, with the right cervical VN supplying the SA node (the pacemaker) of the heart primarily [259].

Orthostatic response analysis involves assessment of the cardiovascular system's response to an orthostatic challenge, usually via standing (i.e. the Active Stand [AS] test) or response to a Head Up Tilt Test (HUTT). The analysed outcome measures include Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), and Heart Rate (HR), with most centres now also incorporating a measure of concomitant cerebral perfusion such as the Tissue Saturation

Index (TSI) as measured via Near- Infrared Spectroscopy (NIRS).[260] The AS test has gained favour in clinical autonomic research due to its relatively quick procedure time (an AS takes approximately 10 minutes to perform), ease of reproducibility and patient tolerance of the procedure [261]. The gold standard for assessment of cardiovascular responses to orthostasis remains the Italian Protocol HUTT [262] however notably a recent evolution to this protocol has demonstrated that a 10-minute HUTT may offer equivalent diagnostic insights as a longer test which would improve its usability [263].

Given the intimate associations that the VN has with maintaining cardiovascular tone, both via BP and HR responses, it is prudent to ensure that stimulating the VN either invasively or transcutaneously, does not inadvertently cause a drop in either BP or HR and potentially affect cerebral perfusion.

Heart Rate Variability

As mentioned in Chapter 1, HRV is a biomarker of autonomic function, reflecting the variation in time intervals between successive heartbeats. There are various indices, both in the time-domain and frequency-domain of analysis and these indices reflect the autonomic modulation of the heart [264].

Time domain indices of HRV include the root-mean square of the difference of successive R-R intervals (RMSSD); standard deviation of the inter-beat interval (IBI) of normal sinus beats (SDNN); the percentage of adjacent N-N intervals that differ from each other by more than

50 ms (pNN50); whereby N-N refers to the interval between normal beats (i.e. whereby ectopic beats have been removed), among others.

Frequency domain indices of HRV include the High Frequency (HF) band, also called the respiratory band, which sits at a frequency of 0.15–0.40 Hz and reflects parasympathetic activity and corresponds to the HR variations related to the respiratory cycle; the Low Frequency (LF) band which corresponds to oscillations at 0.04–0.15 Hz, and reflects baroreflex activity and not cardiac sympathetic innervation; the Very Low Frequency (VLF) and Ultra-Low-Frequency (ULF) bands correspond to more intrinsic physiological functions such as the Renin-Angiotensin system and thermoregulatory mechanisms, they require longer periods of ECG monitoring to accurately detect and less is known about their significance compared to other time- and frequency- domain HRV indices [92,265].

HRV indices reflect parasympathetic, mixed sympathetic, and parasympathetic and circadian rhythms. In population studies, decreased HRV indices have demonstrated predictive value for mortality among older healthy adults [266] and in other populations (such as post-myocardial infarction, patients with diabetes) it has also highlighted patients at particularly high risk of decline [267]. Whether HRV may be affected by invasive or transcutaneous VNS will be explored in subsequent paragraphs.

Preclinical research in this area has noted, in multiple mammalian species, that experimental VNS reduced HR [268–272] and had significant effects on various parameters of HRV [271,273–275] which paved the way for clinical research in this field.

iVNS and Cardiovascular Responses

Various post-iVNS insertion studies have analysed cardiovascular outcomes and HRV indices in populations with iVNS devices for either epilepsy or depression at various timepoints after device insertion, with diverse outcomes noted.

An early study investigated iVNS in patients with refractory epilepsy, revealing that high-frequency stimulation led to a significant decrease in the LF:HF ratio and increased HF power compared to low-frequency stimulation [276]. Again in a later study, a significant increase in LF and HF power during stimulation periods in epilepsy patients undergoing iVNS was again observed, suggesting a modulatory effect on cardiac autonomic function [277]. Additionally it was found that iVNS led to significant improvements in certain HRV indices and concomitant ambulatory blood pressure [278].

However, these initial results were mostly not replicated in further studies of similar populations with comparable stimulation settings, with timeframes ranging from just minutes of stimulation to 1 year of stimulation.[279–283]

Studies examining iVNS in the context of treatment-resistant depression found significant increases in discrete HRV parameters (RMSDD) during stimulation periods, indicating a potential therapeutic effect on cardiac autonomic regulation in this group but warrants further investigation [284].

There has been a relative paucity of published data investigating the effects of iVNS specifically on orthostatic responses to date. Case reports detail bradycardia during iVNS device insertion [285–287] and up to 2 years after iVNS device insertion [288,289]. One study analysed SBP, DBP and HR outcome measures at rest and after a 5-minute HUTT in patients who had iVNS devices implanted for control of refractory seizures [290]. The device parameters were set with the primary aim of reducing seizures. Notably, after six months of iVNS treatment, there was a significant decrease in resting SBP (from 141.31 +/- 20.4 mmHg to 122.83 +/- 15.22 mmHg) and resting HR (from 77.85 +/- 11.0 bpm to 69.33 +/- 6.09 bpm) but there was no exaggerated BP or HR drop during the HUTT, in fact surprisingly there was a significant increase in both SBP, DBP and HR responses during HUTT. Again, as mentioned in previous paragraphs, those with iVNS devices inserted for the management of epilepsy or depression have altered neurochemistry and whether this may influence the effect of iVNS on cerebral autoregulation during an orthostatic challenge remains to be elucidated. Table 5 details the cardiovascular effects noted in patients with iVNS devices inserted.

iVNS and Cardiovascular Effects								
		VNS Stimulation Parameters						
Study	iVNS/tVNS/ site specific	Hz	mA	Pulse width	Time	Analysis parameters	Population	Result
Kamath et al., 1992 [276]	iVNS for refractory epilepsy (left cervical vagus)	2 Hz 30 Hz	0.1mA 1mA	13,000 µs 500,000 µs	Not specified	Baseline 45 minute ECG readings pre implantation and at 2/52 post implant	Refractory epilepsy n=8 High stimulation and low stimulation groups avg age 34±7.8 range 21-47	HiStim group: LF:HF ratio decreased significantly with iVNS Significantly higher HF power in the HiStim compared to LoStim group
Setty et al., 1998 [291]	iVNS for refractory epilepsy (left cervical vagus) implanted for minimum 1/12	30 Hz	Max tolerated threshold	750 µs	30 seconds on 5 minutes off	Pre and post stimulation ECG (7 min baseline, 2.5 min of stimulation and a 7 min post-stimulation)	Refractory epilepsy n=10 (avg age 28 range 14-46) 8 men	No significant effect noted on HRV variables
Handforth et al., 1998 [292]	iVNS for refractory epilepsy (left cervical vagus)	30Hz in high stimulation group	Avg 1.3mA in high simulation group	500 µs	30 seconds on every 5 minutes	ECG analysis in two groups (high vs low stimulation) in refractory epilepsy	Refractory epilepsy High stimulation group n=95 age 32.1±10.8	No significant changes mean heart rate, HRV or occurrences of bradycardia

		I Hz in low stimulation group	Avg 1.2mA in low stimulation group	130 µs	30 seconds on every 3 hours		Low stimulation n= 103 age 34.2±10.1	
Galli et al., 2003 [280]	iVNS for refractory epilepsy (left cervical vagus)	30 Hz	0.25mA adjusted	500 µs	30 seconds on every 5 minutes	24-h analysis of RR variability at baseline (t0), 1 month (t1, short-term VNS) and 36 months after VNS initiation (t2, long-term VNS)	Refractory epilepsy n=7 (4 men) age 47 ±11.2 range 34-63 f	No significant changes in HRV variables, trend toward increased HF on nocturnal readings
Ronkainen et al., 2006 [293]	iVNS for refractory epilepsy (left cervical vagus)	30 Hz	2.9mA avg	500, 000 µs	30 seconds on 5 minutes off	Pre and 1 year post implantation 24h Holter HRV variables	Refractory epilepsy n=14 (eight male and six female age 34.3±9.3; 20–52) compared to matched controls	No significant effects on any HRV indices
Barone et al., 2007 [294]	iVNS for refractory epilepsy (left cervical vagus)	30 Hz	0.75-1.75 mA	500 µs	30 seconds on, 5 seconds off	24h ECG holter at baseline and after 3/12 implantation	Refractory epilepsy 8 patients (age 32 range 9-65 2 men)	No significant change in HRV parameters after 3/12 iVNS

Stemper et al., 2008 [277]	iVNS implanted for management of epilepsy	30 Hz	1.44 mA	500 µs	program med to stimulate for 60 seconds then pause for 5 mins	BP, HR, cBRS and HRV analysis undertaken during rest only, no orthostatic assessment	n=21 (mean age35.4+/-11.8 years, 11 women, 10 men) iVNS devices implanted 13 +/- 4.5 months before study	RR interval, SBP and DBP did not show significant changes during "on" and "off" phases LF and HF power significantly increased during "on" stimulation
Cadeddu et al., 2010 [278]	iVNS devices inserted for control of refractory epilepsy	Not specifie d	Not Specified	Not Specified	Assessed pre- iVNS implant and then after 7.7 ± 2.3 months iVNS	24h ECG and ambulatory BP monitor, with transthoracic echo	10 subjects (4 females and 6 men, mean [±SD] age 29 ± 18 years)	SBP and DBP significantly increased after iVNS implanted Nocturnal HRV indices RMSSD, SDSD and pNN50 all significantly improved
Sperling et al., 2010 [295]	iVNS (left cervical vagus) for treatment resistant depression (post implantation 6-40 months)	15-30Hz	0.25-2.5mA	500 µs	30 seconds on 5 mins off	ECG testing at baseline, switched on and switched off conditions	Patients with major depressive disorder (ICD-10) n=9 (51.6 years, 5 women, 4 men) Compared to age and sex matched controls	RMSSD increased significantly in switched on conditions during stimulation (30 s) in six patients compared to stimulation-free intervals and baseline

Mulders et al., 2015 [296]	iVNS (left cervical vagus) for treatment resistant epilepsy	20-30 Hz	1-2.25 mA	130 - 500 µs	30 - 60 seconds on, 3- 5 mins off	ECG during rest and exercise	n=10 patients with iVNS and n=5 healthy controls age 44 (20–78) years Time since VNS implantation: 45 (8–132) months	Patients showed a consistent decrease in HR during VNS, compared with epochs prior and after VNS. No HRV analysis available because 50 seconds max time recorded. No statistical tests undertaken as low volume of participants
Garamendi et al., 2017 [290]	iVNS implanted for management of epilepsy	25 Hz (range 20-30)	2 mA (1.75 – 2.5)	250 µs	Not specified	HR, BP and HRV tested at rest. HR and BP and BRS tested during head up tilt	n = 15, age 38.9 (7.8). 13 men. Duration of epilepsy >20 years for 60%	Resting supine SBP and DBP lower after 6/12. Higher SBP, DBP and HR values after active stimulation for 6/12
Constantinescu et al., 2019 [297]	iVNS (left cervical vagus) for treatment resistant epilepsy	30 Hz	1-2 mA	500 µs	30 seconds on 5 mins off	ECG before iVNS implantation, 3/12 after both in on and off conditions	n=5 patients with intractable epilepsy	Just 1 of 5 patients showed significant changes during on VNS stimulation in RMSSD values

Table 5 details the cardiovascular effects noted in iVNS

tVNS and Cardiovascular Responses

tVNS devices and their effect on cardiovascular responses have been examined in several experimental paradigms involving multiple auricular positions, left vs. right ear stimulation, and different stimulation settings. Trial outcomes and reporting of data has not been consistent, and some of the proceeding data has directly conflicted with other published results, leading to a degree of ambiguity in interpreting overall results in this area.

Several studies of the cardiovascular effects of tVNS have noted positive results. A significant decrease in the LF/HF ratio during active tVNS, suggesting a shift towards parasympathetic dominance, was demonstrated by one research group [298] and similarly an increase in SDNN after tVNS, particularly in female participants, was noted by a separate research group [299]. Isolated changes in cardiac Baro-Reflex Sensitivity (cBRS) [191] RMSSD [190,192] and Respiratory Sinus Arrythmia (RSA) [300] have been noted in distinct populations however it is noteworthy that isolated improvements in single indices have been reported rather than persistent signals across multiple indices.

An experimental design comparing left and right tVNS at multiple stimulation targets found that the HRV indices SDNN and RMSSD both were most significantly improved when the right cymba conchae and fossa triangularis were stimulated [192]. When specific parameters of stimulation at the left tragus were sequentially analysed, the settings that had the most significant impact on heart rate analysis in young volunteers were 500 μs at 10 Hz. [301]

Studies investigating the effect of tVNS on HRV during a HUTT found that the RSA measure of HRV (HF domain) was significantly increased during an orthostatic manoeuvre [300] and similarly stimulation at the left cymba conchae during HUTT found that responsivity, i.e. degree of change of heart rate and systolic blood pressure during tVNS were significantly higher during orthostasis compared to control [302].

However there have been conflicting and negative results in this areas, with no difference. noted in any HRV indices comparing active to sham stimulation at multiple intensities. [303] In an experimental crossover design employing a variety of amplitudes at the right cymba conchae, there was no positive signal in any HRV measure [304] and similarly tVNS to the right tragus during rest and autonomic nervous system testing, with appreciably different stimulation parameters to what was previously cited in the literature, also did not have any effect on HRV [305]. No significant changes in HRV indices were noted between active and sham tVNS sessions in patients with systemic sclerosis [147] or healthy volunteers [304].

One research group highlighted the importance of individual differences in response to tVNS. They observed that baseline LF/HF ratio predicted the magnitude of response to tVNS, with higher ratios associated with greater decreases during stimulation[190]. Similarly, they found that a higher LF/HF ratio predicted a greater decrease in HRV parameters during tVNS sessions. Another research group found that expiratory-gated tVNS decreased heart rate only in individuals with high pre-existing heart rates, indicating a nuanced effect dependent on individual cardiovascular characteristics [306]. These findings underscore the need for personalized approaches to tVNS therapy based on individual autonomic profiles.

Inconsistent results may be due to the use of different anatomical sites and stimulation parameters being utilised, some with “lead in” times and some without. Reporting of outcomes in this area has also been of variable quality. Recent international consensus has called for standardised reporting of all tVNS research [253] which will standardise experimental processes and help generalise outcomes for future analysis.

tVNS and Cardiovascular Effects								
		VNS stimulation parameters						
Study	VNS site (iVNS / tVNS)	Frequency (Hz)	Amplitude (mA)	Pulse width (µs)	Time sitmulated	Analysis parameters	Population	Result
Clancy et al., 2014 [298]	tVNS on inner and outer surface of the tragus of the ear Sham – on tragus but disconnected Either active or sham tVNS	30Hz	10-50mA	200 µs	Continuous 15 mins stimulation	HRV frequency and spectral analysis Muscle sympathetic nerve activity (MSNA) recordings	Healthy volunteers n=48 age 20-62 years old (M:F 1:1)	Significant decrease in LF/HF ratio during active tVNS Greater response to tVNS in those who had higher sympathetic predominance at baseline (higher LF/HF ratio)
De Couck et al., 2017 [299] Study 1	tVNS cymba conchae left or right ear vs sham (earlobe)	25Hz	0.7 mA average	250 µs	30 seconds on/ 30 sec off 10 minutes	HRV frequency and spectral analysis	Healthy older volunteers n=30 age 23-58	Right stimulation alone significantly increased SDNN compared to baseline

De Couck et al., 2017 [299] Study 2	tVNS cymba conchae right ear	25Hz	1mA average	250 µs	30 seconds on/ 30 sec off 1 hour	HRV frequency and spectral analysis	Healthy older volunteers n=30 age range 30-65	SDNN significantly increased after 35 min and after one hour specifically in female participants LF and LF/HF significantly increased after 35 min of stimulation
Lamb et al., 2017 [300]	tVNS left tragus/ auditory meatus or sham (no current)	20Hz	5.6mA range 3-11.3mA	100 µs	unavailable	Postural HRV via Tilt Table Test Startle Blink Paradigm	Military veterans with PTSD and mild TBI n=12 or healthy control n=10 age 30 ±7	Significantly increased RSA (HF HRV) in tilt during tVNS
Antonino et al., 2017 [191]	tVNS active – tragus- inner and outer surface sham ear lobe	30 Hz	45 ± 1 mA	200 µs	Continuous 15 min	HRV, BP variability, cBRS	Healthy young male volunteers n =13 age= 23 ±1	Active tVNS acutely improved spontaneous cBRS, decreased LF/HF ratio and evoked slight decrease in HR

	Electrodes placed bilaterally 1)active tVNS 2) sham-electrodes placed on tragus -no current 3) electrodes placed on the earlobe current applied							Nil change with two sham conditions
Badran et al., 2018 [301] Study 1	tVNS to the inner side of the left tragus (anode in the ear canal, cathode on the surface of the tragus) of the left ear for 9 different stimulation rounds	1Hz 10 Hz 25 Hz	At 100 µs: tragus 9.28 ± 2.56mA earlobe 6.5 ±1.83mA At 200 µs tragus 5.32 ± 1.60mA earlobe 3.64 ±1.26mA	100 µs 200 µs 500 µs	Stimulation period (60s) recovery period (180s)	Heart rate analysis	Healthy young adult volunteers n=15 (M:F 1:1) age 26.5 ± 4.9	Active stimulation decreased HR more than control stimulation on with these parameters: 500 µs at 25 Hz 500 µs at 10 Hz

	sham = left earlobe crossover design		At 500 µs tragus 3.0 ± 0.93mA earlobe 1.97 ±0.70mA					
Badran et al., 2018 [301] Study 2	tVNS to the inner side of the left tragus of the left ear for 10 stimulation rounds sham = left earlobe crossover design	10 Hz 25 Hz	tragus-2.09 ± 0.97mA earlobe 2.04 ± 0.82mA	500 µs	Stimulation period (60s) recovery period (90s)	Heart rate analysis	Healthy young adult volunteers n=20 (M:F 1:1)	The parameters 500 ms at 10 Hz alone induced a significant decrease in HR
Badran et al., 2018 [190]	tVNS left tragus daily at home for 15 minutes for 2 weeks	30Hz	2-4 mA	200 µs	15 mins daily for 14 days	HRV frequency and spectral analysis	Healthy participants aged ≥ 55 years n=29 Age 64.14 ±0.89	RMSSD, pRR50, SD1 and nSD1, were significantly higher after 2 weeks tVNS

Borges et al., 2019 [303] Study 1	tVNS to left cymba conchae	25Hz	0.5mA 1mA and 1.5mA	200-300 µs	30 sec on / off cycling 10min stimulation	RMSSD	Healthy young volunteers n=61 (16 female) avg age 23.32	Increase in RMSSD during stimulation compared to the resting phases for all mA settings
Borges et al., 2019 [303] Study 2	tVNS to left cymba conchae	25Hz	1mA Compared to 1.78 mA ±1.13	200-300 µs	30 sec on / off cycling 10min stimulation	RMSSD	Healthy young volunteers n=62 (26 females avg age 24.77)	RMSSD values showed a significant overall increase during the stimulation phase No stimulation conditions significantly differed from each other comparing RMSSD values
Borges et al., 2019 [303] Study 3	tVNS to left cymba conchae vs sham (earlobe)	25 Hz	Active 2.5 mA ±0.93) Sham 2.76 mA ±1.01	200-300 µs	30 sec on / off cycling 10min stimulation each	RMSSD	Healthy young volunteers n=60 (31 females, age avg 23.62)	No difference between active and sham stimulation

Sclocco et al., 2019 [179]	tVNS 1) to cymba conchae no current 2) to cymba conchae active during exhalation 3) to cymba conchae active during inhalation 4) sham to earlobe	25 Hz	2) 1.6mA ±2.3 3) 1.7mA ±2.4 4) 1.4mA ±1.1	450 ms pulse width duration of 1 s	32 minutes	-Instantaneous HF-HRV index -four 8-min duration fMRI scans 1) passive control 2) active stimulation exhalation 3) active stimulation inhalation 4) active control	Healthy adult participants n=16 (9 female, age 27.0 ± 6.6)	Exhalation tVNS but not inhalation enhanced cardiovagal modulation i.e. increased instantaneous HF HRV index Exhalation found significantly signal at MRI site of LC/NTS
Tobaldini et al., 2019 [302]	tVNS left cymba conchae Cross-over design two-day protocol, one day with tVNS and a control day, at least 24 h difference	25Hz	1-6mA adjusted to sensory threshold	200 µs	10 min supine stimulator on (rest tVNS on), 15 min orthostatic position with tVNS on (tilt tVNS on)	1) ECG 2) Respiration 3) Non-invasive beat-to-beat arterial blood pressure at rest and during a 75° tilt test	Healthy young volunteers n=13 (5 males, 8 females) age 27 ± 4 years	Clinostasis: tVNS reduced HR, systolic BP variability and cardiac and peripheral sympathetic modulation Responsivity of HR and BP to orthostatic stress during tVNS was significantly

								higher when compared to control
Bretherton et al., 2019 [190] Study 1	tVNS left tragus One week later sham (electrodes on tragus but no current)	30Hz	2-4 mA	200 µs	15 mins	Baroreceptor sensitivity §	Healthy participants aged ≥ 55 years n=14 Age 69.11 ±1.52	Baseline LF/HF ratio power significantly predicted response to tVNS where higher resting LF/HF ratio was associated with greater decreases during tVNS
Bretherton et al., 2019 [190] Study 2	tVNS left tragus no sham	30 Hz	2-4 mA	200 µs	15 mins	Baroreceptor sensitivity, HRV frequency and spectral analysis	Healthy participants aged ≥ 55 years n=51 Age 65.20 ±0.79	Total power, mean RR interval, Δ RR, SDRR were significantly affected during tVNS A higher LF/HF ratio predicted a greater decrease to tVNS

Gauthey et al., 2020 [304]	tVNS Cymba Crossover design	5Hz 20Hz active 5Hz sham	1.5 ± 0.6mA 1.2 ± 0.5mA 5.5 ± 1.6mA	200 µs	10 min stimulation 10 minute washout	Muscle sympathetic nerve activity (MSNA) recorded by microneurography at rest, during apnoea and tVNS HRV power and spectral analysis	Healthy, young male volunteers *n*= 28 (age 27±4)	Acute right cymba tVNS did not induce any effects on HRV nor MSNA variables when compared to active control
Stavrakis et al., 2020 [307]	tVNS to right tragus (active) or right earlobe (sham) Parellel design	20 Hz		200 µs	1 hour tVNS daily over 6 months	5 min ECG at baseline, 3 and 6 months for HRV analysis	Paroxysmal A Fib n=53, 26 in active arm 27 in sham arm age 65.2 +/- 14.5	<13 readings at each time point available due to AF, pacing or PVCs/PACs LF and LF/HF ratio significantly higher in the active arm
Machetanz et al., 2021 [192]	tVNS right n = 7 left n =6 -cymba conchae -cavum conchae	25 Hz at a periodicity of 1 Hz	0.2-2mA 0.096-0.769mA 0.05-0.4mA	100 µs 260 µs 260 µs	90s (i.e., 3 × 30s) at each stimulation site	HRV power and spectral analysis	Healthy adults n=13 (age 24 ± 3, 8 female)	Significant differences between right- and left-sided stimulation for the SDNN and RMSSD

	-outer tragus -inner tragus -crus helicis -fossa triangularis				144 parameter combinations			analysis only (increasing with right ear stimulation) HRV increases were highest at cymba conchae and fossa triangularis, to a lesser extent to stimulation at the inner tragus
Veiz et al., 2021 [308]	tVNS at left tragus (active) or left earlobe (sham) on two separate days (crossover)	30 Hz	20 mA	250 µs	30 minutes tVNS Does not specific if continuous or block stimulation	Continuous ECG and non-invasive BP recording at rest, no orthostatic challenge	Young volunteers n=20 (10 men 10 women) age 23.35 ± 1.46	no difference for RMSSD, SDNN, HF or cBRS during active vs sham stimulation
Paleczny et al., 2021[306]	tVNS to tragus	25 Hz	0.722 mA	1000 µs per phase, interphase interval 30 µs	2 minutes	Expiratory-gated, inspiratory-gated, and non-respiratory-gated tVNS trials were included. HR, SBP, SVR, BRS, HRV recorded	Seventy-two tVNS trials obtained from n= 12 healthy volunteers (age 23 ± 3 years)	HR decreased during expiratory tVNS only in those with high pre-existing HR (>67 bpm)

Sinkovec et al., 2021 [305]	tVNS to right tragus during rest and autonomic nervous system testing (ANST) sham = no stimulation	20Hz	Adjusted individually to "barely perceptible" < 150 µA	1,000 µs	One hour resting tVNS vs sham 15 min ANST	Continuous cardiac measurements with impedance cardiography Non-invasive arterial BP monitor ECG for HRV analysis	Healthy male volunteers n=15 (age 23 range 20-25)	Indices of LV contractility, LV output, and LV work significantly decreased SBP and TPR significantly increased No difference HRV or ANST parameters
Sommer et al., 2023[234]	tVNS to left cymba conchae (active) and earlobe (sham) Internal crossover	25 Hz	1.6 ± 0.8 mA for active stim and 1.5± 0.8mA for sham	250 µs	30 seconds on / 30 seconds off	10 min ECG for analysis at 6 timepoints (T1-T7)	Healthy volunteers, n=32 completed (19 females; 25.5 years, ±2.6 years)	No change in overall HR, pNN50, VLF, LF, HF, LF/HF, or RMSSD
Bellocchi et al., 2023 [147]	tVNS to left cymba concha Sham: 1Hz stim in	25 Hz	0.2 – 5 mA (participant selected)	250 µs	30 seconds on / 30 seconds off	10 min ECG for HRV analysis at time T0, T1, T2	Patients with systemic sclerosis n=35 enrolled n=21 completed (age 58 +- 11, 18 female)	No change in any HRV index

	same location 4/52 treatment, 4/52 washout, internal crossover design							
Stavrakis et al., 2023 [309]	tVNS to left tragus (active) or left earlobe (sham) Parellel design	20 Hz	Active 15.4 +/- 8.6 mA Sham 16.3 +/- 9.1 mA	200 µs	1 hr daily for 2 months	Tilt test at baseline and after 2 months 5 min ECG supine and upright for HRV analysis	All women, diagnosed POTS n=25, 14 assigned to sham 11 to active treatment	Change in HF, LF and LF/HF ratio from sit-stand was significantly attenuated in the active group compared to sham at 2 months

Table 6 details cardiovascular assessments and outcomes during tVNS

Exploring Mechanisms of VNS-mediated Cognitive Enhancement

There are many potential mechanisms through which VNS may exert its cognitive enhancing effects, including direct neurotransmitter release, increased cerebral perfusion to discreet neuroanatomical structures and via modulation of peripheral haemodynamics.

VNS and Local Neurotransmitter Release

The mentioned in Chapter 1, the main neurotransmitters centrally released via the afferent projections of the VN are thought to be GABA and NE via the LC, among many others.

As the primary inhibitory neurotransmitter, higher levels of GABA decrease cortical excitability, and this mechanism is one of the accepted hypotheses for VNS' anti-seizure efficacy [310]. It has been suggested that increased cortical inhibition due to high GABA levels can sharpen task-relevant representations in the cortex and inhibit competing responses, thereby facilitating response selection and inhibition processes [311,312]. Studies have also demonstrated a decline in GABA concentration in frontal and parietal regions in aging populations, areas crucial for cognitive control. [313,314]

In preclinical models, iVNS both produced a significant increase in hippocampal NE concentrations and blockade of hippocampal α2 -receptors reversed the seizure-suppressing effect of VNS, suggesting that VNS induces increases in extracellular hippocampal NE, which are at least partly responsible for its seizure-suppressing effect [315]. The stimulation

parameters required to produce NE in the hippocampus and cortex in these experimental models was also investigated with 0.0 and 0.25 mA proving no effect on NE concentrations, while the 0.5 mA stimulation increased NE concentrations significantly in the hippocampus and 1.0 mA stimulation significantly increased NE concentrations in both the cortex and hippocampus bilaterally [316].

Further experimental studies confirmed that acute VNS increased NE concentration, as well as increases Brain-Derived Neurotrophic Factor (BDNF) expression in the hippocampus and cerebral cortex [317]. As it projects to the LC, it is possible that stimulation of the VN could directly activate the LC, leading to increased NE at projection sites. Regarding radiographic evidence of activation, three fMRI studies with tVNS in healthy humans show increased LC activation during stimulation [47,49,50] It has also been shown that lesioning the LC in preclinical models reduces the anti-seizure VNS efficacy, suggesting that the LC-NE pathway may also be integral to iVNS attenuating seizures [318].

NE and GABA may in fact work synergistically to facilitate executive functioning; GABA by encouraging response inhibition of task irrelevant stimuli and NE via the LC-NE system increasing frontal NE release and thus executive functioning [225].

Interestingly, and similar to previous effects noted with iVNS stimulation levels and responses [193], moderate levels of NE augment prefrontal cortex function, whereas high and low concentrations of NE impair function i.e. NE exhibits an inverted-U relationship between LC-NE activity and optimal performance on attention tasks [319]. However, in general as NE

levels rise executive function improves, likely via enhanced activation of the prefrontal cortex and frontoparietal control network. [320,321] Inhibitory control for action cancellation is specifically enhanced with noradrenergic modulation, likely via this prefrontal cortical network [322,323]. It is noteworthy that electrical stimulation of the nucleus paragigantocellularis in rodent models resulted in a phasic and excitatory response in the LC [324], while electrical stimulation of the nucleus prepositus hypoglossi yielded to the inhibition of spontaneous discharge of LC neurons [325].

VNS and Cerebral Perfusion

Cerebral autoregulation refers to the physiological mechanism that aims to maintain blood flow to the brain approximately constant when blood pressure changes [115]. Cerebral Blood Flow (CBF) or perfusion, is a measure of the rate of delivery of arterial blood to a capillary bed in cortical tissue [326]. The aim of autoregulation is to protect cerebral tissues against hypoxia or oedema because of low or high arterial blood pressures respectively, and autoregulation moderates CBF to obtain this goal. Multiple factors physiologically modify autoregulation including blood carbon dioxide (CO2) levels and hypoxia [327]. While still controversial, the ANS may play a prominent role in cerebral autoregulation in response to such stimuli, inducing vasodilation or constriction, and parasympathetic and sympathetic nerves are anatomically located in the same perineural sheath innervating cerebral arteries [328].

Multiple modalities have been utilised to assess for CBF changes due to VNS, including fMRI, Position Emission Tomography (PET) and Single Photon Emission Tomography (SPECT) studies. Trials of patients with iVNS treatment for epilepsy and depression have demonstrated a variety of CBF modulatory effects at specific cortical and subcortical areas. Increased CBF at the orbitofrontal cortex [329–333], temporal lobe, [333–337] insular cortex, [338–340] bilateral frontal lobes [341], left dorsolateral prefrontal cortex [342] and subcortical structures including thalamus, hypothalamus, basal ganglia and other nuclei [334] have been observed. For a comprehensive review see Chae et al., 2003. [343]

Notably analysis undertaken during acute iVNS has noted bilateral decreased hippocampal CBF [330,332,333]. This has been replicated in tVNS functional imaging studies which have confirmed stimulation and increased CBF at vagally-innervated brain regions during auricular tVNS and notably decreased perfusion at hippocampal regions.[45,46,180] This is in contrast to the laboratory and preclinical work that found NE release in hippocampi after VNS, and may suggest a more nuanced neuromodulatory effect of neurotransmitter release in discrete cortical regions. The means by which iVNS or tVNS may affect cognition acutely is therefore likely to be unrelated to hippocampal-dependent autobiographical memory, and may be via executive or attention-based processes, as suggested by the neuroanatomical regions that show increased perfusion during VNS.

tVNS has also demonstrated efficacy in increasing arousal in comatose patients who respond to auditory signalling and again the cortical and subcortical regions noted on fMRI to be activated were similar to previous iVNS studies, including left superior temporal gyrus, left prefrontal cortex, left insular cortex, left middle frontal gyrus among other cortical and subcortical structures [344].

It is worth considering that intermittently stimulating neurons at different frequencies produces drastically different changes in neuronal behaviour. Low frequency stimulation may induce long term depression (LTD) and less functional connectivity while intermittent high frequency stimulation may produce long term potentiation (LTP) and increased functional neuronal signalling.[337,345] Therefore acute VNS stimulates cortical regions mostly involved in alertness and frontal processing and it is unclear whether chronic stimulation may improve LTP in classic memory-associated regions, including the hippocampus. Preclinical studies have signalled that different stimulation frequencies and patterns can produce different functional connectivity effects [346]. More recently, NIRS has been utilised to monitor CBF and increased frontal perfusion in patients with epilepsy was noted during iVNS when paired with a cognitive task [347].

Both dementia and even its prodromal stage, MCI, are characterised by a reduction in CBF [348,349]. A metanalysis of twenty-six studies investigating CBF in MCI found overall reduced tissue oxygenation, CBF and velocity in MCI compared to healthy controls [350] and studies are underway investigating the CBF changes that may occur with cognitive stimulation in MCI and dementia [351]. Similar findings have been noted in patients with AD, with reduced CBF in many cortical regions including temporal [352–356] parietal [352,357] and other regions including precuneus, frontal and posterior cingulate cortex [356,358].

VNS and Peripheral Haemodynamics

As well as modulating central neurotransmitter release and CBF, VNS has been shown to have positive peripheral modulatory effects in pathological states characterised by impaired

autonomic regulation including POTS [359] specifically patients with POTS and impaired vagal cardiac control, as defined by reduced HRV [116]. tVNS has also shown benefits in modulating blood pressure in induced orthostatic hypotension [302]. These studies suggest VNS may have a role in positively manipulating the peripheral baroreceptor-reflex and thus cerebral autoregulation, and potentially may improve cortical perfusion via this route, however further dedicated studies are required to precisely delineate this relationship.

VNS, Cognition and HRV

HRV can be conceptualised as a biomarker of parasympathetic modulation, and it is associated with the network of cortical and subcorticalregions involved in autonomic nervous system regulation; the CAN [23,103]. The CAN, described in detail in Chapter 1, comprises prefrontal cortical (anterior cingulate, insula, orbitofrontal, and ventromedial cortices), limbic (central nucleus of the amygdala, hypothalamus), and brainstem regions, areas of the brain intimately involved in emotional regulation and executive functioning, leading to the proposal that vagally-mediated HRV may index these aspects of prefrontal cortical function. [103,106]

Higher HRV has been linked to better cognitive function in healthy adults including healthy older individuals [360,361] and a metanalysis found a positive overall correlation (r = 0.09) between vagally-mediated HRV indices and emotional regulation processes (including

executive functioning, emotion regulation, and effortful or self-control) in healthy participants across a number of age groups [362].

Autonomic system dysfunction is common in patients with MCI, with studies suggesting participants with MCI are 5.6 times more likely than controls to have autonomic dysfunction, specifically on assessment of HRV and cardiac reflexes [363]. A metanalysis of MCI and dementia also found autonomic dysfunction, as defined by reduced HRV, was significantly associated with cognitive impairment [364]. Reduced HRV is associated with worse performance on tests of global cognitive function, more than cardiovascular risk factors [365].

Recent metanalyses of HRV in patients with neurodegenerative conditions including MCI, AD, Dementia with Lewy Bodies (DLB), Vascular Dementia, Parkinson's Disease and Multiple Sclerosis found a significant, moderate effect (r = 0.25) indicating that higher HRV was related to better cognitive and behavioural scores, which was not influenced by mean age or cognitive status [366]. These results were mirrored in a similar recent metanalysis of patients with dementia compared to healthy controls, which found significantly lower resting HRV for parasympathetic function and total variability in those with dementia. On subgroup analysis then most striking differences i.e. worse HRV analysis was found in those with MCI or DLB [367].

HRV and CBF are linked via vagal afferents, and a metanalysis revealed that HRV was significantly associated with regional CBF in the ventromedial prefrontal cortex (including anterior cingulate regions) and the amygdala [368]. In both younger and older adults scanned while at rest, higher HRV is associated with higher medial prefrontal cortex and amygdala

functional connectivity [101]. The Neurovisceral Integration Model holds that HRV, executive cognitive function, and prefrontal neural function are integrally associated [103].

Studies have demonstrated the success of behavioural [369] and pharmacological [370] interventions in manipulating HRV. Increases in HRV seen with physical fitness training are associated with improvements in executive function [371]. The links between executive function and cardiac autonomic regulation were further highlighted by a recent study examining the impact of cognitive and motor training on HRV indices. Physical training alone failed to impact HRV in older adults whereas dual cognitive and motor training significantly improved global and parasympathetic autonomic nervous system activity [372]. These studies point towards a duality; the vagal communications between heart and mind can be bidirectionally manipulated to improve both parasympathetic control of HRV and, synergistically, executive cognitive function.

Summary

VNS is a therapeutic approach that may have promise as a cognitive enhancer via modulation of neurotransmitter release and cerebral perfusion. Both GABA and NE have been implicated as neurotransmitters that underpin the effects of VNS, and both have been shown to be important for its anti-seizure efficacy.

Regarding cerebral perfusion, VNS has been found to modulate blood flow to specific brain regions involved in cognition. While acute VNS increases blood flow in areas like the OFC and PFC, it shows a more nuanced effect on hippocampal perfusion. This suggests that VNS's acute cognitive effects may not be primarily mediated through hippocampal-dependent memory processes but rather through executive or attention-based mechanisms.

VNS also influences HRV, a marker of ANS function highly associated with cognitive performance, especially frontal executive function. Higher HRV, reflecting greater parasympathetic modulation, has been linked to better cognitive function, particularly in executive function and emotional regulation. VNS's ability to modulate HRV suggests a potential mechanism through which it enhances cognitive function, possibly by improving autonomic regulation and neural connectivity in brain regions involved in cognition.

VNS holds promise as a cognitive enhancer due to its multifaceted effects on neurotransmitter release, CBF, and autonomic function, and most interestingly both iVNS and tVNS have demonstrated alterations in HRV and CBF during stimulation. Further research is needed to elucidate the precise mechanisms and optimise stimulation parameters, specifically of tVNS, to fully elucidate the underling mechanisms and draw generalisable conclusions from this research.

Chapter 3: Cognitive Impairment due to Alzheimer's Disease

Clinical Presentation

The typical clinical presentation of cognitive impairment, specifically when due to AD involves episodic memory loss, detectable by the individual or a suitable informant. Atypical AD phenotypes, such as Posterior Cortical Atrophy (PCA) with initial visuo-spatial impairment or logopenic-variant primary progressive aphasia with language impairment, are observed less frequently. Rarely, behavioural or dysexecutive variants of AD may occur. AD may manifest as either MCI or established dementia, depending on functional status as detailed below.

The initial assessment of a person with cognitive impairment should include a thorough history and examination, focusing on the timeline, nature, and impact of cognitive symptoms, and identifying any reversible causes of cognitive impairment. While widely used, traditional screening tools like Folstein's MMSE or the MOCA have limitations in assessing domain-specific cognitive performance [373].

Domain-specific neuropsychological assessments, such as Addenbrooke's Cognitive Assessment (ACE-III) [374] and the Repeatable Battery for Assessment of Neuropsychological Status (RBANS) [375], provide more detailed evaluation of specific cognitive domains. For cases where functional loss is challenging to determine from history alone, structured assessments like the Assessment of Motor and Process Skills (AMPS) can objectively identify functional

impairment in daily tasks [376]. The Clinical Dementia Rating Scale (CDR) serves as an informant-based measure of functional impairment [377].

A comprehensive review should be undertaken for all clinical presentations of cognitive impairment, including screening for contributory factors such as medical comorbidities, medication use, neurological examination, mood/anxiety symptoms, and delirium. Laboratory investigations can reveal micronutrient deficiencies, hypercholesterolemia, diabetes, and other pathophysiological processes impacting cognitive function. Assessments for gait, falls, frailty, and sarcopenia should be integrated into a comprehensive memory and geriatric performance assessment.

Mild Cognitive Impairment

MCI is a syndrome defined by objective cognitive changes without significant impairments in instrumental Activities of Daily Living (ADLs) – typically cognitive performance at least 1.5 standard deviations or greater below appropriate age and education-adjusted cut-off [378,379]. Where due to AD, MCI typically presents with impairments in episodic memory that do not interfere with day-to-day function.

Debate regarding progression of MCI to dementia has numerous caveats including the population studied (whether clinical or population-based), the timeframe patients are followed for and whether any biomarker was used to stratify risk of progression. In general, MCI has an annual conversion rate to dementia of 5-20% [380] however cognitive performance may deteriorate, remain stable, or even improve over time [381,382]. Given this degree of

heterogeneity in progression of MCI it is fruitful for clinicians and patients to understand risk of progression to dementia and what factors influence it.

MCI can be sub-classified as amnestic or non-amnestic, and single or multi-domain affected [383]. Patients with amnestic MCI may have a conversion rate of up to 40% over 5 years compared to those with non-amnestic MCI (0.4%) [384] with those at greatest risk having diagnosed multidomain amnestic MCI (i.e. deficits in episodic memory with another domain deficit e.g. visuospatial, executive, language). This has been demonstrated to have the highest rates of progression to dementia over time [385,386]. Conversion rates from MCI to dementia in individuals with MCI and positive biomarkers of AD pathology (AD-MCI) may be up to 11-fold higher compared to individuals with MCI and negative AD biomarkers (non-AD MCI) [387].

It is noteworthy that a diagnosis of MCI has been shown in multiple longitudinal studies to be associated with an increased risk of all-cause mortality, regardless of whether MCI progresses to dementia or not, with those progressing to dementia carrying understandably higher mortality risk [388–390].

Dementia

Dementia is a clinical syndrome where cognitive impairment results in impairments in ADLs and day-to-day function which may be further characterised as mild, moderate and severe [391]. AD is the most common pathology causing dementia in older adults, however other conditions such as DLB, Fronto-Temporal Dementia (FTD), Limbic-predominant Age-related

TDP-43 Encephalopathy (LATE) and cerebrovascular disease (causing cognitive impairment or dementia) give rise to co-pathology, which is encountered frequently in older adults [392].

Alzheimer's Disease

AD impacts approximately 5% of individuals aged 65 and over, with its prevalence rising to around one-third among those aged 90 and above [393]. Beyond its direct influence on heightened morbidity and mortality in older adults, AD is strongly linked to the emergence of various geriatric conditions such as falls, delirium, urinary incontinence, polypharmacy, and physical frailty [394–396]. For geriatric specialists, adopting a Comprehensive Geriatric Assessment (CGA) methodology for evaluating both cognition and these frequently concurrent syndromes is pivotal in providing holistic care to older adults grappling with AD. It's important to note that while this chapter primarily focuses on AD, "pure AD" is more of an exception than the norm in older adults [397]. The presence of co-pathologies contributing to both MCI and dementia is commonly observed in individuals seeking assistance from a Memory Assessment Service (MAS) [391].

AD Pathophysiology

The key pathological mechanisms in AD revolve around the accumulation of amyloid plaque and hyper-phosphorylated tau. Amyloid Precursor Protein (APP) undergoes cleavage by a membrane protein enzyme, typically α-secretase in healthy states, resulting in the release of a non-toxic protein called α-CTF [398]. However, in the presence of pathological amyloid build-up, β- and γ-secretase cleave APP at various points, producing amyloid fibrils, with the most

harmful being amyloid-β 42 (Aβ-42). This Aβ-42 is released into the extracellular space, triggering a conformational shift in other amyloid proteins that aggregate into oligomers, fibrils, and eventually form plaques [399]. Tau, a microtubule binding protein, plays a role in stabilizing axonal structures for nutrient and signal transportation along axons. Hyperphosphorylation of tau (p-tau) leads to the neurofibrillary "tangle" pathology in AD, involving a conformational change resulting in intraneuronal tangles [400]. These tangles disrupt normal axonal messaging, ultimately leading to cell death and atrophy [401,402]. Recent findings propose a synergistic interaction between p-tau and amyloid, contributing to the toxic accumulation of both protein species [403,404] (please see Figure 8).

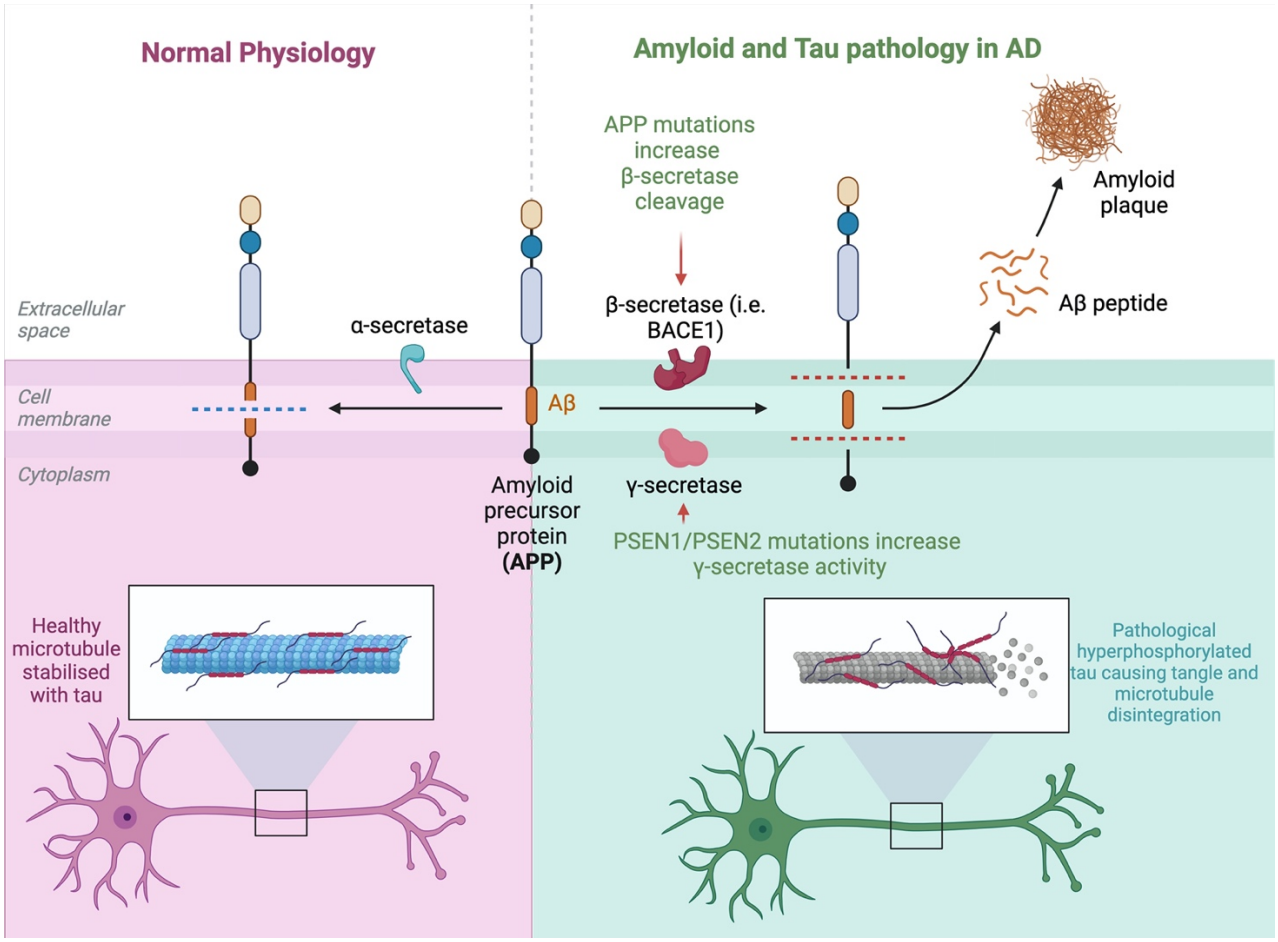


Figure 8: Amyloid and Tau Processing Under Physiological Conditions and in Alzheimer's Disease.

Figures 8 Legend: PSEN 1: Presenilin 1. PSEN 2: Presenilin 2. Aβ: Amyloid beta. BACE-1: Beta-site amyloid precursor protein cleaving enzyme 1.

The degree to which amyloid plaques and tau tangles correlate with cognitive impairment in those with genetic predispositions is well established [405] and illustrated by persons with genetic APP or presenilin (PSEN1 or PSEN 2) mutations all directly causing toxic amyloid build-up and development of cognitive impairment [406]. However as brains age there are often multiple factors causing neurodegeneration and cell death [407] with amyloid and tau pathology only two of multiple pathological causes of cognitive impairment [391]. The extent to which inflammation, metabolic dysfunction, vascular disease, dysfunctional proteostasis, alterations in lipid metabolism, cellular senescence and other neurodegenerative processes affect cognitive impairment in AD is a rapidly advancing field, beyond the scope of this thesis [408–410] (Please see Figure 9).

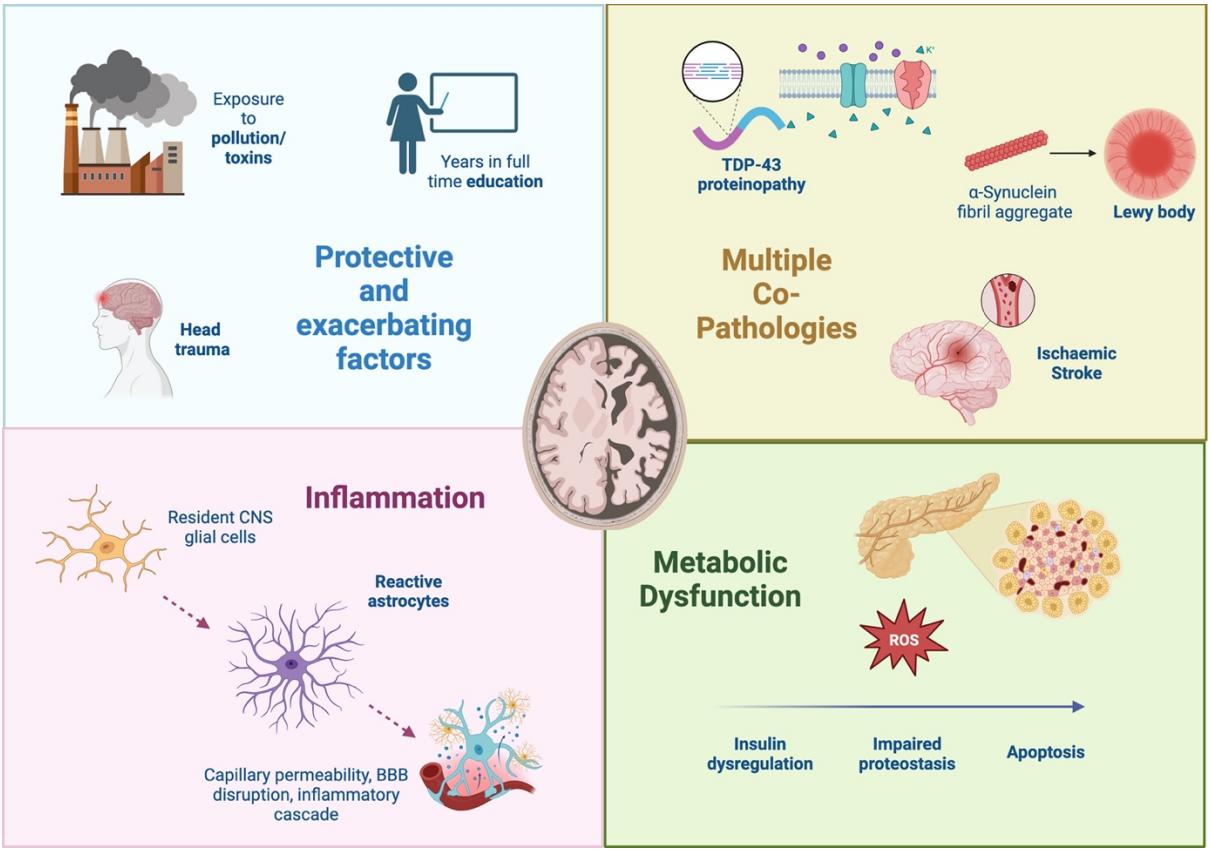


Figure 9 Additional pathophysiological processes known to influence AD pathology in older adults

Figure 9 Legend: TDP-43: TAR (transactive response) DNA binding protein 43. CNS: Central Nervous System. BBB: Blood Brain Barrier

Biomarker-Assisted Definitions of AD

For several decades, the prevailing diagnostic criteria for AD (NINCDS-ADRDA) were based on clinical criteria alone and graded as mild/moderate/severe based on functional dependence [411]. It has since been estimated that up to one-third of those diagnosed based on these criteria have no evidence of underlying AD neuro-pathology when examined post-mortem [412]. The 2018 National Institute of Aging and Alzheimer's Association (NIA-AA)

research criteria advocate for a biological classification of AD in a clinical context based on available biomarkers [413] and initiated the diagnosis of AD based on ATN criteria (A: Amyloid +/ –, T: Tau +/-, N: Neurodegeneration +/-). Recent criteria from the International Working Group assert that biomarker status must be only interpreted in the presence of an appropriate clinical phenotype ("phenotype-positive AD") [391]. These criteria are briefly summarised in Table 7 and Figure 10.

Frameworks for Diagnosing Alzheimer Disease		
Descriptor, year	**Clinical**	**Biological**
NINCDS-ADRDA (1984)	Dementia – memory or cognitive changes plus another cognitive impairment	Nil
IWG Research Criteria (2007)	Designed for research purposes – required diagnosis of "amnestic syndrome of hippocampal type"	CSF biomarkers, MRI atrophy, FDG- PET hypometabolism, amyloid PET positive, or AD autosomal dominant mutation
NIA-AA (2011)	Clinical diagnosis of MCI (amnestic or non-amnestic) or dementia	Amyloid β marker (CSF or PET) or marker of degeneration (CSF tau, p-tau, FDG-PET, or structural MRI)
NIA-AA (2018)	No clinical diagnosis necessary Introduced concept of ATN classification	Amyloid β marker (CSF or PET) and tau marker (CSF or PET)
IWG (2021)	Must have clinical diagnosis of one of: Amnestic variant Posterior cortical atrophy Logopenic variant primary progressive aphasia Behavioural or dysexecutive frontal variant Corticobasal syndrome	Amyloid β marker (CSF or PET) and tau marker (CSF or PET)

NINCDS-ADRDA: National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association. IWG: International Working Group. CSF: Cerebro-Spinal Fluid. NIA-AA: National Institute on Aging and Alzheimer's Association. MRI: Magnetic Resonance Imaging. FDG: Fluoro-Deoxyglucose. PET: Positron Emission Tomography. P-tau: Phosphorylated tau. MCI: Mild Cognitive Impairment.

Table 7 Frameworks for diagnosing Alzheimer Disease

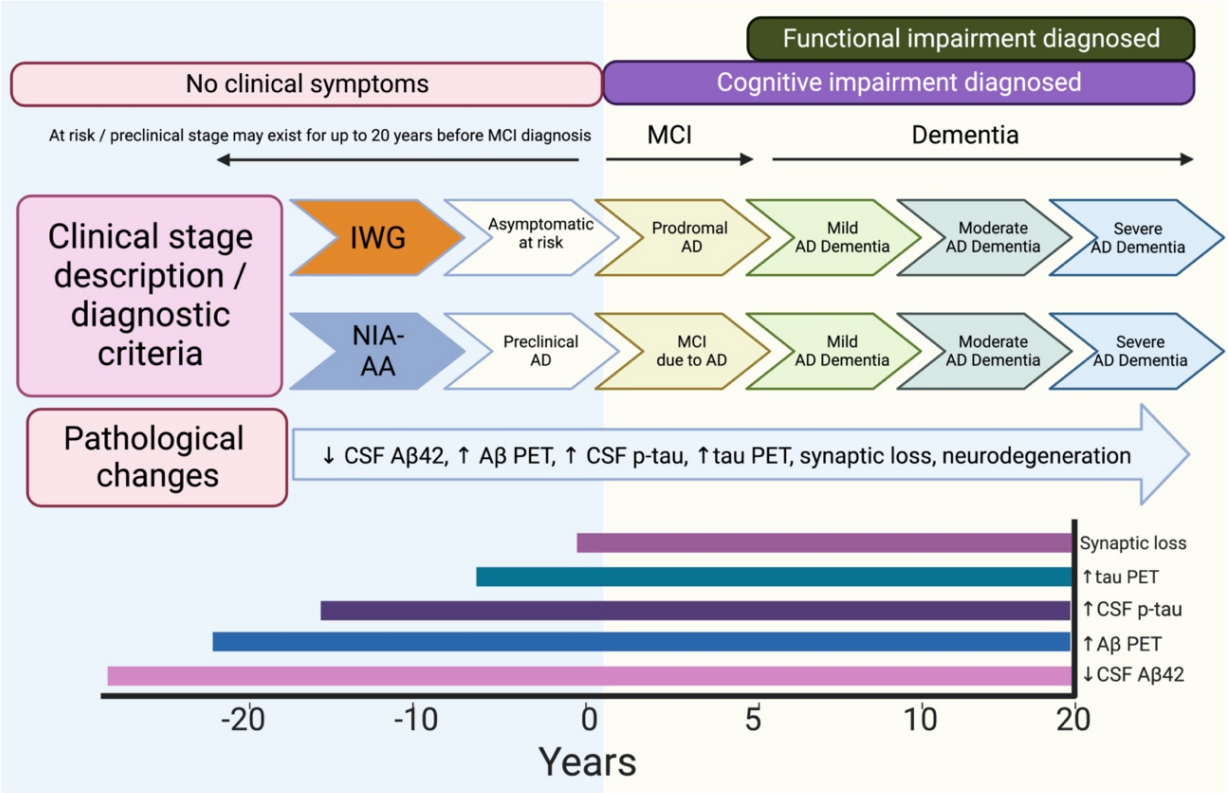


Figure 10. Diagnostic criteria and disease stages of AD over a chronological continuum

Figure 10 Legend: IWG: International Working Group. NIA-AA: National Institute of Aging and Alzheimer's Association. MCI: Mild Cognitive Impairment. CSF: Cerebro-spinal Fluid. Aβ42: Amyloid-beta 42. Aβ-PET: Amyloid-beta Positron Emission Tomography. P-tau: phosphorylated tau

As mentioned the diagnosis of AD based on clinical phenotype alone may not accurately reflect underlying pathological correlates. As a result, there have been substantial developments in the use of diagnostic biomarkers for AD pathology. The most studied biomarkers include Cerebro-Spinal Fluid (CSF) and Positron Emission Tomography (PET) imaging which demonstrate changes in amyloid and p-tau seen with AD pathology. AD Blood Bio - Markers (BBMs) are an area of intense research interest and are likely to become clinically available in the coming years [414] and are explored in greater detail in Chapter 7.

A specific concern in the use of diagnostic biomarkers in older adults with AD is the presence of co-pathology. In later life dementia multiple proteinopathies are more frequent than single causes of neurodegeneration [391]. These include the presence of alpha-synuclein, TDP-43 proteinopathies (including LATE), non-AD tauopathies, vascular pathology and hippocampal sclerosis which may be present in over half of clinically-defined AD [415,416]. Many older adults also have evidence of AD pathology but no cognitive symptoms [417,418].

CSF analysis for AD biomarkers may be particularly useful in predicting conversion to dementia in older adults with MCI [419] with a sensitivity and specificity approaching 95% and 87% respectively [420,421]. However it has been estimated that the risk of developing dementia with positive AD biomarkers depends on age, with a hazard ratio of developing AD dementia at age 65 of 21.4, and a HR of 4.9 age 85 for the same AD biomarker profile[422,423].

Converging evidence for Autonomic Nervous System Dysfunction in Alzheimer's Disease

LC and cognition

There is evolving evidence that the LC-NE system may be implicated early in the pathogenesis of AD. Revision of the original AD staging system from 2011 incorporated the LC as the earliest site of p-tau accumulation [424] and reports have shown that as the Braak stages increases by one unit, the LC volume decreases by 8.4% [425]. Both in human [426,427] and

preclinical studies [428,429] loss of LC neurons leads to increased Aβ deposition. In these human studies, neuronal loss across the rostrocaudal axis of the LC was shown to topographically correspond to the distribution of Aβ plaques within the cerebral cortex of AD brains.

The presence of neurofibrillary tangles in the LC is associated with the upregulation of inflammatory markers, as well as increased microglia infiltration into the LC [430]. LC-lesioned P301S mice exhibit increased activation of microglia and astrocytes in the hippocampus, which is postulated to contribute to hippocampal degeneration in this model [431]. It has been noted that older adults with denser LC innervation (i.e. higher neuromelanin MRI contrast) had overall better performance on a reversal memory tasks [432] and had improved cognitive reserve [433]. Similarly in a post-mortem study of patients with AD, lower LC cell integrity and greater cortical tangle density was associated with greater tau burden beyond the medial temporal lobes and worsening memory decline, identifying LC integrity as a promising indicator of initial AD-related processes [434].

LC cell loss results in amplified microglial and astroglial activation in the frontal cortex and hippocampus in mutant APP mouse models and so the dysregulation of the LC–NE system may play a major role in disease-related inflammation [429]. LC lesioning leads to increased glial inflammation and Aβ deposition in LC projection areas, including the hippocampus and frontal cortex; whereas, non-projection areas such as the paraventricular thalamus remain unaffected [428]. Recent data comparing post mortem brains of cognitively impaired and unimpaired individuals found a post-mortem correlation between LC tangle density and successive Braak staging, with lower LC integrity and greater cortical tangle density associated with greater tau burden beyond the medial temporal lobes [434]. These

neuropathological findings were associated with retrospective memory decline supporting the hypothesis that early LC tau accumulation may relate to AD-disease progression and identified LC integrity as a promising indicator of early AD pathology.

Treatments for Alzheimer's Disease

Non-Pharmacological

In recent years the concept of multifactorial lifestyle interventions to prevent AD and other causes of dementia has been established - given that up to 40% of the population attributable risk for dementia is due to potentially modifiable risk factors [435]. In early AD (MCI or mild dementia due to AD), a comprehensive lifestyle intervention approach, guided by CGA, aims to mitigate the risk posed by remediable factors contributing to cognitive impairment. This includes correcting visual and hearing impairments, alcohol and smoking cessation, implementing a Mediterranean diet, assessment and modification of vascular risks factors, cessation of contributory medications (e.g. benzodiazepines and anticholinergics) and assessment and management of sleep, mood, anxiety, social isolation and loneliness [436].

The landmark Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) study has demonstrated the potential of a multi-domain intervention inclusive of vascular risk factor monitoring, cognitive stimulation, exercise and diet modification to maintain or even improve cognitive function in older adults at risk of dementia [437,438].

A recent Randomised Controlled Trial (RCT) of a peer-supported exercise intervention has demonstrated benefits in executive function, attention and working memory in older adults with MCI [439], adding to results of an earlier meta-analysis in MCI [440] and established dementia[441]. Structured exercise may also mitigate against sarcopenia, frailty and falls in older adults with early AD [442].

Pharmacological

There are no licensed pharmacological treatments for MCI. Cholinesterase inhibitors have for decades been the mainstay of symptomatic treatment of dementia due to AD. Evidence from a comprehensive Cochrane review notes that after 26 weeks, treatment of dementia due to AD with cholinesterase inhibitors was associated with better cognitive function on both the MMSE of +1.05 and the ADAS-Cog of -2.67, improvements in ADLs and on the clinician-rated global assessment [443]. In moderate-severe dementia due to AD, memantine (an NMDA receptor antagonist) is frequently used which exerts moderate benefits in combination with cholinesterase inhibitors – namely a small clinical benefit in clinical global rating (-0.21), slight improvements in ADL performance, behaviour and mood (-1.84 points on the Neuro-Psychiatric Inventory) but no impact on cognitive function [444]. Response to these medications is variable and due to side effects (gastrointestinal upset, fatigue and muscle cramps) they are not tolerated by around one-third of older adults[445].

Recent phase 3 RCTs have demonstrated potential efficacy of immunotherapies targeting aggregated forms of Aβ (aducanumab, lecanemab and donanemab) in slowing clinical

progression of early AD (MCI due to AD and mild dementia due to AD) defined by clinical-biological diagnosis with positive AD biomarkers [446,447]. Typically, the patients included in these RCTs are younger, more educated, with fewer medical comorbidities and less frail than real-world patients. Notably, based on Appropriate Use Recommendations (AURs) for aducanumab and lecanemab, a significant number of older adults presenting to MAS would be eligible for treatment with these agents, should they become available [448–450].

Non- Invasive Brain Stimulation

The recent recommendations from the European task force for Brain Health services noted a potential beneficial effect of non-invasive brain stimulation on cognitive outcomes for persons with MCI or dementia due to AD pathology [422]. In particular, transcranial Direct Current Stimulation (tDCS) and repetitive Transcranial Magnetic Stimulation (rTMS) have been investigated in this sphere.

tDCS is a form of neuromodulation that uses constant, low direct current delivered via electrodes on the head. It is a transcutaneous brain stimulation technique that delivers a low electric current to the scalp, to activate cortical tissues below the cranium. tDCS can be applied with different polarities: anodal stimulation involves applying positive current to increase neuronal excitability, while cathodal stimulation involves applying negative current to decrease excitability [451]. The exact mechanism of action of tDCS is not fully understood, but it is believed to modulate neuronal membrane potentials, which can influence the firing rates of neurons and affect cortical excitability [452]. Anodal stimulation is thought to depolarize neurons, increasing their likelihood of firing, while cathodal stimulation

hyperpolarizes neurons, reducing their firing rates [453]. tDCS is thought to induce neuroplasticity, the brain's ability to reorganize and adapt, by promoting changes in synaptic strength and connectivity. Specifically, tDCS administration to a specific area, such as the prefrontal cortex during task training, may be an effective way to enhance the specific performance of that cortical region [454] , hence one of the limitations of this therapy is its lack of ability to reach deeper cortical and subcortical structures [455]. Metanalyses of tDCS in patients with AD dementia demonstrated improvements in immediate cognitive scores [456,457] however a beneficial effect was not seen on metanalysis of patients with MCI [458].

rTMS is a non-invasive neuromodulation technique that involves the application of repetitive magnetic pulses to specific regions of the brain. These magnetic pulses are generated by a coil placed over the scalp and produce transient electrical currents in the underlying cortical tissue [459]. rTMS can be delivered at different frequencies, typically categorized as low-frequency (≤1 Hz) or high-frequency (≥5 Hz). Low-frequency rTMS is thought to have inhibitory effects on cortical excitability, while high-frequency rTMS is believed to enhance excitability [460]. The intensity of rTMS refers to the strength of the magnetic field generated by the coil and is typically expressed as a percentage of the maximum output of the stimulator. Individualized motor threshold determination is often used to determine the appropriate intensity for each patient, ensuring that the stimulation is subthreshold for motor responses. The coil can be positioned over different scalp locations to target specific cortical regions implicated in the condition being treated [461]. Neuronavigation techniques, such as MRI-guided targeting or EEG-based localization, may be used to optimize coil placement and ensure accurate targeting of the desired brain area [462].

rTMS has been investigated, with promising results shown in cognitive impairment post-stroke [463] and Parkinson's Disease [464]. A recent metanalysis of rTMS in cognitive impairment due to AD indicated a positive overall result (Hedges' g = 0.48) [465] with rTMS of the precuneus thought to be one of the methods by which, via stimulation of the Default Mode Network, medial temporal structures may be activated [466]. rTMS is not portable as it requires large magnets, and at present it also benefits from above mentioned neuronavigation tools such as MRI, which limits its usability in a real-world clinical setting.

Summary

The clinical presentation of cognitive impairment due to AD involves episodic memory loss, though atypical phenotypes are well-characterised. MCI, characterized by objective cognitive changes without significant functional impairment, may progress to dementia at rates influenced by various factors, and for some individuals does not progress to dementia. Finding therapeutic strategies to prevent the decline from MCI to dementia would be beneficial to both patients, caregivers and society at large.

AD pathophysiology centers around amyloid plaque and tau accumulation, leading to neurodegeneration. Biomarker-assisted definitions of AD are increasingly utilized, aiding in more accurate diagnoses. However, the presence of co-pathologies complicates diagnostic interpretation, highlighting the need for comprehensive assessments.

Evidence suggests that dysfunction in the LC-NE system may be involved in the early stages

of AD pathogenesis. The LC, identified as one of the earliest sites of p-tau accumulation in Braak staging, experiences neuronal loss as AD progresses. This loss correlates with increased Aβ deposition, and the presence of neurofibrillary tangles in the LC is associated with inflammation and increased microglia infiltration, likely contributing to hippocampal degeneration.

Up until the emergence of immunotherapies against amyloid, treatments for MCI only included non-pharmacological interventions such as lifestyle modifications. Pharmacological treatments in the form of emerging immunotherapies are licensed for use in early AD dementia and MCI due to AD. Non-invasive brain stimulation techniques such as tDCS and rTMS have shown promise in improving cognitive outcomes, though they have limitations in their usability in a clinical setting. tVNS may be a valuable neuromodulation technique in early AD given its effects on the LC-NE system, and that it is light and portable. Therefor it is timely to establish whether tVNS is safe and feasible in a population with MCI.

Chapter 4: Tolerability, Acceptability and Usability of the NEMOS © tVNS device in a population with MCI

Introduction

In recent years, there has been a growing emphasis on the role of usability in ensuring the safety of medical devices, as the correlation between product usability and safety is recognized across various industries [467,468]. Globally, healthcare regulators are increasingly incorporating usability testing in addition to safety assessment of devices prior to licensing [469,470].

This formalised approach to device usability development is known as human factors engineering (also referred to as usability engineering or user-centred design) where usability is integrated into the entire development lifecycle of the device [471,472]. In Europe, the medical device standards from the International Organisation of Standardisation (ISO) International Electrotechnical Commission (IEC) standard 62366 and in the USA the American National Standards Institute/Association for Advancement of Medical Instrumentation ® ANSI/AAMI standard HE75 stipulate the how to apply usability engineering to medical devices, and specifically include standards on device usability [473,474].

Device feasibility studies offer a unique and essential opportunity to assess many real-world characteristics of a device in a discrete population [475]. Device feasibility studies occur after national regulations and licenses have been granted, but offer valuable insights into properties such as tolerability of side effects, acceptability of the device, ergonomics,

specific usability facets and potentially even potentially further safety information that was not uncovered on initial device testing. Thus safety, tolerability, acceptability and usability of a device all may be assessed within the context of a device feasibility study.

Determining the tolerability and usability of a device may be accomplished via many formats. The aforementioned ISO standard details a lengthy procedure of identifying user interface characteristics, assessing use of device in a specific environment, and then assessing subjective acceptability of the device. One way in which acceptability may be assessed is via subjective rating scales of side effects, unpleasant sensations, likelihood of using the device again among others[476] . These often take the format of a Likert-type scales, rating responses from 1 to 5. These form useful survey data of tolerability and feasibility of an intervention, but they suffer from many biases including social desirability bias, self-report bias, and a tendency for surveyed participants to pick the neutral or middle option on the 5-point scale, all of which can skew results [477–479].

However, subjective ratings of personal skills are insufficient to ensure a device is truly usable, as participants often over- or under-estimate their skills [480]. Therefore, objective rating of skills and function is an integral skill in this process. In clinical contexts, an Occupational Therapist (OT) is the member of a Multi-Disciplinary Team (MDT) trained to objectively assess functional ability and task performance in the care of older adults.[481] There are many methods by which an OT may measure skills and functional ability, however one approach is via the Kettle Test (KT), a brief measurement tool of cognitive and functional performance which has been validated in clinical cohorts [482,483]. One benefit of the KT, which

was advantageous in this study, is that it is easy to teach a clinician who is not an OT how to perform it accurately, and the scoring system is straightforward and reproducible.

One of the stark issues with clinical trials of medicinal products and medical devices is the lack of inclusion of older adults. It has been suggested that just 15 participants per major population group should be sufficient to identify 90-97% of usability problems with a device [468] however older adults or adults with cognitive impairments are frequently entirely excluded from clinical trials, including device trials [484–487]. This is despite data suggesting older adults can adapt and learn to use new technology [488,489].

Therefore, it is currently unclear whether a tVNS device such as NEMOS © is in fact usable and tolerable by a population with MCI. Research to date reporting tolerability of tVNS side effects has been exclusively undertaken in young adults, with a recent metanalysis of the side effect profile of tVNS undertaken, and the average age of study participants was 34.99 ± 15.15 years. [490] There is a paucity of any data investigating the usability of tVNS devices, in either cognitively intact or cognitively impaired cohorts.

Consequently, via The Vagus Nerve Stimulation in Mild Cognitive Impairment (VINCI-AD) study, we aimed to determine the safety and feasibility of the NEMOS © tVNS device as a potential treatment in older adults with MCI. We sought to establish the tolerability and acceptability of the NEMOS © tVNS device using subjective rating scales and an adapted KT to objectively assess the participants' skills and ability to use the tVNS device independently.

In this chapter we will report on the safety, tolerability, acceptability and usability data from the study, which was the primary endpoint. Pre-specified exploratory secondary analysis assessed the effect of acute tVNS on domain-specific cognitive function, peripheral and central haemodynamic responses to orthostatic stress (via the AS), and inflammatory marker response to tVNS, and each of these analyses will be addressed in subsequent thesis chapters respectively.

Methods

Trial Design

A single-site, single-blind, sham-controlled crossover study was designed, for participants with diagnosed amnestic MCI. Each participant was assessed at baseline (no stimulation) followed by randomisation to sham stimulation and active tVNS stimulation over two subsequent study visits. The study comprises a baseline assessment (visit 1) followed 7-10 days later by either an active or sham stimulation study visit and again 7-10 days later by a third visit wherein the final condition (active or sham) was undertaken. The inclusion of both a baseline and sham stimulation condition is performed to control for potential unblinding, which is common in device trials. Participants were enrolled and consented one study clinician (HD).

Please see Figure 11 for an illustration of randomisation and crossover methodology utilised in this study.

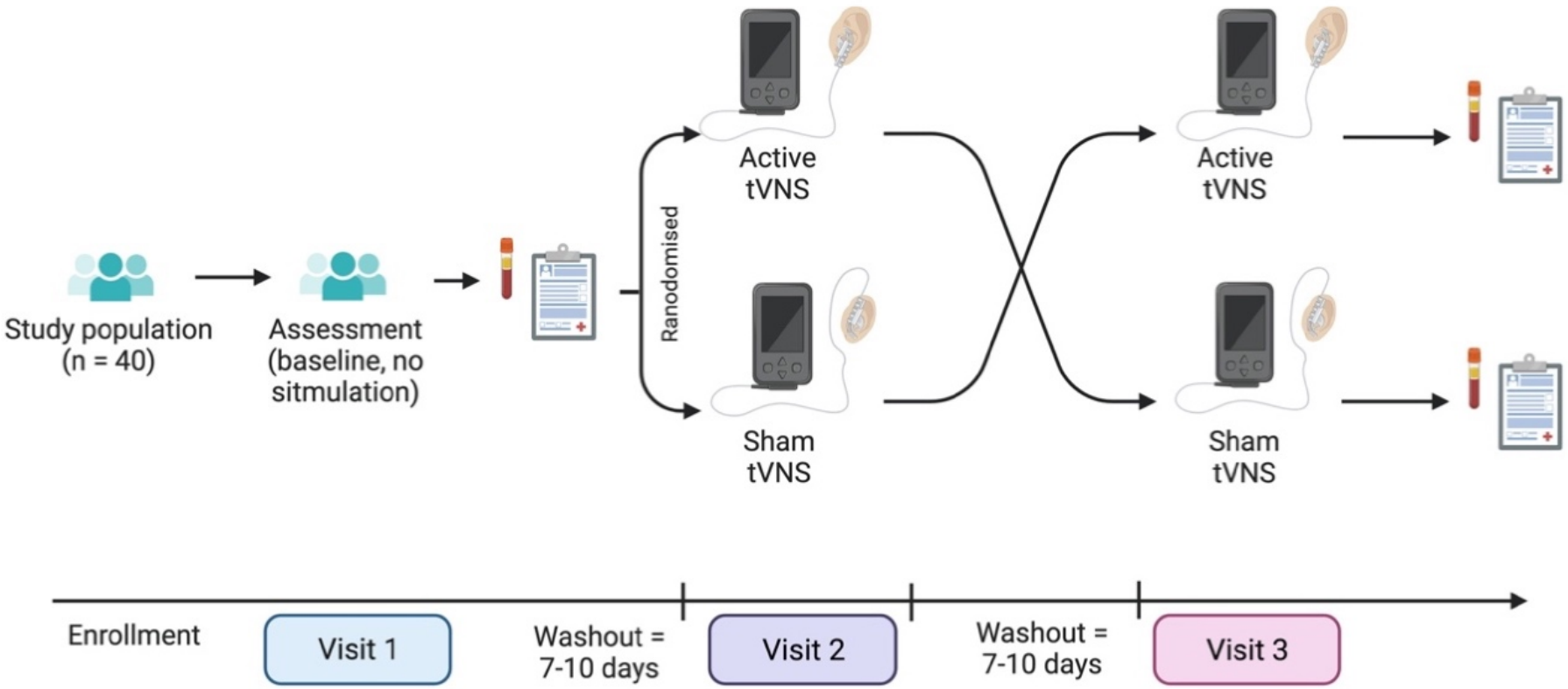


Figure 11: VINCI-AD study design and method of crossover

This study followed the Standard Protocol Items: recommendations for Interventional Trials (SPIRIT) guidelines [491]. Table 8 illustrates the SPIRIT table summarising study procedures, outcome measures, randomisation and assessments for each study visit. Figure 12 details the cognitive, neurocardiovascular and inflammatory response assessments undertaken at each visit.

	Study Period			
	Screening (day -90 to day -7)	**Visit 1 (day 0)**	**Visit 2 (day 7-10)**	**Visit 3 (day 14-17)**
Enrolment				
- **Eligibility Screen**	x			
- **Informed Consent**	x			
Intervention				
- **Baseline assessments (no stimulation)**		x		
- **Active (left cymba conchae) stimulation**			ø	ø
- **Sham (left earlobe) stimulation**			†	†
Assessments				
- **General assessment, vitals, medication changes**		x	x	x
- **Primary Outcome: Safety**				
- Assess for AE/ SAEs			x	x
- Active stand with TSI		x	x	x
- **Primary Outcome: Acceptability and Tolerability**				
- Likert scale questionnaires			x	x
- **Cognitive assessments**				
- FNAT		x	x	x
- SART		x	x	x
- SHQ		x	x	x
- Verbal word learning		x	x	x
- Semantic fluency		x	x	x
- Digit span forwards		x	x	x
- Digit span backwards		x	x	x
- Verbal word recall		x	x	x
- Verbal word recognition		x	x	x
- **Frontal Oxygenation**				
- Cognitive testing TSI		x	x	x
- **Neurocardiovascular assessments**				
- HRV		x	x	x
- **Serum / Plasma venepuncture**				
- Inflammatory cytokine panel		x	x	x
- **Device usability assessment**				
- Kettle test				x

Table 8 Table of VINCI-AD Protocol Timeline

Table 8 legend:
Ø = active stimulation randomisation: participant randomised to either sham or active stimulation at Visit 2 and alternative stimulation site at Visit 3
† = sham stimulation randomisation: participant randomised to either sham or active stimulation at Visit 2 and alternative stimulation site at Visit 3
AE – Adverse event; SAE – Serious adverse event; FNAT – Face Name Association Task; SART – Sustained Attention Response Task; SHQ – Sea Hero Quest Tas; NIRS – functional Near Infrared Spectroscopy; HRV – Heart Rate Variability; TSI – Tissue Saturation Index

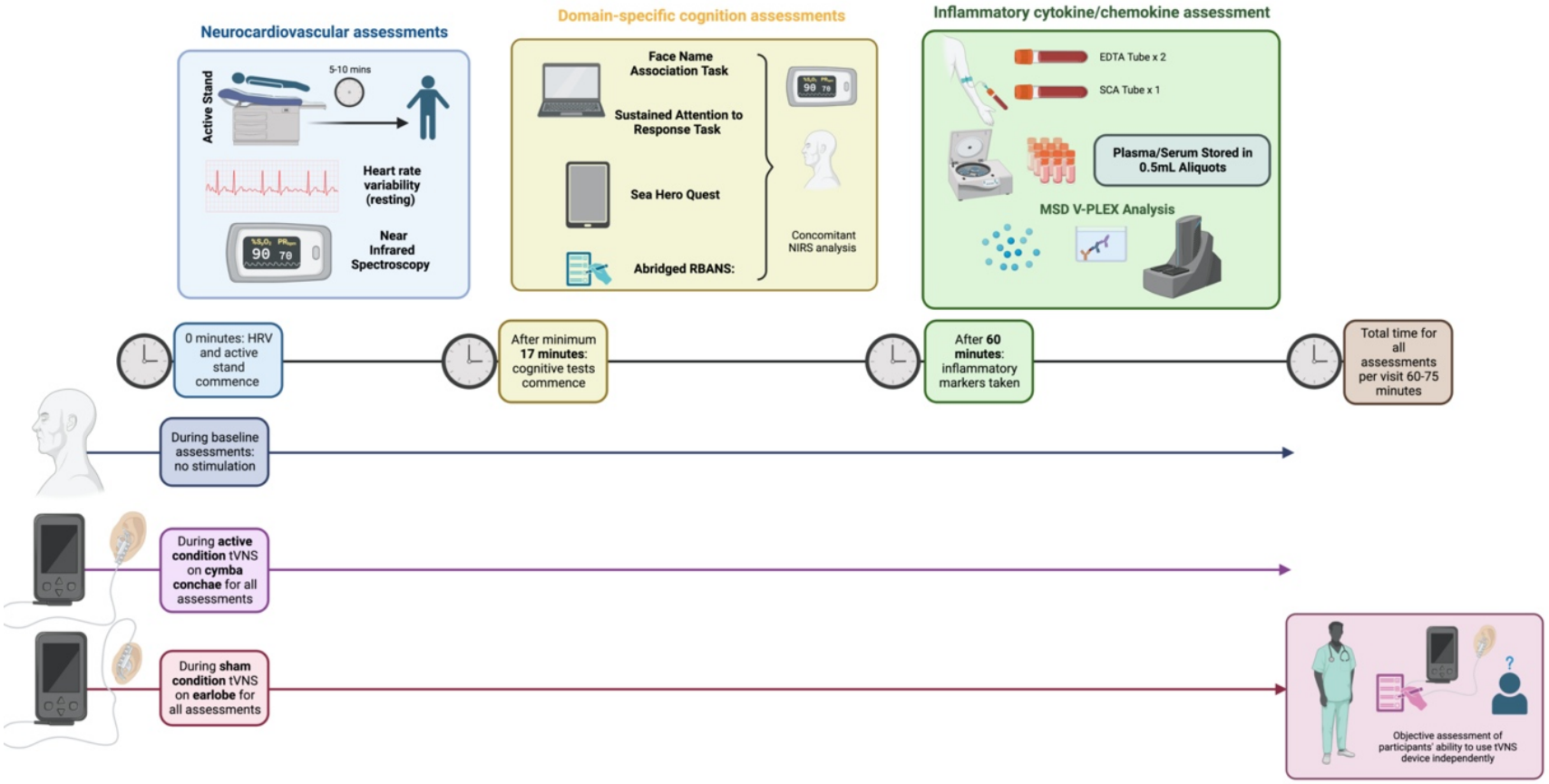


Figure 12: VINCI-AD study protocol and interventions at each study visit

Please note that an in-depth analysis of the cognitive, neurocardiovascular and inflammatory response assessments listed above is explored in detail in subsequent thesis chapters.

Study setting

This study was carried out at the Institute of Memory and Cognition, Tallaght University Hospital, Dublin, Ireland which is a tertiary referral centre at a University teaching hospital. The Institute is coordinated by Consultant Geriatricians and a Consultant Neurologist with consensus diagnosis for cognitive impairment undertaken with specialised Advanced Nurse Practitioner, Occupational Therapist, Speech and Language Therapist and Neuropsychologist input.

Sample size

To establish the safety and feasibility of tVNS in a population of older adults with MCI, VINCI-AD aims to recruit 40 participants for inclusion. This sample size is based on previous literature which investigated the effects of tVNS in healthy young adults [226–229,492] and also Cognitively Unimpaired (CU) older adults [223]. Potential effect sizes from the results of this feasibility study will inform an accurate power calculation for future definitive trials.

Eligibility Criteria

This study recruited both males and females with a diagnosis of amnestic MCI as determined by consensus diagnosis at the Institute of Memory and Cognition. Those eligible for inclusion in VINCI-AD had an index delayed memory score on the RBANS [375] of less than 85 (indicative of amnestic features) or equivalent, and a score of 0.5 on the CDR Global scale [377] indicating mild impairment not impacting daily function. All participants must be on stable medications for one month prior to recruitment, must have sufficient understanding of English language and must be able to provide informed consent. Exclusion criteria include:

1. Significant current depression
2. Uncorrected vision/hearing loss
3. History of brain surgery
4. History of epilepsy with any seizure event in last year
5. Prescribed any pharmacological agents known to significantly increase seizure risk
6. Diagnosed arrhythmia including Atrial Fibrillation
7. Cardiac pacemaker implant
8. Existing left ear deformity or recent ear trauma
9. Alcohol dependence
10. Prescribed Disease Modifying Anti-Rheumatic Drugs (DMARDs) or immunotherapies

Assessment procedures

Participants underwent a baseline visit to assess eligibility by the study clinician (HD). This baseline visit included a detailed clinical review to encompass clinical, demographic and anthropomorphic data, cognitive history and medication review.

Device and Intervention

Participants received active and sham stimulation with the Conformité Européenne (CE) certified tVNS device NEMOS® (Cerbomed GmbH, Erlangen, Germany) device. This device includes an external pulse generator and a cutaneous contact electrode developed to be placed manually at the cymba concha in the auricular tract of the left ear for active stimulation or the earlobe for sham stimulation. The electrode is connected to rechargeable a pulse generator via a connector cable. The device has been programmed to stimulate with 8 Hz stimulation frequency, 250 µsec pulse width and 30 s on/30 s off time whereas the amplitude output is individually adjusted to tolerance (from 0.1 mA to 5 mA).

Participants were instructed in the use of the device, were advised to titrate the current output until a mild tingling sensation in the cymba concha (for active stimulation) or earlobe (for sham stimulation) was felt and to keep the current at a level that would cause no painful sensation. At each stimulation study visit the device is explained to the participant and then fitted by the study researcher. Participants were given oral and written instructions at each stimulation study visit regarding how to set up and fit the device in their ear.

Safety and Tolerability of tVNS in MCI

The risk associated with use of the NEMOS © Cerbomed tVNS device is minimal as published metanalyses of the safety profile show minimal adverse events [490,493]. The most common adverse events reported include local skin tingling, burning, redness, itching, tinnitus or headache which occur in up to 18% of participants, and tends to resolve quickly after discontinuation of the use of the device. Due to the theoretical risk of increasing cardiac vagal-mediated bradycardias or hypotensive episodes by stimulating the right VN, participants had stimulation of the left ear only for active and sham conditions.

Feasibility (Safety, Tolerability and Usability) assessments

During the active and sham stimulation conditions, safety was recorded by completion of contemporaneous recording of any adverse events reported by participants whilst undergoing either active or sham stimulation and Likert-Based Questionnaires were used to assess for established side effects previously reported with tVNS: pain, skin discomfort, tingling, headache and tinnitus. These were administered both during active and sham stimulation. To assess feasibility of tVNS in MCI, we assessed the number of participants completing assessment across the three sessions.

Acceptability of the device was assessed via Likert-based scale questions including if the device felt comfortable, their subjective sense of confidence using the device, would the participant use it again.

To assess usability of the device, a modified KT was designed with senior OT colleagues in our institution. The KT assess a participant's ability to do a specific task independently (in this instance, operate a tVNS device) and subdivides the task into 13 sub-tasks. These are objectively graded by the assessor from 0 (no assistance required, fully independent) to 4 (fully assisted with this aspect of task performance). Scores on the KT range from 0 - 52, with lower scores in this circumstance indicating more independent use of the tVNS device and higher scores reflecting more severe difficulty in using the tVNS device independently.

Following assessment, participants were given contact details of the principal investigator and study coordinator at the hospital who they could contact if they have concerns, or any new symptoms arose. Any adverse events were recorded during the intervention and closely monitored until resolution. Data of participants who discontinued or deviated from the study protocol were retained for intention-to-treat analysis.

Training of Trial Clinician in Administering Usability Assessment Tool

The KT is a brief objective assessment tool designed to assess a patient's skills in a functional context. It is most frequently administered by a trained OT in clinical settings, and can be administered in a wide variety of functional settings (including assessing participant's ability to use everyday devices such as kitchen appliances, bathroom devices, or more advanced technology tools and devices). In this trial scenario the trial clinician (HD) administered all assessment tools. In order to be proficient in independently administering this tool with trial participants, HD undertook three 1-hour training sessions with a senior cognitive OT in our

institution. This was then validated in a small group (n=3) of volunteers to ensure accurate and reproducible administration of the assessment tool. The KT designed for this study is displayed below.

PTID:_____ Date:_____ Time:_____

tVNS Functional Assessment

Scoring & Comments on task performance

Step / Score >	0 Intact	1 trial & error	2 general cue	3 a. specific cue	4 assisted
1. Taking tVNS device out of zip bag					
2. Identifying ear piece, cord, liquid, plastic covers and stimulator					
3. Identifying input area for cord into tVNS stimulator					
4. Manually inserting cord into tVNS stimulator the right way up					
5. Turning on tVNS device using correct button					
6. Choosing ear plug that will fit their ear					
7. Placing small plastic covers on electrodes					
8. Rub earpiece in tVNS stimulator liquid					
9. Placing ear piece into correct ear and correct part of ear					
10. Appropriately pressing correct button to increase tVNS stimulation parameters					
11. Appropriately troubleshooting common problems (device loses contact with skin or falls out of ear)					
12. Choosing correct tVNS stimulation parameters (below pain threshold)					
13. Disassembling tVNS device appropriately and safely					
Total score = /52	(	+	+	+	+)

Figure 13 details the Independent Usability Assessment tool (Kettle Test [KT])

Planned Primary and Secondary Aims and Analysis

The primary aim of the current study was to assess the feasibility (safety, tolerability and usability) of tVNS in MCI. Feasibility was reported via recruitment rates, successful enrolment, and study completion in proportions and percentages. To assess safety, a chi-square test was planned to compare the number of adverse events reported during the active vs sham condition. Tolerability was assessed using Likert scales, which will be compared between active and sham conditions using paired between-condition statistics (t-test for normally distributed or non-parametric equivalent). Descriptive statistics were planned to report acceptability using the modified Kettle test, as well as describing the cohort, to be reported as means (with standard deviations) or medians (with inter-quartile ranges) and proportions (percentages) as appropriate. Statistical analysis were undertaken using MATLAB (The MathWorks, Inc., Natick, MA, USA), STATA (Stata Statistical Software: Release 18, College Station, TX: StataCorp LLC) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Ethics approval and consent to participate

The Joint Research Ethics Committee of St James's Hospital and Tallaght University Hospital approved the current study (REC reference 307). This study was conducted in accordance with the Declaration of Helsinki. All participants were required to provide written informed consent by signing all study form(s), which were witnessed by an appropriately qualified and delegated member of the research team, prior to the completion of all study-specific procedures/ investigations.

Results

Demographics, recruitment and retention

There were 50 patients with MCI screened for inclusion in this trial, with 10 failing screening due to the following reasons; no means of transport to get to and from the hospital (3 participants), recent hospitalisation and not willing to be recruited (3 participants), active excessive alcohol consumption (2 participants), diagnosed Atrial Fibrillation (1 participant) and not willing to undergo nerve stimulation as they found the idea frightening (1 participant) which gave a successful enrolment rate of 80%.

In total there were 40 participants who successfully screened and enrolled into the trial, of whom 40 completed visit 1, 40 completed visit 2 and 38 completed visit 3. The reasons for 2 participants not completing the third hospital visit were intercurrent illness (1 participant) and no means of transport as family unwell (1 participant) which gives a trial completion rate of 95%.

Of the 40 participants, 22 were male, the median age was 71.7 ($\pm$ 6.9), the median Body Mass Index (BMI) was 26.6 (range 20.8 to 35.2), all participants were ethnically white Irish, 22 of the 40 participants (55%) owned and used a smartphone and 27 of the 40 participants (67.5%) had biomarker proven AD-MCI. Please see Table 9 for a summary of the demographic details of the study participants.

Demographic and recruitment details of participants	
Participants screened	50
Screen failed	10 (20%)
Reasons for screen-fail: - No means of transport to and from hospital for visits - Hospitalised recently - Active excessive alcohol use - Diagnosed Atrial Fibrillation (AF) - Not willing to undergo nerve stimulation, found the idea frightening	 3 3 2 1 1
Participants recruited	40 (80%)
Completed first visit Completed second visit Completed third visit - Reasons for lack of completion o Intercurrent illness o No transport	40 (100%) 40 (100%) 38 (95%) - 1 (2.5%) - 1 (2.5%)
Sex - Male - Female	 22 (55%) 18 (45%)
Age	71.7 ± 6.9
BMI (kg/m^2)	26.6 (20.8 – 35.2)
Ethnicity - White Irish	 40 (100%)
Owns and uses a smartphone	22/40 (55%)
Aetiology of MCI:	
- Alzheimer's Disease (AD) o CSF +ve ▪ Aβ-42 (pg/ml) ▪ P-tau (pg/ml) o PET +ve	27/40 (67.5%) - 24 o 514.7 ± 236.1 o 87.2 ± 44.5 - 3 o 1: Amyloid PET o 2: FDG – PET
- Non-AD pathology	6/40 (15%)
- Probable AD, no biomarker (age >80)	3/40 (7.5%)
- Probable AD, no biomarker (refused)	4/40 (10%)

Table 9 details the demographic details of VINCI-AD study participants

Comorbidity and Polypharmacy

Thirty-six of the 40 participants (90%) had a Charlson Comorbidity Index (CCI) of 3 or greater indicating moderate comorbidity. The most common comorbidities encountered were hypertension (55%), anxiety or depression (55%) and dyslipidaemia (42.5%). A summary and breakdown of the known comorbidities of the study participants is listed in Table 10.

Clinical details of participants (n=40)	
Charlson Comorbidity index - 1 - 2 - 3 - 4 - 5 - 6	 - 3/40 (7.5%) - 1/40 (2.5%) - 13/40 (32.5%) - 17/40 (42.5%) - 5/40 (12.5%) - 1/40 (2.5%)
Hypertension	22/40 (55%)
Anxiety / Depression	22/40 (55%)
Dyslipidaemia	17/40 (42.5%)
Ischaemic Heart Disease - without PCI or intervention - with history of PCI or CABG	13/40 (32.5%) - 11/40 (27.5%) - 2/40 (5%)
Dyspepsia / PUD - Without diagnosed ulcers - With diagnosed peptic/duodenal ulcers	12/40 (30%) - 8/40 (20%) - 4/40 (10%)
Arthritis (any site)	6/40 (15%)
Osteopaenia/ Osteoporosis	6/40 (15%)
COPD / Asthma	5/40 (12.5%)
Hypothyroidism	4/40 (10%)
Urinary Incontinence (stress or urge)	4/40 (10%)
Gout	3/40 (7.5%)
BPAD/ SCZ	2/40 (5%)
CKD III or greater	1/40 (2.5%)
CVA/TIA	1/40 (2.5%)
CCF	1/40 (2.5%)
Colitis	1/40 (2.5%)
Diabetes - Insulin-Dependent - Non-Insulin Dependent	 - 0/40 - 1/40 (2.5%)

Table 10 details the clinical details of VINCI-AD study participants

Polypharmacy, as indicated by 5 or more regular prescribed medications, was present in 32.5% of patients, with 12.5% taking 9 or more medications daily. The most commonly prescribed medications were Selective Serotonin Reuptake Inhibitors / Serotonin and Norepinephrine Reuptake Inhibitors (SSRI / SNRI's) with 52.5% of participants taking a regular SSRI / SNRI. Twenty-one participants were also on active treatment for hypertension,

with Calcium – Channel Blockers (CCB's) and ACE-inhibitors / Angiotensin Receptor Blockers (ACEi/ARB's) the most commonly prescribed anti-hypertensives (with 42.5% and 30% prescribed respectively). The medications prescribed for the participants of the study are detailed in Table 11.

Medications	
Polypharmacy: - Number of medications daily: o 0-2 o 3-5 o 6-8 o 9-12 - Number of participants taking ≥ 5 medications daily	 - 12/40 (30%) - 18/40 (45%) - 7/40 (17.5%) - 5/40 (12.5%) - 13/40 (32.5%)
SSRI / SNRI	21/40 (52.5%)
Statin	17/40 (42.5%)
PPI	17/40 (42.5%)
Antihypertensive (any) - CCB - ACEi / ARB - Alpha – blocker - Diuretic (loop)	21/40 (52.5%) - 12/40 (30%) - 17/40 (42.5%) - 3/40 (7.5%) - 1/40 (2.5%)
Antiplatelet agent	11/40 (27.5%)
Sedative (any) - Benzodiazepine - Z-drug	6/40 (15%) - 4/40 (10%) - 4/40 (10%)
Vitamin D replacement (any formulation)	5/40 (12.5%)
L-thyroxine replacement	4/40 (10%)
Inhaled steroid/LABA/LAMA	4/40 (10%)
Bladder stabiliser / antimuscarinic	4/40 (10%)
Codeine/ Tramadol analgesia	3/40 (7.5%)
Mood stabiliser	3/40 (7.5%)
Antihistamine (regular)	3/40 (7.5%)
Beta blocker	3/40 (7.5%)
Anti-psychotic medication	2/40 (5%)
Midodrine / Fludrocortisone	1/40 (2.5%)
DOAC / warfarin	1/40 (2.5%)
Non-insulin anti-diabetic medication	1/40 (2.5%)
Pregabalin / Gabapentin	1/40 (2.5%)
Regular oral steroid	1/40 (2.5%)

Table 11 details the regular medications of study participants

Stimulation settings

In total, the mA amplitude settings tolerated by participants was slightly higher during active stimulation vs. sham (2.31 ± 0.98 vs. 2.14 ± 0.8) which was non-significant. The overall time

stimulated per condition (active or sham) was not significantly different, although there was slightly longer stimulation times for the active stimulation condition prior to some of the cognitive testing (specifically the FNAT, SART and abridged RBANS). We explore the potential effect longer stimulation times may have had on cognitive outcomes in Chapter 5. The stimulation times and settings are detailed in Table 12.

tVNS stimulation to the left auricle (outer ear)			
	Active stimulation (cymba concha)	Sham stimulation (earlobe)	
Amplitude (mA)	2.31 ± 0.98 Range 0.9 – 5	2.14 ± 0.8 Range 0.8 – 4.2	p= 0.33 (ns)
Frequency (Hz)	8	8	n/a
Pulse width (μsec)	250	250	n/a
Waveform	30 sec on / 30 sec off	30 sec on / 30 sec off	n/a
Total time stimulated (mins)	Mean: 60.5 ± 4.4 Range: 49 - 73	Mean: 59.5 ± 5.7 Range: 39 - 82	p= 0.23 (ns)
Time stimulated pre – FNAT (mins)	Mean: 21.3 ± 4.9 Range: 16 – 35	Mean 19.1 ± 2.8 Range: 15 - 28	p= 0.02 (*) r^2 = 0.133
Time stimulated pre – SART (mins)	Mean: 36.4 ± 5.1 Range: 27 - 52	Mean: 33.3 ± 3.5 Range: 22 - 40	p= 0.002 (**) r^2 = 0.215
Time stimulated pre – SeaHero Quest (mins)	Mean: 44.5 ± 5.3 Range: 35 – 59	Mean: 42.2 ± 4.7 Range: 35 - 51	p= 0.06 (ns)
Time stimulated pre – abridged RBANS (mins)	Mean: 51.6 ± 4.73 Range: 43 - 65	Mean: 49.7 ± 4.55 Range: 35 - 58	p= 0.02 (*) r^2 = 0.13

Table 12 details stimulation settings for active and sham tVNS stimulation

Tolerability and Acceptability

To assess tolerability and acceptability, assessment of Adverse Events (AEs) and Serious Adverse Events (SAEs) was recorded after every stimulation condition, both active and sham. There was one AE reported during the active stimulation condition at the cymba conchae for one participant who experienced moderately severe tinnitus for several hours after active stimulation, which resolved completely within 24 hours. This participant had been diagnosed

with episodic tinnitus prior to the study. A letter of referral was made with the participant's permission to their primary care provider alerting them to this event and follow up care with their Otolaryngologist was organised. No SAE were reported.

Likert scales (1= no discomfort, pain, headache etc to 5= severe or intolerable discomfort, pain, headache etc) were devised and used as patient report surveys after both active and sham conditions. The scales can be found in Supplementary Appendix 1. Overall the device was very well-tolerated, with participants experiencing zero or minimal pain (100%), burning (97.4%), headache (97.4%) or tinnitus (97.4%).

The most common side effect was a sensation of tingling, with 24 of 39 participants (61/5%) rating it as mild, tolerable, 12 of 39 participants (30.7%) rating it as moderate and 3 of 39 participants (7.6%) rating it as a heavy, noticeable tingling sensation. No participants rated the tingling sensation as severe or intolerable.

We noted that 26/38 (68.4%) of participants stated they would use the device again without hesitation and a further 11/38 (28.9%) stated they would probably use the device again.

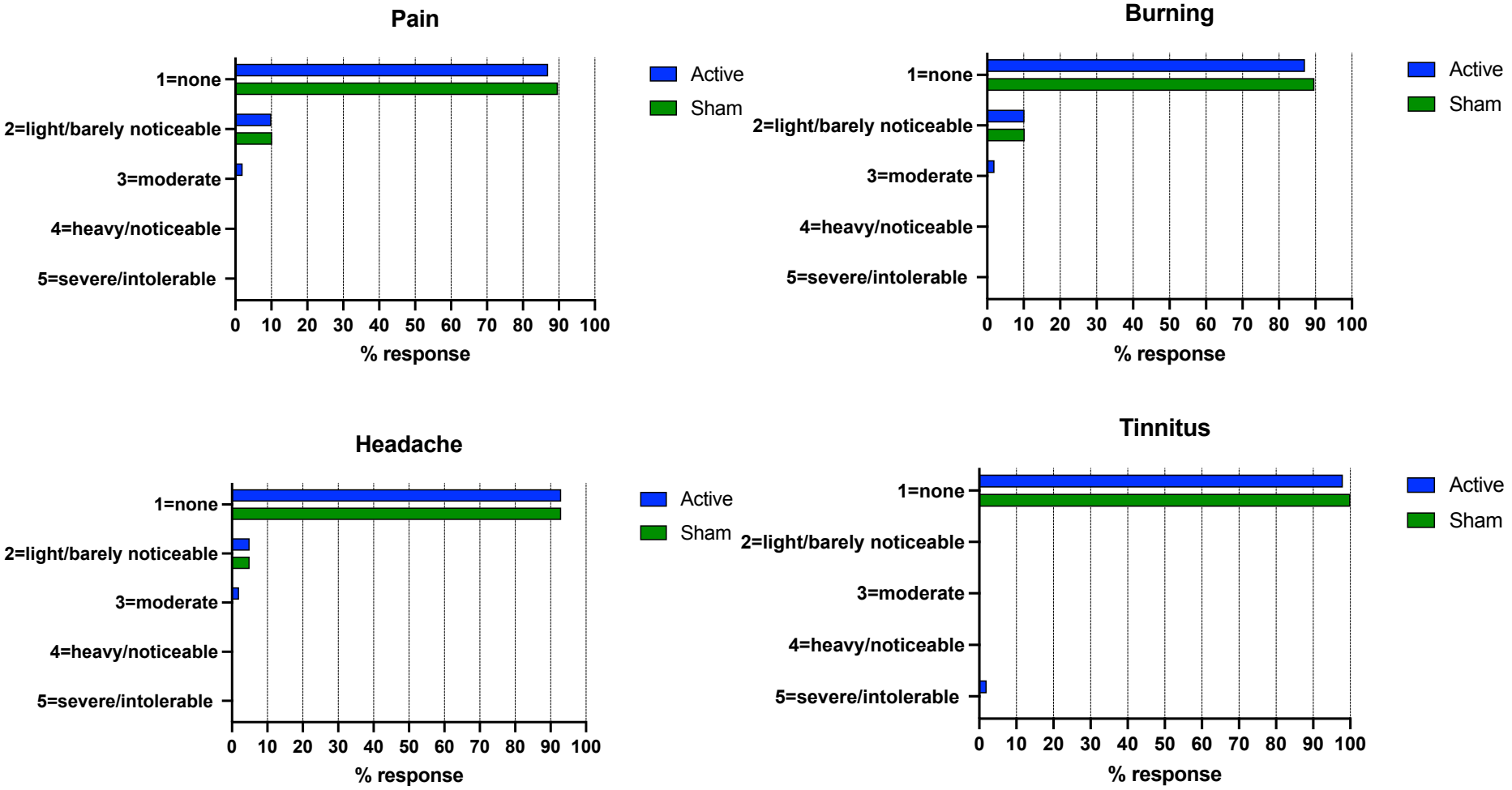
It was noteworthy that participants' lacked self-confidence using the device, having been introduced to the NEMOS© tVNS device on three occasions and worn it for two sessions (one active stimulation at the cymba conchae and one sham stimulation at the earlobe). Twenty -two of 38 participants who completed the three trial visits (57.8%) stated they had no confidence using the device, that they "would be lost and wouldn't know what to do" if

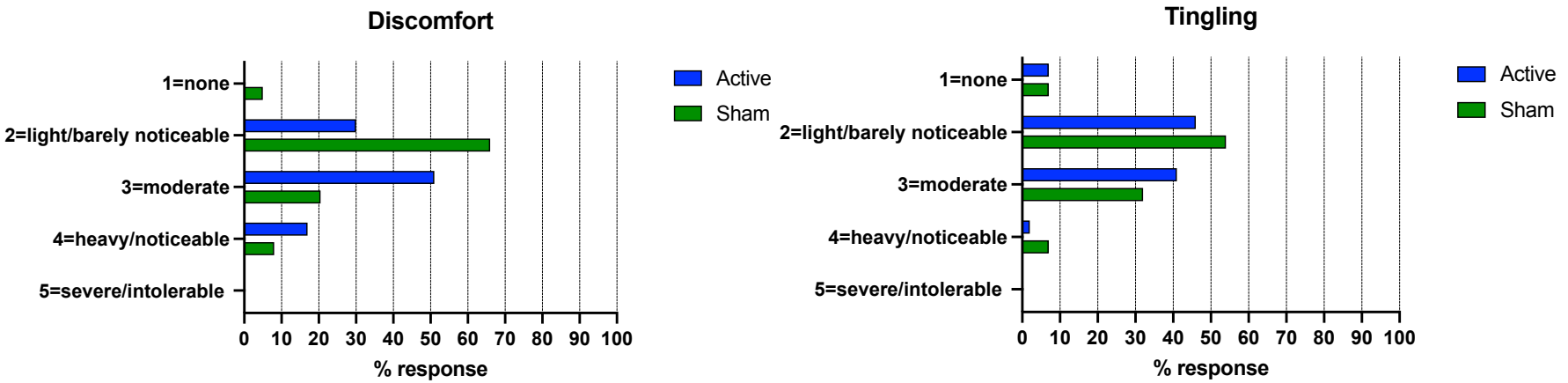
handed the device, with a further 6/38 (15.7%) of participants feeling “not too confident” using the device independently.

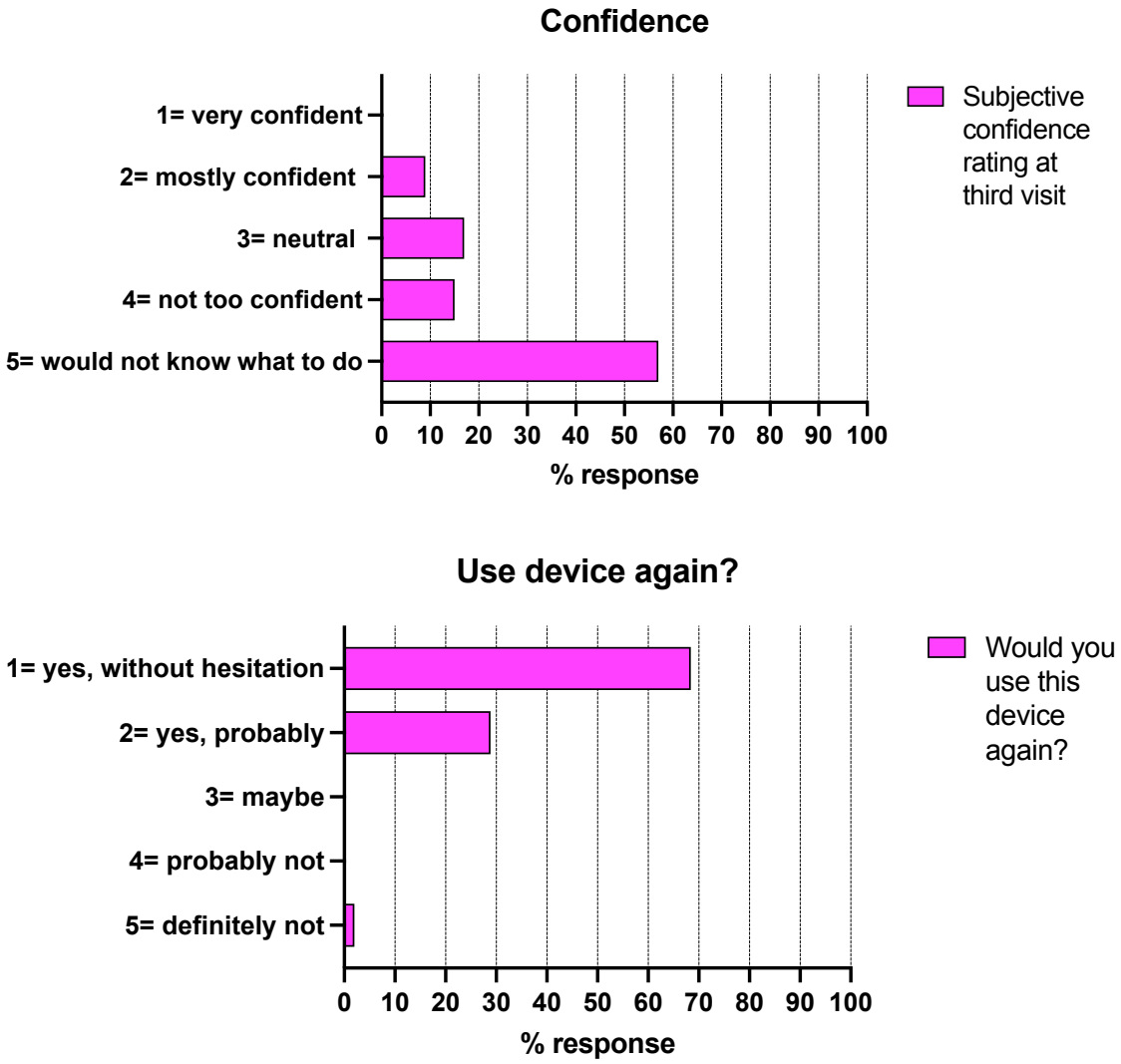
The details of means with Standard Deviation (SD) of the Likert scale assessments of tolerability and acceptability of the NEMOS© tVNS device are shown in Table 13 and graphical illustrations of these results are shown below. Given the non-parametric nature of this data, to compare group means a Wilcoxin-rank sum statistic was used.

Acceptability, tolerability, usability			
	Active stimulation (cymba concha)	Sham stimulation (earlobe)	
Adverse events	1	0	
Serious adverse events	0	0	n/a
Pain (1-5)	1.46 ± 0.51	1.36 ± 0.49	z = 2.9 p= 0.02
Burning (1-5)	1.15 ± 0.43	1.1 ± 0.3	z = 0.388 p = 0.69
Tingling (1-5)	2.38 ± 0.75	2.36 ± 0.71	z = -0.06 p = 0.94
Headache (1-5)	1.1 ± 0.38	1.05 ± 0.22	z = 0.48 p = 0.62
Tinnitus (1-5)	1.1 ± 0.64	1 ± 0	z = 1.01 p = 0.32
Comfortable (1-5)	2.87 ± 0.69	2.31 ± 0.69	z = 3.5 p = 0.01
Descriptive statistics on final visit			
Confidence using device (1-5)	Mostly confident (1-3): 10/38 (26.3%) Not too confident (4): 6/38 (15.7%) Lost, no confidence, wouldn't know what to do (5): 22/38 (57.8%)		
Would use again (1-5)	Yes, without hesitation (1): 26/38 (68.4%) Probably (2): 11/38 (28.9%) Maybe (3): 0 Probably not (4): 0 Definitely not (5): 1/38 (2%)		

Table 13 details the tolerability scores for active and sham tVNS stimulation. A Wilcoxin rank sum test (z) was used to compare non-parametric means


Pain
1=none
2=light/barely noticeable
3=moderate
4=heavy/noticeable
5=severe/intolerable
0 10 20 30 40 50 60 70 80 90 100
% response
Active
Sham
Burning
1=none
2=light/barely noticeable
3=moderate
4=heavy/noticeable
5=severe/intolerable
0 10 20 30 40 50 60 70 80 90 100
% response
Active
Sham
Headache
1=none
2=light/barely noticeable
3=moderate
4=heavy/noticeable
5=severe/intolerable
0 10 20 30 40 50 60 70 80 90 100
% response
Active
Sham
Tinnitus
1=none
2=light/barely noticeable
3=moderate
4=heavy/noticeable
5=severe/intolerable
0 10 20 30 40 50 60 70 80 90 100
% response
Active
Sham


Discomfort
1=none
2=light/barely noticeable
3=moderate
4=heavy/noticeable
5=severe/intolerable
0 10 20 30 40 50 60 70 80 90 100
% response
Active
Sham
Tingling
1=none
2=light/barely noticeable
3=moderate
4=heavy/noticeable
5=severe/intolerable
0 10 20 30 40 50 60 70 80 90 100
% response
Active
Sham



Figures 14 (A-H): Tolerability and acceptability scores during sham and active stimulation, and subjective confidence ratings

Objective Assessment of Device Usability

A modified KT was devised with assistance from senior cognitive OT colleagues in our institution, specifically to assess a participants' ability to use the NEMOS© tVNS device independently. The participants were introduced to the device on three occasions, and the set-up and instructions for use demonstrated on two occasions by the study clinician (HD). Thirteen sub-tasks were devised as outlined in Table 14, and the scores for each participant are also listed. We noted that most participants were fully independent with certain sub-tasks, such as taking the device out of a zipped bag and dis-assembling the device safely. However, most participants needed full assistance with placing the electrodes at the cymba concha of the ear (36 of 38 participants, 94.7%), even with a mirror to assist, and also needed full assistance with troubleshooting, e.g. did not know how to remedy the situation if the device fell from the ear (37 of 38 participants, 97.3%).

Please see Table 14 for the breakdown of participants' independence with the relative subtasks of the KT. A graphical representation of the proportions of participants who scored independently to needing full assistance with the various subtasks of device usability is also given in Figure 15.

Breakdown of KT assessment scores (Device Usability: Objective Assessment)				
	Independent	General Cue	Specific Cue	Full Assistance
Taking tVNS device out of zip bag	38 (100%)	0 (0%)	0 (0%)	0 (0%)
Identifying all parts of device	20 (52.6%)	18 (47.3%)	0 (0%)	0 (0%)
Identifying input area for cord into tVNS stimulator	30 (78.9%)	4 (10.5%)	2 (5.2%)	2 (5.2%)
Manually inserting cord into tVNS stimulator	8 (21%)	12 (31.5%)	10 (26.3%)	8 (21%)
Turning on tVNS device using correct button	12 (31.5%)	10 (26.3%)	10 (26.3%)	6 (15.7%)
Choosing ear piece that will fit their ear	26 (68.4%)	10 (26.3%)	2 (5.2%)	0 (0%)
Placing small plastic covers on electrodes	14 (36.8%)	12 (31.5%)	10 (26.3%)	2 (5.2%)
Rub earpiece in tVNS stimulator liquid	2 (5.2%)	8 (21%)	28 (73.6%)	0 (0%)
Placing ear piece into correct part of ear	0 (0%)	0 (0%)	2 (5.2%)	36 (94.7%)
Increase tVNS stimulation parameters	8 (21%)	12 (31.5%)	10 (26.3%)	8 (21%)
Troubleshooting (device loses contact with skin or falls out of ear)	0 (0%)	0 (0%)	1 (2%)	37 (97.3%)
Choosing correct tVNS stimulation parameters (below pain threshold)	10 (26.3%)	12 (31.5%)	11 (28.9%)	5 (13.1%)
Disassembling tVNS device appropriately and safely	36 (94.7%)	2 (5.2%)	0 (0%)	0 (0%)

Table 14 Kettle Test (independent usability testing) assessment scores

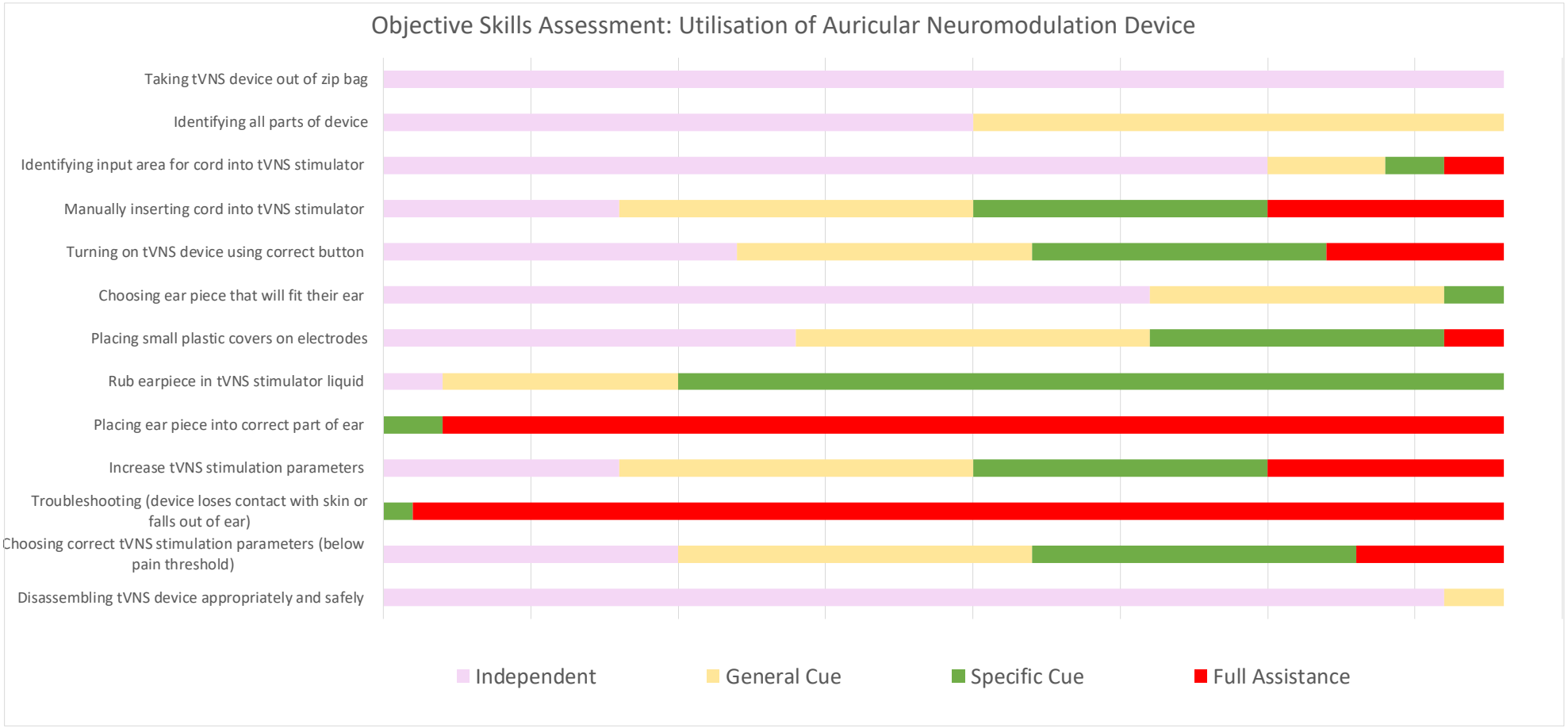


Figure 15: Results of Independent Usability Assessment tool (Kettle Test [KT])

Associations with Demographic, Polypharmacy and Comorbidity data

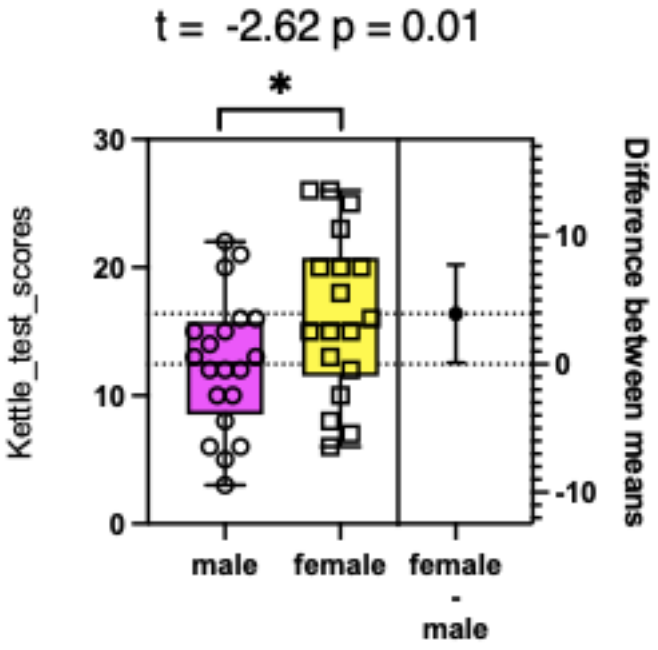
Given the non-parametric distribution of the Likert scale responses, comparison between groups was undertaken using a Wilcoxin rank sum statistic. Comparisons between groups of the KT which was parametrically distributed was undertaken using an independent t-test. The only significant finding on examining the differences in acceptability and tolerability scores across age, sex, BMI, smartphone use, polypharmacy, CCI or AD biomarker positivity was that those with polypharmacy were less likely to state they would use the device again ($z = -2.64$, $p = 0.01$). There were no other significant findings in any of the tolerability or acceptability scores and age, sex, BMI, smartphone use, polypharmacy, CCI or AD biomarker positivity. For statistics and measures of significance, please see Table 15.

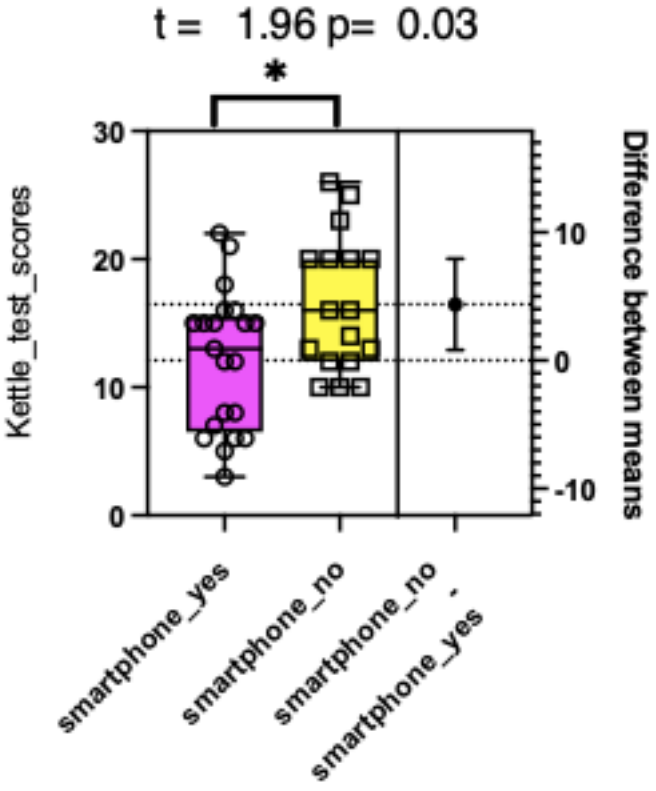
On analysis of usability, significantly more men than women were independent using the NEMOS © tVNS device when analysed using an independent t-test ($t = -2.62$, $p = 0.01$) and it was noted that those who owned and used a smartphone device were significantly more independent using the NEMOS © tVNS device as assessed via the KT ($t = 1.96$, $p = 0.03$). We also noted that those with lower BMI were more independent using the NEMOS © tVNS device ($t = -2.67$, $p = 0.01$) For the relevant statistics used and measures of statistical significance please see Table 15 below. Graphical representation of the significant findings are also illustrated below.

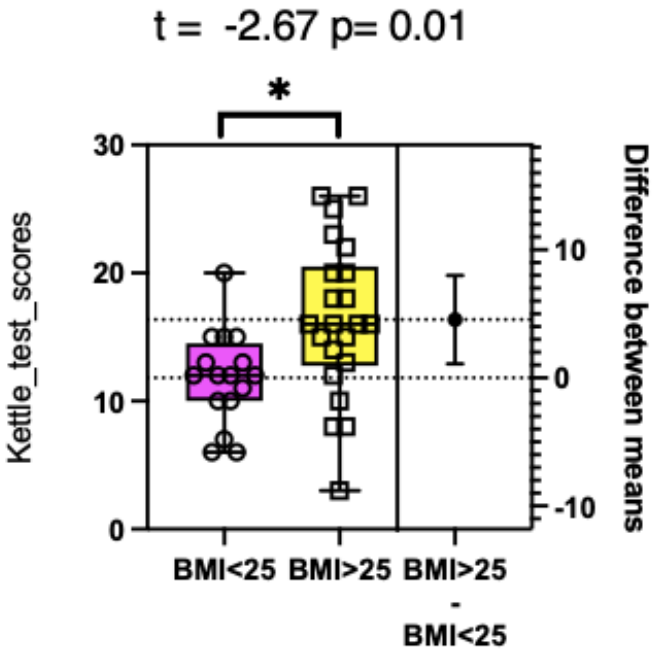
	Tolerability					Acceptability			Usability
	Pain	Burning	Headache	Tingling	Tinnitus	Comfort	Confidence	Use again	Objective KT
Age									
- 55-69	2 (IQR 1.5-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 3-3.5)	5 (IQR 3-5)	1 (IQR 1-2)	14.75 (SD 6.25)
- ≥70	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 2-3)	4 (IQR 2.5-5)	1 (IQR 1-1.5)	14.31 (SD 5.3)
	z = 0.93	z = -1.57	z = -1.18	z = -0.35	z = -0.66	z = 1.28	z = 1.24	z = 0.78	t = 0.22
	p = 0.35	p = 0.11	p = 0.23	p = 0.72	p = 0.5	p = 0.19	p = 0.21	p = 0.43	p = 0.41
Sex									
- M	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 3-3)	5 (IQR 3-5)	1 (IQR 1-2)	12.35 (SD 4.6)
- F	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 2-3)	4 (IQR 3-5)	1 (IQR 1-2)	16.77 (SD 5.7)
	z = 0.35	z = -0.58	z = 0.48	z = -0.18	z = 0.92	z = 1.31	z = 0.75	z = -1.11	**t = -2.62**
	p = 0.72	p = 0.56	p = 0.62	p = 0.85	p = 0.35	p = 0.18	p = 0.44	p = 0.26	**p = 0.01**
BMI									
- <25	2 (IQR 2-2)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	1 (IQR 1-1)	3 (IQR 2.5-3)	5 (IQR 4-5)	1 (IQR 1-1)	11.8 (SD 3.6)
- ≥25	2 (IQR 1-2)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	1 (IQR 1-1)	3 (IQR 2-3)	3 (IQR 2.5-5)	1 (IQR 1-2)	16.36 (SD 6.04)
	z = 0.35	z = 0.02	z = -0.21	z = 0.47	z = 1.19	z = 0.51	z = 1.79	z = -1.2	**t = -2.67**
	p = 0.72	p = 0.98	p = 0.82	p = 0.63	p = 0.23	p = 0.6	p = 0.07	p = 0.22	**p= 0.01**
AD biomarker:									
- positive	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 2-3)	5 (IQR 3-5)	1 (IQR 1-1.5)	14.6 (SD 6.6)
- negative	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	3 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 3-4)	3 (IQR 2-4.5)	1 (IQR 1-2)	14.15 (SD 2.96)
	z = 0.46	z = 0.41	z = 0.06	z = 1.23	z = 1.41	z = 1.7	z = -1.41	z = -0.31	t = -0.23
	p = 0.64	p = 0.68	p = 0.94	p = 0.21	p = 0.15	p = 0.08	p = 0.15	p = 0.75	p =0.81
CCI									
- 1-3	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 2-4)	4 (IQR 3-5)	1 (IQR 1-1)	14.86 (SD 7.3)
- ≥4	2 (IQR 1-2)	1 (IQR 1-1)	1 (IQR 1-1)	2 (IQR 2-3)	1 (IQR 1-1)	3 (IQR 2-3)	5 (IQR 3-5)	1 (IQR 1-2)	14.17 (SD 4.4)
	z = 0.26	z = 1.09	z = 1.1	z = 0.91	z = 1.26	z = 0.87	z = -0.58	z = -1.48	t = 0.36
	p = 0.79	p = 0.27	p = 0.28	p = 0.36	p = 0.21	p = 0.38	p = 0.55	p = 0.13	p = 0.71

	Tolerability					Acceptability			Usability
	Pain	Burning	Headache	Tingling	Tinnitus	Comfort	Confidence	Use again	Objective KT
Polypharmacy - 0-4 - ≥5 meds	 2 (IQR 1-2) 2 (IQR 1-2) z = 0.23 p= 0.81	 1 (IQR 1-1) 1 (IQR 1-1) z = -0.28 p= 0.77	 1 (IQR 1-1) 1 (IQR 1-1) z = 0.03 p= 0.97	 2 (IQR 2-3) 2 (IQR 2-3) z = 0.14 p= 0.88	 1 (IQR 1-1) 1 (IQR 1-1) z = 0.70 p= 0.47	 3 (IQR 2-3) 3 (IQR 2-3) z = 1.11 p= 0.26	 4.5 (IQR 3-5) 5 (IQR 3-5) z = -0.11 p = 0.91	 1 (IQR 1-1) 2 (IQR 2-2) z = -2.64 **p= 0.01**	 14.75 (SD 6.1) 14.3 (SD 5.4) t = -0.22 p= 0.82
Smartphone - Yes - No	 2 (IQR 1-2) 2 (IQR 1-2) z = 0.96 p= 0.33	 1 (IQR 1-1) 1 (IQR 1-1) z = -0.34 p= 0.73	 1 (IQR 1-1) 1 (IQR 1-1) z = 0.67 p= 0.51	 2 (IQR 2-3) 2 (IQR 2-3) z = -0.42 p= 0.67	 1 (IQR 1-1) 1 (IQR 1-1) z = -0.92 p= 0.35	 3 (IQR 2-3) 3 (IQR 2-3) z = -0.24 p= 0.8	 4 (IQR 2-5) 5 (IQR 3-5) z = 0.81 p= 0.42	 1 (IQR 1-2) 1 (IQR 1-1.5) z = -0.51 p= 0.61	 12.91 (SD 5.49) 16.35 (SD 5.2) **t = 1.96** **p= 0.03**

Table 15 details tVNS tolerability, acceptability and usability associations and correlations


t = -2.62 p = 0.01
*
Kettle_test_scores
30
20
10
0
male
female
female - male
10
0
-10
Difference between means


t = 1.96 p= 0.03
*
Kettle_test_scores
30
20
10
0
smartphone_yes
smartphone_no
smartphone_no - smartphone_yes
10
0
-10
Difference between means



Figures 16 (A-C):Associations of KT scores with baseline demographic characteristics

Discussion

In conclusion, we found that the NEMOS © tVNS device was acceptable and tolerable in a cohort of participants with amnestic MCI. In general, participants experienced slightly more symptoms such as tingling or burning during active as compared to sham stimulation, and they found active stimulation more uncomfortable. However, 98% of participants would definitely or probably use the device again, indicating high acceptability of the device.

What was noteworthy, nonetheless, was participants' lack of personal confidence using the device, and objective lack of skills using the device. This was particularly apparent in placing the electrodes in the correct part of the auricle for active stimulation, or troubleshooting common issues such as lack of device contact. More extended teaching sessions familiarising

participants with the device would be needed to ensure accurate use of the device for future trials. Potentially an audio-visual teaching aid that participants could refer to in their own time to remind them of specific issues would also be a useful adjunct, especially if they are to use this device outside of a hospital or laboratory setting. Given that the population studied has cognitive impairment, including a family member or friend in device application training and use may also be fruitful.

Though this trial was not powered to detect a difference between groups, it was interesting to note that men who have and use smartphones were the most independent group using the device. This may indicate that previous familiarity with technology and devices may transfer to independence using a new device. There was no difference in independence or subjective confidence in participants with AD biomarker positivity or high degrees of comorbidity indicating that a wide variety of patients could be included in future trials of neuromodulation devices.

Chapter 5: Exploring the Effects of Acute tVNS on Domain-Specific Cognitive Tasks in a population with MCI

Introduction

The diagnosis of MCI is based on the assessment of objective loss of cognitive function in the absence of loss of functional ability, as assessed by a person's ability to perform their ADLs (see Chapter 3 for an in-depth discussion on MCI, subtypes, and pathological substrates). An assessment of cognitive function usually encompasses the following: episodic memory (immediate and delayed), visuospatial or constructional ability, language, attention and executive function [494]. MCI may be diagnosed in the presence of objective loss of ability in one (single domain) or two or more (multi-domain) areas of tested cognitive ability [379]. If the person's episodic delayed memory is impaired, by definition this is diagnosed as amnestic MCI [495].

Amnestic MCI [496], and more specifically multi-domain amnestic MCI carries the highest risk of conversion to dementia [497] so these diagnostic categories have prognostic importance. It is also important to note that not all persons with MCI develop dementia, with up to 50% of those with MCI stable at 5 years [379] and up to 16% reverting to normal cognition [498]. This state of plasticity or flux is noteworthy, as it suggests that interventions, such as a targeted Brain Health interventions or non-invasive brain stimulation could help plateau cognitive impairment or even reverse it over time.

Discrete cognitive processes are associated with distinct neuroanatomical cortical and subcortical regions, one of the most established being the hippocampus, precuneus,

posterior cingulate and posterior parietal cortex which form part of the functional circuit involved in episodic memory and are classically affected by diseases such as AD [57,499]. Attention is mediated via both forebrain and midbrain "top-down" interactions, involving salience and the ability to pay selective attention [500,501]. Executive function, which involves goal-setting, flexibly task-shifting, self-monitoring and inhibition, involves the pre-frontal cortex, basal ganglia and thalamus [205,502,503]. Appropriate and functional language relies on the operational interplay of Broca's area in the posterior frontal lobe, Wernicke's area of the posterior superior temporal lobe and the angular gyrus to integrate language-related auditory, visual or sensory information [504,505]. Accurate visuospatial perception and integration relies on the occipital lobes and the "what" pathway of the parietal lobe bilaterally [506,507]. These are notable as we consider the potential effects of tVNS, and the neuroanatomical substrates associated with any beneficial effect.

Improved cognition has been noted in populations treated with iVNS for epilepsy and depression [136,202,213,214,218,221,508,509] and a one -year pilot study found that iVNS devices stabilised or improved cognitive performance in participants with AD [203,204]. The cognitive benefits of tVNS in CU populations have been described in detail in Chapter 2, in summary language [241,255], executive function [226,227,229,510] and associative memory [223] all improved during active stimulation in distinct study groups, and metanalysis in this area confirmed the largest effect size for executive function improvements [225]. To our knowledge there is one published study investigating the cognitive effects of tVNS in a population with MCI, which showed over 24 weeks there were improvements in the MOCA- B (designed for populations with low literacy levels) between active and sham stimulation (p=0.03, no effect size given) [224].

Persons with MCI have been noted to have reduced cerebral oxygenation when measured via non-invasive measurements [350] such as NIRS, which uses light-emitting diodes to give an approximation of the balance of oxygenated and deoxygenated blood in a specific tissue, termed the TSI [511,512]. Cerebral TSI estimates in persons with MCI are generally lower than CU persons, but higher than those with established dementia for whom cell atrophy and shrinkage has likely already occurred [513,514]. TSI has been shown to increase during tVNS [515], however TSI changes and tVNS has not to our knowledge ever been tested in a population with MCI.

It is also noteworthy that in the tVNS trials referenced in Chapter 2 and in the metanalysis on this topic [225], the trial design was either a parallel design (with two groups, matched for age and sex, assigned either active or sham stimulation) or some involved internal crossover designs. None accounted for the potential bias of wearing a nerve stimulation device, i.e. none compared outcomes of sham and active stimulation to baseline assessments, nested per individual. We conceptualised a three-way design in this trial (comparing baseline, sham and active conditions) to control for this unblinding bias of the participant wearing a nerve stimulation device. In this manner, any potential efficacy signal would have to be significant compared to baseline and the sham condition in this study.

Methods

Trial Design

Please see Chapter 4 for a detailed description of the Trial Design.

Study Setting

Please see Chapter 4 for a detailed description of the Study Setting.

Sample Size

Please see Chapter 4 for a detailed description of the sample size. Please note that due to the design of the study as a feasibility analysis of a neuromodulatory tVNS device in a population with MCI, a sample size of n=40 was chosen. This trial was not powered to detect between-group changes in cognitive assessments, and any positive results would need replication in an adequately powered sample in future trials.

Eligibility Criteria

Please see Chapter 4 for a detailed description of the eligibility criteria.

Device and Intervention

Please see Chapter 4 for a detailed description of the device and intervention.

Assessment of Domain Specific Cognitive Tasks during tVNS

VINCI-AD was designed as a feasibility study of tVNS device use in a population with MCI. Exploratory secondary analysis was planned to assess the potential benefits of acute tVNS on cognitive performance. Cognitive function was assessed under identical conditions in all participants at all three visits and encompassed the following assessments.

Associative Memory

The FNAT is cross-modal associative memory test [516,517] and was hosted on E-PRIME (Psychology Software Tools, Sharpsburg, USA). The participant was shown 30 face-name pairs. After a brief interlude (less than one minute) participants were then shown a combination of faces, some of which have already been displayed. The assessment involves both accurate recognition of recently displayed faces (Face Recall) and correct recall of associated names (Name Recognition). tVNS has been shown to boost associative memory as assessed by this task in CU older adults [223] but has not been assessed in those with MCI.

The FNAT consists of the following

- Learning phase: Participants were instructed to attend to a series of faces, each with an associated name. They were asked to remember each face-name pair. There were 30 learning trials containing either a male or female face (50/50) and with the name provided underneath each face. Each trial began with a fixation cross in the centre of the screen (Courier New font 24) for 1000ms. A male/female (randomly) and name (Courier New font 32) appeared on screen for 5000 miliseconds (ms). This was followed by a blank screen for 1000ms. After all 30 face/names trials were complete, participants were requested to wait for further instruction.
- Recall phase: Participants were instructed to indicate whether each of the subsequent faces were old i.e. part of the learning phase that they had seen) or new (a novel face). 60 faces (30 'old' and 30 'new') were presented (no name was provided). The face remained on screen until either 1 or 2 key was pressed. There was no time limit with this. If 2 was pressed (i.e. New), the participant moved onto the next trial. If 1 was pressed (i.e. Old), them the face appeared with a choice of 4

names underneath (one of which was correct). Participants had to press 1, 2, 3 or 4. Once a number was pressed (there was again no time limit), the participant moved onto the next trial.

Executive Function

The SART is a computer-based continuous performance response inhibition task [518] hosted on E-PRIME (Psychology Software Tools, Sharpsburg, USA) It is similar to Go/No-Go or Stop/Signal tasks whereby participants are required to withhold response to a single, infrequent target (the digit 3) presented amongst a background of frequent non-targets (0-2, 4-9) to which the participant does respond by pressing the space key on the computer keyboard. In the SART test, each digit appears for 300 ms, with an interval of 800 ms between digits. The cycle of digits 1 to 9 is repeated 23 times, giving a total of 207 trials. The SART test lasts for approximately 4 min. Participants are required to press a keyboard key as soon as possible for each digit presented. In practice, over the course of the test, many participants lose attention and commit mistakes [209]. Two types of mistakes can be detected in the data: errors of commission (i.e., responding to NO–GO trials), which reflect lapses of sustained attention; and errors of omission (i.e., failure to respond to GO trials), reflecting a break from task engagement, also corresponding to lapsing attention [519]

Spatial Navigation

SeaHero Quest ™ (SHQ) is an electronic tablet-based app (Sea Hero Quest; GLITCHERS © London, UK; University College London, London, UK) whereby participants were asked to complete 5 levels of increasing complexity of a nautical-themed spatial navigation task designed to assess speed and accuracy of navigating a boat to a map target [520,521]. The game primarily involves participants virtually navigating a boat through a series of waterways and

rivers to find a target. Before setting off, participants are provided with a map that shows their current location and to where they need to navigate (i.e. the target). In this task participants had to complete 5 different levels of increasing complexity. Levels 1 and 2 were also used as practice. There is no time limit to the task but unlocking a more difficult level is dependent on completing the previous one. The time (seconds) to reach the target is used to measure spatial learning.

Language and working memory

Language assessments included list learning, semantic fluency, list recall and list recognition assessments adapted from the RBANS battery as tVNS has demonstrated benefits in these domains in both healthy [241,255] and clinical populations [218,509]. Working memory was assessed via digit span forward and backward which was be undertaken as part of the adapted RBANS battery.

Assessment of TSI during Domain-Specific Cognitive Tasks and tVNS

In order to explore the potential effects that tVNS may have on cerebral oxygenation, participants wore the portable NIRS analysis device during all cognitive tasks so that the TSI could be measured continuously during cognitive tasks at baseline, during active and during sham stimulation.

Planned Secondary Aims and Planned Analysis

Exploratory secondary aims of this study, including the effects of tVNS on domain-specific cognition, were analysed using a mixed-effects model, with the cognitive test as the dependent variable in the model. Linear regression analysis was used to explore if baseline characteristics had a significant effect on cognitive outcomes. A mixed effects model was also used to analyse the differences between TSI under baseline, active and sham stimulation conditions.

Ethics Approval and Consent to Participate

Please see Chapter 4 for details of Research Ethics Committee approval.

Results

Associative Memory

Associative memory was assessed via the FNAT under baseline, active and sham conditions. All participants (n = 40) completed the FNAT at baseline but one did not complete the test under sham condition, while another one did not complete it under the active condition. The demographic and baseline characteristics of the participants have been described in Chapter 4.

Table 16 details the unadjusted analysis of the FNAT under baseline, active and sham condition, while Table 17 details the mixed effect model adjusted for covariates. The FNAT is analysed both in terms of the Face Recall (and associated Reaction Times [RTs]), and Name Recognition (and RTs). Q-q plots and the Shapiro Wilk (SW) test was used to test for normality of data distribution, and the presence of significant skew or outlier data. In the case of normally distributed data, a mixed effects model was used. In the case of a significant SW test ($p<0.05$) the natural log transformed data was used. As noted previously, for a results to be clinically meaningful, an effect should be observed both during active vs baseline and active vs sham stimulation, and no difference between baseline and sham stimulation seen. Adjustment was made for relevant covariates including age, sex, BMI, comorbidity count and polypharmacy. The natural log transformed data was used for RT's due to data skew.

There was no significant effect seen for Face Recall accuracy in unadjusted or adjusted models. Similarly, there was no significant difference in the log-RT for incorrect responses

during Face Recall during baseline, active or sham stimulation. The log-RT for correct responses was significantly quicker during active stimulation compared to sham (β= -0.15 [-0.27; -0.04]; z= -2.68 p= 0.01), however there was no difference between sham and baseline β= -0.08 [-0.21; 0.02]; z= -1.54; p= 0.12) or, importantly, between active and sham conditions (β= -0.03 [-0.11; 0.05]; z= -0.82; p= 0.42).

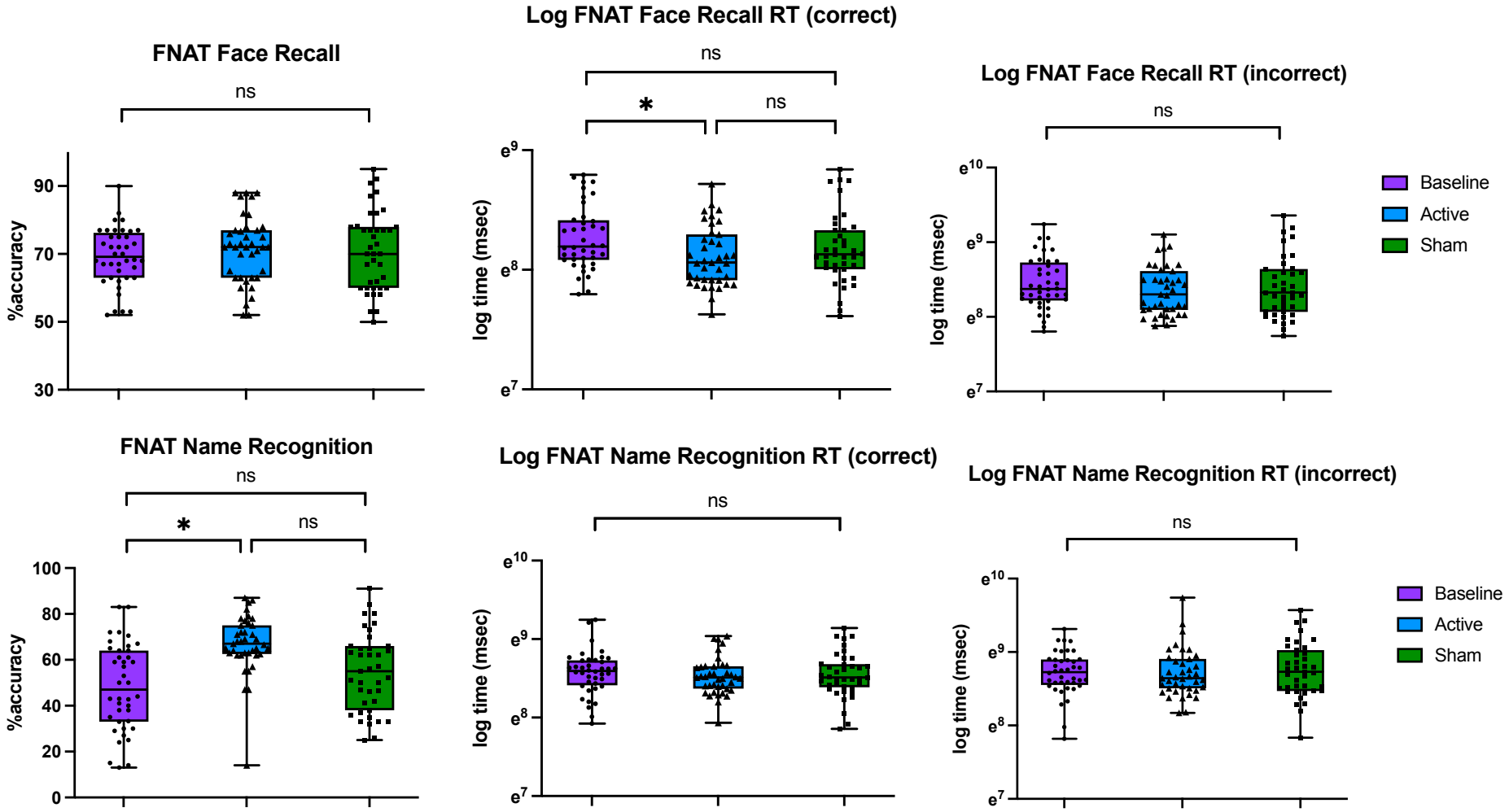
A significant effect was found during active stimulation for Name Recognition accuracy compared to baseline (β= 21.09 [14.04; 28.18], z= 5.83, p=0.01) however there was no effect seen between active and sham conditions (β= 7.33 [-1.67;16.54], z= 1.6, p= 0.11) or between baseline and sham conditions (β= 6.42 [-0.66; 13.5], z= 1.78, p= 0.08). There was no significant difference found in log-RT during Name Recognition for either correct or incorrect responses.

	Mean (SD) or median (IQR) of test results			*Mixed effects model, unadjusted*		
	Baseline	Active	Sham	Baseline vs Active	Baseline vs. Sham	Active v Sham
FNAT Facial Recall Accuracy	69.17 SD 8.63	72.16 SD 10.52	70.98 SD 11.68	β= 2.93 95% CI (-1.51- 7.45) z= 1.3 p=0.19	β= 1.89 95% CI (-2.69 – 6.3) z=0.79 p=0.43	β= 0.56 95% CI (-1.67 – 2.89) z= 0.51 p= 0.62
Log_FNAT Facial Recall RT correct	8.26 (SD 0.26)	8.11 (SD 0.24)	8.17 (SD 0.29)	β= -0.15 95% CI (-0.27 - -0.04) z= -2.59 p= 0.01*	β= -0.09 95% CI (-0.21 – 0.02) z= -1.48 p= 0.14	β= -0.03 95% CI (-0.11- 0.05) z= -0.82 p= 0.42
Log_FNAT Facial Recall RT incorrect	8.44 (SD 0.34)	8.34 (SD0.32)	8.38 (SD0.41)	β= -0.09 95% CI (-0.25 – 0.06) z= -1.16 p= 0.24	β= -0.05 95% CI (-0.21 – 0.14) z= -0.65 p= 0.52	β= -0.02 95% CI (-0.09 – 0.05) z=-0.5 p= 0.62
FNAT_ Name Recognition Accuracy	47 (IQR 33.1 – 64)	68 (IQR 63 – 78)	55 (IQR 38 – 66)	β= 21.01 95% CI (13.59 – 28.43) z= 5.55 p= 0.01*	β= 6.45 95% CI (-0.96 – 13.87) z= 1.7 p=0.09	β= 7.28 95% CI (-1.76 – 16.23) z= 1.59 p= 0.11
Log_FNAT Name Recognition RT (correct)	8.47 (SD 0.59)	8.52 (SD 0.23)	8.54 (SD 0.28)	β= 0.04 95% CI (-0.13 – 0.22) z= 0.5 p= 0.62	β= 0.06 95% CI (-0.11 – 0.24) z= 0.7 p= 0.48	β= -0.01 95% CI (-0.09 – 0.08) z=-0.19 p=0.85
Log_FNAT Name Recognition RT (incorrect)	8.64 (SD 0.55)	8.71 (SD 0.32)	8.75 (SD 0.36)	β= 0.07 95% CI (-0.11 – 0.25) z= 0.73 p= 0.47	β= 0.11 95% CI (-0.07 – 0.29) z= 1.17 p= 0.25	β= -0.02 95% CI (-0.11 – 0.07 z= -0.43 p= 0.66

Table 16 details the unadjusted mixed effects model analysing the effect of stimulation on Face Name Association Task (FNAT) indices

	Mean (SD) or median (IQR) of test results			*Mixed effects model, adjusted for age, sex, BMI, comorbidity, polypharmacy*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
FNAT Facial Recall Accuracy	69.17 SD 8.63	72.16 SD 10.52	70.98 SD 11.68	β= 3.16 95% CI (-0.77 – 7.06) z=1.56 p= 0.12	β= 1.8 95% CI (-2.09 – 5.66) z= 0.91 p=0.36	β= 0.68 95% CI (-1.28 – 2.66) z=0.68 p=0.49
Log_FNAT Facial Recall RT correct	8.26 (SD -.26)	8.11 (SD 0.24)	8.17 (SD 0.29)	β= -0.15 95% CI (-0.27 – -0.04) z= -2.68 p= 0.01*	β= -0.08 95% CI (-0.21 – 0.02) z= -1.54 p= 0.12	β= -0.03 95% CI (-0.11 – 0.05) z=-0.8 p= 0.42
Log_FNAT Facial Recall RT incorrect	8.44 (SD 0.34)	8.34 (SD0.32)	8.38 (SD0.41)	β= -0.09 95% CI (-0.23 – 0.06) z=-1.16 p= 0.25	β= -0.05 95% CI (-0.2 – 0.09) z= -0.69 p= 0.49	β= -0.07 95% CI (-0.09 – 0.06) z= -0.47 p= 0.64
FNAT Name Recognition Accuracy	47 (IQR 33.1 – 64)	68 (IQR 63 – 78)	55 (IQR 38 – 66)	β= 21.09 95% CI (14.04 – 28.18) z= 5.83 p=0.01*	β= 6.42 95% CI (-0.66 – 13.5) z= 1.78 p= 0.08	β= 7.33 95% CI (-1.67 – 16.54) z= 1.6 p= 0.11
Log_FNAT Name Recognition RT (correct)	8.47 (SD 0.59)	8.52 (SD 0.23)	8.54 (SD 0.28)	β= 0.04 95% CI (-0.12 – 0.21) z= 0.49 p= 0.62	β= 0.06 95% CI (-0.11 – 0.23) z= 0.71 p= 0.48	β= -0.01 95% CI (-0.09 – 0.07) z= -0.21 p= 0.83
Log_FNAT Name Recognition RT (incorrect)	8.64 (SD 0.55)	8.71 (SD 0.32)	8.75 (SD 0.36)	β= 0.07 95% CI (-0.11 – 0.24) z= 0.76 p= 0.44	β= 0.11 95% CI (-0.07 – 0.28) z= 1.19 p= 0.23	β= -0.02 95% CI (-0.11 – 0.07) z= -0.43 p= 0.66

Table 17 details the mixed effects model of the effect of stimulation on Face Name Association Task (FNAT) indices, adjusted for covariates



Figures 17 (A-F) graphically depict the results of the mixed effects models for the FNAT indices shown in Table 17

Executive Function

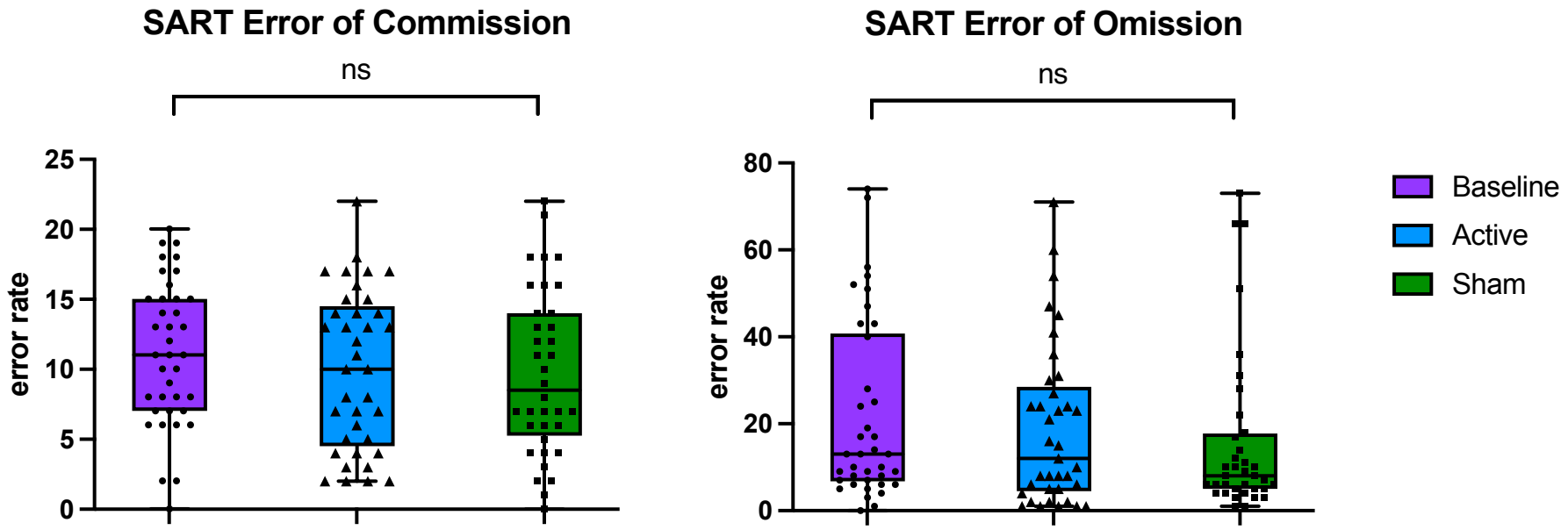
The SART test was used to assess executive function under baseline, active and sham conditions. There was one participant who could not complete the SART test due to it causing eye strain and headache, so the total number of SART assessments under baseline condition was n=39, with n=38 completing it under active condition and n=38 completing it under sham condition. Table 18 details the unadjusted analysis of the SART cognitive task under baseline, active and sham conditions, while Table 19 details the mixed effects models adjusted for covariates. There were no significant differences in the total number pressed, the overall RTs, the Error Of Commission (EOC) rate, the Error Of Omission (EOO) rate or the EOC RT during baseline active or sham conditions.

	Mean (SD) or median (IQR) of test results			*Mixed effects model, unadjusted*		
	Baseline	Active	Sham	Baseline vs Active	Baseline vs. Sham	Active v Sham
SART Total No pressed (absolute)	189.42 (SD 21.9)	187 (SD 29.97)	193.75 (SD 20.12)	β= -2.45 95% CI (-13.31 – 8.47) z= -0.44 p= 0.66	β= 4.32 95% CI (-6.64 – 15.29) z= 0.77 p= 0.43	β= -3.37 95% CI (-8.89 – 2.15) z= -1.2 p= 0.23
SART RT total (sec)	478 (IQR 372.7 – 582.4)	480.8 (IQR 359.8 – 559.2)	452.9 (IQR 372.3 – 520.5)	β= 0.31 95% CI (-48.09 – 48.71) z= 0.01 p= 0.99	β= -20.95 95% CI (-69.71 – 27.78) z= -0.84 p= 0.39	β= 10.58 95% CI (-13.96 – 35.14) z= 0.85 p= 0.39
SART EOO (error rate)	21.78 (SD 20.77)	23 (SD 28.53)	15.9 (19.23)	β= -1.21 95% CI (-3.68 – 1.26) z= -0.96 p= 0.34	β= -1.48 95% CI (-3.98 – 1.01) z= -1.17 p= 0.24	β= 0.13 95% CI (-1.13 – 1.39) z= 0.21 p= 0.87
SART EOC (error rate)	11.2 (5.12)	10 (SD 5.63)	9.72 (SD 5.88)	β= 1.21 95% CI (-9.15 – 11.54) z= 0.23 p= 0.82	β= -5.81 95% CI (-16.25 – 4.62) z= -1.09 p= 0.27	β= 3.5 95% CI (-1.76 – 8.76) z= 1.31 p= 0.19
SART EOC RT (msec)	398.65 (IQR 323.4 – 616.5)	404.2 (IQR 330.6 – 573.5)	398.3 (IQR 327.9 – 520.5)	β= 12.73 95% CI (-70.03 – 95.51) z= 0.3 p= 0.76	β= -34.29 95% CI (-118.24 – 49.65) z= -0.8 p= 0.42	β= 23.41 95% CI (-18.57 – 65.41) z= 1.09 p= 0.27

Table 18 details the unadjusted mixed effects model analysing the effect of stimulation on Sustained Attention Response Task (SART) indices

	Mean (SD) or median (IQR) of test results			*Mixed effects model, adjusted for age, sex, BMI, comorbidity, polypharmacy*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
SART Total No pressed (absolute)	189.42 (SD 21.9)	187 (SD 29.97)	193.75 (SD 20.12)	β= -2.49 95% CI (-12.59 – 7.59) z= -0.49 p= 0.62	β= 3.91 95% CI (-6.25 – 14.07) z= 0.75 p= 0.45	β= -3.2 95% CI (-8.31 – 1.91) z= -1.23 p= 0.22
SART RT total (sec)	478 (IQR 372.7 – 582.4)	480.8 (IQR 359.8 – 559.2)	452.9 (IQR 372.3 – 520.5)	β= -1.12 95% CI (-46.26 – 44.01) z= -0.05 p= 0.96	β= -20.86 95% CI (-66.31 – 24.57) z= -0.9 p= 0.36	β= 9.82 95% CI (-13.45 – 32.71) z= 0.84 p= 0.4
SART EOO(error rate)	21.78 (SD 20.77)	23 (SD 28.53)	15.9 (19.23)	β= -1.32 95% CI (-3.45 – 0.81) z= -1.22 p= 0.22	β= -1.68 95% CI (-3.82 – 0.46) z= -1.54 p= 0.12	β= 0.16 95% CI (-0.92 – 1.26) z= 0.3 p= 0.76
SART EOC (error rate)	11.2 (5.12)	10 (SD 5.63)	9.72 (SD 5.88)	β= 1.17 95% CI (-8.53 – 10.87) z= 0.24 p= 0.81	β= -5.58 95% CI (-15.35 – 4.18) z= -1.12 p= 0.26	β= 3.37 95% CI (-1.55 – 8.2) z= 1.34 p= 0.18
SART EOC RT (msec)	398.65 (IQR 323.4 – 616.5)	404.2 (IQR 330.6 – 573.5)	398.3 (IQR 327.9 – 520.5)	β= 12.65 95% CI (-68.29 – 93.61) z= 0.31 p= 0.75	β= -36.01 95% CI (-118.06 – 46.76) z= -0.86 p= 0.39	β= 24.54 95% CI (-16.76 – 65.22) z= 1.16 p= 0.25

Table 19 details the mixed effects model analysing the effect of stimulation on Sustained Attention Response Task (SART) indices, adjusted for relevant covariates



Figures 18 (A,B) graphically depict the mixed effects models for the Error of Commission (EOC) rate and Error of Omission (EOO) rate of the SART under baseline active and sham stimulation conditions

Spatial Navigation

Spatial Navigation was assessed using the SHQ tablet-based application. As mentioned above, five levels were chosen, the first two (Level 1 and Level 2) are considered introductory levels to familiarise the participants with the application. The next three levels that were chosen for this study are Levels 6,7 and 8. The number of participants who completed the baseline SHQ assessment was n=39, with n=38 completing SHQ under active stimulation and n=38 completing SHQ under sham stimulation.

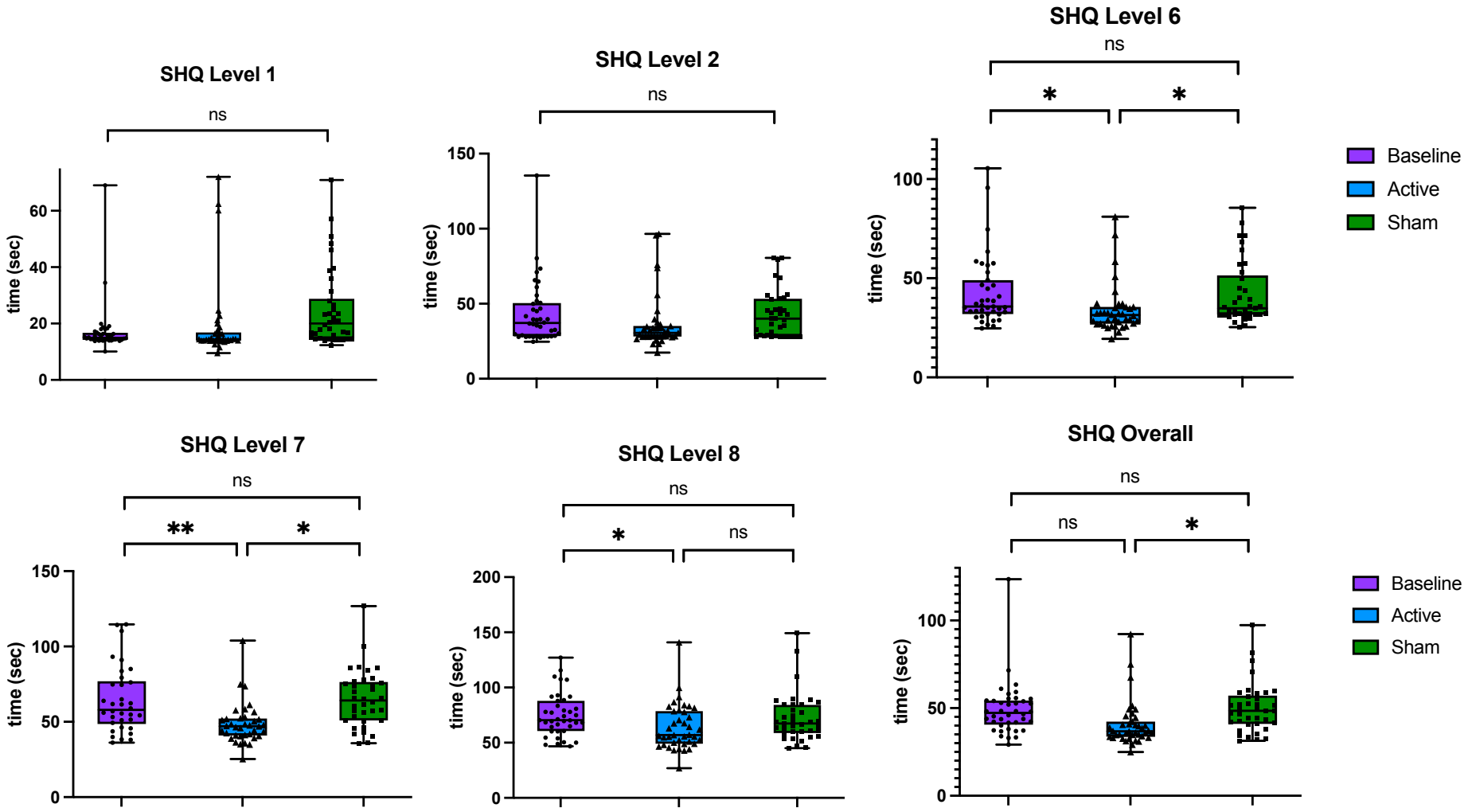
There were no significant differences found between the baseline, active and sham stimulation conditions for SHQ Level 1 or 2. However a significant finding was noted at Level 6, with the active stimulation condition scoring quicker time to target compared to both baseline condition (β= -8.76 [-14.91; -2.56], z= -2.78, p=0.01) and sham condition (β= -4.15 [-7.32; -0.99], z= -2.58, p=0.01) on adjusted mixed effects modelling. Similarly, at Level 7 participants scored quicker times to target during active stimulation compared to baseline (β= -15.82 [-23.96; -7.67], z= -3.81, p= 0.01) and active compared to sham stimulation (β= -8.22 [-13.07l -3.58], z= -3.44, p= 0.01). There was no significant effect observed at the most challenging level (Level 8) between active and sham stimulation (β= -4.23 [-8.91; 0.43], z= -1.78, p= 0.08). There was only an effect seen in the overall values during active compared to sham stimulation (β= -4.96 [-7.55; -1.83], z= -3.22, p= 0.02) but not active stimulation compared to baseline. Table 20 details the unadjusted analysis of the SHQ cognitive task under baseline, active and sham conditions, while Table 21 details the mixed effects model adjusted for covariates.

	Mean (SD) or median (IQR) of test results			*Mixed effects model, unadjusted*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
SHQ_L1 (sec to target)	18.63 SD 18.3	19.39 SD 15.025	24.95 SD 13.95	β= 0.75 95% CI (-6.17 – 7.65) z= 0.21 p= 0.83	β= 6.32 95% CI (-0.66 – 13.31) z= 1.77 p= 0.08	β= -2.76 95% CI (-6.29 – 0.76) z= -1.53 p= 0.13
SHQ_L2 (sec to target)	43.43 SD 20.83	36.743 SD 18.18	43.06 SD 15.94	β= -6.69 95% CI (-14.74 – 1.36) z= -1.63 p= 0.21	β= -0.37 95% CI (-8.53 – 7.78) z= -0.09 p= 0.92	β= -3.19 95% CI (-7.31 – 0.93) z= -1.52 p=0.13
SHQ_L6 (sec to target)	**42.82 SD 17.85**	**34.09 SD 12.47**	**42.25 SD 16.04**	**β= -8.72 95% CI (-15.62 - -1.83) z= -2.48 p= 0.02***	**β= -0.57 95% CI (-7.51 – 6.36) z= -0.16 p= 0.87**	**β= -4.09 95% CI (-7.62 - -0.57) z= -2.28 p=0.03***
SHQ_L7 (sec to target)	**64.23 SD 21.7**	**48.72 SD 13.61**	**65.26 SD 19.09**	**β= -15.51 95% CI (-23.92 - -7.08) z= -3.61 p= 0.01***	**β= 1.02 95% CI (-7.45 – 9.51) z= 0.24 p= 0.81**	**β= -8.29 95% CI -12.88 - -3.71) z= -3.54 p= 0.01***
SHQ_L8 (sec to target)	75.155 SD 19.56	63.91 SD 20.62	72.39 SD 22.06	β= -11.23 95% CI (-20.32 - -2.15) z= -2.42 p= 0.02*	β= -2.78 95% CI (-11.97 – 6.44) z= -0.59 p= 0.55	β= -4.29 95% CI (-8.96 – 0.36) z= -1.81 p= 0.07
SHQ_overall (sec to target)	49.05 SD 15.24	40.64 SD 12.72	46.98 SD 14.17	β= -8.41 95% CI (-14.54 - -2.27) z= -2.69 p=0.02*	β= 0.93 95% CI (-5.24 – 7.11) z= 0.3 p=0.76	β= -4.67 95% CI (-7.81 - -1.55) z= -2.93 p=0.03*

Table 20 details the unadjusted mixed effects model analysing the effect of stimulation on Sea Hero Quest (SHQ) indices

	Mean (SD) or median (IQR) of test results			*Mixed effects model, adjusted for age, sex, BMI, comorbidity, polypharmacy*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
SHQ_L1 (sec to target)	18.63 SD 18.3	19.39 SD 15.025	24.95 SD 13.95	β= 1.01 95% CI (-5.39 – 7.42) z= 0.31 p= 0.75	β= 6.51 95% CI (-0.05 – 12.94) z= 1.98 p=0.08	β= -2.72 95% CI (-5.99 – 0.54) z= -1.64 p= 0.12
SHQ_L2 (sec to target)	43.43 SD 20.83	36.743 SD 18.18	43.06 SD 15.94	β= -6.71 95% CI (-13.87 – 0.65) z= -1.78 p=0.08	β= 0.05 95% CI (-7.32 – 7.41) z= 0.99 p=0.89	β= -3.36 95% CI (-7.08 -0.36) z= -1.77 p=0.08
SHQ_L6 (sec to target)	**42.82 SD 17.85**	**34.09 SD 12.47**	**42.25 SD 16.04**	**β= -8.76 95% CI (-14.91 - -2.56) z= -2.78 p=0.01***	**β= -0.45 95% CI (-6.65 – 5.75) z= -0.14 p= 0.88**	**β= -4.15 95% CI ((-7.32 - -0.99) z= -2.58 p=0.01***
SHQ_L7 (sec to target)	**64.23 SD 21.7**	**48.72 SD 13.61**	**65.26 SD 19.09**	**β= -15.82 95% CI (-23.96 - -7.67) z= -3.81 p= 0.01****	**β= 0.78 95% CI (-7.39 – 8.98) z= 0.19 p= 0.85**	**β= -8.22 95% CI (-13.07 - -3.58) z= -3.44 p= 0.01***
SHQ_L8 (sec to target)	75.155 SD 19.56	63.91 SD 20.62	72.39 SD 22.06	β= -11.22 95% CI (-20.08 - -2.37) z= -2.49 p= 0.02*	β= -2.87 95% CI (-11.85 – 6.11) z= -0.63 p= 0.53	β= -4.23 95% CI (-8.91 – 0.43) z= -1.78 p= 0.08
SHQ_overall (sec to target)	49.05 SD 15.24	40.64 SD 12.72	46.98 SD 14.17	β= -6.54 95% CI (-13.54 – 2.34) z= -2.13 p= 0.12	β= 1.12 95% CI (-4.51 – 6.76) z= 0.39 p=0.69	β= -4.96 95% CI (-7.55 - -1.83) z= -3.22 p= 0.02*

Table 21 details the mixed effects model analysing the effect of stimulation on Sea Hero Quest (SHQ) indices, adjusted for relevant covariates



Figures 19 (A-F) graphically depict the results of the mixed effects models for the SHQ indices shown in Table 21

Language and Working Memory

An abridged RBANS was undertaken to analyse verbal list learning, semantic categorical fluency, digit span forward and backward, list recall and list recognition under baseline, active and sham stimulation conditions. To avoid a carryover or learned effect, a different list of nouns to assess verbal learning and a different category to assess semantic fluency (e.g. fruits and vegetables, or animals) were used at subsequent trial visits. In total, baseline language and working memory assessments were undertaken for n=40 participants, with sham stimulation for n=39 and active stimulation for n=39.

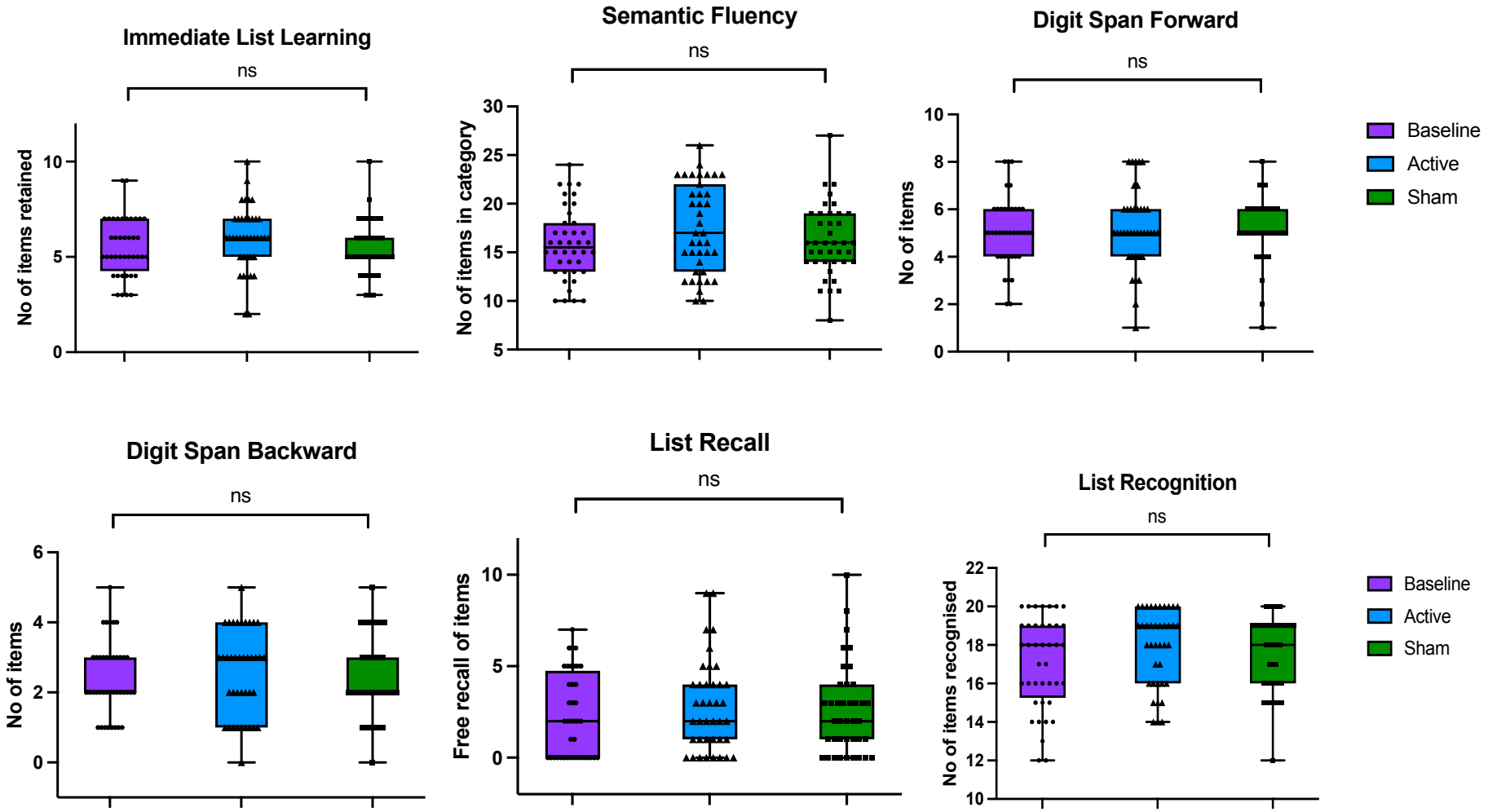
Table 22 details the unadjusted analysis of the language and working memory cognitive assessments under baseline, active and sham conditions, while Table 23 details the analysis of the mixed effects model adjusted for covariates. There were no significant effects seen during baseline, active or sham stimulation for the assessment of list learning, semantic categorical fluency, digit span forward or backward, list recall or list recognition.

	Mean (SD) or median (IQR) of test results			*Mixed effects model, unadjusted*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
Immediate list learning (absolute)	5.525 SD 1.52	6 SD 1.67	5.46 SD 1.45	β=0.47 95% CI (-0.19 – 1.41) z=1.38 p= 0.16	β= -0.06 95% CI (-0.73 – 0.61) z=-0.18 p= 0.85	β=0.26 95% CI (-0.07 – 0.61) z= 1.55 p= 0.12
Semantic fluency (absolute)	15.85 SD 3.68	17.46 SD 4.6	16.17 SD 3.68	β=1.51 95% CI (-0.13 – 3.34) z=1.81 p= 0.07	β=0.32 95% CI (-1.41 – 2.07) z=0.37 p= 0.71	β=0.64 95% CI (-0.24 – 1.52) z= 1.42 p= 0.15
Digit span forward (absolute)	5.075 SD 1.44	5.23 SD 1.62	5.15 SD 1.31	β=0.15 95% CI (-0.48 – 0.79) z= 0.48 p= 0.63	β=0.07 95% CI (-0.48 – 0.79) z= 0.24 p= 0.81	β= 0.04 95% CI (-0.28 – 0.36) z= 0.23 p= 0.81
Digit span backward (absolute)	2.4 SD 1.1	2.56 SD 1.23	2.44 SD 1.16	β=0.16 95% CI (-0.33 -0.66) z=0.65 p= 0.52	β=0.04 95% CI (-0.46 – 0.53) z=0.14 p= 0.89	β=0.06 95% CI (-0.18 – 0.32) z= 0.5 p= 0.62
List recall (absolute)	2.25 SD 2.3	2.82 SD 2.47	2.56 SD 2.44	β=0.57 95% CI (-0.47 – 1.61) z=1.07 p= 0.28	β=0.31 95% CI (-0.73 – 1.35) z= 0.59 p= 0.56	β= 0.12 95% CI (-0.39 – 0.65) z=0.48 p= 0.63
List recognition (absolute)	17.075 SD 2.37	17.92 SD 1.97	17.58 SD 1.94	β=0.84 95% CI (-0.07 – 1.76) z=1.81 p= 0.08	β= 0.51 95% CI (-0.41 – 1.43) z= 1.1 p= 0.27	β=0.16 95% CI (-0.29 – 0.63) z= 0.7 p= 0.48

Table 22 details the unadjusted mixed effects model analysing the effect of stimulation on language and working memory indices

	Mean (SD) or median (IQR) of test results			*Mixed effects model, adjusted for age, sex, BMI, comorbidity, polypharmacy*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
Immediate list learning (absolute)	5.53 SD 1.52	6 SD 1.67	5.46 SD 1.45	β=0.48 95% CI (-0.17 – 1.14) z= 1.43 p= 0.15	β=-0.05 95% CI (-0.72 – 0.61) z=-0.18 p= 0.86	β=0.27 95% CI (-0.06 – 0.61) z=1.59 p= 0.11
Semantic fluency (absolute)	15.85 SD 3.68	17.46 SD 4.6	16.17 SD 3.68	β=1.66 95% CI (-0.33 – 3.29) z=1.79 p= 0.09	β=0.33 95% CI (-1.29 – 1.96) z=0.4 p= 0.69	β= 0.66 95% CI (-0.15 – 1.49) z=1.58 p= 0.11
Digit span forward (absolute)	5.08 SD 1.44	5.23 SD 1.62	5.15 SD 1.31	β=0.16 95% CI (-0.44 – 0.78) z= 0.53 p= 0.59	β=0.09 95% CI (-0.52 – 0.71) z= 0.28 p= 0.78	β=0.04 95% CI (-0.27 – 0.35) z= 0.25 p= 0.81
Digit span backward (absolute)	2.4 SD 1.1	2.56 SD 1.23	2.44 SD 1.16	β=0.17 95% CI (-0.29 – 0.65) z= 0.73 p= 0.46	β=0.04 95% CI (-0.43 – 0.51) z= 0.17 p= 0.86	β=0.06 95% CI (-0.17 – 0.31) z= 0.56 p= 0.57
List recall (absolute)	2.25 SD 2.3	2.82 SD 2.47	2.56 SD 2.44	β= 0.56 95% CI (0.46 – 1.59) z= 1.08 p= 0.28	β= 0.31 95% CI (-0.72 – 1.33) z= 0.58 p= 0.59	β= 0.13 95% CI (-0.39 – 0.64) z= 0.49 p= 0.63
List recognition (absolute)	17.08 SD 2.37	17.92 SD 1.97	17.58 SD 1.94	β=0.87 95% CI (-0.03 – 1.71) z= 2.04 p= 0.09	β=0.52 95% CI (-0.31 – 1.36) z= 1.23 p= 0.28	β= 0.17 95% CI (-0.28 – 0.63) z= 0.74 p= 0.46

Table 23 details the mixed effects model analysing the effect of stimulation on language and working memory indices, adjusted for covariates



Figures 20 (A-F) graphically depict the results of the mixed effects models for the Language and Working Memory indices shown in Table 23

TSI during cognitive tasks under baseline, active and sham tVNS stimulation

The TSI is a measure of tissue oxygenation and has been used to approximate cerebral oxygenation in dynamic contexts. However, one of the limitations of NIRS-TSI technology is the degree of intra-subject variability (both repeated measures within one day, and between days) that exists in various cohorts [522,523]. To overcome this, the relative changes in TSI per assessment within-individual are used instead of absolute TSI values per cognitive task.

Table 24 details the unadjusted mixed effects model analysis of the min, max and mean (with standard deviation [SD]) TSI values per cognitive task under baseline, active and sham stimulation conditions, while Table 25 details the analysis adjusted for relevant covariates. The values were normally distributed. There was no significant effect of active or sham tVNS on TSI values, when analysed with a mixed effects model, both unadjusted, and adjusted for relevant covariates.

	Mean (SD) or median (IQR) of test results			Mixed effects model, unadjusted		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
Associative Memory						
FNAT TSI mean	67.9 SD 3.92	66.56 SD 4.02	67.09 SD 3.92	β=-1.36 95% CI (-3.15 – 0.43) z= -1.49 p= 0.14	β=-0.81 95% CI (-2.57 – 0.95) z= -0.9 p= 0.36	β=-0.26 95% CI (-1.16 – 0.63) z=-0.58 p= 0.56
FNAT TSI min	65.97 SD 4.13	64.79 SD 4.32	65.3 SD 4.24	β=-1.17 95% CI (-3.09 – 0.73) z= -1.2 p=0.22	β=-0.61 95% CI (-2.51 – 1.27) z= -0.64 p= 0.52	β=-0.27 95% CI (-1.22 – 0.68) z= -0.56 p= 0.57
FNAT TSI max	69.34 SD 3.8	67.91 SD 3.71	68.45 SD 3.84	β= -1.43 95% CI (-3.15 – 0.27) z= -1.64 p= 0.1	β=-0.88 95% CI (-2.51 – 0.81) z= -1.03 p= 0.3	β=-0.26 95% CI (-1.12 – 0.59) z= -0.6 p=0.54
Executive Function						
SART TSI mean (SD)	68.66 SD 3.75	67.34 SD 3.61	67.84 SD 3.91	β=-1.32 95% CI (-3.02 – 0.38) z= -1.52 p= 0.12	β=-0.82 95% CI (-2.52 – 0.88) z= -0.94 p= 0.34	β=-0.25 95% CI (-1.11 – 0.61) z= -0.57 p= 0.57
SART TSI min (range, SD)	67.3 SD 4.05	65.95 SD 4.04	66.56 SD 4.16	β= -1.34 95% CI (-3.19 – 0.51) z= -1.43 p=0.15	β=-0.74 95% CI (-2.57 – 1.01) z= -0.79 p= 0.43	β=-0.3 95% CI (-1.23 – 0.64) z= -0.63 p=0.52
SART TSI max (range, SD)	69.63 SD 3.62	68.41 SD 3.48	68.79 SD 3.78	β=-1.23 95% CI (-2.87 – 0.41) z= -1.47 p=0.14	β=-0.85 95% CI (-2.48 – 0.79) z= -1.01 p=0.31	β=-0.19 95% CI (-1.02 – 0.64) z= -0.45 p= 0.65

Spatial Navigation						
SHQ TSI mean	68.21 SD 3.77	66.89 SD 3.5	67.28 SD 4.14	β=-1.31 95% CI (-3.04 – 0.41) z= -1.5 p=0.13	β=-0.92 95% CI (-2.64-0.79) z= -1.05 p= 0.29	β=-0.19 95% CI (-1.07 – 0.67) z=-0.44 p= 0.65
SHQ TSI min	66.71 SD 4.14	65.42 SD 3.88	65.88 SD 4.51	β= -1.28 95% CI (-3.17 – 0.62) z= -1.33 p= 0.18	β=-0.82 95% CI (-2.75 – 1.07) z= -0.85 p= 0.39	β= -0.23 95% CI (-1.19 – 0.73) z= -0.47 p= 0.63
SHQ TSI max	69.46 SD 3.54	68.05 SD 3.34	68.41 SD 4.01	β=-1.41 95% CI (-3.05 – 0.23) z= -1.68 p= 0.09	β=-1.04 95% CI (-2.69 – 0.61) z=-1.24 p= 0.21	β=-0.18 95% CI (-1.02 – 0.66) z= -0.43 p= 0.67
Language and Working Memory						
Cognitive TSI mean	68.28 SD 3.96	66.94 SD 3.61	67.07 SD 3.79	β=-1.34 95% CI (-3.05 – 0.37) z= -1.53 p= 0.13	β=-1.21 95% CI (-2.92 – 0.51) z= -1.38 p= 0.16	β=-0.06 95% CI (-0.94 – 0.81) z=-0.15 p= 0.88
Cognitive TSI min	66.66 SD 4.29	65.41 SD 3.91	65.43 SD 4.29	β= -1.24 95% CI (-3.13 – 0.64) z= -1.29 p= 0.19	β=-1.22 95% CI (-3.11 – 0.68) z= -1.28 p= 0.2	β=-0.01 95% CI (-0.96 – 0.95) z=-0.01 p= 0.98
Cognitive TSI max	69.56 SD 3.9	68.47 SD 3.29	69.48 SD 6.58	β= -1.09 95% CI (-3.26 – 1.07) z= -0.99 p= 0.32	β= -0.08 95% CI (-2.25 – 2.08) z= -0.08 p= 0.93	β=-0.51 95% CI (-1.5-0.58) z= -0.9 p= 0.36

Table 24 details the unadjusted analysis of the min, mean and max TSI values per cognitive task under baseline, active and sham stimulation

	Mean (SD) or median (IQR) of test results			*Mixed effects model, adjusted for age, sex, BMI, comorbidity, polypharmacy, AD status, smoking status*		
	Baseline	Active	Sham	Baseline v Active	Baseline v Sham	Active v Sham
Associative memory						
FNAT TSI mean	67.9 SD 3.92	66.56 SD 4.02	67.09 SD 3.92	β=-1.38 95% CI (-3.08 – 0.32) z= -1.58 p= 0.11	β=-0.81 95% CI (-2.49 – 0.87) z= -0.94 p= 0.34	β= -0.03 95% CI (-0.15 – 0.09) z= -0.53 p= 0.59
FNAT TSI min	65.97 SD 4.13	64.79 SD 4.32	65.3 SD 4.24	β= -1.19 95% CI (-3.02 – 0.62) z= -1.29 p= 0.19	β=-0.61 95% CI (-2.4 – 1.19) z= -0.66 p= 0.51	β=-0.28 95% CI (-1.19 – 0.63) z=-0.62 p=0.53
FNAT TSI max	69.344 SD 3.8	67.91 SD 3.71	68.45 SD 3.84	β=-1.44 95% CI (-3.08 – 0.18) z=-1.73 p= 0.08	β= -0.89 95% CI (-2.54 – 0.71) z= -1.09 p= 0.27	β= -0.26 95% CI (-1.08 – 0.55) z=-0.63 p= 0.52
Executive Function						
SART TSI mean (SD)	68.66 SD 3.75	67.34 SD 3.61	67.84 SD 3.91	β=-1.35 95% CI (-2.97 – 0.26) z=-1.63 p= 0.1	β=-0.88 95% CI (-2.51 – 0.73) z= -1.07 p= 0.28	β=-0.23 95% CI (-1.05 – 0.59) z= -0.55 p= 0.58
SART TSI min (range, SD)	67.3 SD 4.05	65.95 SD 4.04	66.56 SD 4.16	β=-1.38 95% CI (-3.14 – 0.38) z= -1.53 p= 0.12	β= -0.79 95% CI (-2.56 – 0.96) z= -0.89 p= 0.37	β=-0.29 95% CI (-1.19 – 0.61) z= -0.64 p= 0.52
SART TSI max (range, SD)	69.63 SD 3.62	68.41 SD 3.48	68.79 SD 3.78	β=-1.25 95% CI (-2.81 – 0.31) z= -1.58 p= 0.11	β= -0.91 95% CI (-2.47 – 0.64) z= -1.15 p= 0.24	β=-0.16 95% CI (-0.95 – 0.62) z= -0.42 p= 0.67

Spatial Navigation						
SHQ TSI mean	68.21 SD 3.77	66.89 SD 3.5	67.28 SD 4.14	β= -1.34 95% CI (-2.99- 0.29) z= -1.61 p= 0.11	β=-0.95 95% CI (-2.61 – 0.68) z= -1.14 p= 0.25	β=-0.19 95% CI (-1.03 – 0.64) z= -0.45 p= 0.64
SHQ TSI min	66.71 SD 4.14	65.42 SD 3.88	65.88 SD 4.51	β= -1.29 95% CI (-3.1 – 0.51) z= -1.41 p= 0.16	β=-0.82 95% CI (-2.62 – 0.98) z= -0.89 p= 0.27	β=-0.23 95% CI (-1.15 – 0.68) z= -0.51 p= 0.61
SHQ TSI max	69.46 SD 3.54	68.05 SD 3.34	68.41 SD 4.01	β= -1.44 95% CI (-3.02 – 0.13) z= -1.79 p= 0.08	β=-1.09 95% CI (-2.67 – 0.48) z= -1.36 p= 0.17	β=-0.17 95% CI (-1.02 – 0.66) z= -0.41 p= 0.68
Language and Working Memory						
Cognitive TSI mean	68.28 SD 3.96	66.94 SD 3.61	67.07 SD 3.79	β= -1.33 95% CI (-2.98 – 0.32) z= -1.59 p= 0.11	β= -1.21 95% CI (-2.86 – 0.44) z= -1.43 p= 0.15	β=-0.06 95% CI (-0.91 – 0.77) z= -0.15 p= 0.88
Cognitive TSI min	66.66 SD 4.29	65.41 SD 3.91	65.43 SD 4.29	β=-1.24 95% CI (-3.07 – 0.57) z=-1.34 p= 0.18	β= -1.22 95% CI (-3.04 – 0.61) z= -1.31 p= 0.19	β=-0.02 95% CI (-0.94 – 0.91) z= -0.03 p= 0.97
Cognitive TSI max	69.56 SD 3.9	68.47 SD 3.29	69.48 SD 6.58	β=-1.15 95% CI (-3.26 – 0.96) z= -1.07 p= 0.28	β=-0.14 95% CI (-2.25 – 1.97) z= -1.07 p= 0.28	β=-0.51 95% CI (-1.57 – 0.56) z= -0.93 p= 0.35

Table 25 details the min, mean and max TSI values per cognitive task under baseline, active and sham stimulation conditions, adjusted for covariates

Effect of baseline characteristics or TSI values on positive cognitive results

Given the variability in the baseline characteristic of this population, we were interested to explore whether baseline age, sex, BMI, smartphone use, comorbidity, polypharmacy count or AD biomarker positivity influenced the positive cognitive results. We were also interested to explore whether the amount of time stimulated, or the mA of stimulation during active or sham condition influenced any of the positive cognitive outcomes. Table 26 details the analysis of associations between age, sex, BMI, AD positivity, polypharmacy, CCI and smartphone use with the five cognitive outcomes that demonstrated positive results under active stimulation. For parametric data a Pearson correlation (r) or unpaired two tailed students T-test (t) was used, and for non-parametric data a Spearman correlation (p) or Wilcoxin- rank sum (U) was performed. There was no significant association between age, sex, BMI, AD positivity, polypharmacy, CCI or smartphone use with any of the positive cognitive test results

Was there any association between the positive cognitive results under active stimulation and age, sex, BMI, AD positivity, polypharmacy CCI or smartphone use?

	FNAT Facial Recall RT (correct)	FNAT Name Recognition Accuracy	SHQ_L6	SHQ_L7	SHQ_L8	SHQ_overall
Age	p = -0.08 p= 0.62	r = 0.01 p= 0.99	r= -0.21 p=0.27	r = 0.14 p= 0.39	r = -0.09 p= 0.58	r = 0.04 p= 0.77
Sex	U = -1.1 p= 0.29	t = 0.47 p= 0.63	t = 0.57 p= 0.55	t= 0.99 p= 0.33	t= 0.97 p= 0.34	t= 0.47 p= 0.63
BMI	p = -0.11 p= 0.54	r= -0.01 p= 0.98	r= 0.18 p=0.34	r= 0.02 p= 0.91	r= 0.31 p= 0.07	r= 0.22 p= 0.16
AD positivity	U = -0.09 p= 0.93	t = 0.55 p= 0.58	t=0.59 p=0.45	t= 0.79 p= 0.43	t= 0.62 p= 0.54	t= 0.02 p= 0.98
Polypharmacy	U = -0.41 p= 0.68	t = 0.72 p= 0.47	t=0.37 p=0.76	t= 0.29 p= 0.76	t= 0.27 p= 0.78	t= 0.57 p= 0.57
CCI	p= 0.05 p= 0.74	r= -0.19 p= 0.25	r=0.12 p=0.83	r= -0.01 p= 0.99	r= 0.05 p= 0.75	r= 0.08 p= 0.62
Smartphone	U = -1.69 p= 0.09	t= -1.01 p= 0.28	t = 0.73 p=0.31	t= 1.21 p= 0.23	t= 0.52 p= 0.6	t= -1.53 p= 0.11

Table 26 details the associations and correlations between baseline demographic and clinical information and positive cognitive test results

under active tVNS stimulation

We were also interested to explore what effect stimulation time (mins) or amplitude (mA) may have had during active or sham stimulation on any of the cognitive tests that demonstrated positive results. A linear regression model was used, with the cognitive test as the dependent variable, with the model controlled for baseline age and sex. There were no significant effects found for amount of time stimulated (active or sham) or mA amplitude (active or sham) on the cognitive tests with positive outcomes listed above. These results are tabulated below in Tables 27 – 30.

Does the amount of time stimulated pre-testing affect cognitive outcomes during active stimulation?
Linear Model, dependent variable is cognitive test. Controlled for age, sex, polypharmacy, BMI, CCI, AD status

Cognitive test	Beta Coefficient (95% CI)	statistic
FNAT Face Recall Accuracy	-0.29 (-0.99 to 0.401)	p=0.39
FNAT Face Recall RT Correct	-42.91 (-107.01 to 21.18)	p=0.18
FNAT Face Recall RT Incorrect	-54.82 (-168.65 to 59.01)	p=0.33
FNAT Name Recognition Accuracy	-0.36 (-1.44 to 0.71)	p=0.49
FNAT Name Recognition RT correct	-32.3 (-210.8 to 146.1)	p=0.81
FNAT Name Recognition RT incorrect	-31.87 (-211.68 to 147.9)	p=0.72

Cognitive test	Beta Coefficient (95% CI)	statistic
SHQ_L1	-0.22 (-1.18 to 0.75)	p=0.65
SHQ_L2	0.35 (-0.82 to 1.53)	p=0.54
SHQ_L6	-0.14 (-0.97 to 0.68)	p=0.72
SHQ_L7	-0.17 (-1.07 to 0.73)	p=0.7
SHQ_L8	0.18 (-1.15 to 1.51)	p=0.78
SHQ_overall	0.02 (-0.81 to 0.85)	p=0.95

Table 27 details the linear regression models investigating time of active stimulation pre testing and cognitive outcomes

Does the amount of time stimulated pre-testing affect cognitive outcomes during sham stimulation?
Linear Model, dependent variable is cognitive test
Controlled for age, sex, BMI, polypharmacy, CCI, AD status

Cognitive test	Beta Coefficient (95% CI)	statistic
FNAT Face Recall Accuracy	0.72 (-0.52 to 1.96)	p=0.245
FNAT Face Recall RT Correct	29.56 (-114.45 to 173.57)	p=0.679
FNAT Face Recall RT Incorrect	230.69 (-16.65 to 478.04)	p=0.067
FNAT Name Recognition Accuracy	-0.26 (-2.39 to 1.86)	p=0.803
FNAT Name Recognition RT correct	40.07 (-130.73 to 210.9)	p=0.637
FNAT Name Recognition RT incorrect	169.63 (-135.17 to 474.43)	p=0.266

Cognitive test	Beta Coefficient (95% CI)	statistic
SHQ_L1	-0.66 (-1.66 to 0.34)	p=0.182
SHQ_L2	-0.74 (-1.75 to 0.28)	p=0.151
SHQ_L6	0.1 (-1.03 to 1.24)	p=0.854
SHQ_L7	-0.22 (-1.36 to 0.91)	p=0.692
SHQ_L8	0.55 (-1.03 to 2.14)	p=0.484
SHQ_overall	-0.07 (-1.04 to 0.91)	p=0.888

Table 28 details the linear regression models investigating time of sham stimulation pre testing and cognitive outcomes

Does the amplitude of stimulation (mA) affect cognitive outcomes during active stimulation?
Linear Model, dependent variable is cognitive test
Controlled for age, sex, BMI, polypharmacy, CCI, AD status

Cognitive test	Beta Coefficient (95% CI)	statistic
FNAT Face Recall Accuracy	-0.69 (-4.08 to 2.69)	p=0.680
FNAT Face Recall RT Correct	-38.87 (-355.09 to 277.35)	p=0.804
FNAT Face Recall RT Incorrect	3.24 (-552.02 to 558.52)	p=0.991
FNAT Name Recognition Accuracy	-1.01 (-6.22 to 4.22)	p=0.699
FNAT Name Recognition RT correct	-74.53 (-522.02 to 372.9)	p=0.737
FNAT Name Recognition RT incorrect	-305.81 (-1159.9 to 548.28)	p=0.472

Cognitive test	Beta Coefficient (95% CI)	statistic
SHQ_L1	1.72 (-3.56 to 7.01)	p=0.512
SHQ_L2	1.17 (-5.25 to 7.6)	p=0.712
SHQ_L6	1.66 (-2.78 to 6.12)	p=0.452
SHQ_L7	1.08 (-3.88 to 6.05)	p=0.660
SHQ_L8	-1.63 (-8.88 to 5.62)	p=0.650
SHQ_overall	0.67 (-3.85 to 5.21)	p=0.763

Table 29 details the linear regression models investigating amplitude (mA) of active stimulation pre testing and cognitive outcomes

Does the amplitude of stimulation (mA) affect cognitive outcomes during sham stimulation?
Linear Model, dependent variable is cognitive test
Controlled for age, sex, BMI, polypharmacy, CCI, AD status

Cognitive test	Beta Coefficient (95% CI)	statistic
FNAT Face Recall Accuracy	1.41 (-3.04 to 5.87)	p=0.524
FNAT Face Recall RT Correct	144.48 (-365.53 to 654.49)	p=0.569
FNAT Face Recall RT Incorrect	352.82 (-561.01 to 1266.6)	p=0.438
FNAT Name Recognition Accuracy	3.68 (-3.75 to 11.12)	p=0.322
FNAT Name Recognition RT correct	142.5 (-463.68 to 748.85)	p=0.636
FNAT Name Recognition RT incorrect	596.64 (-485.58 to 1678.86)	p=0.271

Cognitive test	Beta Coefficient (95% CI)	statistic
SHQ_L1	4.01 (-2.02 to 10.05)	p=0.186
SHQ_L2	2.74 (-3.55 to 9.04)	p=0.381
SHQ_L6	4.09 (-2.63 to 10.82)	p=0.224
SHQ_L7	-0.21 (-7.1 to 6.72)	p=0.952
SHQ_L8	-2.73 (-12.3 to 6.92)	p=0.568
SHQ_overall	0.83 (-5.04 to 6.72)	p=0.774

Table 30 details the linear regression models investigating amplitude (mA) of sham stimulation pre testing and cognitive outcomes

We were also interested to explore whether various TSI measures had any significant association with cognitive test scores. Tables 31 (A-D) and 32 (A-D) below detail the analysis of the effects of baseline TSI or change in TSI (delta between min and max value per cognitive test analysed) had on any of the domain specific cognitive tests, controlled for baseline age and sex. There was no significant effect found of baseline TSI or delta TSI per cognitive test for any of the cognitive tests undertaken.

Does baseline TSI predict cognitive results under active stimulation?
Linear Model, dependent variable is cognitive test
Controlled for age, sex, polypharmacy, CCI, AD status

Cognitive test	Beta Coefficient (95% CI)	statistic
FNAT Face Recall Accuracy	-0.79 (-1.57 to 0.01)	p=0.054
FNAT Face Recall RT Correct	2.13 (-86.9 to 91.2)	p=0.961
FNAT Face Recall RT Incorrect	-51.99 (-207.2 to 103.21)	p=0.500
FNAT Name Recognition Accuracy	-0.63 (-1.75 to 0.5)	p=0.266
FNAT Name Recognition RT correct	-12.85 (-130.46 to 104.76)	p=0.825
FNAT Name Recognition RT incorrect	49.65 (-190.09 to 289.4)	p=0.676

Cognitive test	Beta Coefficient (95% CI)	statistic
SART_total_no_pressed	1.62 (-1.3 to 4.55)	p=0.266
SART_RT_total	-3.56 (-14.12 to 7.01)	p=0.497
SART_error_commision	0.49 (-0.01 to 0.99)	p=0.053
SART_error_ommision	-1.13 (-3.92 to 1.66)	p=0.415
SART_EOC_RT	10.29 (-12.16 to 32.74)	p=0.357

Cognitive test	Beta Coefficient (95% CI)	statistic
SHQ_L1	0.39 (-0.96 to 1.74)	p=0.559
SHQ_L2	-0.04 (-1.69 to 1.61)	p=0.962
SHQ_L6	-0.46 (-1.43 to 0.51)	p=0.336
SHQ_L7	-0.01 (-1.2 to 1.26)	p=0.997
SHQ_L8	0.09 (-1.8 to 2.08)	p=0.927

SHQ_overall	-0.02 (-1.12 to 1.1)	p=0.975

Cognitive test	*Beta Coefficient (95% CI)*	*statistic*
Immediate_list_learning	0.01 (-0.15 to 0.17)	p=0.883
Semantic_Fluency	-0.25 (-0.65 to 0.16)	p=0.222
Digit_span_forward	0.03 (-0.12 to 0.19)	p=0.693
Digit_span_backward	-0.01 (-0.12 to 0.1)	p=0.858
List_recall	0.03 (-0.21 to 0.26)	p=0.827
List_recognition	-0.04 (-0.22 to 0.13)	p=0.595

Table 31 (A-D) details the linear regression models used to investigate the association between baseline TSI and cognitive results under active stimulation

Does change in TSI during active stimulation predict cognitive results under active stimulation?
Linear Model, dependent variable is cognitive test, independent variable is delta (change in TSI during active stimulation during that test)
Controlled for age, sex, polypharmacy, CCI, AD status

Cognitive test	Beta Coefficient (95% CI)	statistic
FNAT Face Recall Accuracy	-0.08 (-2.2 to 2.06)	p=0.938
FNAT Face Recall RT Correct	11.01 (-218.1 to 240.08)	p=0.923
FNAT Face Recall RT Incorrect	-33.66 (-435.72 to 368.39)	p=0.866
FNAT Name Recognition Accuracy	-0.04 (-3 to 2.91)	p=0.975
FNAT Name Recognition RT correct	-0.55 (-303.4 to 302.31)	p=0.997
FNAT Name Recognition RT incorrect	7.28 (-611.28 to 625.85)	p=0.981

Cognitive test	Beta Coefficient (95% CI)	statistic
SART_total_no_pressed	1.48 (-7.05 to 10.03)	p=0.725
SART_RT_total	-17.71 (-47.5 to 12.14)	p=0.235
SART_error_ommision	-1.83 (-9.9 to 6.23)	p=0.646
SART_error_commision	-0.34 (-1.86 to 1.17)	p=0.644
SART_EOC_RT	-23.51 (-88.2 to 41.21)	p=0.464

Cognitive test	Beta Coefficient (95% CI)	statistic
SHQ_L1	0.13 (-4.08 to 4.34)	p=0.950
SHQ_L2	2.14 (-2.96 to 7.25)	p=0.399
SHQ_L6	2.03 (-0.9 to 4.9)	p=0.167
SHQ_L7	0.25 (-3.7 to 4.24)	p=0.896
SHQ_L8	1.66 (-4.49 to 7.81)	p=0.586
SHQ_overall	1.35 (-2.1 to 4.82)	p=0.430

Cognitive test	Beta Coefficient (95% CI)	statistic
Immediate_list_learning	0.26 (-0.15 to 0.68)	p=0.211
Semantic_Fluency	1.02 (-0.01 to 2.04)	p=0.052
Digit_span_forward	0.32 (-0.08 to 0.71)	p=0.110
Digit_span_backward	0.23 (-0.07 to 0.53)	p=0.132
List_recall	0.08 (-0.52 to 0.69)	p=0.779
List_recognition	0.29 (-0.14 to 0.74)	p=0.177

Table 32 (A-D) details the linear regression models used to investigate the association between change in TSI during active stimulation and cognitive results under active stimulation

Discussion

The design of this trial was to primarily ascertain the feasibility of tVNS in a population with MCI. To explore the secondary aims of the effect of tVNS on domain-specific cognitive assessments, the trial was designed as a three-way internal crossover design to account for the unblinding participants may experience from wearing a nerve stimulation device. Therefore, for any results in this trial to be significant, an effect must be noted both during active stimulation compared to baseline (no stimulation), and during active stimulation compared to sham stimulation.

The improvements seen in the FNAT for Name Recognition were only noted when comparing active and sham, and there was no significant difference between active and sham stimulation condition. A similar result was seen during the SHQ test, whereby for Level 8 and for SHQ overall there were positive results seen comparing active and baseline, or active and sham stimulation, but not both.

The test results which demonstrated significantly improved results during active stimulation when compared to both baseline and sham stimulation were SHQ Level 6 and 7. In general, accurate spatial navigation relies on integrating subcortical inputs with retrosplenial, dorsal anterior cingulate and prefrontal cortex [524,525]. Previous fMRI studies of cymba conchae tVNS have confirmed activation of the insula, precentral gyrus, thalamus, ipsilateral NTS, bilateral STN, dorsal raphe, LC, amygdala, and nucleus accumbens [45,180] but not specifically any areas related to spatial navigation. Whether this improved spatial navigation effect may have been mediated via improved attention via activating subcortical systems such as the LC-NE should

be investigated in further studies, if these signals were reproduced in larger, adequately powered studies.

We noted no significant effect of baseline AD- biomarker positivity, age, or smartphone use on any outcomes, which was noteworthy. Baseline oxygenation as measured via the TSI did not predict results under active stimulation, and the changes in TSI during active stimulation was not associated with positive test results.

There were some limitations to this study. Repeated measures of the same cognitive test 7-10 days apart could result in a learned effect for the cognitive tests used. Given that each participant has been diagnosed with amnestic MCI, this was not predicted to contribute significantly to the overall variance or results. Additionally, the trial design attempted to control for this by randomising visit 2 and visit 3 as either active or sham stimulation, and accounting for visit number in the mixed effects models. This design was also single-blinded, in that the participants were blinded to the active or sham condition but the trial clinician who undertook the assessments was not. We were also mindful that most participants have smartphones and internet access, and could easily use the internet to ascertain which tVNS stimulation condition was sham or active, so they may have been unblinded in this manner.

However, a positive signal was seen in this small sample size indicating that acute tVNS could improve some discrete aspects of cognition, namely spatial navigation, and this should be further investigated in larger powered trials, as a neuromodulatory device if efficacious to help treat MCI, could be useful and beneficial.

Chapter 6 : Exploring the Effects of Acute tVNS on NeuroCardioVascular Outcomes in a population with MCI

Background

Older adults with MCI and dementia are at increased risk for NCVI and OH [526,527]. NCVI/OH may even influence clinical progression and mortality in those with cognitive impairment and dementia [528,529]. The VN is the primary mediator of neuro-cardiovascular reflexes including the baroreceptor response that maintains cerebrovascular perfusion during systemic hypotension or hypertension. Given the prominent role of the VN in NCVI, it is crucial to understand the effect of VN modulation on NVCI phenotypes in older adults with MCI, who may be at greater risk of OH and other NCVI phenotypes. It is thus of paramount importance to establish its safety in older adults with MCI. Key measures to assess the neuro-cardiovascular response to acute tVNS stimulation include measurement of HRV, peripheral (finometry-based beat-to-beat BP and HR assessment) and central (measured via NIRS) responses to Active Stand (AS).

As discussed in Chapter 2, the assessment of BP, HR and central oxygenation responses to an orthostatic challenge can be undertaken via many approaches, one of which is the AS. This procedure involves measuring beat-to-beat peripheral BP and HR responses resting and then during a stand and for 3 minutes post-standing, with many centres globally now also including a measure of non-invasively measured cerebral oxygenation. The benefits of an AS to measure BP, HR and oxygenation responses to an orthostatic challenge are that it is quick (it takes approximately 10 minutes to perform), reproducible, well-tolerated by patients and

provides more advanced and detailed autonomic information than a traditional arm cuff approach. BP responses to the orthostatic challenge of standing during the AS can be classified into the following phenotypes:

- Classical OH may be defined as a sustained decrease of ≥ 20 mmHg in SBP (or ≥ 30 mmHg in those with baseline HTN) or ≥ 10 mmHg (≥ 15 mmHg in those with baseline HTN) in DBP (or SBP < 90 mmHg) occurring between 60 and 180 seconds of standing
- Initial orthostatic hypotension (IOH) which involves a transient decrease in SBP of > 40 mmHg and/or > 20 mmHg in DBP within 15 seconds of standing in the absence of sustained OH
- Delayed recovery involves the inability of SBP to recover to ≤ 20 mmHg of supine baseline values at 30–40 s after standing but not meeting the criteria of classical OH with recovery occurring within 3 minutes of standing
- Orthostatic HTN (OHTN) is defined as a sustained increase of ≥ 20 mmHg in SBP or ≥ 10 mmHg in DBP (or > 140/90 mmHg if patient is normotensive supine), with HTN occurring at all time points occurring 60–180 s after the standing

For an in-depth review of recommendations for AS measurement and analysis approaches please see Finucane et al., (2019).[261]

Theoretically, vagally-mediated transient reductions in cerebral perfusion in those with MCI could be deleterious, as those with MCI have reduced cerebral blood flow compared to CU individuals at baseline [349]. tVNS, however, specifically exploits the peripheral anatomy of the VN, and as previously discussed in Chapter 1, it is the right cervical VN that provides most negatively chronotropic effects via the SA node. Hence only the ABVN distribution of the left

ear is stimulated during tVNS, as the left VN provides cardiac innervation via the AV node which is less likely, when stimulated, to cause bradycardia [26,530].

A recent metanalysis of the cardiovascular effects of stimulating the ABVN noted no significant effect on SBP or DBP values of acute stimulation, however it noted a significant effect on resting HR (i.e. reduced HR) for stimulation of the ABVN territory, and for the subset of studies included that solely analysed electrical stimulation [531]. However the study that contributed most significantly to the weighted balance of the effect size of that metanalysis was one wherein bilateral earlobe situation was employed [532], and the earlobe is most often used in this arena as a site for sham stimulation, so this metanalysis' results should be interpreted cautiously. It is also worth noting that the metanalysis included studies of auricular acupuncture, acupressure and electrical stimulation which may limit its generalisability. A systematic review of the cardiovascular effects of tVNS to the left ear only and of the known distribution of the ABVN (i.e. the tragus, concha and cymba concha, not earlobe) is warranted.

Oxygenation of cerebral tissue can be noninvasively measures using tools such as NIRS devices. As explored in Chapter 2, NIRS devices project near-infrared light into underlying tissue (such as cerebral cortical tissue) and measures the backscatter of this light using detectors. Based on the amount of backscatter, the concentration of oxygenated haemoglobin and deoxygenated haemoglobin can be determined and from this information the Tissue Saturation Index (TSI; a measure of tissue oxygenation) can be determined [512].

TSI has emerged as an objective surrogate marker of cerebral perfusion. NIRS devices are useful clinically and in research settings as they are light and portable. TSI has shown

promise as a marker of dynamic cerebrovascular haemodynamics during standing [533] and has been evaluated as a marker of oxygenation during tVNS and cognitive testing [534].

One of the disadvantages of TSI measurements is that intra-individual variability is high, and one may obtain different TSI measures both on the same day and on different days for one individual [535–537]. As such, the relative rather than absolute changes in TSI during one measurement are likely a more accurate marker of cerebral oxygenation and are used in research settings.

There is a known age-related reduction in resting-state TSI in the prefrontal cortex in older CU adults [514] and it has also been noted that persons with MCI have even lower resting state TSI levels compared to healthy controls [350,513]. Whether frontal TSI could be augmented by tVNS in a population with MCI remains to be explored.

Methods

Trial Design

Please see Chapter 4 for a detailed description of the Trial Design. The method of crossover and study randomisation is detailed in Figure 11 below.

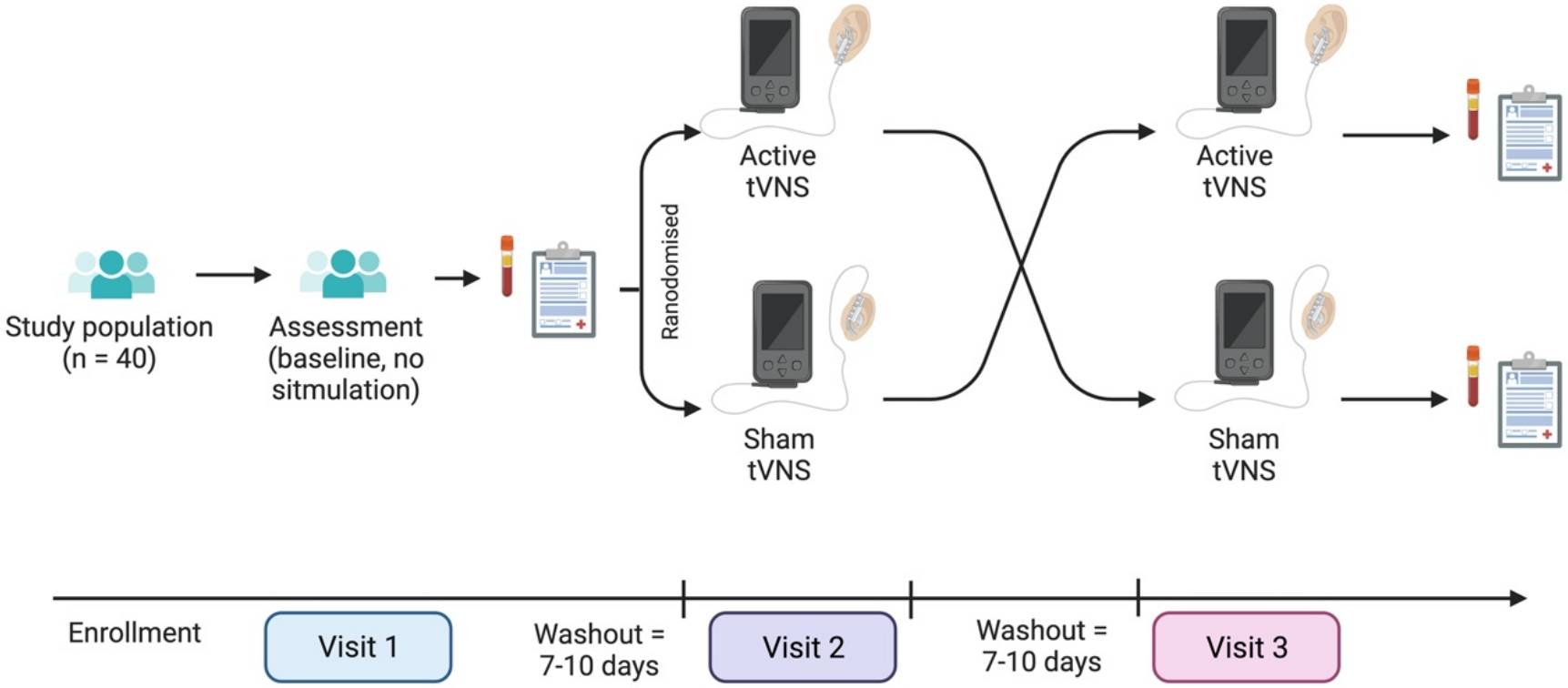


Figure 11 (repeat from Chapter 4)

Study Setting

Please see Chapter 4 for a detailed description of the Study Setting.

Sample Size

Please see Chapter 4 for a detailed description of the Sample Size.

Eligibility Criteria

Please see Chapter 4 for a detailed description of the Eligibility Criteria.

Device and Intervention

Please see Chapter 4 for a detailed description of the Device and Intervention.

Assessment

The risk associated with use of the NEMOS © Cerbomed tVNS device is minimal as published data of the safety profile shows minimal adverse events. The most common adverse events reported include local skin tingling, burning, redness, itching, tinnitus or headache which occur in up to 18% of participants, and tends to resolve quickly after discontinuation of the use of the device [493]. Due to the theoretical risk of increasing cardiac vagal-mediated bradycardias or hypotensive episodes by stimulating the right VN, participants will have stimulation of the left ear only.

During the active and sham stimulation conditions, safety was monitored by contemporaneous recording of any adverse events reported by participants whilst undergoing either active or sham stimulation and also via Likert-Based Questionnaires to assess for established side effects previously reported with tVNS: pain, skin discomfort, tingling, headache and tinnitus which were administered both during active and sham stimulation as detailed in Chapter 4.

Active Stand

Given the potential risk of amplifying an OH response, we were interested in assessing the response to AS as part of the safety assessments in this trial. Detailed assessment of subjective (OH symptoms including faintness, light-headedness), peripheral (beat-to-beat blood pressure) and central (TSI) haemodynamic responses to active stand were measured at all three visits per participant. Participants underwent AS from supine to standing. After lying supine for five minutes, participants were asked to stand as fast as they could. Beat-to-beat blood pressure and heart rate responses (assessed via the Finapres© NOVA) were monitored during AS and for three minutes after.

To standardise assessments, all assessments were taken at the same time each day, and all routine medications were taken as usual each morning. Participants were asked to avoid nicotine and caffeine for 12 hours prior to testing. The phenotypes of BP and HR response to standing as outlined above (Classical OH, IOH, delayed recovery, OHTN) were recorded per participant.

Oxygenation

During AS, cerebral oxygenation (as estimated via TSI) was measured using NIRS-based analysis software. TSI analysis was undertaken using the PortaLite OxySoft© NIRS device (PortaLite, Artinis Medical Systems B.V., Elst, The Netherlands) and the light emitting diode was placed on clean skin on the right forehead, devoid of moisture or oil, and secured with a black headband. TSI readings were transferred via Bluetooth in real-time to give an approximation of tissue oxygenation during rest and active stand.

Following assessment, participants were given contact details of the principal investigator and study coordinator at the hospital who they could contact if they had concerns, or any new symptoms were to arise. Any adverse events were diarised during the intervention and closely monitored until resolution. Data of participants who discontinue or deviate from the study protocol were retained for intention to treat analysis.

Figure 12 (Repeated from Chapter 4) below details the neurocardiovascular, cognitive and inflammatory assessments undertaken at each study visit.

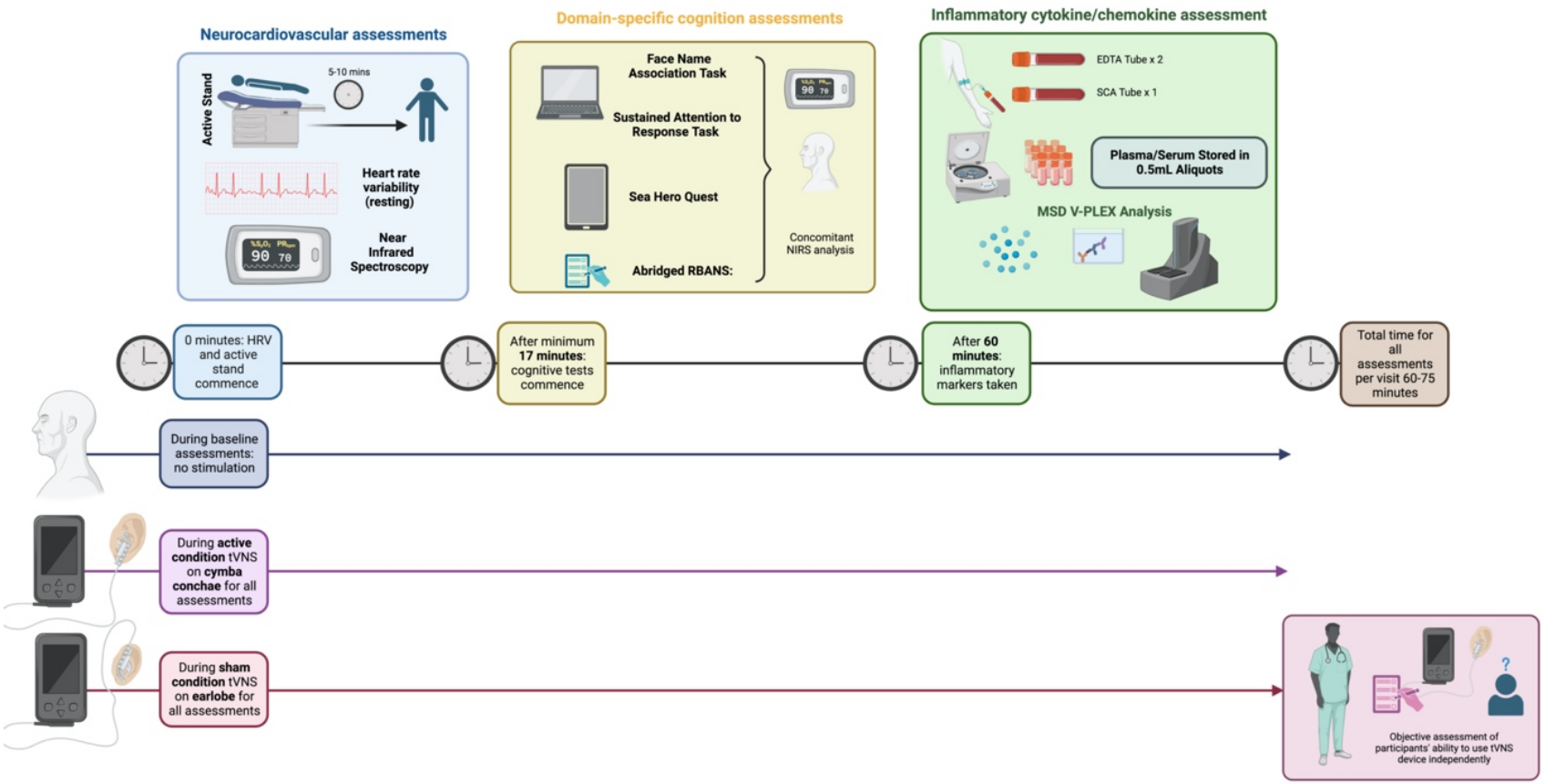


Figure 12 (Repeated from Chapter 4)

Heart Rate Variability

HRV assessments were undertaken using a 3 – lead continuous ECG measure via the Finapres© device. HRV analysis was undertaken during five minutes of rest pre- AS. Various HRV measures were taken, however specifically the RMSSD index is of primary interest as it is most highly correlated with older adults' cognition and executive function on metanalysis [366].

Ethics Approval and Consent to Participate

Please see Chapter 4 for details of Research Ethics Committee approval.

Covariates

As noted in the VINCI-AD study protocol [538], at the baseline assessment visit a comprehensive medical history, including demographic, behavioural, medical history and current medications was obtained and secured on an encrypted database to allow for covariate analysis.

Data processing and Statistical analysis

Data quality

Patients with incomplete neurocardiovascular signals were excluded from analyses. BP, HR, and TSI signals were visually inspected and screened for signal quality by two study investigators (HD, AZM) with poor signals not included in further analyses.

Signal processing

SBP, DBP, HR, and TSI signals were analysed by MATLAB R2019a (The Math Works, Inc., MATLAB, Version 2019a, Natick, MA) applying a standardized analytical process. The Finometer height correction signal was visually analysed and was used to derive the time of stand.

Features extracted from the AS responses are detailed below. A resting baseline (approximately −60 to −30 seconds before standing), values at 10 seconds, 20 seconds, 30 seconds, 40 seconds, 60 seconds, 120 seconds and the Steady-State (mean of value from 120 to 180 seconds) values were obtained. For TSI, SBP and DBP signals, nadir and overshoot values were defined, while for HR the maximum, minimum and time from min to max were extracted. The recovery rate from nadir to overshoot (or maximum and minimum for HR) was also derived for all signals. Values are reported as relative from baseline to ensure accuracy of signal reporting, unless otherwise stated.

Absolute values, unadjusted and adjusted mixed effects models for SBP, DBP and HR can be found in Supplementary Appendix 2.

HRV signal analysis was also undertaken in MATLAB R2019a. The ECG signal resting for 5 minutes pre AS was manually visually inspected by two study investigators (HD and AZM) for data quality and inclusion, with ectopic beats identified and removed. Signals with less than 200 seconds of identifiable QRS complexes were removed. The Inter-Beat-Interval (IBI) obtained from the arterial cuff measurements and R-R interval from the 3-lead ECG measurements were both analysed and used to identify interference or ectopic beats. Given the maximum amount of ECG signal available for HRV analysis was 5 minutes, we opted to

perform time-domain HRV analysis as this is most likely to be accurate in 5 minute HRV recordings [539].

Statistical Analysis

For descriptive statistics, the mean with Standard Deviation (SD) was used for parametric data and the median with Inter-Quartile Range (IQR) used for non-parametrically distributed data. A mixed-effects model was used to examine the difference in all SBP, DBP, TSI and HR measurements at specific time intervals during the AS, per condition (baseline, active stimulation and sham stimulation) accounting for covariates. In this nested method, each person acts as their own control. For a statistical test to be clinically meaningful in this study, there would have to be a significant difference both from baseline to active stimulation, and from active to sham stimulation.

Exploratory secondary aims of this study, including the effects of tVNS on HRV, were again analysed using a mixed-effects model, with the HRV index as the dependent variable in the model. Linear regression analysis was used to explore if covariates had a significant effect on HRV outcomes. Statistical analysis were undertaken using MATLAB (The MathWorks, Inc., Natick, MA, USA), STATA (Stata Statistical Software: Release 18, College Station, TX: StataCorp LLC) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results

Baseline characteristics of participants and relevant covariates are reported in Chapter 4. In total there were 118 individual participant visits, with one participant not returning for a sham stimulation visit and one participant unavailable for an active stimulation visit,.

Active Stand

Finometer-derived (SBP, DBP and HR) data was available for AS analysis for 112 participant visits. Six signals were excluded from AS analysis due to excessively noisy signals that were not suitable for accurate interpretation. TSI signals during AS were available for 118 participants.

SBP

SBP data was derived for each condition (baseline [no stimulation], active stimulation and sham stimulation) prior to standing, at 10-second intervals after standing and then the mean of the values from 120-180 seconds was used for the steady state values. There were no significant differences observed in the SBP values at 10, 20, 30, 40, 60, 120 seconds or during steady state (mean of values from 120-180 seconds) during baseline (no stimulation), active stimulation or sham stimulation. This was at baseline and after controlling for multiple covariates including baseline HTN, age, sex, antihypertensive Rx and antidepressant Rx. There were no significant differences observed in the SBP nadir values, the time to nadir, the

SBP overshoot or SBP recovery rate values between baseline, active stimulation, and sham stimulation as analysed in a mixed effects linear model. There was an association with quicker time to nadir values for active stimulation (active stimulation 11.46 ± 2.9 seconds, baseline 14.17 ± 5.93 seconds and sham stimulation 13.01 ± 5.21 seconds) [β= -2.12 (95% CI- 4.31- – 0.06); z= -1.9; p=0.06]) but this did not reach statistical significance. Table 33 details the SBP data and the unadjusted mixed effects modelling used to compare values across the three conditions, while Table 34 depicts the mixed effects model for this data adjusted for covariates. These results are graphically depicted in Figures 21 (A,B).

	Mean (SD) or median (IQR) of test results			***Mixed effect model - unadjusted***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active Stim	Baseline v Sham Stim	Active Stim v Sham Stim
Systolic Blood Pressure (SBP)						
SBP pre-stand mmHg	152.97 SD 19.77	147.42 SD 19.18	145.87 SD 26.96	β= -5.53 95% CI (-15.45 - 4.39) z= -1.09 p= 0.27	β= -7.195% CI (-17.01- 2.81) z=-1.40 p=0.161	β=0.78 95% CI (-4.32- 5.88) z= 0.30 p=0.76
SBP 10 sec (relative) in mmHg	-23.12 SD 21.15	-26.72 SD 15.42	-25.1 SD 25.6	β= -3.59 95% CI (-13.01 – 5.83) z=-0.75 p= 0.455	β= -1.97 95% CI (-11.47 – 7.5) z= -0.41 p=0.68	β= - 0.82 95% CI (-5.67 – 4.02) z= -0.33 p= 0.74
SBP 20 sec (relative) in mmHg	-21.24 SD 22.62	-21.25 SD 18.1	-22.7 SD 23.99	β= 0.002 95% CI (-9.69 – 9.7) z= 0.00 p=1.0	β=-1.45 95% CI (-11.22 – 8.31) z= -0.29 p= 0.77	β= 0.72 95% CI (-4.25 – 5.7) z= 0.29 p=0.78
SBP 30 sec (relative) in mmHg	-18.71 SD 20.76	-16.4 SD 17.28	-17.61 SD 20.34	β= 2.31 95% CI (-6.42 – 11.04) z= 0.52 p=0.61	β= 1.09 95% CI (-7.1 – 9.89) z= 0.24 p=0.81	β= 0.62 95% CI (-3.87 – 5.1) z= 0.27 p=0.79
SBP 40 sec (relative) in mmHg	-17.8 SD 20.83	-16.83 SD 16.42	-16.31 SD 18.45	β=0.97 95% CI (-7.39 – 9.33) z=0.23 p=0.82	β=1.48 95% CI (-6.93 – 9.91) z=0.35 p=0.73	β=-0.25 95% CI (-4.55 – 4.04) z= -0.12 p= 0.91
SBP 60 sec (relative) in mmHg	-14.78 SD 24.03	-16.5 SD 16.5	-14.49 SD 20.14	β= -1.72 95% CI (-10.9 – 7.46) z=-0.37 p= 0.71	β=0.29 95% CI (-8.96 – 9.5) z= 0.06 p= 0.95	β= -1.01 95% CI (-5.72 – 3.71) z= -0.42 p=0.67

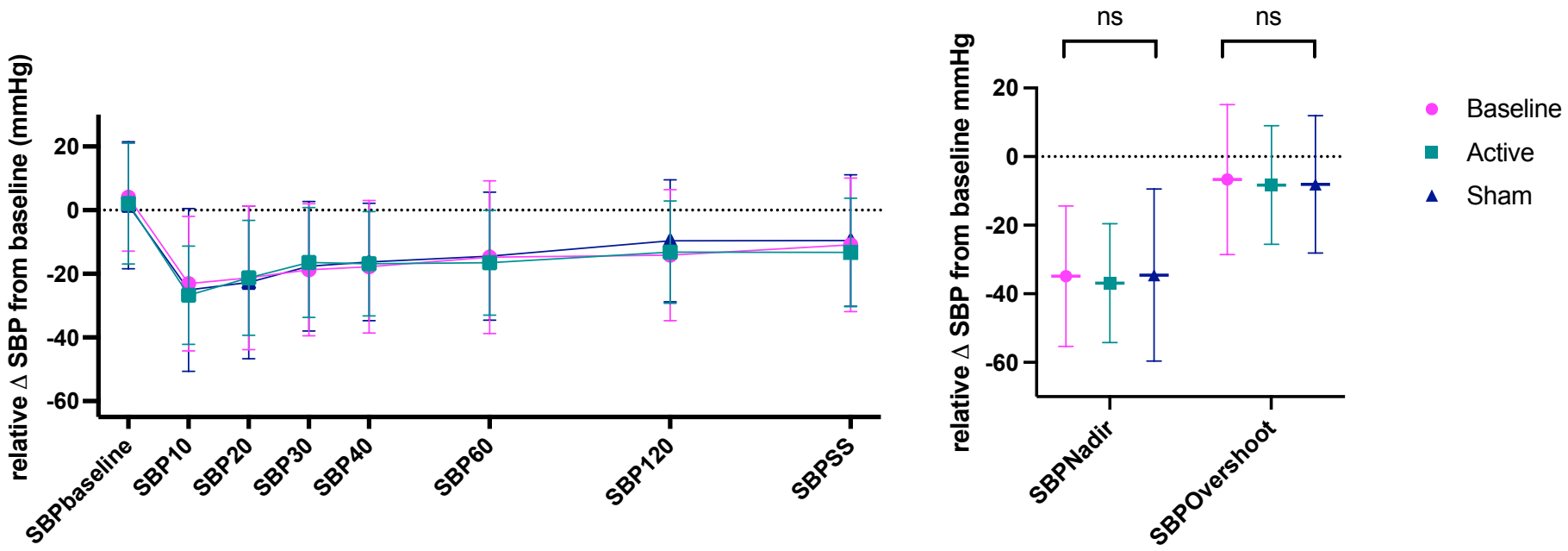
SBP 120 sec (relative) in mmHg	-14.1 SD 20.62	-13.15 SD 16.12	-9.61 SD 19.16	β= 0.91 95% CI (-7.47 – 9.2) z=0.21 p= 0.83	β= 4.45 95% CI (-4.06 – 12.96) z=1.03 p=0.31	β= -1.74 95% CI (-6.09 – 2.6) z=-0.79 p= 0.43
SBP steady state (120-180 sec) (relative) in mmHg	-10.9 SD 21.01	-13.29 SD 17.02	-9.57 SD 20.65	β= -2.32 95% CI (-11.1 – 6.47) z= -0.52 p= 0.61	β= 1.41 95% CI (-7.44 – 10.27) z= 0.31 p= 0.75	β= -1.86 95% CI (-6.38 – 2.64) z= -0.81 p= 0.42
SBP nadir in mmHg	-34.87 SD 20.49	-36.88 SD 17.3	-34.55 SD 25.1	β= - 2 95% CI (-11.62 – 7.61) z=-0.41 p= 0.683	β=0.32 95% CI (-9.21 – 9.8) z= 0.07 p= 0.95	β= -1.16 95% CI (-6.1- 3.77) z= -0.46 p= 0.65
SBP recovery rate (seconds)	1.18 (IQR 0.81-2.15)	1.27 (IQR 0.66 – 1.87)	0.97 (IQR 0.58 – 1.32)	β= -0.09 95% CI (-0.49 – 0.31) z= -0.44 p= 0.66	β= -0.31 95% CI (-0.72 – 0.1) z= -1.5 p= 0.14	β=- 0.11 95% CI (-0.11 – 0.32) z=1.02 p=0.31
SBP overshoot (relative) in mmHg	-6.7 SD 21.83	-8.28 SD 17.32	-8.1 SD 20.01	β= -1.35 95% CI (-10.37 – 7.67) z= -0.29 p= 0.76	β= -1.58 95% CI (-10.61 – 7.44) z= -0.34 p= 0.73	β=-0.12 95% CI (-4.78 – 4.55) z= -0.05 p= 0.96
SBP time to nadir (seconds)	14.17 SD 5.93	11.46 SD 2.9	13.01 SD 5.21	β=-2.67 95% CI (-4.91 - -0.43) z= -2.34 p=0.02*	β= -1.12 95% CI -3.34 – 1.09) z= -0.99 p= 0.32	β= -0.77 95% CI (-2.07 – 0.53) z= -1.16 p= 0.24

Table 33 details the unadjusted mixed effects model analysing the difference in Systolic Blood Pressure (SBP) across baseline, active and sham stimulation conditions

	Mean (SD) or median (IQR) of test results			***Mixed effects model adjusted for baseline HTN, age, sex, antihypertensive Rx, antidepressant Rx***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active Stim	Baseline v Sham Stim	Active Stim v Sham Stim
Systolic Blood Pressure (SBP)						
SBP pre-stand mmHg	152.97 SD 19.77	147.42 SD 19.18	145.87 SD 26.96	β=1.49 95% CI (-5.03 – 8.02) z=0.45 p= 0.65	β=-0.99 95% CI (-7.48 – 5.5) z=-0.3 p= 0.77	β= 1.23 95% CI (-2.01 – 4.4) z=0.75 p=0.455
SBP 10 sec (relative) in mmHg	-23.12 SD 21.15	-26.72 SD 15.42	-25.1 SD 25.6	β= -5.77 95% CI (-14.9 – 3.37) z=-1.24 p= 0.22	β= -3.81 95% CI (-13.01 – 5.38) z= -0.81 p= 0.42	β= -0.99 95% CI (-5.61 -3.6) z=-0.42 p= 0.67
SBP 20 sec (relative) in mmHg	-21.24 SD 22.62	-21.25 SD 18.1	-22.7 SD 23.99	β= -3.16 95% CI (-12.66 – 6.3) z= -0.65 p= 0.51	β= -4.62 95% CI (-14.16 – 4.9) z= -0.95 p=0.34	β= 0.71 95% CI (-4.07 – 5.51) z= 0.29 p= 0.77
SBP 30 sec (relative) in mmHg	-18.71 SD 20.76	-16.4 SD 17.28	-17.61 SD 20.34	β= 0.29 95% CI (-8.52 – 9.1) z=0.06 p= 0.95	β=-0.99 95% CI (-9.83 – 7.85) z=-0.22 p= 0.82	β= 0.63 95% CI (-3.78 – 5.06) z= 0.28 p= 0.78
SBP 40 sec (relative) in mmHg	-17.8 SD 20.83	-16.83 SD 16.42	-16.31 SD 18.45	β= -0.18 95% CI (-8.73 – 8.3) z= -0.04 p=0.96	β= 0.28 95% CI (-8.31 – 8.87) z= 0.06 p=0.95	β= -0.23 95% CI (-4.52- 4.06) z=-0.1 p= 0.92
SBP 60 sec (relative) in mmHg	-14.78 SD 24.03	-16.5 SD 16.5	-14.49 SD 20.14	β= -2.61 95% CI (-11.91 – 6.6) z=-0.55 p=0.58	β=-0.77 95% CI (-10.11 – 8.5) z= -0.16 p=0.87	β= -0.92 95% CI(-5.59 – 3.74) z=-0.39 p=0.7

SBP 120 sec (relative) in mmHg	-14.1 SD 20.62	-13.15 SD 16.12	-9.61 SD 19.16	β= -0.1 95% CI (-8.59 – 8.3) z= -0.02 p=0.98	β= 3.61 95% CI (-4.96 – 12.18) z=0.83 p=0.41	β=-1.85 95% CI (-6.14 – 2.44) z=-0.84 p=0.39
SBP steady state (120-180) (relative) in mmHg	-10.9 SD 21.01	-13.29 SD 17.02	-9.57 SD 20.65	β= -2.85 95% CI (-11.57 – 5.87) z=-0.64 p=0.52	β= 0.63 95% CI (-8.12 – 9.3) z=0.14 p=0.89	β= -1.74 95% CI (-6.12 – 2.63) z= -0.78 p=0.43
SBP nadir in mmHg	-34.87 SD 20.49	-36.88 SD 17.3	-34.55 SD 25.1	β= -4.69 95% CI (-14.1 – 4.61) z= -0.99 p=0.32	β= -2.71 95% CI (-11.96 – 6.53) z= -0.57 p=0.57	β= -0.97 95% CI (-5.67 – 3.72) z=-0.41 p=0.68
SBP recovery rate (seconds)	1.18 (IQR 0.81-2.15)	1.27 (IQR 0.66 – 1.87)	0.97 (IQR 0.58 – 1.32)	β= -0.09 95% CI (-0.51 – 0.32) z=-0.44 p=0.65	β= -0.31 95% CI (-0.73 – 0.11) z= -1.45 p=0.14	β= 0.11 95% CI (-0.1 – 0.32) z=1.00 p=0.32
SBP overshoot (relative) in mmHg	-6.7 SD 21.83	-8.28 SD 17.32	-8.1 SD 20.01	β= -2.48 95% CI (-11.68 – 6.72) z= -0.53 p= 0.59	β= -2.48 95% CI (-11.69 – 6.77) z= -0.52 p= 0.6	β= -0.02 95% CI (-4.69 – 4.66 z= -0.01 p=0.99
SBP time to nadir (seconds)	14.17 SD 5.93	11.46 SD 2.9	13.01 SD 5.21	β= -2.12 95% CI (-4.31 – 0.06) z= -1.9 p=0.06	β= -0.53 95% CI (-2.71 – 1.64) z= -0.48 p=0.63	β=-0.79 95% CI (-1.9 – 0.32) z=-1.39 p= 0.16

Table 34 details the mixed effects model analysing the difference in Systolic Blood Pressure (SBP) across baseline, active and sham stimulation conditions, adjusted for covariates



Figures 21 (A,B) detail the Systolic Blood Pressure results of the Active Stand (AS) during baseline, active and sham stimulation

DBP

DBP data was derived for each condition (baseline [no stimulation], active stimulation and sham stimulation) prior to standing, at 10-second intervals after standing and then the mean of the values from 120-180 seconds was used for the steady state values. There were no significant differences observed in the DBP values at 10, 20, 30, 40, 60, 120 seconds or during steady state (mean of values from 120-180 seconds) during baseline (no stimulation), active stimulation or sham stimulation. This was at baseline and after controlling for multiple covariates including baseline HTN, age, sex, antihypertensive Rx and antidepressant Rx as analysed in a mixed effects linear model. There were no significant differences observed in the DBP nadir values, the time to nadir, the DBP overshoot or DBP recovery rate values between baseline, active stimulation, and sham stimulation. Table 35 details the summary DBP data and the unadjusted mixed effects model used to compare values across the three conditions, while Table 36 details this analysis adjusted for covariates. These results are graphically depicted in Figures 22 (A,B).

	Mean (SD) or median (IQR) of test results			***Mixed effects model - unadjusted***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Diastolic Blood Pressure (DBP)						
DBP pre-stand mmHg	85.09 SD 16.38	79.83 SD 13.09	80.5 SD 17.13	β= -5.25 95% CI (-12.2 – 1.7) z= -1.47 p= 0.14	β=-4.57 95% CI (-11.58 – 2.4) z= -1.47 p= 0.19	β= -0.33 95% CI (-3.94 – 3.27) z= -0.18 p= 0.86
DBP 10 sec (relative) in mmHg	-12.66 SD 10.12	-11.21 SD 11.31	-11.71 SD 16.2	β= 1.45 95% CI (-4.23 – 7.13) z= 0.5 p=0.61	β= 0.95 95% CI (-4.77 – 6.67) z= 0.33 p=0.74	β= 0.25 95% CI (-2.66 – 3.17) z= 0.17 p= 0.86
DBP 20 sec (relative) in mmHg	-9.3 SD 11.85	-7.01 SD 12.31	-8.02 SD 13.82	β=2.25 95% CI (-3.39 – 7.91) z= 0.78 p=0.43	β= 1.24 95% CI (-4.44 – 6.94) z= 0.43 p= 0.67	β=0.51 95% CI (-2.39 – 3.42) z= 0.35 p= 0.73
DBP 30 sec (relative) in mmHg	-6.34 SD 12.13	-3.61 SD 11.87	-4.12 SD 11.71	β=2.72 95% CI (-2.6 – 8.05) z=1 p=0.31	β=2.22 95% CI (-3.14 – 7.58) z=0.81 p=0.42	β=0.26 95% CI (-2.48 – 3.01) z= 0.19 p= 0.85
DBP 40 sec (relative) in mmHg	-5.6 SD 12.5	-3.49 SD 11.32	-3.13 SD 11.45	β=2.11 95% CI (-3.16 – 7.38) z=0.78 p= 0.43	β= 2.46 95% CI (-2.84 – 7.77) z= 0.91 p= 0.36	β=-0.16 95% CI (-2.89- 2.55) z=-0.12 p=0.9

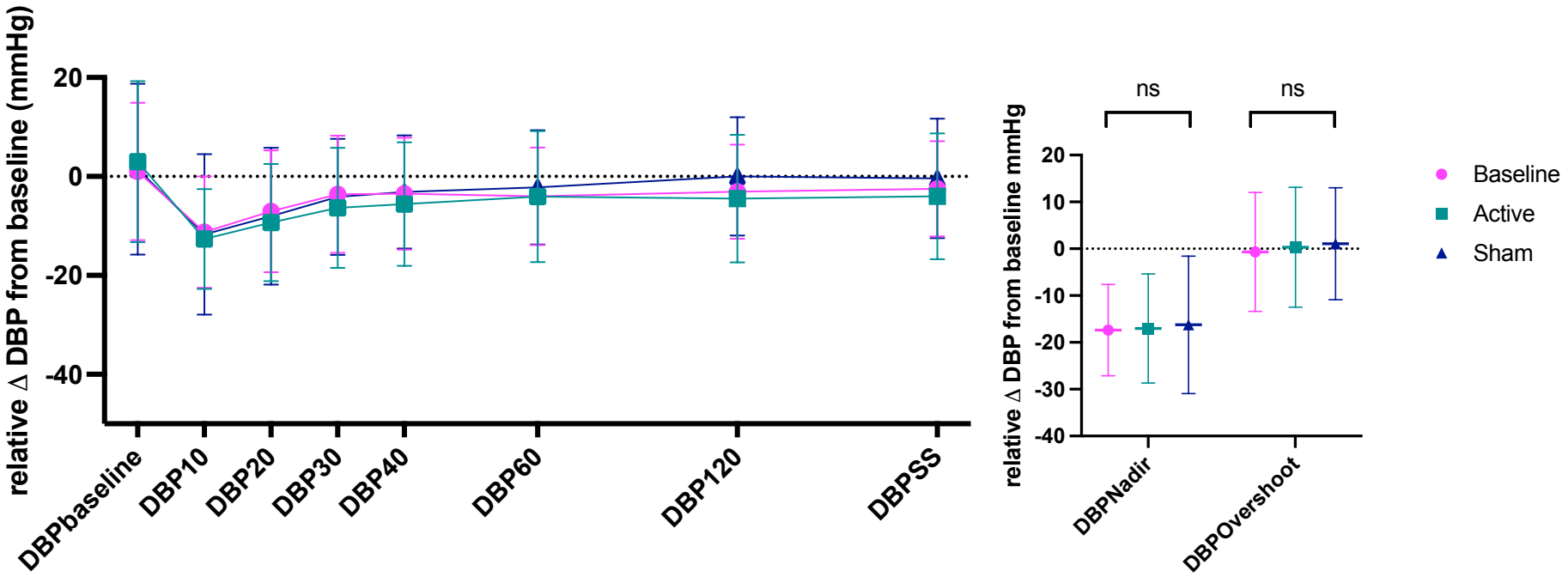
DBP 60 sec (relative) in mmHg	-4.06 SD 13.23	-3.99 SD 9.9	-2.18 SD 11.55	β=0.07 95% CI (-5.14 – 5.3) z= 0.04 p=0.98	β=1.88 95% CI (-3.39 – 7.14) z= 0.7 p= 0.48	β=-0.89 95% CI (-3.58 – 1.78) z= -0.65 p= 0.51
DBP 120 sec (relative) in mmHg	-4.46 SD 12.9	-3.05 SD 9.52	0.01 SD 11.94	β=1.41 95% CI (-3.77 – 6.58) z=0.53 p= 0.59	β=4.47 95% CI (-0.77 – 9.72) z= 1.67 p= 0.09	β= -1.5 95% CI (-4.19 – 1.19) z=-1.09 p=0.27
DBP steady state (120-180) (relative) in mmHg	-4.03 SD 12.75	-2.46 SD 9.66	-0.41 SD 12.09	β=1.56 95% CI (-3.62 – 6.75) z=0.59 p=0.55	β=3.62 95% CI (-1.61-8.84) z=1.36 p=0.17	β=-1.01 95% CI (-3.69 – 1.66) z= -0.74 p=0.45
DBP nadir in mmHg	-17.37 SD 9.74	-17.03 SD 11.65	-16.25 SD 14.69	β= 0.34 95% CI (-5.15 – 5.83) z= 0.12 p= 0.9	β= 1.11 95% CI (-4.34 – 6.56) z= 0.4 p= 0.69	β=-0.39 95% CI (-3.21 – 2.42) z= -0.27 p= 0.79
DBP recovery rate (seconds)	0.63 (IQR 0.45 – 1.31)	0.65 (IQR 0.43 – 1.06)	0.66 (IQR 0.42 – 1.09)	β=-0.03 95% CI (-0.3 – 0.22) z= -0.29 p= 0.77	β=-0.09 95% CI (-0.35 – 0.16) z= -0.71 p= 0.46	β=0.02 95% CI (-0.11 – 0.16) z= 0.41 p= 0.68
DBP overshoot (relative) in mmHg	-0.67 SD 12.71	+0.31 SD 12.8	+1.06 SD 11.94	β= 0.97 95% CI (-4.68 – 6.64) z= 0.34 p= 0.74	β= 1.73 95% CI (-3.89 – 7.37) z=0.6 p= 0.55	β=-0.38 95% CI (-3.29 – 2.53) z= -0.26 p= 0.79
DBP time to nadir (seconds)	11.36 (IQR 9.36 – 14.35)	10.42 (IQR 9.19 – 12.73)	10.67 (IQR 8.55 – 13.9)	β= -0.75 95% CI (-2.95 – 1.46) z= -0.66 p= 0.51	β= -0.29 95% CI (-1.9 – 2.48) z= 0.26 p= 0.79	β= -0.52 95% CI (-1.65 – 0.62) z= -0.89 p= 0.37

Table 35 details the unadjusted mixed effects model analysing the difference in Diastolic Blood Pressure (DBP) across baseline, active and sham stimulation conditions

	Mean (SD) or median (IQR) of test results			***Mixed effects model adjusted for baseline HTN, age, sex, antihypertensive Rx, antidepressant Rx***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Diastolic Blood Pressure (DBP)						
DBP pre-stand mmHg	85.09 SD 16.38	79.83 SD 13.09	80.5 SD 17.13	β= -1.22 95% CI (-6.32 – 3.91) z=-0.47 p= 0.64	β= -1.36 95% CI (-6.48 – 3.7) z=-0.52 p=0.6	β=0.07 95% CI (-2.49 – 2.63) z= 0.05 p= 0.96
DBP 10 sec (relative) in mmHg	-12.66 SD 10.12	-11.21 SD 11.31	-11.71 SD 16.2	β= 0.49 95% CI (-4.73 – 5.72) z= 0.19 p= 0.85	β= 0.01 95% CI (-5.24 – 5.25) z= 0.00 p=0.99	β= 0.24 95% CI (-2.37 – 2.87) z= 0.18 p= 0.85
DBP 20 sec (relative) in mmHg	-9.3 SD 11.85	-7.01 SD 12.31	-8.02 SD 13.82	β= 1.08 95% CI (-4.24 – 6.4) z= 0.4 p= 0.69	β= -0.22 95% CI (-5.57 – 5.12) z= -0.08 p= 0.93	β= 0.65 95% CI (-2.02 – 3.33) z= 0.48 p= 0.63
DBP 30 sec (relative) in mmHg	-6.34 SD 12.13	-3.61 SD 11.87	-4.12 SD 11.71	β= 1.96 95% CI (-3.26 – 7.18) z= 0.74 p= 0.46	β= 1.34 95% CI (-3.91 – 6.58) z= 0.5 p= 0.62	β=0.32 95% CI (-2.31 – 2.94) z= 0.24 p= 0.81
DBP 40 sec (relative) in mmHg	-5.6 SD 12.5	-3.49 SD 11.32	-3.13 SD 11.45	β=1.74 95% CI (-3.35 – 6.83) z= 0.67 p= 0.5	β= 1.88 95% CI (-3.23 – 7.01) z= 0.72 p= 0.47	β= -0.06 95% CI (-2.62 – 2.49) z= -0.05 p= 0.96
DBP 60 sec (relative) in mmHg	-4.06 SD 13.23	-3.99 SD 9.9	-2.18 SD 11.55	β=0.03 95% CI (-4.83 – 4.9) z= 0.01 p= 0.99	β=1.42 95% CI (-3.46 – 6.3) z= 0.57 p= 0.57	β=-0.69 95% CI (-3.14 – 1.75) z= -0.56 p= 0.58

DBP 120 sec (relative) in mmHg	-4.46 SD 12.9	-3.05 SD 9.52	0.01 SD 11.94	β=1.47 95% CI (-3.53 – 6.46) z= 0.58 p=0.57	β=4.4 95% CI (-0.62 – 9.48) z=1.72 p= 0.08	β=-1.45 95% CI (-4.01 – 1.09) z= -1.12 p=0.26
DBP steady state (120-180) (relative) in mmHg	-4.03 SD 12.75	-2.46 SD 9.66	-0.41 SD 12.09	β= 1.84 95% CI (-2.97 – 6.66) z= 0.75 p= 0.45	β= 3.39 95% CI (-1.45 – 8.2) z= 1.37 p= 0.17	β= -0.76 95% CI (-3.19 – 1.67) z= - -0.61 p= 0.54
DBP nadir in mmHg	-17.37 SD 9.74	-17.03 SD 11.65	-16.25 SD 14.69	β=-0.04 95% CI (-5.15 – 5.07) z= -0.02 p= 0.98	β= -0.001 95% CI -5.09 – 5.08) z= -0.00 p= 1.00	β= -0.02 95% CI (-2.6 – 2.56) z= -0.01 p= 0.98
DBP recovery rate (seconds)	0.63 (IQR 0.45 – 1.31)	0.65 (IQR 0.43 – 1.06)	0.66 (IQR 0.42 – 1.09)	β= -0.07 95% CI (-0.32 – 0.19) z= -0.54 p= 0.59	β=-0.11 95% CI (-0.36 – 0.14) z= -0.86 p= 0.39	β=0.02 95% CI (-0.11 – 0.15) z=0.31 p= 0.75
DBP overshoot (relative) in mmHg	-0.67 SD 12.71	+0.31 SD 12.8	+1.06 SD 11.94	β=0.63 95% CI (-4.66 – 5.92) z=0.23 p= 0.82	β=0.82 95% CI (-4.45 – 6.09) z=0.3 p= 0.76	β= -0.09 95% CI (-2.77 – 2.58) z= -0.07 p= 0.94
DBP time to nadir (seconds)	11.36 (IQR 9.36 – 14.35)	10.42 (IQR 9.19 – 12.73)	10.67 (IQR 8.55 – 13.9)	β= -0.73 95% CI (-2.86 – 1.4) z= --0.67 p= 0.5	β= 0.25 95% CI (-1.87 – 2.37) z= 0.23 p= 0.81	β= -0.49 95% CI (-1.58 – 0.61) z= -0.87 p= 0.38

Table 36 details the mixed effects model analysing the difference in Diastolic Blood Pressure (DBP) across baseline, active and sham stimulation conditions, adjusted for covariates



Figures 22 (A,B) detail the Diastolic Blood Pressure results of the Active Stand (AS) during baseline, active and sham stimulation

Heart Rate

HR data was derived for each condition (baseline [no stimulation], active stimulation and sham stimulation) prior to standing, at 10-second intervals after standing and then the mean of the values from 120-180 seconds was used for the steady state values. There were no significant differences observed in the HR values at 10, 20, 30, 40, 60, 120 seconds or during steady state (mean of values from 120-180 seconds) during baseline (no stimulation), active stimulation or sham stimulation. This was at baseline and after controlling for multiple covariates including age, sex, baseline HR, polypharmacy and beta-blocker Rx as analysed in a mixed effects linear model. There were no significant differences observed in the HR minimum or maximum values, the HR recovery rate or the time between HR minimum and maximum values across the three conditions. Table 37 details the summary HR data and the unadjusted mixed effects model used to compare values across the three conditions, while Table 38 depicts this analysis adjusted for covariates. These results are graphically depicted in Figures 23 (A-D).

	Mean (SD) or median (IQR) of test results			***Mixed effects model – unadjusted***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Heart Rate						
HR pre-stand in bpm	70.32 SD 11.53	69.48 SD 12.76	68.89 SD 12.51	β=-0.84 95% CI (-6.32 – 4.63) z= -0.3 p= 0.76	β= -1.43 95% CI (-6.91 – 4.04) z= -0.51 p= 0.6	β=0.29 95% CI (-2.49 – 3.09) z=0.21 p= 0.83
HR 10 sec (relative) in bpm	+5.51 SD 4.1	+4.95 SD 7.78	+5.95 SD 10.51	β= -0.55 95% CI (-4.04 – 2.93) z= -0.31 p= 0.75	β= 0.45 95% CI (-3.06 – 3.96) z= 0.25 p= 0.8	β=-0.5 95% CI (-2.3 – 1.2) z= -0.55 p= 0.58
HR 20 sec (relative) in bpm	+5.34 SD 4.45	+5.73 SD 9.21	+6.79 SD 6.93	β= 0.38 95% CI (-2.77 – 3.5) z= 0.24 p=0.81	β=1.45 95% CI (-1.73 – 4.6) z=0.89 p=0.37	β=-0.53 95% CI (-2.15-1.09) z=-0.64 p=0.52
HR 30 sec (relative) in bpm	+5.69 SD 4.61	5.21 SD 8.21	+7.67 SD 7.61	β= -0.48 95% CI (-3.57 – 2.61) z= -0.31 p= 0.76	β=1.98 95% CI (-1.13 – 5.1) z=1.25 p= 0.21	β=-1.22 95% CI (-2.82 – 0.36) z= -1.51 p= 0.13
HR 40 sec (relative) in bpm	+6.53 SD 5.22	+5.87 SD 6.93	+8.26 SD 7.89	β=-0.66 95% CI (-3.66 – 2.34) z=-0.43 p= 0.66	β= 1.72 95% CI (-1.29 – 4.74) z= 1.12 p= 0.26	β= -1.19 95% CI (-2.73 – 0.35) z=-1.51 p= 0.13
HR 60 sec (relative) in bpm	+7.62 SD 6.4	+7.18 SD 6.29	+7.9 SD 7.53	β==0.44 95% CI (-3.46 – 2.56) z=-0.29 p=0.77	β=0.28 95% CI (-2.75 – 3.31) z= 0.18 p= 0.86	β= -0.36 95% CI)-1.91 – 1.18) z= -0.46 p= 0.65

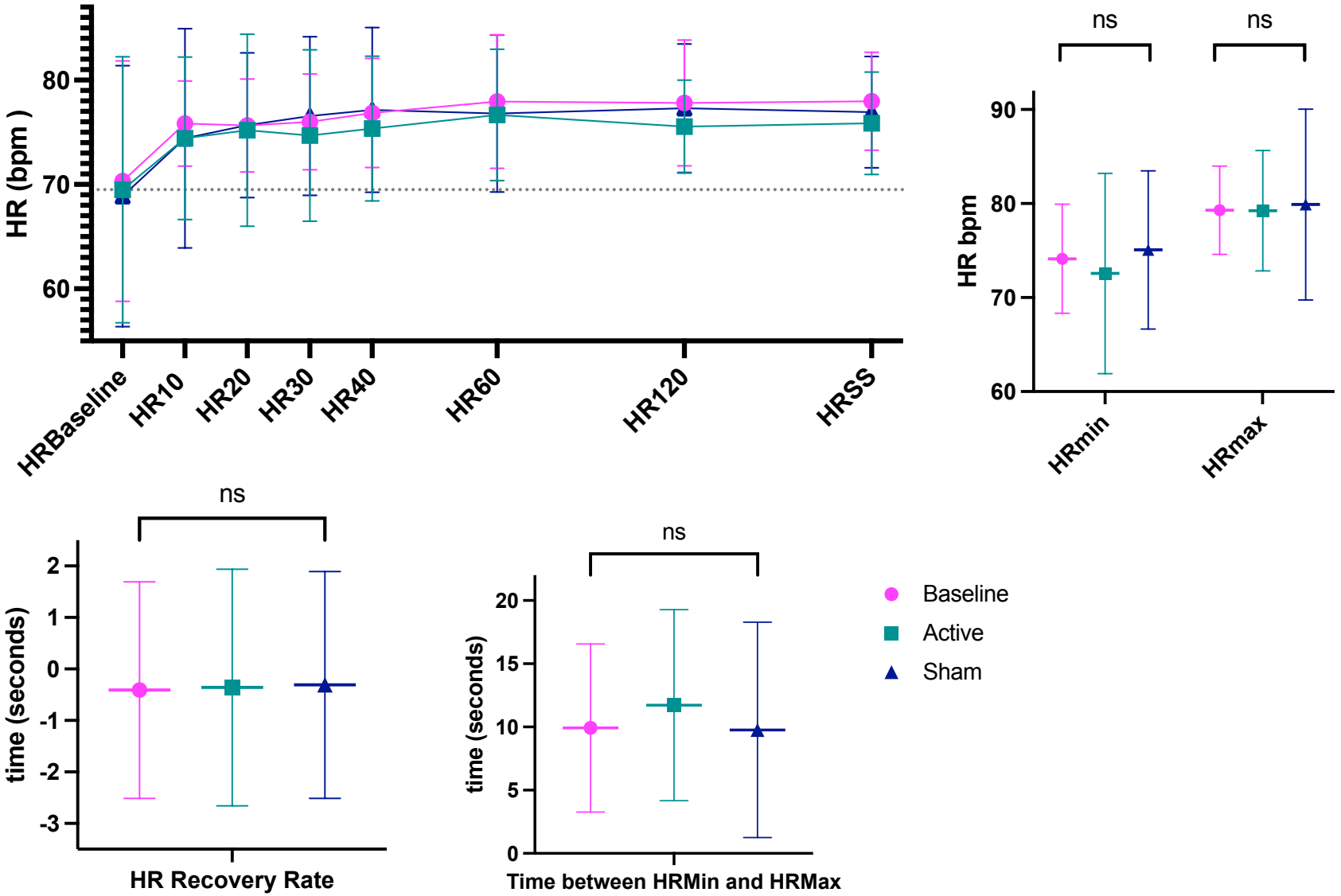
HR 120 sec (relative) in bpm	+7.5 SD 6.04	+6.07 SD 4.46	+8.42 SD 6.17	β=-1.43 95% CI (-3.93 – 1.07) z= -1.12 p= 0.26	β= 0.92 95% CI (-1.61 – 3.47) z= 0.71 p= 0.47	β= -1.18 95% CI (-2.47 – 0.11) z= -1.78 p= 0.07
HR steady state (120-180) (relative) in bpm	+7.66 SD 4.69	+6.38 SD 4.9	+8.04 SD 5.34	β= -1.28 95% CI (-3.51 – 0.93) z= -1.14 p= 0.26	β=0.37 95% CI (-1.87 – 2.63) z= 0.33 p= 0.74	β=-0.83 95% CI (-1.98 – 0.31) z=-1.43 p= 0.15
HR min (relative) in bpm	+4.13 SD 5.79	+3.57 SD 10.65	+7.07 SD 8.41	β=-0.55 95% CI (-4.33 – 3.2) z=-0.29 p= 0.77	β= 2.94 95% CI (-0.87 – 6.74) z=1.51 p= 0.13	β= -1.74 95% CI (-3.68 – 0.2) z=-1.76 p=0.07
HR max (relative) in bpm	+9.3 SD 4.7	+10.23 SD 6.4	+11.9 SD 10.15	β= 0.93 95% CI (-2.34 – 4.2) z= 0.56 p= 0.57	β= 2.6 95% CI (-0.7 – 5.9) z=1.54 p= 0.12	β= -0.82 95% CI (-2.52 – 0.87) z= -0.95 p= 0.34
HR recovery rate (seconds)	-0.41 (IQR -0.66 to -0.15)	-0.36 (IQR -0.64 to -0.19)	-0.31 (IQR -0.43 to -0.14)	β= -0.11 95% CI (-0.4 – 0.18) z=-0.71 p= 0.48	β=0.06 95% CI (-0.24 – 0.36) z= 0.38 p= 0.71	β= -0.08 95% CI (-0.24 – 0.07) z= -1.05 p= 0.29
Time between min and max HR (seconds)	9.92 SD 6.65	11.72 SD 7.55	9.77 SD 8.52	β=1.79 95% CI (-1.58 – 5.18) z= 1.04 p= 0.29	β= -0.15 95% CI (-3.56 – 3.26) z=-0.09 p= 0.93	β=0.98 95% CI (-0.76 – 2.72) z= 1.1 p=0.27

Table 37 details the unadjusted mixed effects model analysing the difference in Heart Rate (HR) during baseline, active and sham stimulation conditions

	Mean (SD) or median (IQR) of test results			***Mixed effects model adjusted for age, sex, baseline HR, polypharmacy, beta-blocker Rx***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Heart Rate						
HR pre-stand in bpm	70.32 SD 11.53	69.48 SD 12.76	68.89 SD 12.51	β= -1.22 95% CI (-6.34 – 3.9) z= -0.47 p= 0.64	β= -2.21 95% CI (-7.35 – 2.91) z= -0.85 p= 0.39	β=0.49 95% CI (-2.21 – 3.11) z= 0.37 p= 0.71
HR 10 sec (relative) in bpm	+5.51 SD 4.1	+4.95 SD 7.78	+5.95 SD 10.51	β=-0.59 95% CI (-3.96 – 2.77) z=-0.35 p= 0.73	β= 0.24 95% CI (-3.15 – 3.63) z= 0.14 p= 0.89	β=-0.42 95% CI (-2.15 – 1.31) z= -0.47 p= 0.63
HR 20 sec (relative) in bpm	+5.34 SD 4.45	+5.73 SD 9.21	+6.79 SD 6.93	β= 0.38 95% CI (-2.6 – 3.38) z= 0.25 p= 0.79	β= 1.21 95% CI (-1.8-4.22) z= 0.79 p= 0.43	β= -0.41 95% CI (-1.95 – 1.13) z= -0.52 p= 0.6
HR 30 sec (relative) in bpm	+5.69 SD 4.61	+5.21 SD 8.21	+7.67 SD 7.61	β= -0.49 95% CI (-3.55 – 2.36) z=-0.32 p= 0.75	β= 1.86 95% CI (-1.21 – 4.94) z= 1.19 p= 0.23	β= -1.17 95% CI(-2.75 – 0.39) z= -1.47 p= 0.14
HR 40 sec (relative) in bpm	+6.53 SD 5.22	+5.87 SD 6.93	+8.26 SD 7.89	β= -0.66 95% CI (-3.63 – 2.3) z=-0.44 p= 0.66	β=1.62 95% CI (-1.37 – 4.6) z=1.06 p=0.29	β=-1.13 95% CI (-2.67- 0.39) z=-1.46 p= 0.14
HR 60 sec (relative) in bpm	+7.62 SD 6.4	+7.18 SD 6.29	+7.9 SD 7.53	β= -0.47 95% CI (-3.47 – 2.52) z=-0.31	β=0.2 95% CI (-2.82 – 3.22) z= 0.13	β= -0.37 95% CI (-1.87 – 1.2) z= -0.43

				p= 0.76	p= 0.89	p= 0.67
HR 120 sec (relative) in bpm	+7.5 SD 6.04	+6.07 SD 4.46	+8.42 SD 6.17	β=-1.34 95% CI (-3.78 – 1.1) z= -1.07 p= 0.28	β=0.91 95% CI (-1.57 – 3.39) z=0.71 p= 0.48	β=-1.12 95% CI (-2.38 – 0.14) z= -1.74 p= 0.08
HR steady state (120-180) (relative) in bpm	+7.66 SD 4.69	+6.38 SD 4.9	+8.04 SD 5.34	β=-1.19 95% CI (-3.32 – 0.95) z=-1.09 p=0.27	β= 0.35 95% CI (-1.82 – 2.52) z= 0.32 p= 0.75	β=-0.78 95% CI (-1.88 – 0.33) z= -1.38 p= 0.16
HR min (relative) in bpm	+4.13 SD 5.79	+3.57 SD 10.65	+7.07 SD 8.41	β= -0.53 95% CI (-4.21 – 3.16) z=-0.28 p= 0.78	β=2.75 95% CI (-0.97 – 6.46) z=1.45 p= 0.14	β=-1.63 95% CI (-3.53 – 0.27) z= -1.69 p= 0.09
HR max (relative) in bpm	+9.3 SD 4.7	+10.23 SD 6.4	+11.9 SD 10.15	β=0.88 95% CI (-2.35 – 4.13) z= 0.54 p= 0.59	β=2.49 95% CI (-0.77 – 5.7) z= 1.5 p= 0.13	β=-0.79 95% CI (-2.47 – 0.88) z= -0.93 p= 0.35
HR recovery rate (seconds)	-0.41 (IQR -0.66 to -0.15)	-0.36 (IQR -0.64 to -0.19)	-0.31 (IQR -0.43 to -0.14)	β= -0.97 95% CI (-0.38 – 0.18) z= -0.68 p= 0.49	β=0.06 95% CI (-0.23 – 0.35) z= 0.41 p= 0.68	β=-0.07 95% CI (-0.23 – 0.06 z= -1.04 p= 0.29
Time between min and max HR (seconds)	9.92 SD 6.65	11.72 SD 7.55	9.77 SD 8.52	β=1.66 95% CI (-1.55 – 4.88) z= 1.01 p= 0.31	β= -0.35 95% CI (-3.59 – 2.88) z= -0.21 p= 0.83	β= 1.01 95% CI (-0.64 – 2.66) z= 1.2 p=0.23

Table 38 details the mixed effects model analysing the difference in Heart Rate (HR) across baseline, active and sham stimulation conditions, adjusted for covariates



Figures 23 (A-D) detail the Heart Rate response results of the Active Stand (AS) during baseline, active and sham stimulation

Tissue Saturation Index

TSI data was derived for each condition (baseline [no stimulation], active stimulation and sham stimulation) prior to standing, at 10-second intervals after standing and then the mean of the values from 120-180 seconds was used for the steady state values. There were no significant differences observed in the TSI values at 10, 20, 30, 40, 60, 120 seconds or during steady state (mean of values from 120-180 seconds) during baseline (no stimulation), active stimulation or sham stimulation. This was at baseline and after controlling for multiple covariates including age, sex, BMI, AD status, polypharmacy, CCI and smoking status as analysed in a mixed effects linear model. There were no significant differences observed in the TSI nadir values, the time to nadir, the TSI overshoot or TSI recovery rate values between baseline, active stimulation, and sham stimulation. Table 39 details the summary TSI data and the unadjusted mixed effects model used to compare values across the three conditions, while Table 40 details this analysis adjusted for covariates. These results are graphically depicted in Figures 24 (A-D).

	Mean (SD) or median (IQR) of test results			***Mixed effects model, unadjusted***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Tissue Saturation Index (TSI)						
TSI pre-stand (in %)	66.57 SD 3.61	65.79 SD 4.02	65.85 SD 3.7	β= -0.78 95% CI (-2.46 – 0.89) z= -0.92 p= 0.35	β=-0.71 95% CI (-2.37 – 0.93) z= -0.85 p= 0.39	β= -0.02 95% CI (-0.86 – 0.81) z= -0.06 p= 0.95
TSI 10 sec (relative) in %	-0.9 SD 1.61	-0.95 SD 0.96	-1.22 SD 1.25	β= -0.04 95% CI (-0.62 – 0.53) z=-0.15 p= 0.88	β=-0.31 95% CI (-0.88 – 0.25) z= -1.08 p= 0.28	β=0.13 95% CI (-0.15 – 0.42) z=0.92 p=0.35
TSI 20 sec (relative) in %	-1.09 SD 1.77	-1.07 SD 1.11	-0.89 SD 1.27	β= 0.02 95% CI (-0.6 0 0.65) z= 0.08 p=0.94	β=0.19 95% CI (-0.42 – 0.82) z= 0.63 p= 0.53	β=-0.08 95% CI (-0.4 – 0.22) z= -0.55 p= 0.58
TSI 30 sec (relative) in %	-0.8* SD 1.52	-0.93 SD 1.14	-0.6 SD 1.11	β= -0.12 95% CI (-0.68 – 0.44) z= -0.42 p= 0.67	β= 0.2 95% CI (-0.35 – 0.76) z= 0.71 p= 0.47	β= -0.16 95% CI (-0.44 – 0.12) z= -1.13 p= 0.25
TSI 40 sec (relative) in %	-0.81* SD 1.57	-0.96 SD 1.15	-0.89 SD 1.17	β= -0.14 95% CI (-0.72 – 0.43) z= -0.49 p= 0.62	β= -0.07 95% CI (-0.64 – 0.5) z=-0.25 p= 0.8	β= -0.03 95% CI (-0.32 – 0.25) z= -0.24 p= 0.81
TSI 60 sec (relative) in %	-0.91 SD 1.52	-0.97 SD 1.34	-0.98 SD 1.24	β= -0.05 95% CI (-0.66 – 0.55) z= -0.17 p= 0.87	β=-0.05 95% CI (-0.65 – 0.54) z= -0.18 p= 0.86	β=0.001 95% CI(-0.3 – 0.31 z= 0.01 p=0.99

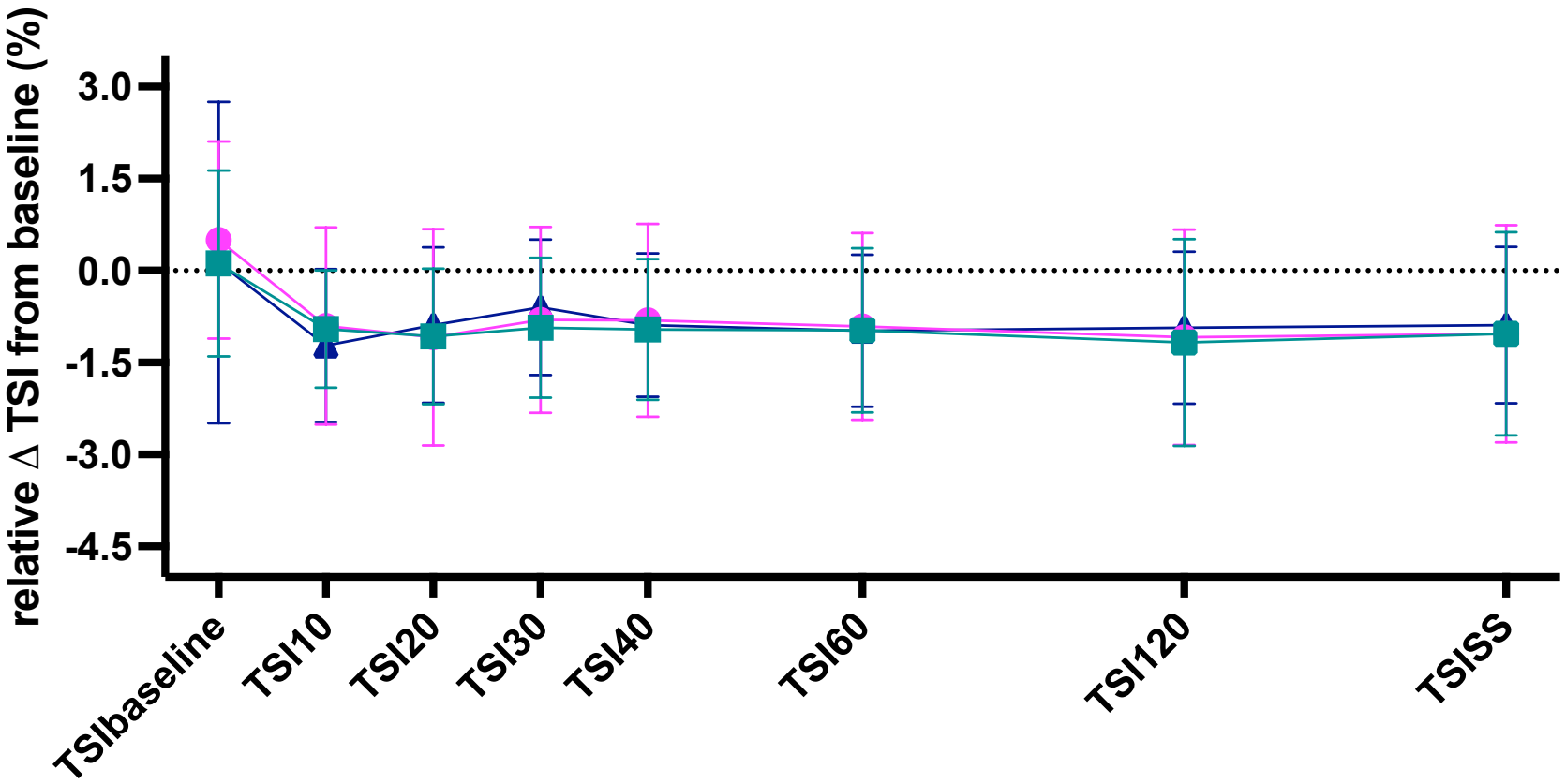
TSI 120 sec (relative) in %	-1.09 SD 1.76	-1.17 SD 1.69	-0.93 SD 1.24	β=-0.08 95% CI (-0.78 – 0.61) z= -0.24 p= 0.81	β= 0.16 95% CI (-0.52 – 0.85) z= 0.46 p= 0.64	β=-0.12 95% CI (-0.47 – 0.22) z= -0.7 p= 0.48
TSI steady state (relative) in %	-1.03 SD 1.77	-1.03 SD 1.66	-0.89 SD 1.28	β= 0.01 95% CI (-0.69 – 0.71) z= 0.02 p= 0.98	β= 0.14 95% CI (-0.56 – 0.83) z= 0.39 p= 0.69	β= -0.07 95% CI (-0.41 – 0.29) z= -0.37 p= 0.71
TSI recovery rate (seconds)	0.08 (IQR 0.03 – 0.17)	0.08 (IQR 0.02 – 0.14)	0.07 (IQR 0.04 – 0.19)	β=-0.01 95% CI (-0.05 – 0.04) z= -0.35 p= 0.72	β=0.02 95% CI (-0.03 – 0.06) z= 0.79 p= 0.42	β=-0.01 95% CI (-0.03 – 0.01) z= -1.14 p= 0.25
TSI nadir (relative) in %	-1.8 SD 1.9	-1.77 SD 1.06	-1.92 SD 1.44	β= 0.03 95% CI (-0.64 – 0.71) z= 0.09 p=0.92	β= -0.12 95% CI (-0.78 – 0.55) z= -0.35 p= 0.73	β= 0.07 95% CI (-0.26 – 0.41) z= 0.43 p=0.66
TSI time to nadir (seconds)	11.78 (IQR 9.07 – 19.1)	14.12 (IQR 10.9 – 20.4)	13.06 (IQR 9.5 – 18.7)	β= 1.37 95% CI (-1.57 – 4.32) z= 0.91 p= 0.36	β= 0.29 95% CI (-2.61 – 3.21) z= 0.2 p= 0.84	β= 0.53 95% CI (-0.93 – 2.01) z= 0.71 p= 0.47
TSI overshoot (relative) in %	-0.43 (IQR -1.09 – 0.32)	-0.45 (IQR -1.15 – 0.42)	-0.11 (IQR -0.65 – 0.66)	β= -0.1 95% CI (-0.72 – 0.52) z= -0.31 p= 0.75	β=0.09 95% CI (-0.52 – 0.72) z= 0.31 p= 0.76	β=-0.09 95% CI (-0.41 – 0.21) z= -0.63 p= 0.53

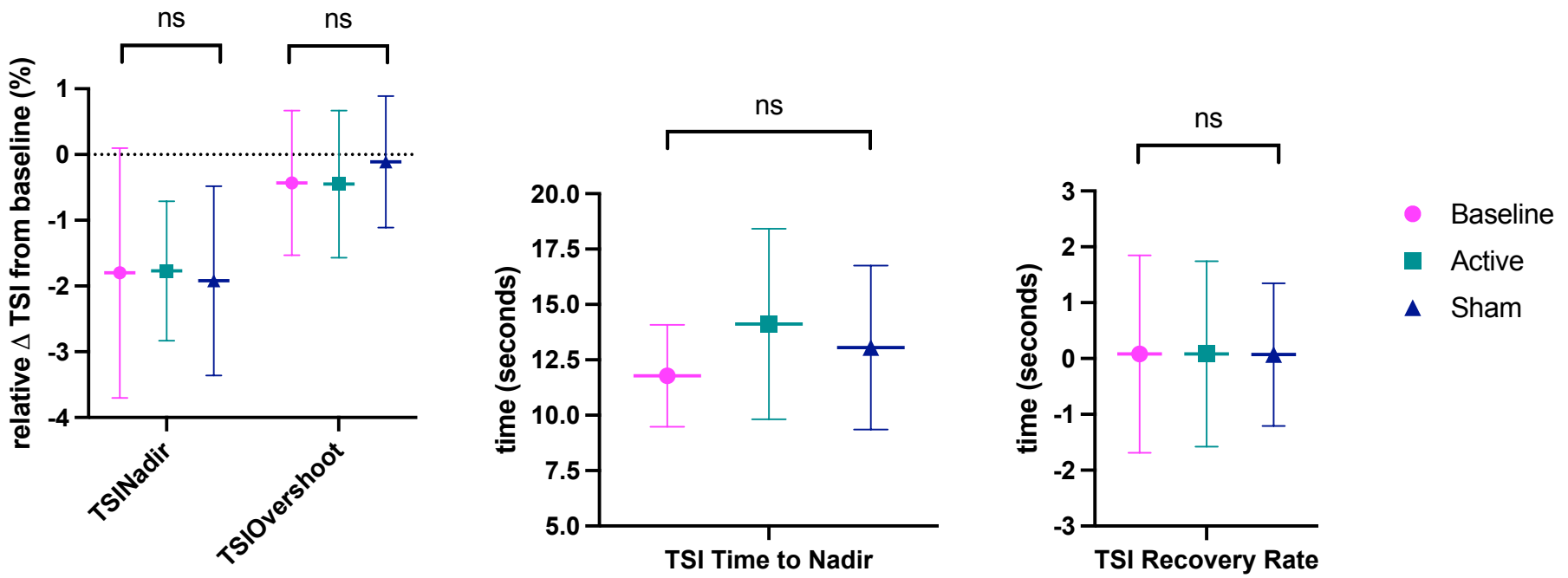
Table 39 details the unadjusted mixed effects model analysing the difference in Tissue Saturation Index (TSI) during baseline, active and sham stimulation conditions

	Mean (SD) or median (IQR) of test results			***Mixed effects model adjusted for baseline age, sex, BMI, AD status, polypharmacy, CCI, smoking***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Tissue Saturation Index (TSI)						
TSI pre-stand (in %)	66.57 SD 3.61	65.79 SD 4.02	65.85 SD 3.7	β= -0.83 95% CI (-2.36 – 0.69) z= -1.07 p= 0.28	β= -0.77 95% CI (-2.28 – 0.73) z= -1.01 p= 0.31	β= -0.02 95% CI (-0.79 – 0.75) z= -0.06 p= 0.95
TSI 10 sec (relative) in %	-0.9 SD 1.61	-0.95 SD 0.96	-1.22 SD 1.25	β= -0.05 95% CI (-0.63 – 0.51) z= -0.2 p= 0.84	β= -0.33 95% CI (-0.89 – 0.23) z= -1.14 p= 0.25	β=0.13 95% CI (=1.15 – 0.42) z= 0.94 p= 0.35
TSI 20 sec (relative) in %	-1.09 SD 1.77	-1.07 SD 1.11	-0.89 SD 1.27	β= 0.02 95% CI (-0.59 – 0.62) z= 0.05 p= 0.96	β=0.18 95% CI (-0.42 – 0.77) z= 0.57 p= 0.57	β= -0.08 95% CI (-0.38 – 0.22) z= -0.52 p= 0.6
TSI 30 sec (relative) in %	-0.8 SD 1.52	-0.93 SD 1.14	-0.6 SD 1.11	β=-0.12 95% CI (-0.66 – 0.43) z= -0.42 p= 0.67	β= 0.19 95% CI (-0.34 – 0.72) z= 0.7 p= 0.48	β= -0.15 95% CI (-0.43 – 0.12) z= -1.11 p= 0.27
TSI 40 sec (relative) in %	-0.81 SD 1.57	-0.96 SD 1.15	-0.89 SD 1.17	β= -0.13 95% CI (-0.69 – 0.43) z= -0.46 p= 0.64	β= -0.08 95% CI (-0.63 – 0.47) z=-0.29 p= 0.77	β= -0.02 95% CI (-0.31 – 0.26) z= -0.17 p= 0.86
TSI 60 sec (relative) in %	-0.91 SD 1.52	-0.97 SD 1.34	-0.98 SD 1.24	β= -0.05 95% CI (-0.62 – 0.51) z= -0.18 p= 0.86	β= -0.07 95% CI (-0.63 – 0.49) z= -0.24 p= 0.81	β= 0.01 95% CI (-0.27 – 0.29) z= 0.06 p= 0.95

TSI 120 sec (relative) in %	-1.09 SD 1.76	-1.17 SD 1.69	-0.93 SD 1.24	β=-0.06 95% CI (-0.68 – 0.55) z=-0.22 p= 0.83	β= 0.14 95% CI (-0.46 – 0.75) z= 0.48 p= 0.63	β= -0.11 95% CI (-0.42 – 0.19) z= -0.69 p= 0.48
TSI steady state (relative) in %	-1.03 SD 1.77	-1.03 SD 1.66	-0.89 SD 1.28	β=0.014 95% CI (-0.6 – 0.63) z= 0.04 p= 0.97	β=0.11 95% CI (-0.49 – 0.72) z= 0.37 p= 0.71	β=-0.05 95% CI (-0.36 -0.25) z= -0.32 p= 0.74
TSI recovery rate (seconds)	0.08 (IQR 0.03 – 0.17)	0.08 (IQR 0.02 – 0.14)	0.07 (IQR 0.04 – 0.19)	β= -0.01 95% CI (-0.05 – 0.03) z= -0.34 p= 0.73	β= 0.02 95% CI (-0.22 – 0.06) z= 0.9 p=0.37	β= -0.012 95% CI (-0.03 – 0.01 z=-1.23 p= 0.21
TSI nadir (relative) in %	-1.8 SD 1.9	-1.77 SD 1.06	-1.92 SD 1.44	β= 0.03 95% CI (-0.63 – 0.69) z= 0.09 p= 0.93	β=-0.11 95% CI (-0.76 – 0.54) z= -0.34 p= 0.73	β=0.07 95% CI (-0.25 – 0.39) z= 0.43 p= 0.67
TSI time to nadir (seconds)	11.78 (IQR 9.07 – 19.1)	14.12 (IQR 10.9 – 20.4)	13.06 (IQR 9.5 – 18.7)	β=1.29 95% CI (-1.63 – 4.21) z= 0.87 p= 0.39	β= 0.28 95% CI (-2.59 – 3.16) z= 0.19 p=0.85	β= 0.49 95% CI (-0.95 – 1.95) z= 0.67 p= 0.5
TSI overshoot (relative) in %	-0.43 (IQR -1.09 – 0.32)	-0.45 (IQR -1.15 – 0.42)	-0.11 (IQR -0.65 – 0.66)	β= -0.06 95% CI (-0.66 – 0.53) z= -0.21 p= 0.83	β=0.11 95% CI (-0.475 – 0.7) z= 0.38 p= 0.71	β=-0.09 95% CI (-0.38 – 0.21) z= -0.59 p= 0.56

Table 40 details the mixed effects model analysing the difference in Tissue Saturation Index (TSI) during baseline, active and sham stimulation conditions, adjusted for covariates


relative Δ TSI from baseline (%)
3.0
1.5
0.0
-1.5
-3.0
-4.5
TSIbaseline
TSI10
TSI20
TSI30
TSI40
TSI60
TSI120
TSISS



Figures 24 (A-D) detail the Tissue Saturation Index (TSI) response results of the Active Stand (AS) during baseline, active and sham stimulation

Associations

We found no significant moderating effects of baseline AD status on SBP, DBP or TSI responses to AS, however there was a difference at baseline in baseline HR values between those with underlying AD and those without, whereby those with AD had lower resting HR values than those without known underlying AD (or for other reasons did not undergo AD testing, please see Chapter 4 for breakdown of underlying aetiology). Those with known AD had a baseline HR of 67.58 ± 10.5 bpm, and those without underlying AD had baseline HR values of 76.48 ± 11.7 bpm. This association on linear regression withstood controlling for significant covariates including age, sex, BMI, baseline HR, polypharmacy and beta-blocker Rx (β= -8.96 [-17.51 - -0.41]) t = -2.14; p=0.04). AD status did not predict any other HR outcomes during AS. These data are displayed in Tables 41, 42 and 43 and graphically depicted in Figure 25 below.

Does AD status predict TSI % at baseline or during AS?
Linear Model, dependent variable is TSI
Controlled for age, sex, BMI, smoking, polypharmacy

	Beta Coefficient (95% CI)	statistic
TSI baseline	-0.18 (-2.77 – 2.39)	t = -0.14 p= 0.89
TSI 10 seconds	-0.01 (-1.15 – 1.17)	t = -0.61; p = 0.98
TSI 20 seconds	-0.69 (-1.88 – 0.49)	t = -1.18; p= 0.24
TSI 30 seconds	-0.37 (-1.39 – 0.66)	t= -0.73; p= 0.47
TSI 40 seconds	-0.29 (-1.42 – 0.84)	t= -0.52; p=0.6
TSI 60 seconds	-0.09 (-1.11 – 0.92)	t= -0.19; p -0.85
TSI 120 seconds	-0.73 (-1.88 – 0.42)	t = -1.29; p=0.2
TSI SS seconds	-0.58 (-1.76 – 0.59)	t = -1.01; p = 0.32
TSI Nadir	-0.43 (-1.72 – 0.87)	t = -0.68; p=0.5
TSI time to nadir	1.81 (-3.5 – 7.14)	t= 0.7; p= 0.49
TSI overshoot	-0.33 (-1.54 – 0.89)	t = -0.55; p = 0.59
TSI recovery rate	-0.01 (-0.05 – 0.06)	t= 0.18; p= 0.86

Table 41 details the linear regression model used to investigate associations between AD status and TSI at baseline and during Active Stand (AS)

Does AD status predict SBP (mmHg) at baseline or during AS?
Linear Model, dependent variable is SBP
Controlled for age, sex, BMI, baseline HTN, antidepressant and antihypertensive Rx

	Beta Coefficient (95% CI)	statistic
SBP baseline	0.17 (-10.6 – 11.01)	t = 0.03; p =0.98
SBP 10 seconds	-15.42 (-33.8 – 2.99)	t= -1.72; p= 0.09
SBP 20 seconds	-8.73 (-27.05 – 9.59)	t=-0.57; p=0.34
SBP 30 seconds	-4.92 (-23.18 – 13.3)	t = -0.55; p=0.58
SBP 40 seconds	-12.08 (-30.49 – 6.33)	t=-1.35; p=0.19
SBP 60 seconds	-9.32 (-28.94 – 10.3)	t=-0.98; p=0.34
SBP 120 seconds	-6.63 (-25.3 – 12.03)	t= -0.73; p=0.47
SBP SS	-7.69 (-25.26 – 9.87)	t= -0.9; p=0.38
SBP Nadir	-12.76 (-29.93 – 4.41)	t= -1.53; p=0.14
SBP time to nadir	-1.82 (-6.13 – 2.49)	t=-0.87; p=0.39
SBP overshoot	-12.21 (-31.79 – 7.37)	t=-1.28; p=0.21
SBP recovery rate	-0.14 (-0.9 – 0.61)	t=-0.39; p=0.69

Table 42 details the linear regression model used to investigate associations between AD status and SBP at baseline and during Active Stand

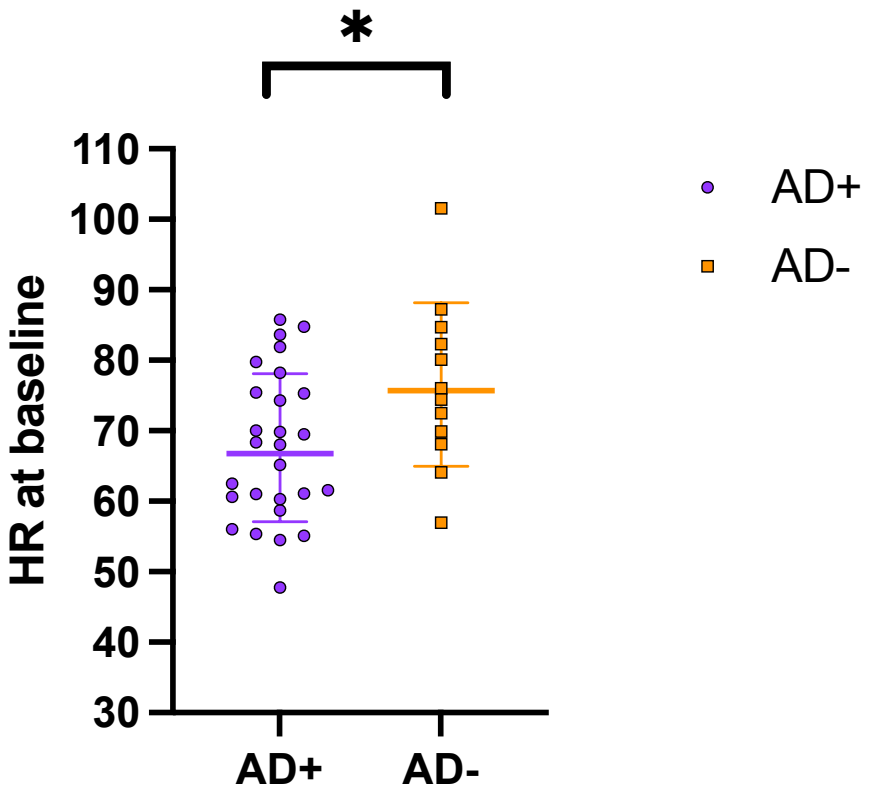
(AS)

Does AD status predict HR (bpm) at baseline or during AS?
Linear Model, dependent variable is HR
Controlled for age, sex, BMI, baseline HR, polypharmacy and B-blocker Rx

	Beta Coefficient (95% CI)	statistic
HR baseline	Unadjusted -8.9 (-16.56 - -1.24) Adjusted -8.96 (-17.51 - -0.41)	 t= -2.35; p=0.02* t = -2.14 p=0.04*
HR 10 seconds	1.93 (-0.99 – 4.87)	t = 1.35; p= 0.19
HR 20 seconds	-1.16 (-4.71 – 2.37)	t= -0.67; p= 0.51
HR 30 seconds	-2.33 (-5.88 – 1.21)	t=-1.34; p =0.19
HR 40 seconds	-1.31 (-5.52 – 2.91)	t= -0.63; p= 0.53
HR 60 seconds	-1.38 (-6.72 – 3.95)	t = -0.53; p =0.6
HR 120 seconds	0.55 (-4.61 – 5.71)	t = 0.22; p= 0.83
HR SS	0.27 (-3.69 – 4.23)	t=0.14; p = 0.89
HR min	-2.07 (-6.28 – 2.13)	t = -1.01; p = 0.32
HR max	-1.11 (-5.19 – 2.96)	t = -0.56; p = 0.58
HR time between min-max	2.81 (-2.17 – 7.79)	t – 1.15; p = 0.26
HR recovery rate	0.11 (-0.39 – 0.62)	t= 0.44; p=0.66

Table 43 details the linear regression model used to investigate associations between AD status and HR at baseline and during Active Stand

(AS)



Figures 25 depicts the difference seen in resting Heart Rate (HR) for participants with diagnosed AD compared to those without known AD

There were no significant differences in the incidence of classical OH, delayed recovery (at 30 seconds, 40 seconds or overall), IOH or OHTN between conditions. Fewer participants appeared to have delayed BP recovery during active stimulation (6 [18.7%]) compared to sham (8 [25%]) or baseline (12 [37.5%]) but this did not reach statistical significance (x^2 =4.01; p=0.13). This data is summarised in Table 44 (A, B) and graphically depicted in Figures 26 (A-F).

Table 44 A. Comparisons for all data points (intention to treat) (n =39)

	Condition			
	Baseline n=39	Active n=36	Sham n=36	*Test statistic; significance*
Classic OH	8 (20.5%)	10 (27.7%)	7 (19.4%)	x^2 =0.78; p =0.68
Delayed Recovery (overall)	16 (44.4%)	6 (16.6%)	10 (27.7%)	x^2 =5.44; p=0.06
Delayed Recovery at 30 seconds	11 (28.2%)	5 (13.8%)	7 (19.4%)	x^2 =2.35; p=0.31
Delayed Recovery at 40 seconds	11 (28.2%)	5 (13.8%)	6 (16.6%)	x^2 =2.65; p=0.26
Initial OH	11 (28.2%)	6(16.6%)	7 (19.4%)	x^2 =1.56; p=0.46
Orthostatic HTN	3 (7%)	3 (8%)	2 (5%)	x^2 =0.19, p-0.91

Table 44 B. Comparisons only for participants with complete data at all 3 time points (n= 32)

	Condition			
	Baseline n=32	Active n=32	Sham n=32	*Test statistic; significance*
Classic OH	6 (18.7%)	7 (21.8%)	6 (18.7%)	x^2 =0.13; p =0.94
Delayed Recovery (overall)	12 (37.5%)	6 (18.7%)	8 (25%)	x^2 =4.01; p=0.13
Delayed Recovery at 30 seconds	8 (25%)	5 (15.6%)	6 (18.7%)	x^2 =0.92; p=0.63
Delayed Recovery at 40 seconds	8 (25%)	5 (15.6%)	6 (18.7%)	x^2 =0.92; p=0.63
Initial OH	10 (31.2%)	6 (18.7%)	7 (21.8%)	x^2 =1.49: p=0.48
Orthostatic HTN	3 (9.3%)	3 (9.3%)	2 (6.2%)	x^2 =0.27; p=0.87

Table 44 (A,B) details the comparisons across baseline, active and sham stimulation of incidence of Orthostatic Hypotension (OH) phenotypes

Heart Rate Variability

There were 99 participants' data available for HRV analysis, with 19 individual readings not suitable for analysis due to a high degrees of baseline interference and poor data quality. There were no significant differences found for the values of RMSSD, SDNN or pnn50 for any condition (baseline [no stimulation], active stimulation or sham stimulation} when analysed using a mixed effects model, both unadjusted and adjusted for baseline HR, age, sex, BMI, AD status, polypharmacy and beta-blocker Rx. The unadjusted and adjusted models are presented in Table 45 and 46. AD status did not predict any HRV indices at baseline, during active or sham stimulation when modelled via a linear regression analysis, which is presented in Table 47.

	Mean (SD) or median (IQR) of test results			***Mixed effects model, unadjusted***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Heart Rate Variability						
RMSSD	20.98 (IQR 14.01 – 33.23)	18.05 (IQR 11.23 – 25.8)	23.45 (IQR 14.57 – 35.15)	β = -0.73 (95% CI = -47.01 – 45.55) z = -0.03 p =0.97	β = -10.61 (95% CI = -57.29 – 36.06) z = -0.45 p =0.65	β = 4.96 (95% CI = -19.09 – 38.92) z = 0.4 p =0.68
SDNN	35.05 (IQR 24.23 – 44.71)	30.98 (IQR 22.17 – 42.39)	35.98 (IQR 22.62 – 40.68)	β = -2.47 (95% CI = -29.43 – 24.49) z = -0.18 p =0.85	β = -10.13 (95% CI = -37.33 – 17.05) z = -0.73 p =0.45	β = 3.75 (95% CI = -10.2 – 17.08) z = 0.53 p =0.59
pNN50	0.02 (IQR 0.01 – 0.09)	0.01 (IQR 0 – 0.05)	0.01 (0.01 – 0.13)	β = -0.02 (95% CI = -0.09 – 0.05) z = -0.68 p =0.53	β = -0.02 (95% CI = -0.11 – 0.04) z = -0.68 p =0.49	β = 0.01 (95% CI = 0.03 – 0.03) z = 0.06 p =0.96

Table 45 details the HRV indices examined under baseline, active and sham conditions, unadjusted

	Mean (SD) or median (IQR) of test results			***Mixed effects model, adjusted for baseline HR, age, sex, BMI, AD status, polypharmacy, B-Blocker Rx***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Heart Rate Variability						
RMSSD	20.98 (IQR 14.01 – 33.23)	18.05 (IQR 11.23 – 25.8)	23.45 (IQR 14.57 – 35.15)	β = -0.22 (95% CI = -42.78 – 42.3) z = -0.01 p =0.99	β = -9.86 (95% CI= -52.62 – 32.9) z = -0.45 p =0.65	β = 4.77 (95% CI= -17.3 – 26.9) z = 0.43 p =0.67
SDNN	35.05 (IQR 24.23 – 44.71)	30.98 (IQR 22.17 – 42.39)	35.98 (IQR 22.62 – 40.68)	β = 0.61 (95% CI = -23.52 – 24.73) z = 0.05 p =0.96	β = -7.31 (95% CI = -31.37 – 17.14) z = -0.57 p =0.56	β = 3.85 (95% CI = -8.69 – 16.39) z = 0.6 p =0.54
pNN50	0.02 (IQR 0.01 – 0.09)	0.01 (IQR 0 – 0.05)	0.01 (0.01 – 0.13)	β = -0.02 (95% CI = -0.09 – 0.04) z = -0.65 p =0.52	β = -0.02 (95% CI= -0.09 – 0.04) z = -0.53 p =0.59	β = -0.01 (95% CI = -0.04 – 0.03) z = -0.11 p =0.91

Table 46 details the HRV indices examined under baseline, active and sham conditions, adjusted for covariates

Does AD status predict HRV indices at baseline or during stimulation?
Linear Model, dependent variable is HRV index. Controlled for age, sex, BMI, baseline HR, polypharmacy, beta-blocker Rx

	Beta Coefficient (95% CI)	statistic
RMSDD (baseline)	0.01 (-1.11 – 0.14)	t=0.19; p=0.85
RMSDD (active stim)	0.12 (-0.05 – 0.3)	t=1.47; p =0.15
RMSDD (sham stim)	0.13 (-0.03 – 0.29)	t= 1.66; p=0.11
SDNN (baseline)	-1.57 (-16.54 – 13.39)	t=-0.22; p = 0.83
SDNN (active stim)	-0.61 (-14.78 – 13.56)	t=-0.09; p = 0.93
SDNN (sham stim)	2.72 (-10.07 – 15.52)	t=0.44; p=0.66
pnn50 (baseline)	-0.02 (-0.05 – 0.02)	t= -1.03; p=0.31
pnn50 (active stim)	-0.03 (-0.08 – 0.06)	t=-1.77; p=0.09
pnn50 (sham stim)	-0.02 (-0.07 – 0.03)	t= -0.96; p=0.34

Table 47 details the linear regression model used to investigated the associations between HRV indices under baseline active and sham conditions and AD status

Discussion

In the neurocardiovascular arm of this study we found no significant effect of tVNS active or sham stimulation on the parameters measured, including SBP, DBP, HR or TSI at rest or during AS. This reassuringly suggests that in a population with amnestic MCI that is of older age (mean age 71.7 $\pm$ 6.9) of whom 67.5% (27 / 40) have AD as their known underlying pathological diagnosis, that non-invasively stimulating the distribution of the ABVN acutely does not deleteriously affect their response to an orthostatic challenge, either peripherally or centrally. We also noted no significant effect of acute tVNS stimulation on any HRV parameter studied, as measured via five minutes of uninterrupted ECG recording.

There are some limitations to this research. Notably, this study only assesses the effects of active tVNS (less than 60 minutes) on any neurocardiovascular index. Previously research in this area has shown effects on HRV indices when stimulated with tVNS devices daily for two-weeks [190]. The amount of time stimulated in this study may have been too short to capture significant effects on neurocardiovascular outcomes. However these results do support the hypothesis that acute tVNS is safe from a cardiovascular perspective in a population that is known to have high prevalence of NCVI [540] . The next steps in confirming and solidifying tVNS' safety profile would involve at-home stimulation, multiple times a days for a number of weeks with planned neurocardiovascular outcomes measured as primary endpoints. The challenges of undertaking detailed domiciliary-based research in older adults are well-documented [541,542] but remote monitoring of clinical patients and research trial participants has become more acceptable in recent years [543,544] and may render such a study design more feasible.

There was a high incidence of OH at baseline in this study population, with 18.7% presenting with classical OH at baseline, a further 31.2% meeting criteria for IOH not fulfilling classical OH criteria, and 37.5% had delayed recovery either at 30 or 40 seconds post standing i.e. 35 of 40 (87.5%) of the participants studied had some degree of OH at baseline. This data is in keeping with the known literature in this area, which proposes that MCI is correlated with NCVI and the degree of NCVI may influence MCI progression to dementia [528]. The degree of cerebral vascular burden for each participant was not available for analysis as a covariate in this study, and it would be interesting in future studies to see if a higher degree of NCVI is associated with cerebral white matter vascular burden and NCVI in amnestic MCI.

Participants with confirmed underlying AD pathology had significantly slower resting HR than those without confirmed underlying AD, without any significantly slower HR responses to an orthostatic challenge during AS. This finding is noteworthy as higher resting HR has been found to be associated with an increased risk of all-cause dementia, vascular dementia but not AD in large prospective cohort studies.[545] Though the participants in this study technically did not have resting bradycardia, it would be fruitful to further investigate this in wider cross-sectional and longitudinal studies to see if this finding is replicated, as many current AD treatments may cause bradyarrhythmias [546,547] which could be clinically significant.

Overall we found no significant effect on neurocardiovascular indices and parameters during active or sham tVNS at the cymba conchae, indicating that acute non-invasive stimulation of this specific site of the ABVN is likely safe in this population. Further studies should

investigate neurocardiovascular parameters after more prolonged stimulation in a variety of research and domiciliary settings.

Chapter 7 : Exploring the Effects of Acute tVNS on Inflammation, and novel exploration of Blood Biomarkers of Alzheimer's Disease in MCI

VNS and inflammation

The discovery of the neural control of inflammation, particularly the molecular mechanisms of VN regulation of systemic cytokine levels via the "inflammatory reflex" [548] have made it possible to consider VNS in the treatment of inflammation and inflammatory diseases [549]. This neuro-immune reflex mechanism afferently senses inflammation and injury, and the efferent VN projections regulate cytokine production and release [550].

Preclinical work has reported ACh-mediated anti-inflammatory effects on peripheral macrophages, facilitated by peripheral nicotinic receptors (nAChR's). [551] Transection of the VN (i.e. vagotomy) in rodent models subjected to Lipopolysaccharide (LPS)-induced endotoxemia leads to a more destructive Systemic Inflammatory Response Syndrome (SIRS), with higher levels of the pro-inflammatory cytokine TNF-α released from macrophages. Fascinatingly, electrical stimulation of the distal branches of the transected VN attenuated this aggressive SIRS response. [552]

Further preclinical studies found that splenectomy and transection of the splenic nerve abolished the effects of VNS on systemic TNF-α released in response to endotoxemia and polymicrobial sepsis [553] [554] It has since been established that the Alpha -7 – ACh Receptor (α7nAChR) subtype is responsible for the anti-inflammatory effects of ACh, as demonstrated in α7nAChR knockout murine models. [555]

Physiology

The α7nAChR is intimately linked with the cholinergic activation pathway [555]. Centrally, LC cell loss results in amplified microglial and astroglial activation in the frontal cortex and hippocampus in mutant APP mouse models, suggesting that the dysregulation of the LC–NE system may play a major role in disease-related inflammation [429]. LC lesioning leads to increased glial inflammation and Aβ deposition in LC projection areas, including the hippocampus and frontal cortex; whereas, non-projection areas such as the paraventricular thalamus remain unaffected.[428]

Animal disease models

Preclinical models have demonstrated that VNS reduced circulating cytokines in colitis [556], post selective vagotomy [557], in preclinical models of menopause and systemic inflammation [558] and in a murine model of Rheumatoid Arthritis (RA) [559]. Preclinical experimental models of Acute Respiratory Distress Syndrome (ARDS) and sepsis have also noted that acute VNS decreases lung injury and cellular dysfunction.[560]

Clinical Studies and Rheumatological Disease

iVNS studies

Induction levels of pro-inflammatory cytokines by VNS-stimulated mononuclear cells isolated from patients treated with iVNS for refractory epilepsy found significant decreases in IL-8 after 6 months of iVNS in comparison to the pre-stimulation state [561]. A subsequent group of investigators analysed levels of TNF-α post iVNS insertion for epilepsy and found

significantly lower circulating levels post insertion. [562] Similarly an investigation of iVNS devices in patients with epilepsy and no diagnosed inflammatory conditions demonstrated significant inhibition of peripheral blood production of TNF, IL-1β,and IL-6 in those with iVNS devices [563]

Rheumatological diseases

iVNS devices were implanted in a small group of patients (n=18) with RA with significant inhibition of TNF production noted for up to 84 days and reduced RA disease severity indexes suggesting that VNS, targeting the inflammatory cascade, may reduce inflammation in inflammatory disease states [563].

A recent study investigating tVNS treatment for four days in patients with Systemic Lupus Erythematosus (SLE) noted a significant decrease in pain and fatigue compared with sham stimulation. [564] A double blind RCT has been registered aiming to study the change in spondylo-arthritis disease activity after 12 weeks of tVNS treatment vs sham, with the authors aiming to recruit 120 adult patients with treatment-resistant spondylo-arthritis.[565] Non-invasive VNS demonstrated a reduction in circulation cytokines in a small population with Primary Sjogren's Syndrome [566] and similar small pilot studies of tVNS in populations with RA [567–570] have noted improvements in cytokines or disease severity scores, with larger scale studies needed to confirm a therapeutic benefit of non-invasive stimulation in these clinical populations.

Whether the VNS-mediated reduction in symptoms in those with inflammatory conditions may be due moderation of cardiac vagal tone has also been hypothesised. An investigation

of the effect of tVNS on cardiac vagal tone and disease activity scores in 20 patients with Psoriatic Arthritis and 17 patients with Ankylosing Spondylitis demonstrated that in the Ankylosing Spondylitis group cardiac vagal tone was significantly increased 30 min after tVNS in comparison to baseline accompanied by a significant reduction in HR. A significant reduction in disease severity score was demonstrated in the Psoriatic Arthritis group, raising the possibility of further investigation into the cardiac-neural inflammatory link. [571]

These studies offer an exciting route via which manipulating the inflammatory vagal axis might be beneficial in inflammatory disease states. However, overall these studies are small and not powered to detect absolute changes in either symptom burden or circulating cytokines in specific disease states, and further dedicated and powered-RCTs would be fruitful to establish whether there is a role for non-invasively stimulating the VN in managing inflammatory conditions.

Blood Biomarkers of AD

In the past decade there has been unparalleled progress in the development and validation of Blood Bio-Markers (BBMs) in AD [414]. BBMs are likely to have a transformative role in facilitating early, precise diagnosis as well as informing prognostication for those living with AD as they are cheaper, less invasive and more accessible than traditional diagnostic tools (CSF sampling and PET imaging). BBMs are also likely to have a crucial role in identifying individuals suitable for access to clinical trials and emerging Disease-Modifying Therapies (DMTs) - such as anti-amyloid immunotherapies. [447,572,573] Recent appropriate use guidelines

for BBMs encourage their cautious use in specialised memory clinics - with confirmation of results using CSF/PET where possible. [574]

Plasma p-tau 217

As mentioned n Chapter 3, amyloid and tau pathology are two of the key hallmarks of neuropathology in AD. Whilst the amyloid marker Aβ42/Aβ40 is an established biomarker of AD pathology in CSF, its performance as a BBM is limited by peripheral amyloid (Aβ) production which reduces diagnostic sensitivity.[575]

Meanwhile, p-tau species have emerged as promising BBMs for AD detection, with several p-tau epitopes (p-tau181, p-tau217 and p-tau231) - changing at different stages of the AD continuum.[576–578] Currently, p-tau217 is the most promising BBM for detection of AD pathology [579,580] and may differentiate AD from other dementias.[581,582] Plasma p-tau217 can detect cortical Aβ accumulation and may mediate the association between Aβ plaques and subsequent tau tangle pathology.[583,584] Plasma p-tau217 may also have prognostic utility - levels are associated with future AD progression as measured by clinical decline and hippocampal/cortical atrophy.[581,585]

Neurofilament light

Neurofilament light (NfL) is a biomarker that is reflective of axonal degeneration and injury irrespective of cause and it can be measured in CSF and plasma.[586] The levels of NfL are especially increased in ALS, FTD and atypical parkinsonian disorders.[587,588] However, NfL levels are also increased in AD, and studies on autosomal dominant AD show that the rate of

change in blood NfL increases approximately 15 years before symptom onset.[589] It has been noted that higher levels of NfL are associated with faster disease progression and higher cerebral atrophy rates in most neurodegenerative disorders.[587,589] In several neurological diseases, including Multiple Sclerosis, effective DMTs can normalise NfL levels and NfL levels pre-treatment can predict response.[590]

Glial biomarkers

Glial cells are a type of astrocyte, the resident inflammatory cells of the CNS, and are widely distributed in the human brain. The Glial Fibrillary Acidic Protein (GFAP) is a cytoskeletal structure and is astrocyte specific. Its purpose is to equip astrocytes with the ability to quickly remodel and reorganise.[591] In pathological conditions, astrocytes change morphology, and overexpress specific pro-inflammatory proteins, such as GFAP. This phenomenon is termed “astrocyte reactivity” which may modulate pathological CNS responses[592] Several animal and human cell studies have shown that reactive astrocytes penetrate and surround Aβ plaques, possibly contributing to the amyloid-pathological process.[593,594]

GFAP expression peaks in early development, with a slow decline over the life course in normal ageing.[595] A secondary rise of GFAP expression is related to the development of pathology, such as AD or acquired gliosis, commonly also seen in Traumatic Brain Injury (TBI).[596] CSF GFAP, with other biomarkers such as a positive Aβ 42/40 ratio in combination with APOEe4 status, can differentiate likely AD from non-AD pathology.[597] Both CSF and,

more recently, plasma GFAP are gaining recognition as an emerging reliable biomarker of astrocyte reactivity and a surrogate marker of inflammation related to AD.[598]

In this exploratory arm of this study, we were interested in exploring the effects, if any, that acute (less than 60 minutes) tVNS stimulation to the left ear may have on circulating whole blood cytokines and chemokines and what the BBM results were for these 40 participants. A panel of cytokines and chemokines were selected as they have been shown to be prevalent in persons with MCI in international literature.[599,600]

Methods

Trial Design

Please see Chapter 4 for a detailed description of the Trial Design. Please note the venepuncture and blood sampling occurred at the end of each trial visit, i.e. after >60 minutes during baseline (no stimulation), active stimulation or sham stimulation. Please see Figure 12 in Chapter 4 for an illustration of the trial investigations undertaken at each trial visit.

Study Setting

Please see Chapter 4 for a detailed description of the Study Setting.

Sample Size

Please see Chapter 4 for a detailed description of the Sample Size.

Eligibility Criteria

Please see Chapter 4 for a detailed description of the Eligibility Criteria.

Device and Intervention

Please see Chapter 4 for a detailed description of the Device and Intervention.

Assessment Procedures of Inflammatory responses and Blood Biomarkers: Biological Sampling and Processing

Plasma and serum samples were obtained by aseptic venepuncture and collected in 9mL EDTA or serum tubes, which were centrifuged (1.8 g x 10 minutes) with plasma and serum aliquoted into 0.5mL sterile cryovials. Once processed, plasma/serum were stored at -80oC for future analysis. All plasma/serum samples were processed by trained staff on-site (study clinician HD) within 30 minutes to minimise impact of sample handling on pre-analytical variability.

Electrochemiluminescence Immunoassays for BBM

A high-sensitivity Electrochemoluminescence (ECL) assay from MesoScaleDiscovery (MSD) (S-PLEX; K51APFS) was used to assess levels of p-tau217 in both plasma and CSF samples. The commercially available S-PLEX assay uses ECL technology. Plates are coated with Streptavidin and a biotinylated antibody. Analytes of interest (in this case p-tau phosphorylated at threonine 217) are captured in standard sandwich format and a "TURBO BOOST®" antibody used for detection, which is enhanced with "TURBO-TAG®" reagents. This technology allows femtogram/mL measurement of analytes typically not detected by conventional ECL immunoassays.[601,602]

Samples (paired plasma and CSF) were thawed on ice and measured across five individual plates according to the manufacturer's instructions. Samples were randomised across the plates and the assessor blinded to the clinical status of each donor. Once completed, each plate was read on a MSD QuickPlex SQ 120 Analyser and Discovery Workbench 4.0 Software used to analyse results.

In parallel to analysing samples for p-tau217, separate plasma samples from each participant were analysed in single-plex for p-tau181 and in multi-plex for total tau (t-tau), NfL and GFAP using ultra-sensitive S-PLEX p-tau181 and S-PLEX neurology kits from MSD (K-156AGMS/ K-15639S) respectively. Experimental layout and analyses were identical as for p-tau217 and conducted according to manufacturer's instructions.

Electrochemiluminescence Immunoassays for Cytokines / Chemokine

A custom-battery of 10 cytokines and chemokines was analysed using the MSD V-PLEX ECL assay according to standard protocols and includes: IFN- γ, IL-10, IL12p70, IL-6, TNF- α, Eotaxin, IP-10, MCP-1, MIP-1 β, IL-17A based on known associations with MCI as referenced above.

Statistical analysis

Descriptive statistics were used to describe laboratory results across three stimulation conditions (baseline, active and sham stimulation). The natural log of the cytokines and

chemokines was used for comparisons and regressions due to data skew. Multilevel mixed effects models, accounting for relevant covariates, were used to compare the natural log of the cytokine and chemokine results across the three time points of the trial.

Given the MSD S-Plex ECL assay for BBMs for AD is commercially available but not yet validated for use in a clinical cohort, there are no established cut offs for positive or negative values for the BBMs. As such, absolute values are used, and multilevel linear regression models employed to establish associations with cognitive or neurocardiovascular data points.

Statistical analysis were undertaken using MATLAB (The MathWorks, Inc., Natick, MA, USA), STATA (Stata Statistical Software: Release 18, College Station, TX: StataCorp LLC) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results

Inflammatory Cytokines and Chemokines

Whole blood cytokines and chemokines were processed at baseline (n=40), active (n=39) and sham stimulation (n=39). The total stim stimulated pre venepuncture is detailed in Chapter 4, in summary for active stimulation there was a mean of 60.5 minutes (SD 4.4) total stimulation, and for sham stimulation there was a mean of 59.5 minutes (SD 5.7) total stimulation with no significant difference in time of stimulation between both conditions.

Table 48 details the unadjusted mixed effects model comparing cytokines and chemokines across the three conditions, and Table 49 details the mixed effects model comparing cytokines and chemokines across three conditions adjusted for relevant covariates. There was no significant differences found in any of the cytokines or chemokines, IFN- γ, IL-10, IL12p70, IL-6, TNF- α, Eotaxin, IP-10, MCP-1, MIP-1 β, IL-17A, across the three conditions studied, in unadjusted or adjusted models. These results are graphically depicted in Figures 27 (absolute values) and Figure 28 (natural log values) for ease of visualisation.

	Mean (SD) or median (IQR) of test results			***Mixed effects model, unadjusted***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Inflammatory Cytokines (natural log scale)						
IFN γ	1.63 (SD 0.66)	1.73 (SD 0.72)	1.69 (SD 0.56)	β=0.09 95% CI (-0.18 – 0.37) z= 0.65 p= 0.52	β=0.06 95% CI (-0.22 – 0.34) z= 0.41 p= 0.68	β=0.02 95% CI (-0.12 – 0.16) z= 0.24 p=0.81
IL-10	-1.31 (SD -0.85)	-1.35 (SD 0.82)	-1.16 (SD 0.69	β=-0.04 95% CI (-0.39 – 0.3) z= -0.25 p= 0.79	β=0.14 95% CI (-0.2 – 0.49) z=0.81 p= 0.42	β= -0.09 95% CI (-0.27 – 0.08) z=-1.06 p= 0.29
IL12p70	-1.85 (SD 1.08)	-1.82 (SD 1.01)	-1.72 (SD 1.04)	β= 0.02 95% CI (-0.43 – 0.48) z= 0.1 p=0.92	β=0.12 95% CI (-0.33 – 0.58) z= 0.55 p= 0.58	β=-0.05 95% CI (-0.28 – 0.17) z= -0.45 p= 0.66
IL-6	0.35 (SD 1.12)	0.35 (SD1.15)	0.16 (SD 1.29)	β=-0.01 95% CI (-0.52 – 0.51) z=0.00 p=1.0	β=-0.18 95% CI (-0.7 – 0.33) z= -0.7 p= 0.48	β=0.09 95% CI (-0.17 – 0.35) z= 0.69 p= 0.49
TNF-α	0.36 (SD 0.45)	0.34 (SD0.39)	0.33 (SD 0.36)	β=-0.02 95% CI (-0.19 – 0.15) z= -0.25 p= 0.8	β=-0.03 95% CI (-0.21 – 0.15) z= -0.33 p= 0.74	β=0.01 95% CI (-0.08 – 0.09) z=0.09 p= 0.93

Eotaxin	5.3 (SD 4.2)	5.31 (SD 0.46)	5.23 (SD 0.48)	β= 0.02 95% CI (-0.18 – 0.22) z= 0.18 p= 0.86	β=-0.07 95% CI (-0.26 – 0.13) z= -0.67 p= 0.5	β=0.04 95% CI (-0.05 – 0.14) z= 0.84 p= 0.4
IP-10	5.74 (SD 0.54)	5.69 (SD 0.58)	5.58 (SD 0.63)5	β=-0.05 95% CI (-0.31 – 0.2) z= -0.39 p= 0.69	β=-0.15 95% CI (-0.42 – 0.09) z= -1.22 p= 0.22	β=0.05 95% CI (-0.08 – 0.18) z= 0.82 p= 0.41
MCP-1	4.59 (SD 0.47)	4.58 (SD 0.49)	4.53 (SD 0.47)	β=-0.01 95% CI (-0.22 – 0.19) z= -0.13 p= 0.89	β=-0.06 95% CI (-0.27- 0.15) z=-0.56 p=0.57	β= 0.02 95% CI (-0.08 – 0.12) z= 0.42 p= 0.67
MIP-1β	4.23 (SD 0.43)	4.19 (SD 0.47)	4.15 (SD 0.41)	β= -0.04 95% CI (-0.23 – 0.15) z= -0.4 p= 0.69	β= -0.09 95% CI (-0.28 – 0.12) z= -0.87 p= 0.38	β=0.02 95% CI (-0.07 – 0.11) z= 0.47 p= 0.64
IL-17a	0.195 (SD 0.94)	0.21 (SD 0.66)	0.019 (SD 0.93)	β= 0.01 95% CI (-0.38 – 0.4) z= 0.07 p= 0.94	β= -0.17 95% CI (-0.57 – 0.21) z= -0.88 p= 0.38	β=0.09 95% CI (-0.1 – 0.29) z= 0.95 p=0.34

Table 48 unadjusted mixed effects model comparing inflammatory cytokines and chemokines across baseline, active and sham conditions

	Mean (SD) or median (IQR) of test results			***Mixed effects model adjusted for baseline age, sex, BMI, polypharmacy and CCI***		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Inflammatory Cytokines (natural log scale)						
IFN γ	1.63 (SD 0.66)	1.73 (SD 0.72)	1.69 (SD 0.56)	β=0.08 95% CI (-0.18 – 0.36) z= 0.62 p= 0.53	β=0.05 95% CI (-0.21- 0.33) z=0.41 p= 0.68	β=0.01 95% CI (-0.12 – 0.15) z=0.21 p= 0.84
IL-10	-1.31 (SD -0.85)	-1.35 (SD 0.82)	-1.16 (SD 0.69	β=-0.05 95% CI (-0.38 – 0.26) z=-0.34 p= 0.73	β=0.14 95% CI (-0.18 – 0.46) z=0.85 p= 0.39	β=0.09 95% CI (-0.26 – 0.06) z=-1.19 p= 0.23
IL12p70	-1.85 (SD 1.08)	-1.82 (SD 1.01)	-1.72 (SD 1.04)	β=0.01 95% CI (-0.41 – 0.42) z=0.02 p=0.98	β=0.12 95% CI (-0.29 – 0.54) z=0.58 p= 0.56	β=-0.06 95% CI (-0.27 – 0.15) z= -0.55 p=0.58
IL-6	0.35 (SD 1.12)	0.35 (SD1.15)	0.16 (SD 1.29)	β=0.01 95% CI (-0.5 – 0.51) z= 0.01 p= 0.99	β=-0.18 95% CI (-0.69 – 0.32) z= -0.73 p=0.46	β=0.09 95% CI (-0.16 – 0.34) z=-0.73 p=0.46
TNF-α	0.36 (SD 0.45)	0.34 (SD0.39)	0.33 (SD 0.36)	β=-0.02 95% CI (-0.18 – 0.14) z= -0.29 p= 0.77	β=-0.03 95% CI (-0.19 – 0.14) z= -0.34 p= 0.74	β=0.01 95% CI (-0.08 – 0.09) z= 0.05 p=0.96

Eotaxin	5.3 (SD 4.2)	5.31 (SD 0.46)	5.23 (SD 0.48)	β=0.02 95% CI (-0.17 – 0.21) z= 0.17 p= 0.87	β=-0.07 95% CI (-0.26 – 0.12) z= -0.69 p=0.49	β=0.04 95% CI (-0.05 – 0.14) z=0.85 p=0.39
IP-10	5.74 (SD 0.54)	5.69 (SD 0.58)	5.58 (SD 0.63)5	β= -0.04 95% CI (-0.29 – 0.2) z= -0.38 p= 0.71	β=-0.15 95% CI (-0.41 – 0.09) z= -1.25 p= 0.21	β=0.06 95% CI (-0.07 – 0.18) z=0.86 p= 0.39
MIP-1β	4.23 (SD 0.43)	4.19 (SD 0.47)	4.15 (SD 0.41)	β=-0.03 95% CI (-0.21 – 0.15) z= -0.36 p= 0.72	β= -0.08 95% CI (-0.25 – 0.09) z= -0.91 p= 0.36	β= 0.03 95% CI (-0.06 – 0.11) z= 0.55 p= 0.58
IL-17a	0.195 (SD 0.94)	0.21 (SD 0.66)	0.019 (SD 0.93)	β=-0.01 95% CI (-0.36 – 0.35) z= -0.02 p= 0.98	β= -0.18 95% CI (-0.54 – 0.18) z= -0.98 p=0.33	β=0.08 95% CI (-0.09 – 0.27) z= 0.95 p=0.34

Table 49 adjusted mixed effects model comparing inflammatory cytokines and chemokines across baseline, active and sham conditions, adjusted for covariates

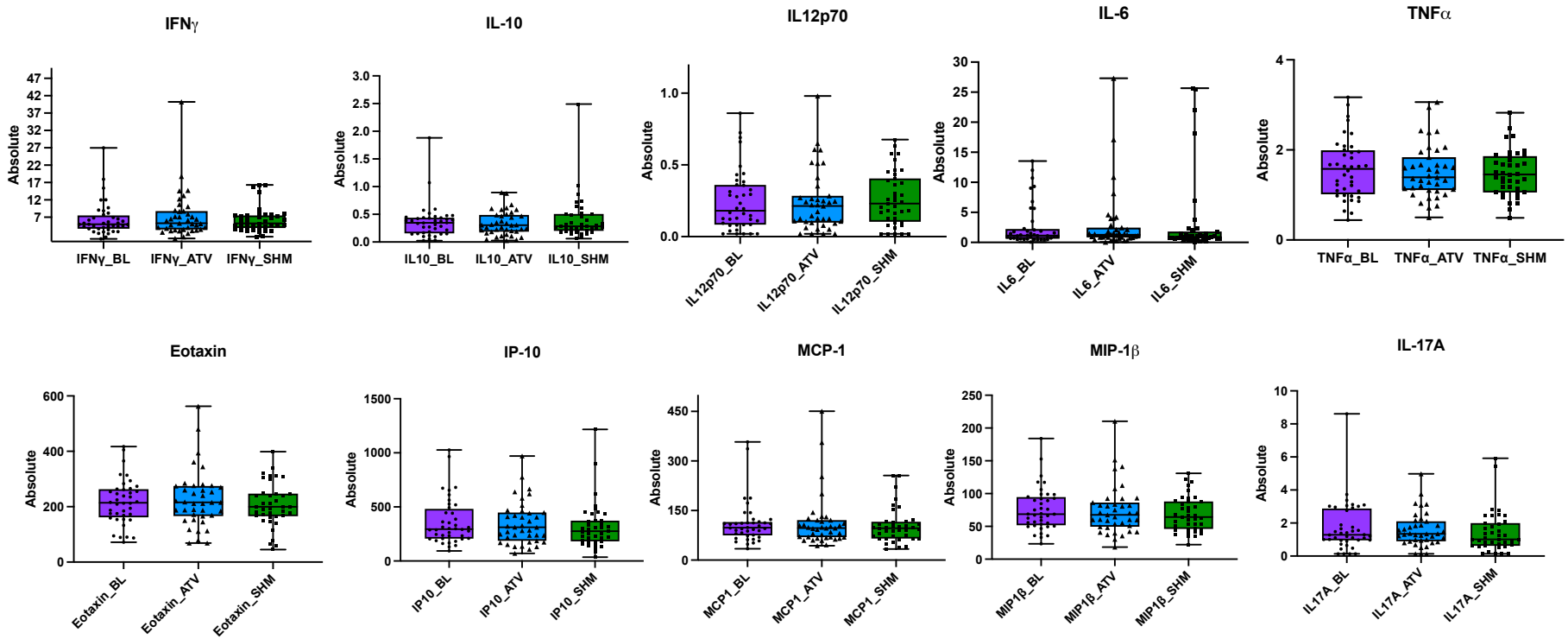


Figure 27 graphically depicts the absolute values of inflammatory cytokines and chemokines investigated under baseline, active and sham stimulation conditions

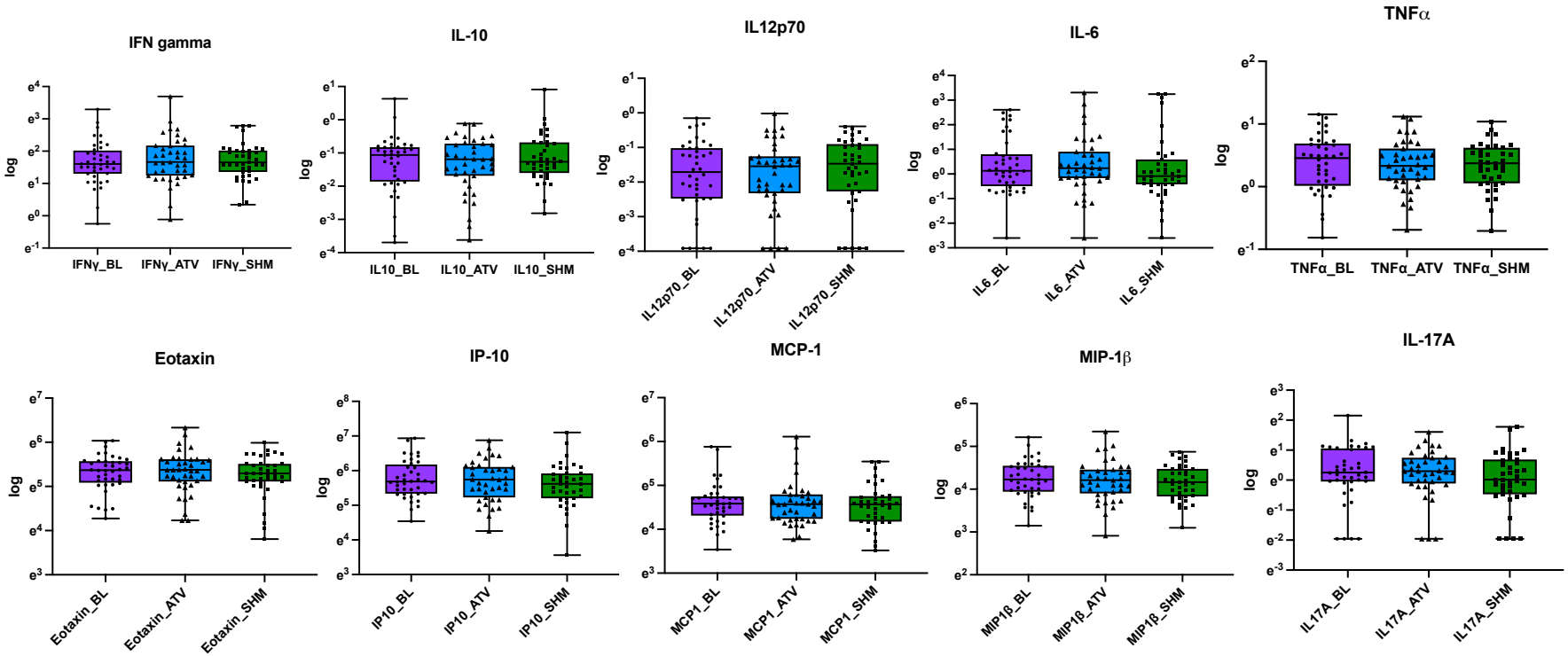


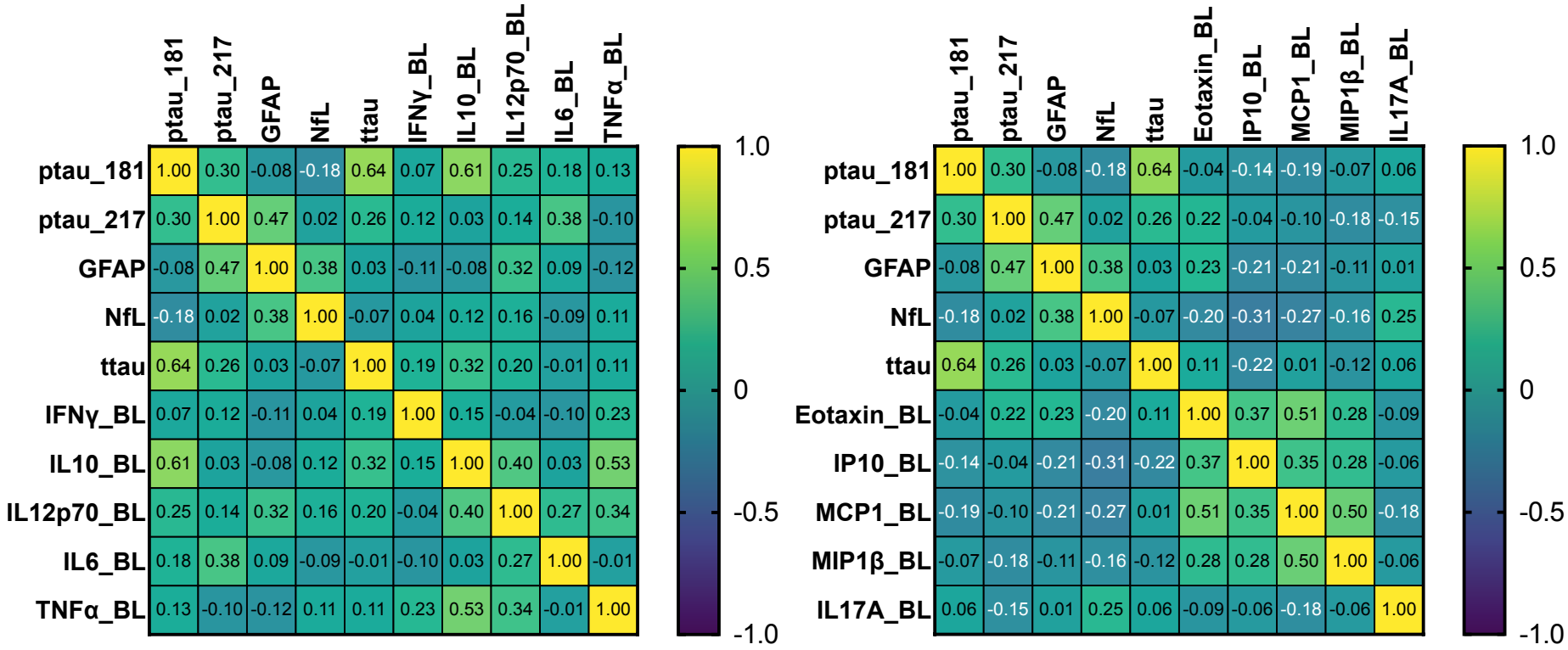
Figure 28 graphically depicts the natural logarithm values of inflammatory cytokines and chemokines investigated under baseline, active and sham stimulation conditions

AD Blood Biomarkers

BBMs were processed at baseline only per participant, with n=38 samples available for analysis. Multilevel linear regression models were used to explore associations between baseline BBM values and cognitive test results, adjusting for age, sex, BMI, comorbidity, polypharmacy and colinear plasma biomarkers.

Associative Memory

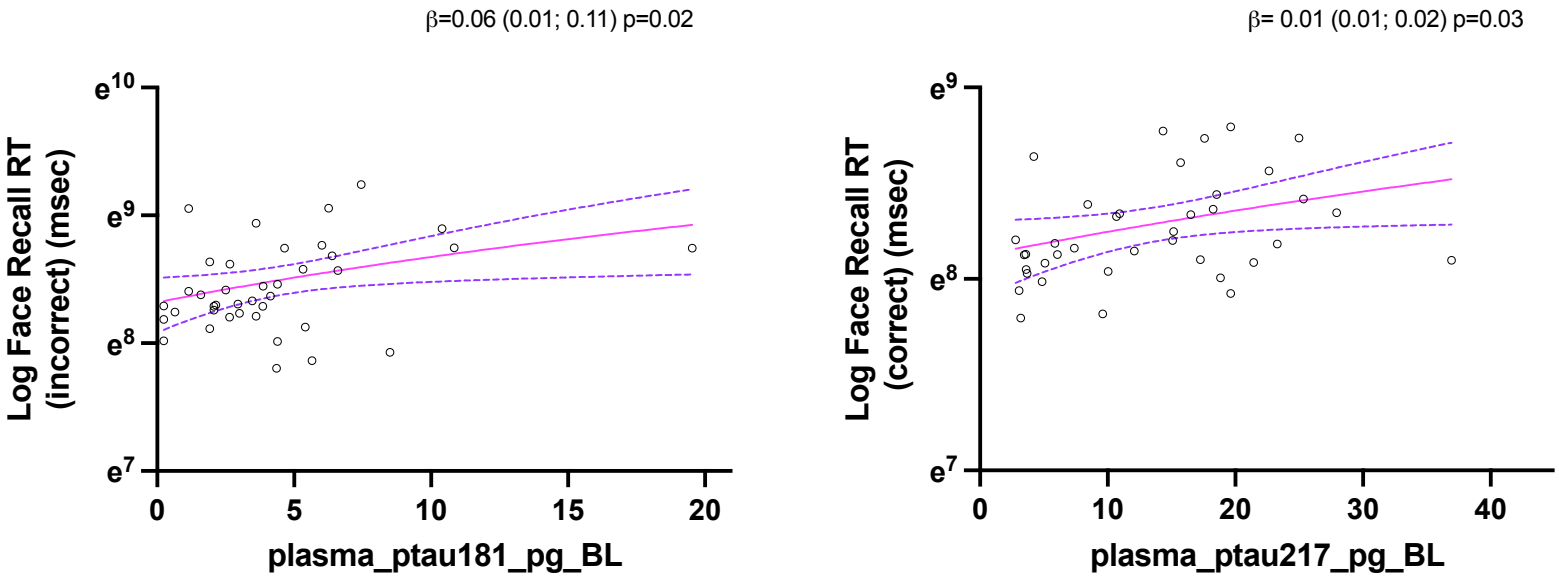
Plasma p-tau 181 and Face Recognition incorrect RTs was significantly associated (β= 0.06 (0.01 – 0.11) p=0.02*) and plasma p-tau 217 and Face Recognition correct RTs were also significantly associated (β=0.01 (0.01 – 0.02) p=0.03*). These results are displayed in Table 50 and Figures 30 (A,B).



Figures 29 (A,B) depicts correlation matrices for ptau 181, ptau 217, GFAP, NfL, t-tau, IFN- γ, IL-10, IL12p70, IL-6, TNF- α, Eotaxin, IP-10, MCP-1, MIP-1 β, IL-17A, with strongest correlation between IL-10 and ptau 181.

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
FNAT Facial Recall Accuracy	0.48 (-0.31 - 1.29) p=0.22	0.22 (-1.42 – 0.97) p=0.71	-0.08 (-0.43 – 0.26) p=0.62	-0.03 (-0.42 – 0.38) p=0.8	-0.04 (-0.11 - 0.14) p=0.13	-0.05 (-0.13 - 0.03) p=0.22	-0.69 (-2.01 – 0.62) p=0.31	-0.18 (-1.68 – 1.31) p=0.81	-0.01 (-0.01 - 0.01) p=0.94	0.01 (-0.01 - 0.01) p=0.97
Log_FNAT Facial Recall RT correct	0.23 (-0.03 – 1.51) p=0.3	117.27 (-49.93 – 284.47) p=0.16	0.01 (-0.01 – 0.03) p=0.05	**0.01 (0.01 – 0.02) p=0.03***	0.47 (-7.79 – 8.71) p=0.91	-2.59 (-14.53 – 9.34) p=0.66	0.67 (-4.55 – 5.83) p=0.79	2.31 (-3.61 – 8.2) p=0.43	0.01 (-0.01 - 0.01) p=0.52	0.01 (-0.01 - 0.01) p=0.71
Log_FNAT Facial Recall RT incorrect	0.03 (-0.01 – 0.06) p=0.04	**0.06 (0.01 – 0.11) p=0.02***	36.84 (-34.18 – 107.86) p=0.3	29.03 (-66.43 – 124.49) p=0.54	-1.06 (-14.28 – 12.25) p=0.87	-0.59 (-20.01 – 18.83) p=0.95	-1.44 (-9.82 – 6.92) p=0.72	1.1 (-8.52 – 10.74) p=0.81	0.01 (-0.01 - 0.01) p=0.77	0.01 (-0.01 - 0.01) p=0.71
Log_FNAT_ Name Recognition Accuracy	0.23 (-1.55 – 2.03) p=0.79	-0.98 (-3.89 – 1.91) p=0.49	0.46 (-0.28 – 1.22) p=0.21	0.7 (-0.31 -1.72) p=0.17	-0.06 (-0.51 - 0.38) p=0.77	0.03 (-0.45 – 0.37) p=0.85	-0.05 (-0.14 – 0;03) p=0.19	-0.07 (-0.17 – 0.03) p=0.16	-0.04 (-0.19 - 0.11) p=0.59	0.01 (-0.01 - 0.01) p=0.96
Log_FNAT Name Recognition RT (correct)	-0.54 (-0.78 – 0.86) p=0.58	1.17 (-1.58 – 3.93) p=0.43	-0.74 (-1.4 – 2.91) p=0.76	5.72 (-86.5 – 97.95) p=0.9	-1.18 (-13.46 – 11.08)	-5.01 (23.77 – 13.75) p=0.59	3.18 (-4.51 – 10.88) p=0.41	5.08 (-4.22 – 14.32) p= 0.27	-0.01 (-0.01 - 0.01) p=0.61	-0.01 (-0.01 - 0.01) p=0.81
Log_FNAT Name Recognition RT (incorrect)	-0.87 (-1.21 – 0.34) p=0.63	0.06 (-0.34 – 0.49) p=0.99	0.34 (-1.52 – 1.23) p=0.91	3.65 (-117.93 – 125.24) p=0.95	4.69 (-10.66 – 20.05) p=0.53	3.23 (-21.5 – 27.97) p=0.79	4.91 (-4.62 – 14.61) p=0.3	3.97 (-8.3 – 16.24) p=0.51	0.01 (-0.01 - 0.01) p=0.68	0.01 (-0.01 - 0.01) p=0.37

Table 50 depicts the adjusted and unadjusted regression models investigating Associative Memory outcomes at baseline with BBMs, models adjusted for age, sex, BMI, comorbidity, polypharmacy, and AD biomarkers



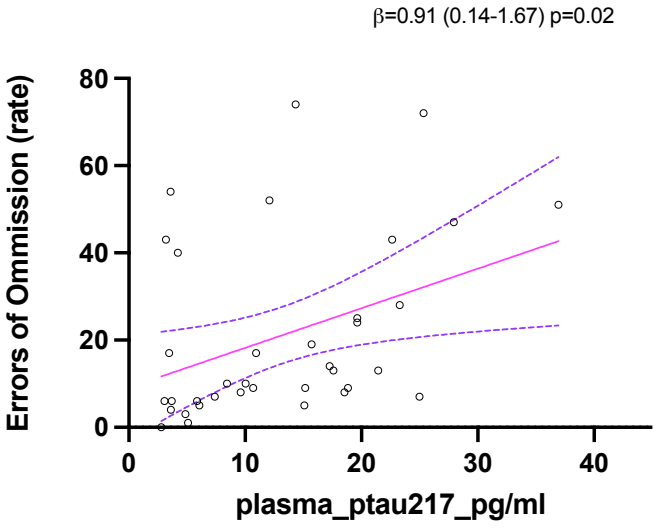
Figures 30 (A,B) depict the adjusted regression models for ptau 181 and log of Face Recall RT (incorrect), and ptau 217 and log of Face Recall RT (correct)

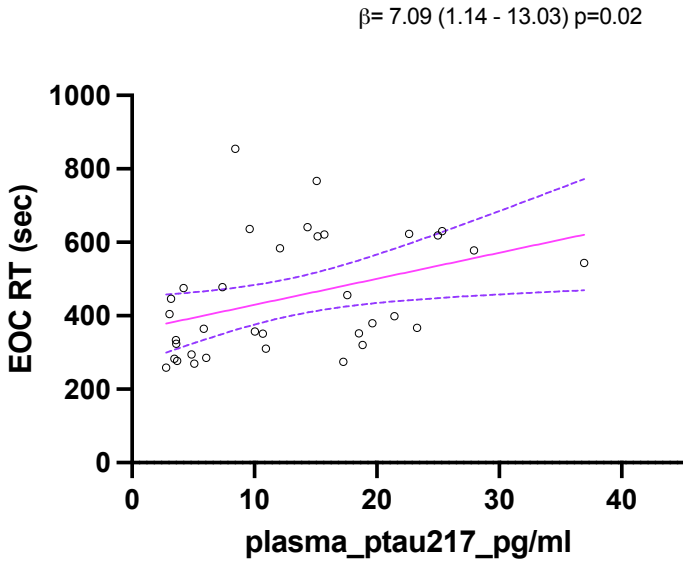
Executive Function

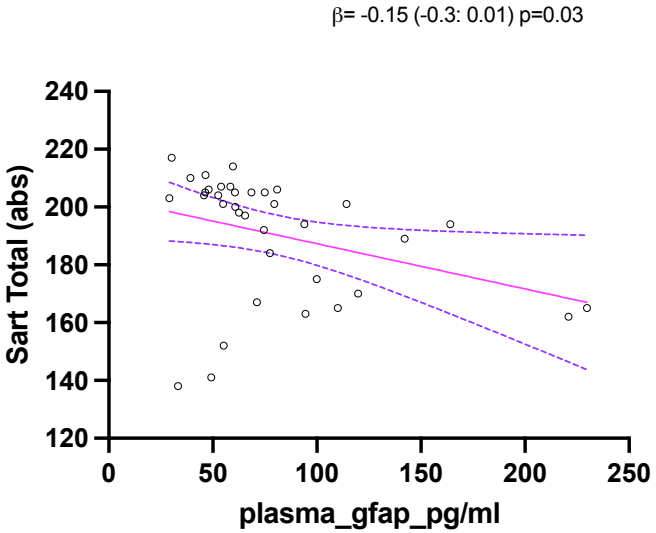
Plasma GFAP was significantly negatively associated with total number pressed during the SART at baseline (β=-0.15 [-0.3 - -0.01] p=0.03*) while p-tau 217 was significantly associated with worse error rate (errors of omission) (β=0.91 [0.14 – 1.67] p=0.02) and slower error rate RTs (β=7.09 [1.14 – 13.03] p=0.02). These results are depicted in Table 60 and illustrated in Figures 31 (A-C).

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
SART_total	0.01 (-0.02 – 0.03) p=0.76	-0.01 (-0.03 – 0.02) p=0.79	-0.78 (-1.59 – 0.02) p=0.06	-0.69 (-1.82 – 0.42) p=0.21	--0.13 (-0.35 – 0.08) p=0.2	**-0.15 (-0.3 - -0.01) p=0.03***	-0.4 (-0.99 – 0.19) p=0.18	-0.41 (-1.02 – 0.19) p=0.17	-0.01 (-0.01 -0.01) p=0.88	-0.01 (-0.01 - 0.01) p=0.87
SART_total RT	0.01 (-0.01 – 0.01) p=0.22	0.01 (-0.01 – 0.01) p=0.11	3.86 (-0.38 – 8.12) p=0.07	3.82 (-2.44 – 10.08) p=0.22	0.61 (-0.18 – 1.4) p=0.12	0.11 (-1.1 – 1.33) p=0.84	0.07 (-0.05 – 0.21) p=0.24	0.03 (-0.11 – 0.17) p=0.67	0.01 (-0.01 - 0.01) p=0.76	0.01 (-0.01 - 0.01) p=0.71
SART_EOC	0.06 (-0.05 – 0.18) p=0.26	0.02 (-0.11 – 0.15) p=0.69	0.12 (-0.07 – 0.31) p=0.21	0.08 (-0.19 – 0.36) p=0.52	0.32 (-1.26 – 1.89) p=0.69	0.16 (-1.45 - 1.76) p=0.84	-1.46 (-4.06 – 1.13) p=0.27	-0.33 (-3.26 – 2.58) p=0.81	-0.04 (-0.6 – 0.51) p=0.86	-0.43 (-1.04 – 0.17) 0.16
SART_EOO	-0.01 (-0.02 – 0.02) p=0.96	0.01 (-0.02 – 0.03) p=0.72	0.78 (-0.25 – 1.82) p=0.13	**0.91 (0.14 – 1.67) p=0.02**	0.16 (0.02 – 0.31) p=0.02*	0.14 (-0.06 – 0.34) p=0.16	0.35 (-0.27 – 0.96) p=0.26	0.43 (-0.19 – 1.06) p=0.17	0.01 (-0.01 - 0.01) p=0.84	0.01 (-0.01 - 0.01) p=0.89
SART_EOC RT	0.01 (-0.01 – 0.01) p=0.14	0.01 (-0.01 – 0.01) p=0.19	7.17 (-1.06 – 15.96) p=0.1	**7.09 (1.14 – 13.03) p=0.02**	1.02 (-0.09 – 2.15) p=0.07	-0.03 (-1.72 – 1.62) p=0.92	0.04 (-0.03 – 0.12) p=0.27	0.04 (-0.03 – 0.13) p=0.25	0.02 (-0.01 – 0.02) p=0.14	0.02 (-0.01 – 0.02) p=0.15

Table 51 depicts the adjusted and unadjusted regression models investigating Executive Function outcomes at baseline with BBMs, models adjusted for age, sex, BMI, comorbidity, polypharmacy, and AD biomarkers


β=0.91 (0.14-1.67) p=0.02
Errors of Ommission (rate)
0
20
40
60
80
0
10
20
30
40
plasma_ptau217_pg/ml


β= 7.09 (1.14 - 13.03) p=0.02
EOC RT (sec)
0
200
400
600
800
1000
0
10
20
30
40
plasma_ptau217_pg/ml


β= -0.15 (-0.3: 0.01) p=0.03
Sart Total (abs)
120
140
160
180
200
220
240
0
50
100
150
200
250
plasma_gfap_pg/ml

Figures 31 (A-C) depict the adjusted regression models, depicting the associations between ptau 217 and SART EOC rate (A), ptau 217 and SART EOC RTs (B) and GFAP with SART total number (C).

Spatial Navigation

Plasma p-tau 217 demonstrated a significant association with the overall time to target for all SHQ trials, with higher p-tau 217 levels associated with slower times to target overall (β=0.18 [0.07 – 0.28] p=0.02*). These results are depicted in Table 61 and displayed graphically in Figure 32.

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
SHQ L1	-0.01 (-0.04 – 0.03) p=.91	0.01 (-0.03 – 0.06) p=0.42	0.1 (0.01-0.19) p=0.03*	0.05 (-0.02 – 0.16) p=0.16	0.02 (-0.48 – 0.53) p=0.91	0.24 (-0.24 – 0.73) p=0.32	0.92 (-0.05 – 1.79) p= 0.02*	0.64 (-0.19 – 1.47) p=0.13	0.07 (-0.12 – 0.26) p=0.43	0.07 (-0.11 – 0.26) p=0.44
SHQ L2	-0.02 (-0.05 – 0.02) p=0.25	-0.01 (-0.04 – 0.03) p=0.71	0.14 (0.06 – 0.22) p=0.01*	0.09 (0.11 – 0.18) 0.2	0.4 (-0.04 – 0.84) p=0.07	-0.05 (-0.44 – 0.33) p=0.77	0.94 (0.16 – 1.72) p= 0.02*	0.8 (-0.07 – 1.5) p=0.09	-0.01 (-0.16 – 0.15) p=0.93	0.02 (-0.15 – 0.19) p=0.78
SHQ L6	-0.02 (-0.06 – 0.02) p=0.25	0.01 (-0.04 – 0.04) p=0.93	0.14(-0.02– 0.19) p=0.02*	0.08 (-0.01 – 0.18) p=0.11	0.39 (-0.12 – 0.91) p=0.13	0.01 (-0.44 – 0.44) p=0.98	0.96 (0.06 – 1.85) p=0.03*	0.8 (-0.04 – 1.6) 0.06	0.05 (-0.14 – 0.23) p=0.61	0.06 (-0.15 – 0.26) p=0.58
SHQ L7	-0.03 (-0.07 – 0.01) p=0.08	-0.03 (-0.07-0.01) p=0.07	0.06 (-0.02 – 0.15) p=0.17	0.05 (-0.02 – 0.13) p=0.16	0.04 (-0.05 – 0.86) p= 0.08	0.28 (-0.13 – 0.69) p=0.17	0.27 (-0.43 – 0.97) p=0.44	0.21 (-0.48 – 0.9) p=0.54	-0.14 (-0.31 – 0.02) p=0.08	-0.14 (-0.31 – 0.02) p=0.08
SHQ L8	-0.02 (-0.05 – 0.12) p=0.22	-0.01 (-0.04 – 0.21) p=0.49	0.01 (-0.06 – 0.09) p-0.67	0.04 (-0.03 – 0.11) p=0.25	-0.05 (-0.46 – 0.34) p=0.78	0.09 (-0.28 – 0.46) p=0.62	0.39 (-0.27 - 1.06) p=0.24	0.55 (-0.13 – 1.22) p=0.11	-0.08 (-0.23 – 0.05) p=0.24	-0.07 (-0.22 – 0.06) p=0.29
SHQ overall	-0.03 (-0.07 – 0.15) p=0.18	-0.08 (-0.05 – 0.04) p=0.72	0.11 (0.01 – 0.22) p=0.04	**0.18 (0.07 – 0.28) p=0.02***	0.39 (-0.18 – 0.96) p=0.17	0.62 (-0.06 – 1.18) p=0.72	1.01 (0.07 - 1.94) p=0.03*	0.05 (-0.02 – 0.12) p=0.13	-0.01 (-0.21 – 0.21) p=0.99	0.01 (-0.21 – 0.23) p=0.92

Table 52 depicts the adjusted linear regression models for the Spatial Navigation cognitive tasks and BBMs, adjusted for age, sex, BMI comorbidity and AD biomarkers

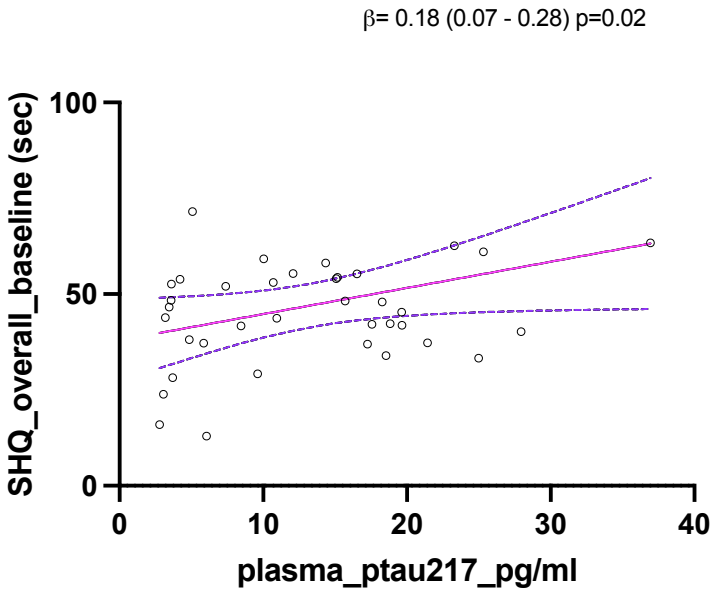


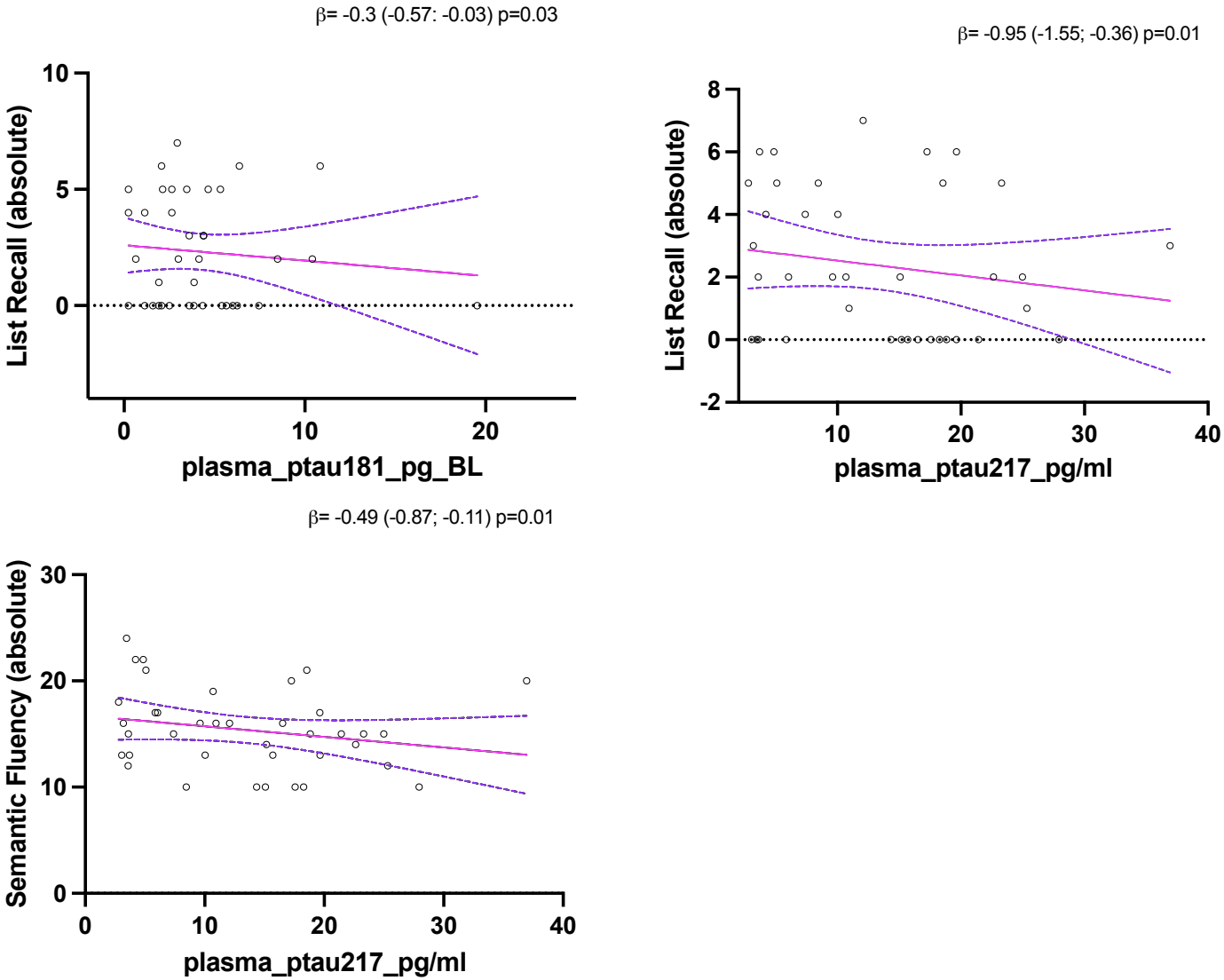
Figure 32 depicts the association between p-tau 217 and overall time to target at baseline for the SHQ trials

Language and Working Memory

Plasma p-tau 181 and plasma p-tau 217 were both negatively associated with list recall during baseline language assessment (β= -0.3 [-0.57 ; -0.03] p=0.03*); (β=-0.95 [-1.55 ;- 0.36] p=0.01*) with plasma p-tau 217 also negatively associated with absolute values on semantic fluency assessment (β=-0.49 [-0.87 ; -0.11] p=0.01*). These results are depicted in Table 62 and displayed graphically in Figures 33 (A-C).

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
List learning	-0.28 (-0.72 – 0.15) p=0.2	-0.25 (-0.68 – 0.16) p=0.22	-0.73 (-1.74 – 0.27) p=0.15	-0.69 (-1.64 – 0.24) p= 0.14	-1.64 (-7.18 – 3.88) p=0.55	-1.05 (-6.79 – 3.97) p=0.67	-10.04 (-18.57 – 1.51) p= 0.06	-8.97 (-17.51 – 0.43) p=0.06	0.29 (-1.61 – 2.19) p=0.76	0.23 (-1.63 – 2.11) p=0.8
Semantic fluency	0.01 (-0.15 – 0.18) p=0.83	-0.01 (-0.17 – 0.17) p=0.98	-0.45 (-0.84 - -0.07) p=0.02*	**-0.49 (-0.87 - -0.11) p=0.01****	-1.13 (-3.26 – 0.99) p=0.29	-6.78 (-2.73 – 1.3) p=0.51	-2.59 (-5.93 – 0.74) p=0.12	-1.78 (-5.33 – 1.72) p=0.32	0.32 (-0.41 – 1.05) p=0.38	0.44 (-0.32 – 1.2) p=0.25
Digit forward	0.14 (-0.31 – 0.61) p=0.53	0.06 (-0.4 – 0.52) p=0.79	-0.25 (-1.33 – 0.82) p=0.64	-0.48 (-1.51 – 0.54) p=0.35	-1.76 (-7.62 – 4.08) p=0.55	1.05 (-4.4 – 6.51) p=0.7	-7.06 (-16.21 – 2.08) p=0.12	-5.2 (-14.61 – 4.21) p=0.27	1.22 (-0.77 – 3.22) p=0.23	0.8 (-1.22 – 2.83) p=0.43
Digit backward	0.41 (-0.18 – 0.99) p=0.17	0.34 (-0.25 – 0.95) p=0.25	-0.7 (-2.08 – 0.67) p=0.31	-1.25 (-2.58 – 0.08) p=0.06	-2.85 (-10.34 – 4.63) p=0.45	1.15 (-6.01 – 8.32) p=0.75	-6.96 (-18.73 – 4.79) p=0.24	-2.07 (-14.49 – 10.34) p=0.74	-0.58 (-1.32 – 0.15) p=0.12	-0.72 (-2.16 – 0.71) p=0.32
List recall	-0.21 (-0.49 – 0.06) p=0.13	**-0.3 (-0.57 – -0.03) p=0.03***	-0.77 (-1.42 - - 0.12) p=0.02*	**-0.95 (-1.55 - -0.36) p=0.01****	0.86 (-2.72 - 4.45) p=0.63	-0.57 (-3.88 – 2.74) p=0.73	-1.14 (-6.81 – 4.52) p=0.69	-1.72 (-7.45 – 4.01) p=0.55	-0.64 (-1.86 – 0.59) p= 0.31	-0.55 (-1.78 – 0.67) p=0.37
List recognition	0.06 (-0.25 – 0.38) p=0.7	-0.04 (-0.38 – 0.28) p= 0.77	-0.29 (-1.04 – 0.44) p=0.43	-0.59 (-1.32 – 0.13) p=0.11	-3.71 (-7.2 – 0.84) p=0.12	-2.28 (-6.18 – 1.6) p=0.24	-3.72 (-6.45 – 0.01) p=0.87	-2.54 (-5.42 – 0.04) p=0.75	0.24 (-1.15 – 1.64) p=0.73	-0.06 (-1.51 – 1.39) p=0.94

Table 53 depicts the adjusted linear regression models for the Language and Working Memory cognitive tasks and BBMs, adjusted for age, sex, BMI comorbidity and AD biomarkers



Figures 33 (A-C) depict regression models for p-tau 181 and list recall (A), p-tau 217 and list recall (B) and p-tau 217 and semantic fluency (C)

BBMs and NeuroCardioVascular indices

Given the known association between AD and NCVI, in this new era of biomarker- supported AD diagnosis we were interested to explore what effect plasma biomarkers have on SBP, DBP, HR and TSI responses to active stand. We took these values at baseline only and included in each regression model significant covariates as outlined below.

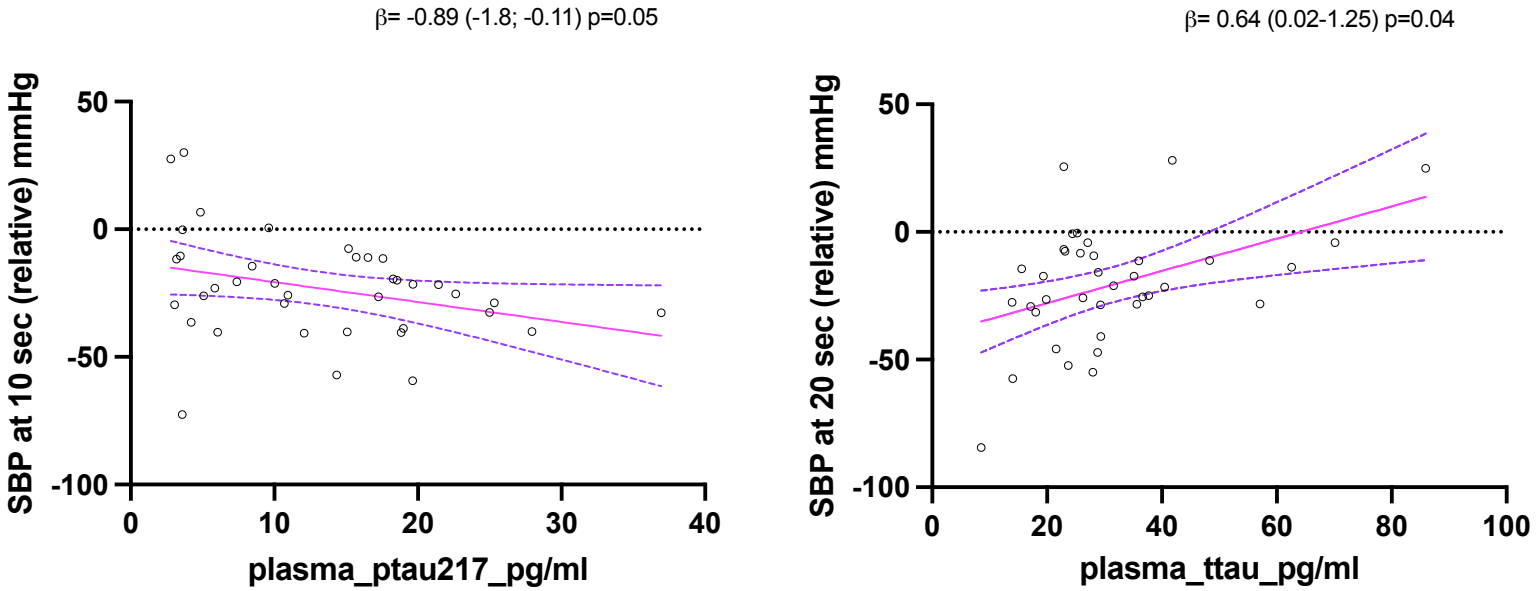
Systolic Blood Pressure

Plasma p-tau 217 was negatively associated with relative SBP values in mmHg at 10 seconds post standing, with higher p-tau values associated with lower SBP values (β=-0.89 [-1.8 – 0.11] p=0.05*). Paradoxically, higher t-tau values were associated with higher relative SBP values at 20 seconds post standing (β=0.64 [0.02-1.25] p=0.04*). The clinical significance of the t-tau association is unclear and would need further testing in larger populations to see if it is replicated. These results are displayed in Table 64 and graphically shown in Figures 34 (A,B).

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
SBP pre-stand mmHg	-0.08 (-1.88 – 1.71) p=0.92	-0.24 (-3.37 – 2.89) p=0.87	-0.23 (-1.01 – 0.54) p=0.54	-0.17 (-1.29 – 0.94) p=0.75	-0.07 (-0.22 – 0.06) p=0.28	-0.04 (-0.26 – 0.18) p=0.69	-0.04 (-0.13 – 0.05) p=0.36	-0.04 (-0.15 – 0.07) p=0.46	0.06 (-0.34 – 0.47) p=0.76	0.07 (-0.58 – 0.73) p=0.81
SBP 10 sec (relative) in mmHg	0.65 (-1.32 – 2.63) p=0.51	-1.39 (-2.87 – 2.59) p=0.91	-0.71 (-1.54 – 0.12) p=0.09	**-0.89 (-1.8 – 0.11) p=0.05**	-0.07 (-0.23 – 0.08) p=0.35	-0.08 (-0.27 – 0.11) p=0.38	0.14 (0.04 – 0.23) p=0.02	0.11 (0.02 – 0.21) p=0.06	0.34 (-0.1-0.77) p=0.13	0.49 (-0.07 – 1.04) p=0.09
SBP 20 sec (relative) in mmHg	2.19 (0.19 – 4.19) p=0.03*	0.65 (-2.26 – 3.57) p=0.64	-0.01 (-0.94 – 0.91) p=0.97	-0.39 (-1.4 – 0.64) p=0.43	-0.02 (-0.19 – 0.14) p=0.77	0.01 (-0.2 – 0.21) p=0.96	0.05 (-0.05 – 0.16) p=0.29	0.09 (-0.01 – 0.19) p=0.1	0.63 (0.19 – 1.06) p=0.01	**0.64 (0.02-1.25) p=0.04***
SBP 30 sec (relative) in mmHg	2.09 (0.27 – 3.91) p=0.02*	0.54 (-2.27 – 3.34) p=0.69	-0.01 (-0.86 – 0.84) p=0.98	-0.27 (-1.27 – 0.72) p=0.58	-0.04 (-0.21 – 0.11) p=0.53	-0.05 (-0.25 – 0.14) p=0.56	0.02 (-0.07 – 0.12) p=0.55	0.07 (-0.03 – 0.17) p=0.17	0.56 (0.16 – 0.96) p=0.01	0.57 (-0.02 – 1.62) p=0.06
SBP 40 sec (relative) in mmHg	1.64 (-0.21 – 3.54) p=0.08	1.44 (-1.52 – 4.41) p=0.32	-0.12 (-0.86 – 0.72) p=0.77	-0.31 (-1.3 – 0.75) p=0.56	-0.04 (-0.19 – 0.12) p=0.63	-0.04 (-0.25 – 0.17) p=0.68	0.11 (0.01-0.21) p=0.04	0.06 (-0.02 – 0.16) p=0.16	0.34 (-0.08 – 0.77) p=0.11	-0.22 (-0.41-0.84) p=0.47
SBP 60 sec (relative) in mmHg	2.17 (0.04 – 4.32) p=0.04*	2.48 (-0.79 – 5.77) p=0.13	0.23 (-0.75 – 1.21) p=0.63	0.32 (-0.84 – 1.52) p=0.56	-0.09 (-0.26 – 0.09) p=0.33	-0.12 (-0.35 – 0.11) p=0.32	0.1 (-0.02 – 0.21) p=0.09	0.02 (-0.08 – 0.14) p=0.62	-0.13 (-0.37 – 0.64) p=0.59	-0.19 (-0.88 – 0.49) p=0.57

SBP 120 sec (relative) in mmHg	1.5 (-0.34 – 3.34) p=0.12	1.6 (-1.4 – 4.74) p=0.27	-0.11 (-0.93 – 0.72) p=0.79	-0.47 (-1.56 – 0.62) p=0.38	-0.01 (-0.16 – 0.14) p=0.88	0.03 (-0.18 – 0.25) p=0.75	0..03 (-0.06 – 0.13) p=0.44	0.06 (-0.04 – 0.18) p=0.22	0.31 (-0.12 – 0.73) p=0.14	0.14 (-0.51 – 0.79) p=0.65
SBP steady state (relative) in mmHg	1.66 (-0.23 – 3.56) p=0.08	2.02 (-0.91 – 4.95) p=0.16	-0.17 (-1.03 – 0.69) p=0.68	-0.59 (-1.06 – 0.48) p=0.28	-0.02 (-0.18 – 0.13) p=0.77	0.06 (-0.14 – 0.25) p=0.52	0.02 (-0.08 – 0.12) p=0.75	0.05 (-0.05 – 0.16) p=0.31	0.37 (-0.06 – 0.81) p=0.08	0.18 (-0.49 – 0.81) p=0.53
SBP nadir in mmHg	1.7 (-0.15 – 3.55) p=0.07	0.82 (-1.92 – 3.62) p=0.54	-0.32 (-1.16 – 0.51) p=0.44	-0.74 (-1.73 – 0.25) p=0.14	-0.04 (-0.19 – 0.12) p=0.63	0.02 (-0.17 – 0.22) p=0.82	0.05 (-0.04 – 0.15) p=0.23	0.07 (-0.02 – 0.18) p=0.12	0.52 (0.12-0.93) p=0.01	0.52 (-0.06 – 1.12) p=0.08
SBP recovery rate (seconds)	0.01 (-0.06 – 0.09) p=0.67	-0.03 (-0.21 – 0.04) p=0.19	0.01 (-0..02 – 0.05) p=0.34	0.01 (-0.03 – 0.06) p=0.56	0.01 (-0.01 – 0.01) p=0.34	0.01 (-0.01 – 0.01) p=0.82	0.01 (-0.01 – 0.01) p=0.62	0.01 (-0.01 – 0.01) p=0.74	0.02 (0.01 – 0.05) p=0.04	0.01 (-0.01 – 0.03) p=0.12
SBP overshoot (relative) in mmHg	1.83 (-0.08 – 3.75) p=0.06	0.53 (-2.44 – 3.51) p=0.72	-0.05 (-0.93 – 0.82) p=0.89	-0.23 (-1.3 – 0.82) p=0.64	-0.02 (-0.18 – 0.14) p=0.79	-0.04 (-0.25 – 0.17) p=0.69	0.06 (-0.03 – 0.16) p=0.18	0.1 (-0.01 0 0.21) p=0.06	0.52 (0.09 – 0.94) p=0.02	0.56 (-0.07 – 1.19) p=0.08
SBP time to nadir (seconds)	-0.39 (-0.93 – 0.15) p=0.15	0.05 (-0.85 – 0.96) p=0.9	-0.22 (-0.45 – 0.02) p=0.06	-0.16 (-0.49 – 0.15) p=0.29	-0.01 (-0.05 – 0.03) p=0.52	-0.01 (-0.07 – 0.05) p=0.73	0.01 (-0.01 – 0.04) p=0.3	0.01 (-0.02 – 0.04) p=0.42	-0.09 (-0.21 – 0.03) p=0.14	-0.08 (-0.28 – 0.11) p=0.36

Table 54 depicts the linear regression models, depicting the associations between SBP values during AS and BBMs, adjusted for age, sex, BMI, comorbidity, polypharmacy, baseline HTN and AD biomarkers



Figures 34 (A,B) depict the associations between p-tau 217 and SBP response at 10 seconds post standing (A) and plasma t-tau and SBP response at 20 seconds post standing (B)

Diastolic Blood Pressure

There were no significant associations found on multiple linear regression modelling, on adjusted or unadjusted models, of DBP values post standing and plasma AD biomarkers.

	plasma ptau 181		plasma ptau 217		plasma GFAP		plasma NfL		plasma total tau	
	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p
DBP pre-stand mmHg	0.29 (-0.59 – 1.18_ p=0.51	-0.24 (-1.64 – 1.1) p=0.73	-0.18 (-0.72 – 0.37) p=0.56	-0.17 (-0.71 – 0.36) p=0.51	-0.05 (-0.16 – 0.36) p=0.49	-0.04 (-0.14 – 0.36) p=0.51	0.03 (-0.02 – 0.06) p=0.26	0.03 (-0.02 – 0.06) p=0.23	0.23 (-0.16 – 0.44) p=0.21	0.13 (-0.16 – 0.44) p=0.35
DBP 10 sec (relative) in mmHg	0.83 (-0.18 – 2.75) p=0.26	0.53 (-2.44 – 3.51) p=0.72	-0.05 (-0.93 – 0.82) p=0.89	-0.23 (-1.3 – 0.82) p=0.64	-0.02 (-0.18 – 0.14) p=0.79	-0.06 (-0.25 – 0.17) p=0.59	0.06 (-0.03 – 0.16) p=0.18	0.2 (-0.01 0 0.21) p=0.2	0.52 (0.09 – 0.94) p=0.21	0.56 (-0.07 – 1.19) p=0.35
DBP 20 sec (relative) in mmHg	-0.02 (-0.06 – 0.02) p=0.25	0.01 (-0.04 – 0.04) p=0.93	0.14(-0.02– 0.19) p=0.02*	0.08 (-0.01 – 0.18) p=0.11	0.39 (-0.12 – 0.91) p=0.13	0.01 (-0.44 – 0.44) p=0.98	0.96 (0.06 – 1.85) p=0.03*	0.8 (-0.04 – 1.6) 0.06	0.05 (-0.14 – 0.23) p=0.61	0.06 (-0.15 – 0.26) p=0.58
DBP 30 sec (relative) in mmHg	0.14 (-0.31 – 0.61) p=0.53	0.06 (-0.4 – 0.52) p=0.79	-0.25 (-1.33 – 0.82) p=0.64	-0.48 (-1.51 – 0.54) p=0.35	-1.76 (-7.62 – 4.08) p=0.55	1.05 (-4.4 – 6.51) p=0.7	-7.06 (-16.21 – 2.08) p=0.12	-5.2 (-14.61 – 4.21) p=0.27	1.22 (-0.77 – 3.22) p=0.23	0.8 (-1.22 – 2.83) p=0.43
DBP 40 sec (relative) in mmHg	0.48 (-0.31 - 1.29) p=0.22	0.22 (-1.42 – 0.97) p=0.71	-0.08 (-0.43 – 0.26) p=0.62	-0.03 (-0.42 – 0.38) p=0.8	-0.04 (-0.11 -0.14) p=0.13	-0.05 (-0.13 - 0.03) p=0.22	-0.69 (-2.01 – 0.62) p=0.31	-0.18 (-1.68 – 1.31) p=0.81	-0.01 (-0.01 -0.01) p=0.94	0.01 (-0.01 - 0.01) p=0.97
DBP 60 sec (relative) in mmHg	0.04 (-0.07 – 0.15) p=0.87	0.03 (-0.15 – 0.22) p=0.89	-0.02 (-0.07 – 0.02) p=0.6	-0.06 (-0.13 - -0.01) p=0.4	-0.01 (-0.01 – 0.01) p=0.91	0.01 (-0.01 – 0.02) p=0.18	-0.01 (-0.01 – 0.01) p=0.27	-0.01 (-0.01 – 0.01) p=0.28	0.02 (-0.01 – 0.04) p=0.21	0.02 (-0.02 – 0.05) p=0.41

DBP 120 sec (relative) in mmHg	-0.02 (-0.68 – 0.66) p=0.68	-0.04 (-1.14 – 0.72) p=0.65	0.02 (-0.27 – 0.1) p=0.37	0.05 (-0.18 – 0.3) p=0.62	0.02 (-0.05 – 0.02) p=0.32	0.02 (-0.07 – 0.03) p=0.35	-0.01 (-0.03 – 0.01) p=0.42	-0.01 (-0.03 – 0.02) p=0.57	-0.05 (-0.15 – 0.04) p=0.27	0.03 (-0.11 – 0.18) p=0.58
DBP steady state (120-180) (relative) in mmHg	0.17 (-0.04 – 4.32) p=0.67	0.48 (-0.79 – 5.77) p=0.23	0.23 (-0.75 – 1.21) p=0.63	0.32 (-0.84 – 1.52) p=0.56	-0.09 (-0.26 – 0.09) p=0.33	-0.12 (-0.35 – 0.11) p=0.32	0.1 (-0.02 – 0.21) p=0.09	0.02 (-0.08 – 0.14) p=0.62	-0.13 (-0.37 – 0.64) p=0.59	-0.19 (-0.88 – 0.49) p=0.57
DBP nadir in mmHg	-0.03 (-0.08 – 0.02) p=0.45	-0.03 (-0.12 – 0.06) p=0.58	-0.01 (-0.03 – 0.01) p=0.78	0.01 (-0.02 – 0.03) p=0.82	-0.01 (-0.01 – 0.01) p=0.28	-0.01 (-0.01 – 0.01) p=0.22	-0.01 (-0.01 – 0.01) p=0.87	0.01 (-0.01 – 0.01) p=0.73	-0.01 (-0.02 – 0.01) p=0.78	-0.01 (-0.02 – 0.02) p=0.63
DBP recovery rate (seconds)	0.07 (-0.11 – 0.26) p=0.38	0.05 (-0.81 – 0.51) p=0.68	-0.06 (-0.13 – 0.02) p=0.35	-0.11 (-0.2 - -0.01) p=0.31	-0.01 (-0.03 – 0.01) p=0.09	0.01 (-0.02-0.02) p=0.72	-0.01 (-0.01 – 0.01) p=0.32	-0.01 (-0.01 – 0.01) p=0.44	0.02 (-0.02 – 0.06) p=0.39	0.01 (-0.04 – 0.06) p=0.67
DBP overshoot (relative) in mmHg	-0.02 (-1.54 – 1.49) p=0.97	0.59 (-1.81 – 2.99_) p=0.61	0.09 (-0.75 – 1.02) p=0.82	0.13 (-0.75 – 1.02) p=0.76	-0.1 (-0.27 – 0.07) p=0.36	-0.1 (-0.27 – 0.07) p=0.25	0.04 (-0.03 – 0.15) p=0.25	0.06 (-0.03 – 0.15) p=0.17	-0.18 (-0.06 -0.32) p=0.32	-0.18 (-0.06 - 0.32) p=0.46
DBP time to nadir (seconds)	-0.51 (-1.94 – 0.91) p=0.47	-0.24 (-2.54 – 2.05) p=0.89	0.23 (-0.37 – 0.84) p=0.43	0.6 (-0.24 – 1.44) p=0.15	-0.03 (-0.14 – 0.07) p=0.52	-0.09 (-0.26 – 0.07) p=0.26	-0.01 (-0.08 – 0.06) p=0.75	0.01 (-0.07 – 0.09) p=0.77	-0.14 (-0.46 – 0.19) p=0.41	-0.18 (-0.68 – 0.32) p=0.45

Table 55 depicts the linear regression models, depicting the associations between DBP values during AS and BBMs, adjusted for age, sex, BMI, comorbidity, polypharmacy, baseline HTN and AD biomarkers

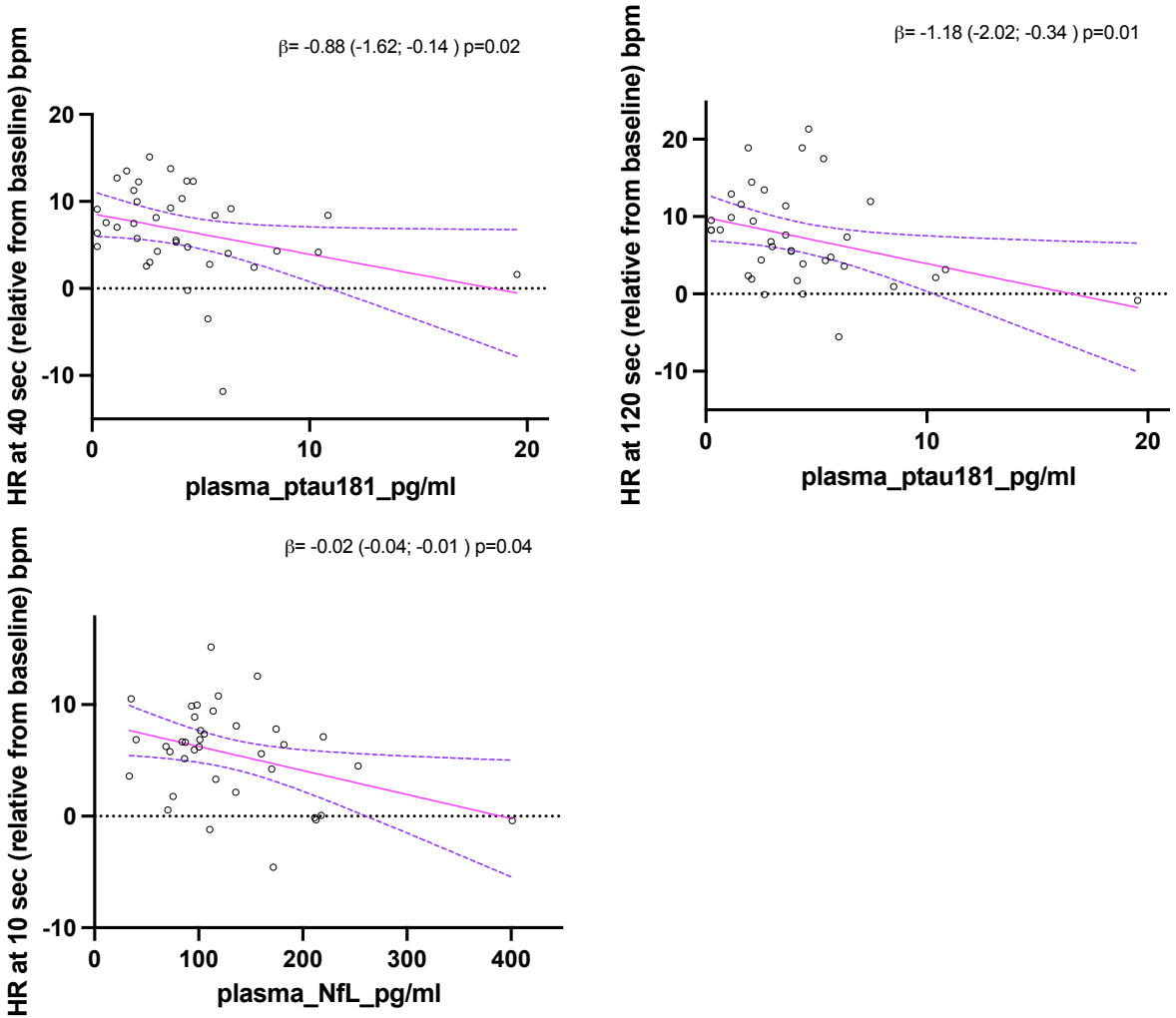
Heart Rate

Plasma NfL was significantly negatively associated with relative HR responses on standing at 10 seconds (β= -0.02 [-0.04; -0.01] p=0.04), with plasma p-tau 181 also demonstrating significant negative associations with HR responses at 40 and 120 seconds respectively (β=-0.88 (-1.62; -0.14) p=0.02); (β=-1.18 [-2.02; - 0.34] p=0.01*) with higher p-tau 181 levels strongly associated with slower HR responses. These results are displayed in Table 66 and Figures 35 (A-C).

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
HR pre-stand in bpm	0.28 (-0.81 – 1.38) p=0.59	0.45 (-1.39 – 2.31) p=0.62	-0.16 (-0.64 – 0.31) p=0.48	-0.15 (-0.81 – 0.51) p=0.63	-0.02 (-0.01 – 0.07) p=0.65	-0.05 (-0.18 – 0.08) p=0.43	0.03 (-0.02 – 0.08) p=0.22	0.05 (-0.02 – 0.11) p=0.13	0.09 (-0.15 – 0.34) p=0.46	0.05 (-0.33 – 0.44) p=0.75
HR 10 sec (relative) in bpm	0.23 (-0.15 – 0.61) p=0.22	-0.17 (-0.73 – 0.38) p=0.53	0.12 (-0.03 – 0.29) p=0.12	0.05 (-0.14 – 0.25) p=0.58	0.01 (-0.02 – 0.03) p=0.76	0.01 (-0.03 – 0.04) p=0.66	-0.02 (-0.04- -0.01) p=0.02	**-0.02 (-0.04 - -0.01) p=0.04**	0.05 (-0.02 – 0.14) p=0.18	0.07 (-0.05 – 0.18) p=0.25
HR 20 sec (relative) in bpm	-0.05 (-0.48 – 0.36) p=0.78	-0.38 (-1.06 – 0.3) p=0.26	-0.02 (-0.19 – 0.17) p=0.89	0.06 (-0.17 – 0.31) p=0.57	-0.01 (-0.04 – 0.02) p=0.58	-0.01 (-0.06 – 0.02) p=0.41	-0.01 (-0.03 – 0.01) p=0.33	-0.01 (-0.03 – 0.02) p=0.57	-0.01 (-0.1 – 0.09) p=0.93	0.06 (-0.07 – 0.21) p=0.34
HR 30 sec (relative) in bpm	-0.25 (-0.68 – 0.16) p=0.23	-0.46 (-1.14 – 0.22) p=0.18	-0.02 (-0.27 – 0.1) p=0.37	0.05 (-0.18 – 0.3) p=0.62	-0.02 (-0.05 – 0.02) p=0.32	-0.02 (-0.07 – 0.03) p=0.35	-0.01 (-0.03 – 0.01) p=0.42	-0.01 (-0.03 – 0.02) p=0.57	-0.05 (-0.15 – 0.04) p=0.27	0.03 (-0.11 – 0.18) p=0.58
HR 40 sec (relative) in bpm	-0.46 (-0.93 - -0.01) p=0.04	**-0.88 (-1.62 - -0.14) p=0.02**	-0.06 (-0.27 – 0.14) p-0.53	-0.06 (-0.2 – 0.32) p=0.62	-0.01 (-0.05 – 0.03) p=0.61	-0.01 (-0.07 – 0.03) p=0.47	-0.01 (-0.03 – 0.02) p=0.47	-0.01 (-0.04 – 0.02) p=0.49	-0.06 (-0.17 – 0.05) p=0.26	0.02 (-0.07 – 0.23) p=0.28
HR 60 sec (relative) in bpm	-0.54 (-1.12 – 0.02) p=0.06	-0.97 (-1.97 –	-0.06 (-0.32 -0.2) p=0.64	-0.01 (-0.34 –	0.01 (-0.03 – 0.06) p=0.62	0.01 (-0.06 –	-0.01 (-0.04 – 0.02) p=0.54	-0.02 (-0.05 –	-0.03 (-0.17 – 0.1) p=0.63	0.12 (-0.08 –

		0.02) p=0.05		0.35) p=0.96		0.08) p=0.7		0.01) p=0.23		0.33) p=0.22
HR 120 sec (relative) in bpm	-0.44 (-0.99 – 0.1) p=0.11	**-1.18 (-2.02 – -0.34) p=0.01***	0.08 (-0.16 – 0.32) p=0.51	0.23 (-0.06 – 0.53) p=0.12	-0.01 (-0.05 – 0.03) p=0.56	-0.03 (-0.09 – 0.02) p=0.21	-0.02 (-0.05 – 0.01) p=0.13	-0.02 (-0.05 – 0.01) p=0.09	-0.02 (-0.15 – 0.1) p=0.7	0.12 (-0.05 – 0.3) p=0.16
HR steady state (relative) in bpm	-0.19 (-0.63 – 0.24) p=0.37	-0.51 (-1.21 – 0.19) p=0.14	0.08 (-0.11 – 0.27) p=0.4	0.09 (-0.15 – 0.34) p=0.44	-0.01 (-0.03 – 0.03) p=0.92	-0.01 (-0.05 – 0.04) p=0.76	-0.02 (-0.04 – 0.01) p=0.09	-0.02 (-0.04 – 0.01) p=0.17	-0.01 (-0.11 – 0.09) p=0.81	0.03 (-0.11 – 0.18) p=0.62
HR min (relative) in bpm	-0.37 (-0.91 – 0.16) p=0.17	-0.63 (-1.42 – 0.14) p=0.11	-0.12 (-0.35 – 0.11) p=0.3	0.11 (-0..16 – 0.39) p=0.4	-0.02 (-0.06 – 0..01) p=0.24	-0.04 (-0.1 – 0.01) p=0.11	-0.01 (-0.04 – 0.02) p=0.41	-0.01 (-0.03 – 0.02) p=0.59	-0.08 (-0.21 – 0.03) p=0.15	0.04 (-0.12 – 0.21) p=0.58
HR max (relative) in bpm	0.13 (-0.31 – 0.58) p=0.53	-0.15 (-0.9 – 0.59) p=0.68	0.12 (-0.65 – 0.31) p=0.19	0.11 (-0.15 – 0.38) p=0.37	0.02 (-0.01 – 0.05) p=0.22	0.01 (-0.03 – 0.06) p=0.55	-0.01 (-0.02 – 0.02) p=0.6	-0.01 (-0.03 – 0.02) p=0.41	0.04 (-0.06 – 0.13) p=0.45	0.06 (-0..09-0.22) p=0.41
HR recovery rate (seconds)	-0.03 (-0.08 – 0.02) p=0.23	-0.03 (-0.12 – 0.06) p=0.52	-0.01 (-0.03 – 0.01) p=0.29	0.01 (-0.02 – 0.03) p=0.82	-0.01 (-0.01 – 0.01) p=0.18	-0.01 (-0.01 – 0.01) p=0.12	-0.01 (-0.01 – 0.01) p=0.87	0.01 (-0.01 – 0.01) p=0.73	-0.01 (-0.02 – 0.01) p=0.07	-0.01 (-0.02 – 0.02) p=0.63
Time between min and max HR (seconds)	0.56 (-0.03 – 1.75) p=0.06	0.59 (-0.37 – 1.56) p=0.22	0.28 (0.02 – 0.54) p=0.03	0.04 (-0.29 – 0.39) p=0.78	0.03 (-0.02 – 0.08) p=0.16	0.03 (-0.02 – 0.11) p=0.25	0.01 (-0.03 – 0.04) p=0.91	0.01 (-0.03 – 0.04) p=0.93	0.09 (-0.04 – 0.23) p=0.17	-0.04 (-0.24 – 0.16) p=0.68

Table 56 depicts the linear regression models, depicting the associations between HR values during AS and BBMs, adjusted for age, sex, BMI, comorbidity, polypharmacy, baseline HR and AD biomarkers



Figures 35 (A-C) illustrate the associations between p-tau 181 and HR response at 40 (A) and 120 seconds (B) post stand, and (C) depicts plasma NfL association with HR response at 10 seconds post stand

Tissue Saturation Index

There were no significant associations found on multiple linear regression modelling, on adjusted or unadjusted models, of TSI values post standing and plasma AD biomarkers.

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
TSI pre-stand (in %)	0.08 (-0.48 – 0.64) p=0.76	0.25 (-0.09 – 0.61) p=0.14	0.09 (-0.05 – 0.24) p=0.21	0.08 (-0.12-0.29) p=0.43	-0.01 (-0.03 – 0.02) p=0.78	-0.01 (-0.04 – 0.02) p=0.33	-0.02 (-0.03 – -0.01) p=0.04*	-0.02 (-0.04 - -0.01) p=0.07	0.03 (-0.05 – 0.1) p=0.51	0.01 (-0.07 – 0.09) p=0.77
TSI 10 sec (relative) in %	-0.02 (-0.18 – 0.12) p=0.71	-0.08 (-0.32 – 0.14) p=0.45	0.04 (-0.02 – 0.11) p=0.14	0.01 (-0.07 – 0.09) p=0.87	0.01 (-0.01 – 0.03) p=0.21	0.01 (-0.01 – 0.03) p=0.1	0.01 (-0.01 – 0.01) p=0.14	0.01 (-0.01 – 0.01) p=0.51	0.02 (-0.03 – 0.04) p=0.89	0.01 (-0.03 – 0.04) p=0.84
TSI 20 sec (relative) in %	0.06 (-0.1 – 0.23) p=0.43	-0.17 (-0.37 – 0.03) p=0.09	0.07 (-0.01 – 0.14) p= 0.05	0.03 (-0.04 – 0.11) p=0.4	0.01 (-0.01 – 0.03) p=0.25	0.01 (-0.01 – 0.02) p=0.28	0.01 (-0.01 – 0.01) p=0.12	0.01 (0.01 – 0.01) p=0.25	0.03 (-0.01 – 0.06) p=0.06	0.01 (-0.07 – 0.12) p=0.12
TSI 30 sec (relative) in %	0.03 (-0.12 - 0.18) p=0.6	-0.13 (-0.33 – 0.05) p=0.16	0.04 (-0.01 – 0.11) p=0.11	0.05 (-0.02 – 0.12) p=0.17	0.01 (0.01 – 0.02) p=0.04*	0.01 (-0.01 – 0.02) p=0.67	0.01 (-0.01 – 0.01) p=0.26	0.01 (-0.01 – 0.01) p=0.59	0.02 (-0.01 – 0.05) p=0.24	0.02 (-0.01 – 0.05) p=0.09
TSI 40 sec (relative) in %	0.03 (-0.11 – 0.18) p=0.62	-0.15 (-0.36 – 0.05) p=0.12	0.07 (0.01 – 0.13) p=0.01	0.07 (0.01 – 0.15) p=0.05	0.01 (-0.01 – 0.03) p=0.62	0.01 (-0.01 – 0.02) p=0.8	0.01 (-0.01 – 0.01) p=0.2	0.01 (-0.01 – 0.01) p=0.36	0.02 (-0.01 – 0.06) p=0.13	0.3 (-0.01 – 0.06) p=0.08
TSI 60 sec (relative) in %	-0.03 (-0.17 – 0.1) p=0.88	-0.02 (-0.17 – 0.1) p=0.32	0.02 (-0.03 – 0.09) p=0.33	0.03 (-0.02 – 0.1) p=0.41	0.01 (-0.01 – 0.02) p=0.21	0.01 (-0.01 – 0.02) p=0.83	0.01 (-0.01 – 0.01) p=0.35	0.01 (-0.01 – 0.01) p=0.5	0.01 (-0.02 – 0.03) p=0.71	0.01 (-0.02 – 0.03) p=0.65

TSI 120 sec (relative) in %	-0.04 (-0.22 – 0.12) p=0.57	-0.17 (-0.42 – 0.05) p=0.13	0.02 (-0.04 – 0.1) p=0.41	0.06 (-0.02 – 0.15) p=0.18	0.01 (-0.01 – 0.01) p=0.34	0.01 (-0.01 – 0.02) p=0.89	0.01 (-0.04 – 0.06) p=0.06	0.02 (-0.03 – 0.07) p=0.09	0.01 (-0.03 – 0.05) p=0.58	0.02 (-0.02 – 0.05) p=0.38
TSI steady state (relative) in %	-0.02 (-0.19 – 0.14) p=0.77	-0.15 (-0.41 – 0.08) p=0.19	0.05 (-0.02 – 0.12) p=0.16	0.07 (-0.01 – 0.16) p=0.12	0.01 (-0.01 – 0.01) p=0.31	0.01 (-0.01 – 0.02) p=0.55	0.01 (-0.01 – 0.01) p=0.12	0.01 (-0.01 – 0.01) p=0.15	0.01 (-0.03 – 0.05) p=0.53	0.01 (-0.02 – 0.05) p=0.43
TSI recovery rate (seconds)	-0.01 (-0.01 – 0.01) p=0.85	-0.01 (-0.02 – 0.01) p=0.46	-0.01 (-0.01 – 0.01) p=0.81	-0.01 (-0.01 – 0.01) p=0.56	-0.01 (-0.01 – 0.01) p=0.22	-0.01 (-0.02 – 0.01) p=0.63	0.01 (-0.01 – 0.01) p=0.37	-0.01 (-0.01 – 0.01) p=0.45	0.01 (-0.01 – 0.01) p=0.25	0.01 (-0.01 – 0.01) p=0.29
TSI nadir (relative) in %	0.02 (-0.15 – 0.21) p=0.74	-0.01 (-0.35 – 0.15) p=0.4	0.06 (-0.01 – 0.13) p=0.11	0.02 (-0.06 – 0.11) p=0.61	0.01 (-0.01 – 0.02) p=0.29	0.01 (-0.01 – 0.03) p=0.2	0.01 (-0.02 – 0.01) p=0.14	0.01 (-0.01 – 0.01) p=0.27	0.01(-0.02 – 0.05) p=0.49	0.02 (-0.02 – 0.05) p= 0.37
TSI time to nadir (seconds)	-0.57 (-1.2 – 0.06) p=0.07	0.01 (-1.09-1.12) p=0.97	-0.18 (-0.46 – 0.08) p=0.17	-0.09 (-0.5 – 0.31) p=0.64	-0.02 (-0.07 – 0.03) p=0.42	-0.01 (-0.07 – 0.04) p=0.65	0.01 (-0.2 – 0.04) p= 0.62	0.01 (-0.02 0.05) p=0.51	-0.13 (-0.27 – 0.01) p=0.04*	-0.13 (-0.29 – 0.11) p=0.07
TSI overshoot (relative) in %	-0.01 (-0.16 – 0.15) p=0.93	-0.18 (-0.39 – 0.02) p=0.08	0.05 (-0.01 - 0.12) p=0.07	0.05 (-0.02 – 0.13) p=0.12	0.01 (-0.01 – 0.03) p=0.23	0.01 (-0.01 – 0.03) p=0.22	0.01 (-0.01 – 0.01) p=0.2	0.01 (-0.01 – 0.01) p=0.39	0.02 (-0.01 – 0.05) p=0.21	0.02 (-0.01 – 0.06) p=0.12

Table 57 depicts the linear regression models, depicting the associations between HR values during AS and BBMs, adjusted for age, sex, BMI, comorbidity, polypharmacy, smoking status and AD biomarkers

Heart Rate Variability

There were no significant associations found on multiple linear regression modelling, on adjusted or unadjusted models, of HRV indices at baseline, and plasma AD biomarkers.

	plasma ptau 181		plasma ptau 217		plasma GFAP		plasma NfL		plasma total tau	
	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p
RMSDD	-0.01 (-0.2 – 0.01) p=0.18	-0.01 (-0.2 – 0.01) p=0.65	-0.01 (-0.01 – 0.01) p=0.78	-0.01 (-0.01 – 0.01) p=0.98	-0.01 (-0.01 – 0.01) p=0.86	-0.01 (-0.01 – 0.01) p=0.11	0.01 (-0.01 - 0.01) p=0.56	0.01 (-0.01 - 0.01) p=0.05	-0.01 (-0.01 – 0.01) p=0.15	-0.01 (-0.01 – 0.01) p=0.81
SDNN	0.81 (-1.2 – 2.83) p=0.42	-0.48 (-3.69 – 2.72) p=0.75	0.25 (-0.64 – 1.16) p=0.56	0.53 (-0.58 – 1.65) p=0.33	-0.01 (-0.16 – 0.16) p=0.99	-0.02 (-0.22 – 0.21) p=0.84	0.03 (-0.07 – 0.13) p=0.52	0.03 (-0.07 – 0.14) p=0.48	0.14 (-0.33 – 0.62) p=0.54	0.36 (-0.34 – 1.06) p=0.29
pnn50	-0.05 (-0.09 – 0.01) p=0.11	-0.04 (-0.08 – 0.02) p=0.16	-0.01 (-0.01 – 0.01) p=0.78	-0.01 (-0.01 – 0.01) p=0.51	-0.02 (-0.01 – 0.01) p=0.65	-0.01 (-0.01 – 0.01) p=0.63	-0.01 (-0.04 – 0.03) p=0.88	-0.01 (-0.03 – 0.03) p=0.89	-0.01 (-0.01 – 0.01) p=0.21	-0.01 (-0.01 – 0.01) p=0.13

Table 58 depicts the linear regression models, depicting the associations between HRV values at baseline and BBMs, adjusted for age, sex, BMI, comorbidity, polypharmacy, baseline HR and AD biomarkers

Associations of BBMs with cognitive responses under active tVNS stimulation

Given the significant improvements seen during active simulation detailed in Chapter 5, we sought to clarify whether any of the AD BBMs demonstrated significant associations with responses under active stimulation. Multilevel regression modelling was used, this time analysing the cognitive test results (not RTs) under active stimulation alone

Plasma p-tau 181 was significantly associated with SART error of commission rate, with higher p-tau 181 levels associated with higher error scores (β=0.75 [0.07 ; 1.58] p=0.05*). Remarkably, p-tau 217 was associated with multiple cognitive test results, with higher p-tau 217 results associated with SART error of omission rate (β=1.67 [0.41; 2.95] p=0.01*), SHQ level 2 (β=0.96 [0.06;1.86] p=0.03*), SHQ Level 7 (β=0.73 [0.04; 1.43] p=0.03*), SHQ overall (β=0.73 [0.07 ; 1.39] p=0.03*), semantic fluency (β=-0.28 [-0.51 ; -0.01] p=0.05*) digit span backwards (β=-0.07 [-0.13 ; -0.01] p=0.04*) and list recognition (β= -0.11 [-0.2 ; -0.01] p=0.02*). These results are displayed in Table 69 and graphically shown in Figures 36 (A,B).

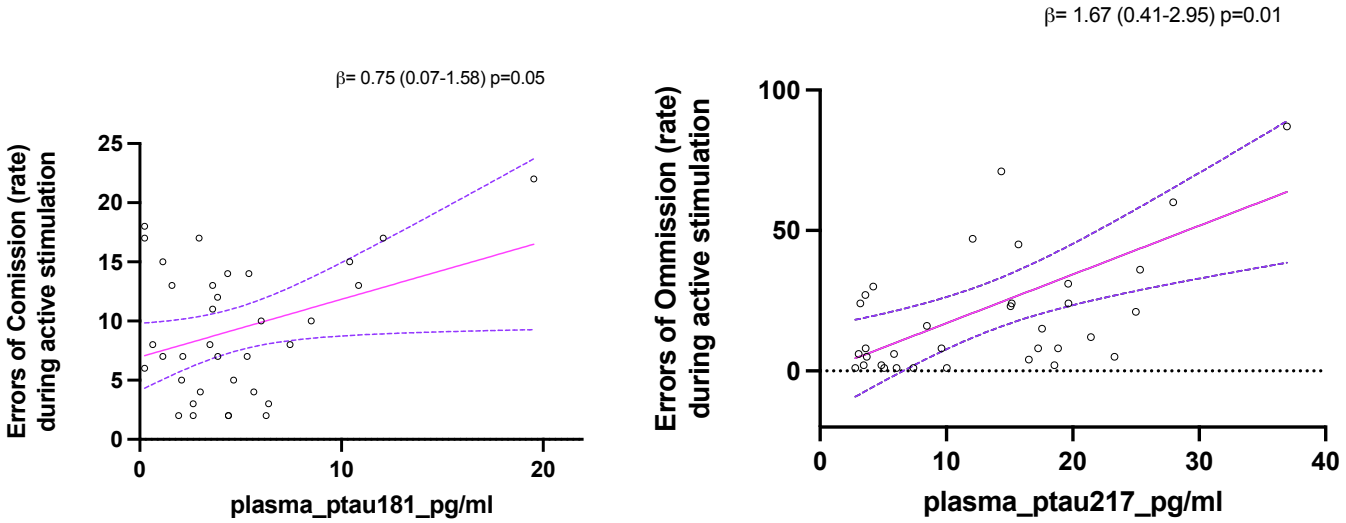
Do plasma AD biomarkers predict cognitive responses under active tVNS stimulation?

	plasma ptau 181		**plasma ptau 217**		**plasma GFAP**		**plasma NfL**		**plasma total tau**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
FNAT Face Recall (active)	0.51 (-0.44 – 1.45) p=0.29	0.64 (-0.88 – 2.17) p=0.39	-0.12 (-0.53 – 0.28) p=0.55	-0.16 (-0.72 – 0.39) p=0.55	-0.03 (-0.11 – 0.04) p=0.3	-0.02 (-0.12 – 0.09) p=0.78	-0.02 (-0.06 – 0.03) p=0.38	-0.01 (-0.05 – 0.05) p=0.96	0.03 (-0.19 – 0.25) p=0.76	-0.04 (-0.37 – 0.28) p=0.76
Log FNAT Name Recognition (active)	0.08 (-1.32 – 1.49) p=0.9	0.05 (-2.29 – 2.41) p=0.96	0.25 (-0.33 – 0.85) p=0.38	0.27 (-0.59 – 1.13) p=0.52	0.02 (-0.08 – 0.14) p=0.59	0.06 (-0.11 – 0.23) p=0.46	-0.03 (-0.1 – 0.03) p=0.34	-0.05 (-0.13 – 0.03) p=0.22	-0.11 (-0.43 – 0.2) p=0.46	-0.13 (-0.64 – 0.36) p=0.58
SART EOC (active)	0.29 (-0.23 – 0.82) p=0.26	**0.75 (0.07 – 1.58) p=0.05***	0.11 (-0.11 – 0.33) p=0.31	-0.06 (-0.35 – 0.24) p=0.69	0.01 (-0.04 – 0.04) p=0.93	0.02 (-0.04 – 0.08) p=0.49	-0.01 (-0.04 – 0.02) p=0.42	-0.01 (-0.03 – 0.03) p=0.9	-0.01 (-0.12 – 0.12) p=0.94	-0.17 (-0.35 – 0.01) p=0.07
SART EOO (active)	-0.12 (-2.88 – 2.62) p=0.92	0.45 (-3.04 – 3.95) p=0.79	1.73 (0.73 – 2.74) p=0.01*	**1.67 (0.41 – 2.95) p=0.01***	0.31 (0.12 – 0.49) p=0.01	0.21 (-0.04 – 0.46) p=0.11	0.07 (-0.06-0.21) p=0.27	0.04 (-0.07 – 0.16) p=0.46	0.16 (-0.47 – 0.79) p=0.61	-0.12 (-0.88 – 0.64) p=0.76
SHQ L1 (active)	-0.51 (-1.94 – 0.91) p=0.47	-0.24 (-2.54 – 2.05) p=0.89	0.23 (-0.37 – 0.84) p=0.43	0.6 (-0.24 – 1.44) p=0.15	-0.03 (-0.14 – 0.07) p=0.52	-0.09 (-0.26 – 0.07) p=0.26	-0.01 (-0.08 – 0.06) p=0.75	0.01 (-0.07 – 0.09) p=0.77	-0.14 (-0.46 – 0.19) p=0.41	-0.18 (-0.68 – 0.32) p=0.45

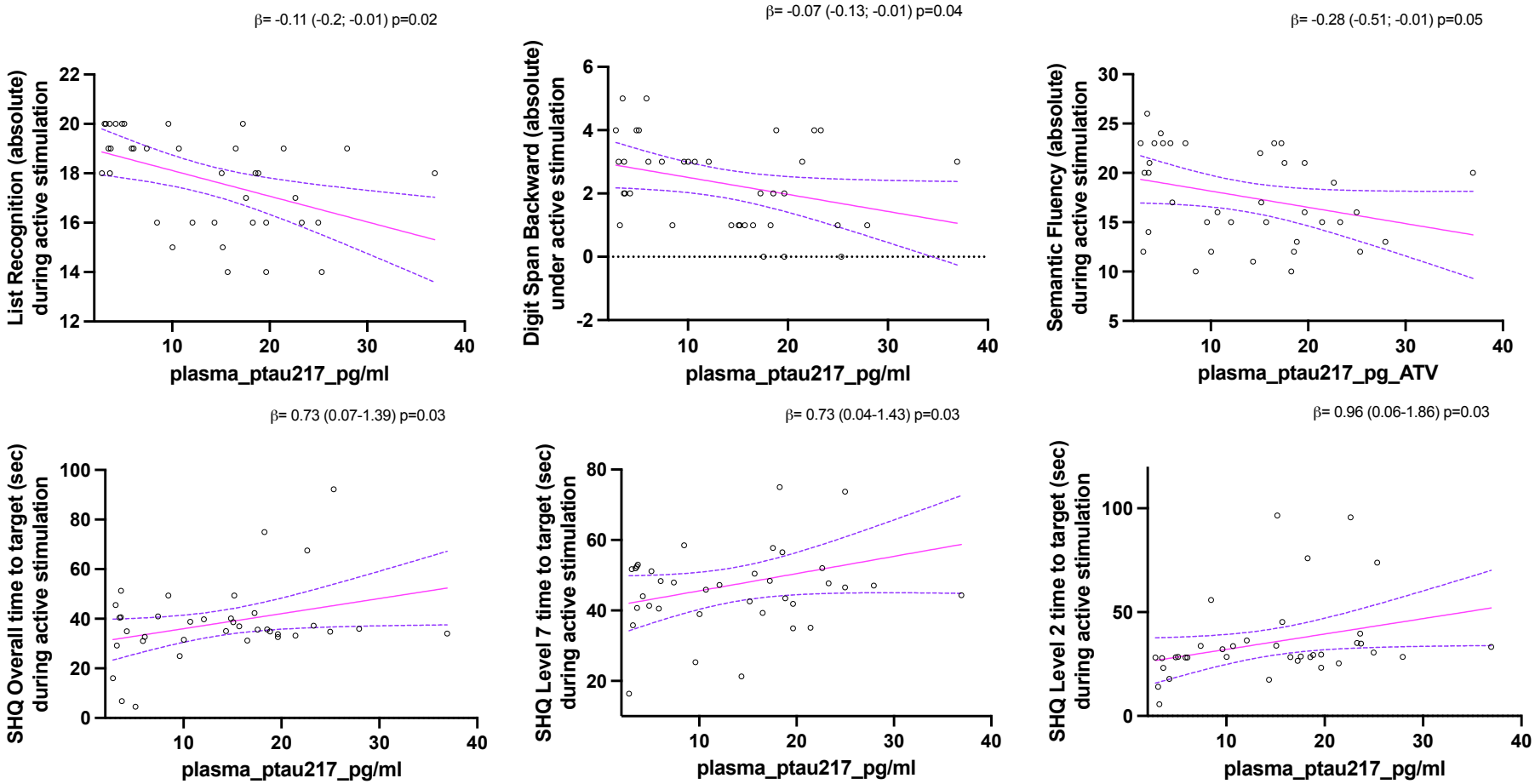
SHQ L2 (active)	-0.79 (-2.53 – 0.93) p=0.35	-0.83 (-3.28 – 1.62) p=0.49	0.48 (-0.24 – 1.21) p=0.18	**0.96 (0.06-1.86) p=0.03***	0.04 (-0.08 – 0.18) p=0.48	-0.04 (-0.22-0.13) p=0.62	0.07 (-0.07 – 0.16) p=0.07	0.08 (-0.01 – 0.16) p=0.06	0.02 (-0.37 – 0.42) p=0.91	0.11 (-0.42 – 0.64) p=0.66
SHQ L6 (active)	-0.71 (-1.92 – 0.47) p=0.23	-0.34 (-2.37 – 1.68) p=0.73	0.27 (-0.22 – 0.77) p=0.27	0.45 (-0.25 – 1.16) p=0.2	0.02 (-0.07 -0.11) p=0.73	-0.03 (-0.17 – 0.11) p=0.71	0.02 (-0.04 – 0.08) p=0.51	0.02 (-0.04 – 0.09) p=0.43	-0.05 (0.33 – 0.22) p=0.69	-0.09 (-0.51 – 0.31) p=0.62
SHQ L7 (active)	-0.72 (-1.97 – 0.52) p=0.24	0.02 (-1.92 – 1.98) p=0.97	0.32 (10.99 – 0.85) p=0.21	**0.73 (0.04-1.43) p=0.03 ***	-0.03 (-0.12 – 0.07) p=0.6	-0.08 (-0.22 – 0.06) p=0.24	0.02 (-0.04 – 0.08) p=0.46	0.03 (-0.03 – 0.11) p=0.27	-0.29 (-0.57 - -0.01) p=0.04	-0.37 (-0.78 – 0.03) p=0.07
SHQ L8 (active)	-1.03 (-2.97 – 0.9) p=0.28	-0.41 (-3.52 – 2.71) p=0.78	-0.03 (-0.87 – 0.81) p=0.93	0.89 (0.28 – 1.99) p=0.19	-0.12 (-0.26 – 0.03) p=0.11	-0.18 (-0.41 – 0.04) p=0.11	-0.02 (-0.11 – 0.08) p=0.76	0.02 (-0.08 – 0.13) p=0.66	-0.32 (-0.76 – 0.11) p=0.14	-0.32 (-0.99 – 0.35) p=0.33
SHQ overall (active)	-0.73 (-1.93 – 0.45) p=0.21	-0.32 (-2.11 – 1.47) p=0.71	0.26 (-0.24 – 0.77) p=0.29	**0.73 (0.07 – 1.39) p=0.03***	-0.02 (-0.11 – 0.07) p=0.64	-0.08 (-0.22 – 0.05) p=0.21	0.02 (-0.04 – 0.08) p=0.49	0.04 (-0.02 – 0.1) p=0.21	-0.14 (-0.42 – 0.13) p=0.31	-0.16 (-0.55 – 0.21) p=0.37
List learn (active)	-0.01 (-0.16 – 0.14) p=0.88	-0.11 (-0.38 – 0.15) p=0.38	-0.01 (-0.07 – 0.05) p=0.73	-0.03 (-0.13 – 0.06) p=0.45	-0.01 (-0.01 – 0.01) p=0.81	0.01 (-0.01 – 0.01) p=0.56	0.01 (-0.01 – 0.02) p=0.18	-0.01 (-0.01 – 0.02) p=0.19	0.01 (-0.02-0.05) p=0.41	0.02 (-0.02 – 0.08) p=0.31
Semantic fluency (active)	0.03 (-0.39 – 0.47) p=0.86	0.07 (-0.63 – 0.78) p=0.83	-0.16 (-0.34 – 0.01) p=0.06	**-0.28 (-0.51 - -0.01) p=0.05***	-0.01 (-0.04 – 0.02) p=0.63	0.01 (-0.03 – 0.06) p=0.56	0.01 (-0.01 – 0.02) p=0.72	0.01 (-0.01 – 0.03) p=0.61	0.02 (-0.07 – 0.12) p=0.6	0.04 (-0.11 – 0.19) p=0.57

Digit forward (active)	0.07 (-0.07 – 0.23) p=0.31	0.06 (-0.19 – 0.32) p=0.62	-0.01 (-0.07 – 0.06) p=0.91	-00.04 (-0.14 – 0.05) p=0.34	-0.01 (-0.02 – 0.01) p=0.58	0.01 (-0.01 – 0.03) p=0.35	-0.01 (-0.01 – 0.01) p=0.38	-0.01 (-0.01 – 0.01) p=0.46	0.02 (-0.01 – 0.06) p=0.21	0.01 (-0.04 – 0.06) p=0.75
Digit backward (active)	0.04 (-0.07 – 0.15) p=0.44	0.03 (-0.15 – 0.22) p=0.7	-0.02 (-0.07 – 0.02) p=0.3	**-0.07 (-0.13 - -0.01) p=0.04***	-0.01 (-0.01 – 0.01) p=0.91	0.01 (-0.01 – 0.02) p=0.13	-0.01 (-0.01 – 0.01) p=0.27	-0.01 (-0.01 – 0.01) p=0.28	0.02 (-0.01 – 0.04) p=0.13	0.02 (-0.02 – 0.05) p=0.41
List recall (active)	-0.12 (-0.35 – 0.11) p=0.28	-0.11 (-0.48 – 0.26) p=0.55	-0.08 (-0.18 – 0.01) p=0.06	-0.12 (-0.26 – 0.02) p=0.08	-0.01 (-0.01 – 0.02) p=0.95	0.01 (-0.02 – 0.03) p=0.58	-0.01 (-0.01 – 0.01) p=0.63	-0.01 (-0.01 – 0.01) p=0.41	-0.03 (-0.08 – 0.02) p=0.25	-0.01 (-0.08 – 0.08) p=0.94
List recognition (active)	0.07 (-0.11 – 0.26) p=0.38	0.05 (-0.21 – 0.31) p=0.68	-0.06 (-0.13 – 0.02) p=0.14	**-0.11 (-0.2 - -0.01) p=0.02***	-0.01 (-0.03 – 0.01) p=0.09	0.01 (-0.02-0.02) p=0.72	-0.01 (-0.01 – 0.01) p=0.16	-0.01 (-0.01 – 0.01) p=0.44	0.02 (-0.02 – 0.06) p=0.37	0.01 (-0.04 – 0.06) p=0.72

Table 59 depicts multiple linear regression models for cognitive test results and AD BBMs, adjusted for age, sex, BMI, comorbidity, polypharmacy and AD biomarkers.



Figures 36 (A,B) depict the linear regressions showing an association between higher p-tau 181 levels and higher EOC rates during active tVNS (A) and higher ptau 217 levels and higher EOO rates during active tVNS (B)



Figures 37 (A-F) depict associations of plasma p-tau 217 and list recognition (A), digit span backwards (B), semantic fluency (C), SHQ overall time to target (D), SHQ Level 7 time to target (E) and SHQ Level 2 time to target (F).

Associations of inflammatory cytokines and chemokines with cognitive responses under active tVNS stimulation

There is a known association between higher circulating inflammatory cytokines and chemokines and worse cognitive test scores, both on cross-sectional and longitudinal data.[603,604] Multiple linear regression models were used to explore if any relationship existed between higher systemic inflammatory cytokines and chemokines and responses to cognitive tasks during active tVNS.

Higher IFNγ was associated with slower time to target during SHQ L1 (13.54 [2.73 ; 24.37] p=0.02*), SHQ Level 7 (β= 12.5 [1.43 ; 23.4] p=0.02), SHQ L8 (β=19.66 [6.13; 33.19] p=0.01*) and SHQ overall (β=10.76 [1.96; 19.76] p=0.02*).

Higher TNF-α was associated with slower time to target during SHQ level 2 (β=25.03 [0.89; 49.18] p=0.04*) and lower semantic fluency absolute scores ((β= 6.05 [0.02 ; 12.31] p=0.05*).

Higher MIP-1β levels were associated with higher SART error of commission rates (β= 7.04 [1.74;12.33] p=0.01*) while higher MCP-1 levels were associated with slower time to target during SHQ L6 during active stimulation (β=12.99 [0.89; 25.09] p=0.03*). Tables 70 (A, B) depict these data which are displayed graphically in Figures 38 (A-F) and Figures 39 (A,B).

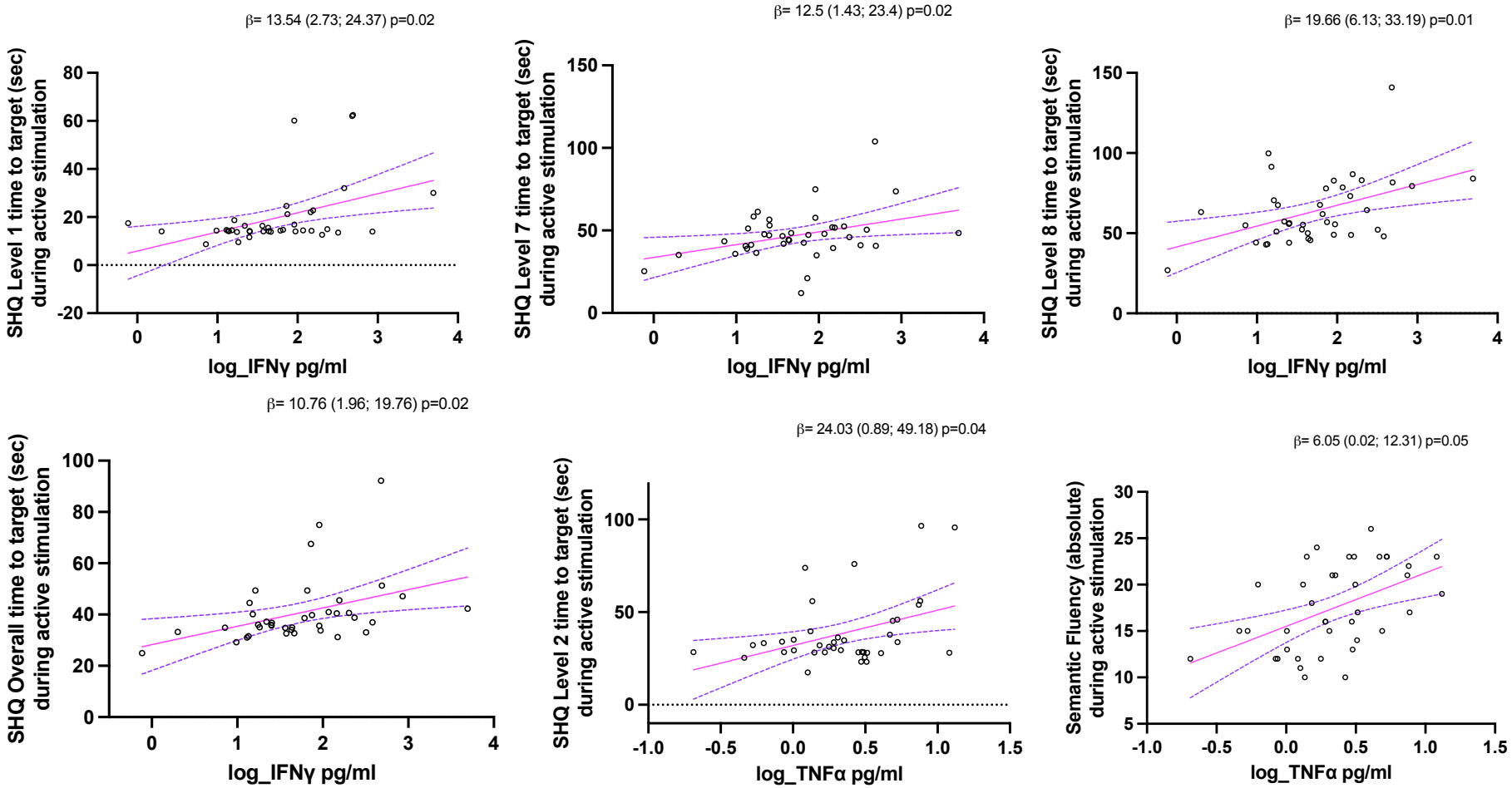
Do inflammatory cytokines predict cognitive responses under active stimulation?

	IFNγ		**IL-10**		**IL-12p70**		**IL-6**		**TNF-α**	
	Unadjusted β coeff (95%CI); p	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*	*Unadjusted β coeff (95%CI); p*	*Adjusted β coeff (95%CI); p*
FNAT FR (active)	1.75 (-3.09 – 6.61) p=0.46	6.34 (-1.37 – 14.05) p=0.19	1.75 (-2.44 – 5.94) p=0.4	5.16 (-0.91 – 11.21) p=0.09	1.15 (-2.24 – 4.58) p=0.5	2.87 (-1.36 – 7.11) p=0.17	-0.57 (-3.47 – 2.61) p=0.7	-1.11 (-6.25 - 4.03) p=0.66	-2.34 (-11.76 – 6.08) p=0.52	-9.13 (-22.31 – 4.83) p=0.19
Log FNAT NR (active)	3.51 (-3.2 – 10.21) p=0.29	5.89 (-5.51 – 17.31) p=0.29	-0.52 (-6.54 – 5.45) p=0.86	-2.91 (-11.89 – 1.06) p=0.51	1.4 (-3.43 – 6.23) p=0.56	0.41 (-5.87 – 6.68) p=0.89	2.23 (-1.96-6.44) p=0.28	0.2 (-7.45 – 7.81) p=0.98	6.34 (-6.04-18.72) p=0.31	5.3 (-15.17 – 25.86) p=0.56
SART EOC (active)	1.34 (-1.27 – 3.98) p=0.3	3.34 (-0.71 – 7.37) p=0.1	-0.08 (-3.21 – 1.54) p=0.48	0.21 (-2.98-3.32) p=0.88	-0.35 (-2.32 – 1.61) p=0.71	-0.29 (-2.67 – 2.08) p=0.8	-0.76 (-2.34 – 0.84) p=0.34	-2.26 (-4.85 – 0.33) p=0.08	-3.73 (-8.32 – 0.88) p=0.11	-5.82 (-12.82 – 1.17) p=0.09
SART EOO (active)	3.76 (-9.6 – 17.1 (p=0.57	-12.06 (-35.98 – 11.82) p=0.31	-19.11 (-37.48 - -0.74) p=0.06	-2.45 (-4.25 – 1.24) p=0.07	4.03 (-5.45 – 13.93) p=0.41	6.37 (-7.71 – 10.45) p=0.35	4.26 (-3.91 – 12.45) p=0.29	-2.31 (-17.65 – 13.05) p=0.75	13.76 (-10.45 – 37.56) p=0.25	39.71 (-1.62 – 81.06) p=0.06
SHQ L1 (active)	6.81 (0.02 – 13.41) p=0.04	**13.54 (2.73 – 24.37) p=0.02***	-5.05 (-10.98 – 0.76) p=0.08	-5.58 (-14.09 – 2.92) p=0.18	-3.18 (-8.01 – 1.64) p=0.18	-3.84 (-9.78 – 2.09) p=0.19	-1.52 (-5.88 – 2.76) p=0.46	1.29 (-5.92 8.54) p=0.71	-3.54 (-16.22 – 9.23) p=0.57	0.38 (-19.05 – 19.08) p=0.96

SHQ L2 (active)	29.04 (13.45 – 44.86) p=0.01	2.12 (-11.32 – 15.54) p=0.74	-3.67 (-10.98 – 3.55) p=0.31	-10.37 (-20.94 – 0.19) p=0.06	2.76 (-3.14 – 8.62) p=0.35	3.04 (-4.33 – 10.42) p=0.4	0.09 (-5.16 – 5.31) p=0.97	0.49 (-8.54 – 9.49) p=0.91	13.43 (-1.36 – 28.22) p=0.07	**25.03 (0.89-49.18) p=0.04***
SHQ L6 (active)	3.33 (-2.34 – 9.02) p=0.24	6.91 (-2.02 – 15.93) p=0.12	-1.52 (-6.55 – 3.49) p=0.54	-3.62 (-10.72 – 3.3) p=0.12	0.18 (-3.92 – 4.29) p=0.92	-1.05 (-5.95 – 3.85) p=0.66	-0.54 (-4.18 – 3.04) p=0.75	2.67 (-4.09 – 9.39) p=0.41	8.17 (-2.34 – 18.71) p=0.12	12.32 (-3.87 – 28.51) p=0.12
SHQ L7 (active)	8.05 (2.34 – 13.45) p=0.01	**12.5 (1.43 – 23.4) p=0.02**	-2.21 (-7.66 – 3.23) p=0.41	-0.01 (-6.17 – 6.15) p=0.99	1.22 (-3.23 – 5.73) p=0.58	-0.12 (8.35 – 8.13) p=0.97	-0.41 (-4.37 – 3.54) p=0.83	-7.37 (-29.34 – 15.14) p=0.5	-0.04 (-13.45 – 12.23) p=0.98	-1.01 (-17.23 – 15.22) p=0.89
SHQ L8 (active)	12.98 (4.42 – 21.76_ p=0.01*	**19.66 (6.13 – 33.19) p=0.01***	-4.24 (-12.35 – 3.85) p=0.28	-3.61 (-14.25 – 7.02) p=0.49	2.24 (-4.45 – 9.01) p=0.5	3.38 (-4.04 - 10.89) p=0.35	-2.47 (-8.34 – 3.54) p=0.42	-5.69 (-14.7 – 3.32) p=0.21	5.23 (-12.23 – 22.34) p=0.55	-0.59 (-24.5 – 23.41) p=0.96
SHQ overall (active)	7.14 (1.73 – 12.55) p=0.01*	**10.76 (1.96 – 19.76) p=0.02***	-3.38 (-8.34 – 1.65) p=0.18	-1.32 (-6.23 – 1.57) p=0.12	0.73 (-3.44 – 4.93) p=0.72	0.42 (-4.42 – 5.26) p=0.85	-0.98 (-4.65 – 2.76) p=0.58	-0.46 (-6.34 – 5.42) p=0.87	4.09 (-5.76 – 15.54) p=0.38	7.03 (-8.81 – 22.72) p=0.36
List learn (active)	0.32 (-0.45 – 1.01) p=0.41	0.57 (-0.8 – 1.95) p=0.39	0.01 (-0.65 – 0.68) p=0.96	-0.28 (-1.36 – 0.79) p=0.58	0.17 (-0.37 – 0.71) p=0.52	0.11 (-0.64 – 0.86) p=0.77	-0.04 (-0.52 – 0.43) p=0.84	-0.2 (-1.11 – 0.71) p=0.65	1.12 (-0.24 – 2.45) p=0.11	1.7 (-0.76 – 4.17) p=0.16
Semantic fluency (active)	0.65 (-1.47 – 2.76) p=0.54	-0.66 (-4.06 – 2.71) p=0.68	1.52 (-0.27 – 3.24) p=0.9	0.09 (-2.54 – 2.77) p=0.94	0.67 (-0.81 – 2.16 p=0.36	-0.12 (-1.97 – 1.73) p=0.89	0.76 (-0.54 – 2.06) p=0.24	-0.31 (-2.56 – 1.94) p=0.77	5.75 (2.34 – 9.13) p=0.01*	**6.05 (0.02 – 12.31) p=0.05***

Digit forward (active)	-0.76 (-2.34 – 1.07) p=0.54	-0.96 (-2.99 – 1.02) p=0.32	-0.21 (-0.45 – 0.32) p=0.51	-0.17 (-2.12 – 1.71) p=0.86	0.17 (-0.36 – 0.71) p=0.52	1.4 (-0.45 – 3.24) p=0.13	-0.03 (-0.5 – 0.44) p=0.89	-0.42 (-2.24 – 1.41) p=0.63	0.74 (-0.62 – 2.12) p=0.28	-0.51 (-1.47 – 0.45) p=0.28
Digit backward (active)	1.65 (-3.45 – 5.85) p=0.89	1.84 (-9.45 – 13.45) p=0.73	-0.06 (-0.54 – 0.44) p=0.84	-0.04 (-0.65 – 0.03) p=0.89	-0.11 (-0.51 – 0.29) p=0.59	4.78 (-5.66 – 15.62) p=0.35	-0.24 (-0.58 – 0.11) p=0.16	-2.83 (-13.45 – 7.54) p=0.35	-0.06 (-1.14 – 0.98) p=0.91	-4.27 (-9.62 – 1.12) p=0.12
List recall (active)	0.76 (-0.56 – 3.65) p=0.51	0.65 (-0.8 – 1.85) p=0.65	0.74 (-0.21 – 1.71) p=0.12	-0.28 (-1.36 – 0.79) p=0.58	0.24 (-0.56 – 1.05) p=0.53	0.11 (-0.64 – 0.86) p=0.77	-0.04 (-0.76 – 0.66) p=0.89	-0.2 (-1.11 – 0.71) p=0.65	1.61 (-0.41 – 3.63) p=0.12	1.7 (-0.76 – 4.17) p=0.16
List recognition (active)	1.23 (-0.56 – 3.65) p=0.43	1.08 (-0.65 – 3.7) p=0.45	0.4 (-0.37 – 1.18) p=0.3	1.83 (-1.44 – 4.01) p=0.81	-0.15 (-1.92 – 1.31) p=0.87	-0.15 (-1.62 – 1.31) p=0.98	0.63 (-1.7 – 1.8) p=0.74	0.53 (-1.7 – 1.88) p=0.94	-0.3 (-1.27 – 3.75) p=0.71	-0.27 (-1.27 – 3.75) p=0.78

Table 60 (A) depicts multiple linear regression models for cognitive test results under active stimulation and levels of IFNγ, IL-10, IL-12p7-0, IL6, TNF-α, adjusted for age, sex, BMI, comorbidity, polypharmacy and inflammatory markers



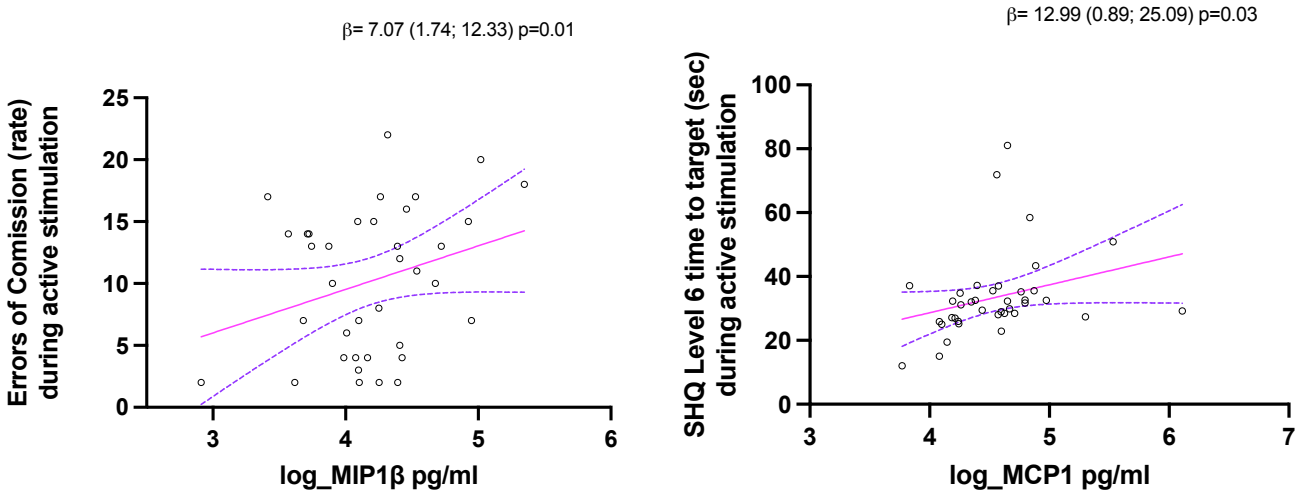
Figures 38 (A-F) depict the associations on adjusted linear regression models between IFNγ and SHQ Level 1 (A), IFNγ and SHQ Level 7(B), IFNγ and SHQ Level 8 (C), IFNγ and SHQ overall time to target (D), TNF-α and SHQ Level 2 (E), and TNF-α and semantic fluency (F) under active stimulation

	Eotaxin		IP-10		MCP-1		MIP-1β		IL-17A	
	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p	Unadjusted β coeff (95%CI); p	Adjusted β coeff (95%CI); p
FNAT Face Recall (active)	1.98 (-9.45 – 13.45) p=0.73	1.84 (-9.45 – 13.45) p=0.73	-2.2 (-12.94 – 10.43) p=0.76	-3.52 (-14.57 – 7.45) p=0.51	3.56 (-6.54 – 10.43) p=0.61	4.78 (-5.66 – 15.62) p=0.35	-0.01 (-14.98 – 13.92) p=0.98	-2.83 (-13.45 – 7.54) p=0.35	-0.02 (-12.98 – 11.87) p=0.9	-4.27 (-9.62 – 1.12) p=0.12
Log FNAT Name Recognition (active)	11.12 (-4.76 – 25.87) p=0.43	13.08 (-3.65 – 29.7) p=0.12	0.89 (-12.45 – 13.87) p=0.99	1.83 (-14.44 – 18.01) p=0.81	-0.01 (-12.09 – 12.99) p=0.99	-0.15 (-15.61 – 15.31) p=0.98	1.23 (-12.09 – 15.98) p=0.67	4.53 (-10.7 – 19.88) p=0.54	-0.02 (-9.99 – 8.56) p=0.98	-0.27 (-8.27 – 7.76) p=0.94
SART EOC (active)	2.01 (-1.89 – 5.98) p=0.45	4.01 (-1.99 – 9.92(p=0.17	0.89 (-9.45 – 7.43) p=0.91	0.57 (-4.98 – 6.13) p=0.88	-0.45 (-9.98 – 7.98) p=76	-2.54 (-10.7 - 0.15) p=0.12	2.73 (-0.25 – 5.65) p=0.32	**7.04 (1.74-12.33) p=0.01***	-1.3 (-4.5 – 0.02) p=0.09	-0.88 (-3.59 – 1.83) p=0.51
SART EOO (active)	-2.2 (-34.98 – 31.09) p=0.9	-2.31 (-37.29 – 32.68) p=0.89	12.98 (-10.99 – 23.35) p=0.45	19.17 (-13.68 – 52.05) p=0.23	-10.8 (-45.98 – 7.98) p=0.27	-32.91 (-64.08 – 1.78) p=0.06	0.09 (-22.98 – 20.5) p=0.98	2.86 (-28.45 – 34.17) p=0.85	-0.05 (-12.98 – 10.87) p=0.89	-3.69 (-19.65 – 12.-3) p=0.64
SHQ L1 (active)	-0.01(-10.24 – 9.49) p=0.92	-3.18 (-19.01 – 12.65) p=0.68	-2.9 (-20.61 – 10.22) p=0.53	-5.19 (-20.61 – 10.22) p=0.49	5.83 (-6.81 – 22.49) p=0.58	7.83 (-6.81 – 22.49) p=0.28	-0.9 (-10.9 – 9.66 P=0.91	-7.51 (-22.02 – 6.99) p=0.29	-0.1 (-10.12 – 8.99) p=0.78	-0.08 (-7.98 – 7.49) p=0.98
SHQ L2 (active)	0.01 (-12.98 – 14.12) p=0.99	0.46 (-19.2 – 20.12) p=0.96	-0.9 (-13.87 – 15.98) p=0.9	-2.15 (-21.3 – 16.99) p=0.81	-2.77 (-13.98 – 11.54) p=0.89	-3.08 (-21.3 – 15.6) p=0.72	-6.7 (-20.77 – 6.98) p=0.51	-10.55 (-28.47 – 7.49) p=0.23	0.34 (-8.76 – 9.87) p=0.81	0.28 (-9.11 – 9.61) p=0.95

SHQ L6 (active)	-4.9 (-14.44 – 7.99) p=0.39	-5.94 (-19.09 – 7.28) p=0.35	-5.65 (-13.98 – 5.65) p=0.48	-7.03 (-20.44 – 6.35) p=0.28	6.56 (-1.5 – 15.67) p=0.1	**12.99 (0.89 – 25.09) p=0.03***	-2.65 (-12.76 – 0.65) p=0.17	-14.31 (-28.41 – 0.21) p=0.06	0.76 (-9.76 – 10.78) p=0.76	0.54 (-5.76 – 6.82) p=0.85
SHQ L7 (active)	-1.81 (-18.9 – 13.98) p=0.88	-1.47 (-18.05 – 15.07) p=0.85	3.1 (-4.5-9.54) p=0.78	4.02 (-10.91 – 18.95) p=0.58	1.1 (-10.65 – 12.43) p=0.98	2.22 (-15.95 – 20.44) p=0.9	-0.54 (-8.02 – 7.17) p=0.89	-0.54 (-8.02 – 7.17) p=0.88	-0.14 (-1.1 – 0.98) =0.67	-0.13 (-1.07 – 0.88) p=0.76
SHQ L8 (active)	-0.99 (-12.8-16.21) p=0.91	-2.34 (-22.32-17.45) p=0.81	1.12 (16.32 – 17.32) p=0.93	0.94 (-18.33 – 20.23) p=0.92	5.65 (-3.34 – 8.43) -0.45	10.83 (-7.47 – 29.81) p=0.23	-0.91 (-12.87 – 13.76) p=0.9	-0.98 (-19.13 – 17.15) p=0.91	-1.5 (-12.4 – 8.43) p=0.79	-1.54 (-11.04 – 7.82) p=0.73
SHQ overall (active)	-1.54 (-2.9 – 5.36) p=0.81	-2.54 (-15.43 – 10.36) p=0.69	-0.48 (-6.21 – 8.93) p=0.89	-2.63 (-15.21 – 9.93) p=0.67	4.35 (-3.54 – 16.23) p=0.48	6.34 (-5.54 – 18.23) p=0.28	-3.54 (-7.34 – 2.43) p=0.45	-6.54 (-18.92 – 5.21) p=0.26	-0.3 (-6.46 – 5.82) p=0.91	-0.28 (-6.46 – 5.82) p=0.92
List learn (active)	-0.87 (-1.99 – 1.02) p=0.45	-0.96 (-2.99 – 1.02) p=0.32	-0.18 (-2.12 – 1.71) p=0.87	-0.17 (-2.12 – 1.71) p=0.86	1.46(-0.45 – 3.24) p=0.2	1.4 (-0.45 – 3.24) p=0.21	-0.32 (-2.24 – 1.41) p=0.76	-0.42 (-2.24 – 1.41) p=0.63	-0.51 (-1.47 – 0.45) p=0.28	-0.51 (-1.47 – 0.45) p=0.28
Semantic fluency (active)	-0.6 (-5.52 – 4.35) p=0.8	-0.59 (-5.52 – 4.35) p=0.81	0.56 (-4.31 – 5.32) p=0.83	0.51 (-4.31 – 5.32) p=0.83	-0.87 (-5.5 – 3.66) p=0.76	-0.92 (-5.5 – 3.66) p=0.68	1.6 (-1.83 – 5.34) p=0.56	2.71 (-1.83 – 7.24) p=0.23	0.87 (-1.62 – 2.7) p=0.45	0.53 (-1.82 – 2.91) p=0.64
Digit forward (active)	0.87 (-2.02 – 4.93) p=0.35	0.91 (-2.02 – 5.93) p=0.22	0.62 (-1.72 – 3.3) p=0.72	0.62 (-1.72 – 3.3) p=0.72	-1.05 (-5.95 – 3.85) p=0.66	-1.05 (-5.95 – 3.85) p=0.66	1.8 (-1.1 – 5.94) p=0.65	2.7 (-1.09 – 6.94) p=0.56	2.2 (-3.97 – 7.81) p=0.67	2.32 (-3.97 – 7.81) p=0.72

Digit backward (active)	-0.3 (-2.1 – 1.4) p=0.76	-0.38 (-2.02 – 1.23) p=0.62	-0.01 (-0.01 – 0.01) p=0.99	-0.11 (-1.61 – 1.51) p=0.98	0.01 (-0.01 – 0.01) p=0.87	0.32 (-1.18 – 1.87) p=0.66	-0.65 (-1.23 – 1.45) p=0.56	-0.71 (-2.28 – 0.79) p=0.34	-0.05 (-0.98 – 0.76) p=0.78	-0.06 (-0.84 – 0.71) p=0.68
List recall (active)	-1.4 (-12.56 – 14.92) p=0.98	-1.47 (-18.05 – 15.07) p=0.85	1.2 (-12.09 – 13.09) p=0.87	4.02 (-10.91 – 18.95) p=0.58	2.1 (-14.93 – 16.54) p=0.98	2.22 (-15.95 – 20.44) p=0.9	-0.65 (-0.76 0 7.54) p=0.92	-0.54 (-8.02 – 7.17) p=0.88	-0.01 (-0.01 – 0.01) p=0.94	-0.13 (-1.07 – 0.88) p=0.76
List recognition (active)	0.65 (-0.1 – 0.56) p=0.65	0.57 (-0.8 – 1.95) p=0.39	-0.1 (-1.63 – 1.54) p=0.67	-0.28 (-1.36 – 0.79) p=0.58	0.15 (-0.67 – 0..87) p=0.67	0.11 (-0.64 – 0.86) p=0.77	-0.03 (-0.1 – 0.1) p=0.91	-0.2 (-1.11 – 0.71) p=0.65	1.1 (-0.3 – 2.34) p=0.45	1.7 (-0.76 – 4.17) p=0.16

Table 60 (B) depicts multiple linear regression models for cognitive test results under active stimulation and levels of Eotaxin, IP-10, MCP-1, MIP-1β, IL-17A, adjusted for age, sex, BMI, comorbidity, polypharmacy and inflammatory markers



Figures 39 (A,B) depict the associations on adjusted linear regression models between MIP-1β and SART EOC during active stimulation (A) and MCP-1 and SHQ Level 6 time to target under active stimulation (B).

Discussion

There was no significant effect noted on whole blood cytokines or chemokines during acute active or sham tVNS stimulation. One of the reasons for this may be that a longer duration of ABVN stimulation may be needed to affect the vagal inflammatory arc, and future studies could investigate the effects of tVNS stimulation over longer periods of time.

Interestingly, higher levels of IFNγ, TNF-α, MIP-1β and MCP-1 were associated with worse cognitive performance, specifically under active tVNS stimulation. This raises the question of whether a higher inflammatory milieu might adversely predict cognitive responses to tVNS, though this would need to be more thoroughly investigated in adequately powered future studies to establish if this is replicated and not due to chance.

In this study, higher levels specifically of p-tau 217 adversely predicted cognitive responses under active tVNS stimulation across a range of domain-specific cognitive tests, most notably those of spatial navigation and working memory. P-tau 217 is emerging as a promising biomarker of amyloid pathology in AD, with an Area Under the Curve (AUC) of 0.92-0.96 for detection of Aβ on CSF.[605] Whether higher p-tau 217 levels index worse cognitive impairment is not yet known, however these fascinating results detailed above indicate that higher ptau-217 levels are associated with worse cognitive performance, but also indicate lower likelihood of response to tVNS stimulation. This may be via less synaptic recruitment, higher p-tau 217 indicating higher degrees of atrophy already, or may be via another unknown mechanism.

As previously referenced, persons with dementia[118,526,540] but also MCI[528] have high rates of NCVI, however these studies were performed before widespread use of AD biomarkers was available. There is a paucity of data investigating the prevalence and effects AD BBMs have on NCV indices. We note in this small study that higher p-tau 217 values were associated with lower SBP values at 10 seconds post standing, p-tau 181 was associated with slower HR responses at 40 and 120 seconds post standing and NfL was associated with slower HR responses at 10 seconds post standing. There were no notable effects on TSI or DBP values during AS.

Further larger studies would be worthwhile to fully characterise the effects neurodegenerative markers have on NCVI, and if possible correlate this data with a biomarker of known atrophy such as standardised MTA scores per patient, to elucidate if hippocampal atrophy as the final biomarker of AD related pathology might index the spectrum of NCVI changes in AD.

Chapter 8 Summary

Strengths

One of the exciting output from this pilot trial is that it establishes that an auricular neuromodulation device is tolerable and acceptable for use in an older population with MCI. Most study participants would use the device again, and most did not find it painful or the side effects bothersome. We also found that the device was both centrally and peripherally safe, and did not adversely affect any neuro-cardiovascular signal during an orthostatic challenge. Even though there was a high degree of orthostatic instability in this group, with 35/40 (87.5%) of participants experiencing some degree of neurocardiovascular instability at baseline (via classical OH, initial OH or delayed recovery) there was no significant effect seen for active stimulation on any of these indices. This is important, as over stimulating the VN in an older population could be deleterious in terms of cerebral oxygenation and falls risk.

This study found a significant effect during two of the Spatial Navigation subset of tasks during the domain-specific cognitive assessment, with an effect observed for active simulation compared to both sham and baseline. Though the cortical and subcortical areas involved in spatial navigation have not classically been shown on tVNS fMRI imaging to be active during auricular stimulation, potentially a globally cognitive enhancing effect of tVNS may have been responsible for this result. It is noteworthy that those with higher circulating cytokines (IFNγ, TNF-α, MIP-1β and MCP-1) had worse cognitive responses during active stimulation, and whether high degrees of inflammation may preclude a benefit in this area remains to be explored.

It was also noteworthy that higher p-tau 217 levels were significantly associated with worse cognitive test scores across a range of cognitive tests. P-tau 217 is emerging as a highly sensitive biomarker of central Aβ pathology in AD, and in the future it may be used to stratify admission to anti-amyloid clinical trials. Whether it may also have a use in determining which participants may benefit from non-invasive VNS modulation remain to be explored

A strength of this study is the robust nature of its design. By nesting each analysis within each participant as their own control over three study visits, it is possible to reduce many biases. It is noteworthy that for some cognitive tests there was no significant difference noted between active and sham conditions, indicating a non-specific effect of stimulation, as opposed to a specific effect of active stimulation (see SHQ overall results). It is plausible that any degree of peripheral nerve stimulation may induce a placebo effect strong enough to cause a significant improvements for some participants. However this naturally calls into question previous trials in this area that have only used parallel designs, whereby separate participants are randomised to either active or sham stimulation. A three-armed crossover device trial such as this, reduces the placebo-bias of sham-induced improvements.

The group of participants who completed this trial were relatively comorbid, with 23/40 (57.5%) having a CCI ≥ 4 and 12/40 (30%) taking more than 6 medications per day. This naturally introduces variance between participants, however this is a relative strength of this trial. This population reflect a real-world cohort of predominantly older adults with MCI. Most persons globally with MCI have other health conditions and have high

comorbidity[606,607] and the real-world feasibility of a neuromodulation device in this cohort is important to explore. It is also a relative strength of this study that of 120 potential participant visits (n=40, 3 study visits) there were 118 completed study visits by close of recruitment. This demonstrates older participants' generosity with their time and engagement with scientific research.

Another relative strength of this study is that there was no age limit to recruitment, and so participants up to the age of 85 participated in this study. Arbitrary upper age cut offs that preclude older adults from participating in research are ageist[484] and result in results from scientific trials that are challenging to generalise to an older population.[608] It is important to include multimorbid older adults in trials, both to accurately represent the clinical population that the investigated medicine or device is likely to be used in, but also ethically it is crucial to include older adults in medical research.

There were different modalities of technology utilised in this study, from the tVNS NEMOS © device itself, to the PortaLite NIRS measurement device, there was both an electronic tablet and a standard laptop computer used to undertake most of the cognitive assessments. Despite the older age, relative comorbidity and pre-existing diagnosis of amnestic MCI of this population, most were able to use most subtasks of the tVNS device independently (as measured via the KT) and most completed all electronic tablet and computer-based cognitive tests without any issue. This highlights what has previously been noted in empirical research; that older adults are capable of "digital self-management[488]"and are able to use many different forms of technology, thus shouldn't be excluded from scientific advancements.

Limitations

There are some limitations to this research it is important to acknowledge. Firstly, this study only investigates the acute effects of approximately 60 minutes tVNS stimulation. As with any neuromodulatory device or intervention, it is crucial to establish whether effects if any, are lasting, and it is of critical importance to establish whether a device is tolerable and usable in a domiciliary home environment. Future studies should aim to trial this device in a similar population at home for several weeks, with planned interim and final analysis. Such a trial design may benefit from remote monitoring to assist with device placement and compliance such as via video conferencing software.

The population studied were 100% white Irish, and this is an obvious limitation to this study as recent research shows that not only are people from ethnic minorities more likely to have incipient cognitive impairment, they are less likely to receive a timely diagnosis and they are routinely excluded from clinical trials in this area. [609–611] Specific quotas for recruitment of a certain percentage of minority ethnic groups may be useful in future to try improve the numbers of poorly represented populations in clinical trial research.

Another limitation is that this trial is entirely quantitative, and some research shows that mixed methods involving some qualitative approaches, for example a focus group, with thematic analysis of interviews would be useful to establish the acceptability of the device. [612,613] This may also provide future direction for how to improve both study design but also the device itself. This could also be used to design participant information literature,

especially for a future trial, if participants are taking the device home for several weeks, a pamphlet or booklet that they could refence for how to troubleshoot and use the device may be useful.

One other limitation of this study is that baseline clinical information such as Medial Temporal Atrophy (MTA) grade scores from baseline screening CT or MRI scans were not available for all patients. This would have been useful as a covariate, as it is an accurate marker of hippocampal atrophy, and any effects of tVNS in cognitive outcomes are likely to rely on intact neural tissue (i.e. less atrophy) to be efficacious. It has been reported that less than half of all patients attending a memory assessment service in the UK get any cranial imaging as part of their diagnostic treatment pathway [614] and though rates are likely higher in Ireland with dedicated Regional Specialist Memory Centre (RSMC) services being established[615], the availability of dedicated neuroradiology staff to quantify specific atrophy scoring systems is highly variable geographically and this can affect the baseline characteristic data available when undertaking empirical research.

A final limitation is the lack of a clear biomarker of target engagement of the VN. As detailed in Chapter 2, there is a relative paucity of large, good quality research clearly indicating the precise anatomical locations of the ABVN, and given it is a relatively small nerve, there is likely a degree of inter-individual variability in cutaneous distribution, degree of myelination, specific ganglia its cell body might sit in and whether stimulating it specifically is activating central VN structures. Several biomarkers of tVNS activity have been proposed, including pupillometry [616,617], salivary amylase [185] and the p300 potential EEG signal.[181,237] Each of these biomarkers have their own strengths and weaknesses, and require local expertise,

knowledge and skill to use reliably. If we were to run this study again, I would like to include the measure of salivary amylase as a marker of central noradrenergic activity, as a determinant of central VN activity would be very useful to clearly define the biological effects of auricular neuromodulation, and prove target engagement of stimulating the VN.

Future Directions

A dedicated, and adequately powered clinical trial of tVNS in AD-MCI investigating cognitive outcomes as its primary outcome measure, whereby trial participants would use an auricular tVNS nerve stimulation device at home for a number of weeks would be the next step in thoroughly investigating this neuromodulatory device. Power calculations for a mixed effect model, accounting for fixed and random effects should be used, utilising the resource of a biostatistician to ensure that all variance and covariate analysis has been accounted for.

Given the fascinating results also seen for rTMS in MCI and early AD and with tDCS for later stage cognitive impairment due to AD (see Chapter 3), in future a comparison of various modes of non-invasive brain stimulation in discrete patients groups would be useful, as currently each have demonstrated a degree of promise in this area. The logistical challenges in attempting a direct head-to-head study of different neuromodulation techniques may be arduous but worthwhile, as this is an area that is likely to expand in years to come and patients and their families deserve to have thorough, scientifically-valid research underpinning decisions to undertake specific therapies.

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Supplementary Appendix 1

Tallaght University Hospital
Ospidéal Ollscoile Thamhlachta
An Academic Partner of Trinity College Dublin

Age Related Memory Service

PTID:_____

Date:_____

Time:_____

Assessment of Tolerability of tVNS (active or sham)

Please rate the following on a scale of 1 to 5, 1=no pain, no discomfort 5 = very uncomfortable, intolerable

1. **Are you experiencing any pain?**

   1= no pain 2 = slight pain 3= moderate pain 4= heavy pain 5= severe intolerable pain

2. **Are you experiencing any burning?**

   1= no burning 2 = slight burning 3= moderate burning 4= heavy burning 5= severe intolerable burning sensation

3. **Are you experiencing any tingling sensation?**

   1= no tingling sensation 2 = slight tingling sensation 3= moderate tingling sensation 4= heavy tingling sensation 5= severe intolerable tingling sensation

4. **Are you experiencing any headache?**

   1= no headache 2 = slight headache 3= moderate headache 4= heavy headache 5= severe headache

5. **Are you experiencing any tinnitus or ringing in the ear?**

   1= no tinnitus 2 = slight tinnitus 3= moderate tinnitus 4= heavy tinnitus 5= severe tinnitus

Any other symptoms:

_____

_____

PTID:_____

Date:_____

Time:_____

Assessment of Usability of tVNS NEMOS device (active or sham)

Please rate the following on a scale of 1 to 5

1. **Was the device comfortable?**

   1= very comfortable 2 = comfortable 3= neutral 4= moderately uncomfortable 5= severe uncomfortable

2. **Did you feel confident using the device?**

   1= very confident 2 = mostly confident 3= neutral 4= not confident 5= lost, would not know what to do with it

3. **Would you use it again?**

   1= yes without hesitation 2 = probably 3= maybe 4= probably not 5= definitely not

Any other symptoms:

_____

_____

Supplementary Appendix 2

	Mean (SD) or median (IQR) of test results			*Mixed effect model - unadjusted*		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active Stim	Baseline v Sham Stim	Active Stim v Sham Stim
Systolic Blood Pressure (SBP) ABSOLUTE VALUES						
SBP pre-stand mmHg	152.65 (IQR 139.98 – 162.11)	146.64 (IQR 131.18 – 159.22)	147.73 (IQR 130.59 – 162.29)	β= -1.06 95% CI (-13.3 – 11.16) z= -0.17 p= 0.86	β= -3.76 95% CI (-16.1 – 8.54) z= -0.6 p= 0.54	β= 1.34 95% CI (-4.89 – 7.56) z= 0.42 p= 0.67
SBP 10 sec (absolute) in mmHg	127.99 (IQR 114.15 – 145.77)	122.12 (IQR 110.78 – 134.67)	126.52 (IQR 109.884 – 136.81)	β= -6.57 95% CI (-17.73 – 4.58) z= -1.15 p= 0.24	β= -8.19 95% CI (-19.43 – 3.04) z= -1.43 p= 0.15	β= 0.77 95% CI (-4.96 – 6.54) z= 0.26 p= 0.79
SBP 20 sec (absolute) in mmHg	130.33 (IQR 111.17 – 151.86)	128.03 (IQR 115.63 – 143.62)	127.72 (IQR 100.12 – 146.16)	β= -3.01 95% CI (-15.02 – 9.01) z= -0.49 p= 0.62	β= -5.98 95% CI (-18.06 – 6.12) z= -0.97 p= 0.33	β= 1.46 95% CI (-4.68 – 7.06) z= 0.47 p= 0.64
SBP 30 sec (absolute) in mmHg	132.37 (IQR 114.99 – 153.57)	130.31 (IQR 118.41 – 147.53)	128.64 (IQR 107.92 0 146.52)	β= -0.97 95% CI (-13.46 – 11.51) z= -0.15 p= 0.87	β= -3.26 95% CI (-15.84 – 9.31) z= -0.51 p= 0.61	β= 1.13 95% CI (-5.23 – 7.51) z= 0.35 p= 0.72
SBP 40 sec (absolute) in mmHg	134.07 (IQR 116.28 – 155.25)	129.55 (IQR 110.67 – 153.77)	132.91 (IQR 107.07 – 146.51)	β= -2.44 95% CI (-15.71 – 10.29) z= -0.38 p= 0.71	β= -3.15 95% CI (-15.97 – 9.66) z= -0.48 p= 0.62	β= 0.34 95% CI (-6.15 – 6.81) z= 0.1 p= 0.91
SBP 60 sec (absolute) in mmHg	136.09 (IQR 118.04 – 158.1)	131.62 (IQR 109.56 – 149.62)	134.21(IQR 115.32 – 146.51)	β= -8.54 95% CI (-21.32 0 3.92) z= -1.35 p= 0.17	β= -0.64 95% CI (-7.65 – 5.65) z= -0.2 p= 0.84	β= -7.34 95% CI (-19.99 – 5.34) z= -1.15 p= 0.25

SBP 120 sec (absolute) in mmHg	135.35 (IQR 125.3 – 161.79(	134.72 (IQR 111.54 – 152.07)	137.32 (IQR 115.32 – 152.35)	β= -1.01 95% CI (-13.45 – 11.91) z= -0.15 p= 0.87	β= -0.54 95% CI (-13.43 – 12.46) z= -0.08 p= 0.93	β= -0.23 95% CI (-6.7 – 6.22) z= -0.07 p= 0.94
SBP steady state (120-180 sec) (absolute) in mmHg	136.47 (IQR 125.02 – 160.62)	132.83 (IQR 112.98 – 147.37)	135.83 (IQR 115.32 – 154.52)	β= -6.14 95% CI (-18.51 – 6.23) z= -0.97 p= 0.33	β= -5.43 95% CI (-17.93 – 7.01) z= -0.86 p= 0.39	β= -0.35 95% CI (-6.66 – 5.94) z= -0.11 p= 0.91
SBP nadir in mmHg (absolute)	117.26 (IQR 94.37 – 135.99)	110.99 (IQR 102.89 – 124.47)	118.72 (IQR 92.81 – 130.94)	β= -3.01 95% CI (-14.78 – 8.75) z= -0.5 p= 0.61	β= -2.66 95% CI (-14.42 – 9.11) z= -0.44 p= 0.65	β= -0.2 95% CI (-6.19 – 5.82) z= -0.06 p= 0.92
SBP recovery rate (seconds)	1.18 (IQR 0.81-2.15)	1.27 (IQR 0.66 – 1.87)	0.97 (IQR 0.58 – 1.32)	β= -0.15 95% CI (-0.56 – 0.25) z= -0.73 p= 0.46	β= -0.26 95% CI (-0.67 – 0.14) z= -1.27 p= 0.21	β= 0.06 95% CI (-0.15 – 0.26) z= 0.53 p= 0.59
SBP overshoot (absolute) in mmHg	145.97 (IQR 129.97 – 168.57)	142.12 (IQR 121.18 – 156.76)	142.36 (IQR 117.57 – 158.33)	β= -6.73 95% CI (-19.79 – 6.33) z= -1.01 p= 0.31	β= -7.11 95% CI (-20.18 – 5.94) z= -1.07 p= 0.28	β= 0.19 95% CI (-6.47 – 6.56) z= 0.06 p= 0.95
SBP time to nadir (seconds)	11.36 (IQR 9.36 – 14.35)	10.42 (IQR 9.19 – 12.73)	10.67 (IQR 8.55 – 13.9)	β= -2.54 95% CI (-4.65 - -0.41) z= -2.34 p= 0.05	β= -1.54 95% CI (-3.68 – 0.55) z= -1.45 p= 0.15	β= -0.48 95% CI (-1.85 – 0.89) z= -0.69 p= 0.49

Supplementary Table 1: SBP values (absolute) under baseline, active and sham stimulation conditions, analysed via an unadjusted mixed effects model

	Mean (SD) or median (IQR) of test results			*Mixed effects model adjusted for baseline HTN, age, sex, antihypertensive Rx, antidepressant Rx*		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active Stim	Baseline v Sham Stim	Active Stim v Sham Stim
Systolic Blood Pressure (SBP) ABSOLUTE VALUES						
SBP pre-stand mmHg	152.65 (IQR 139.98 – 162.11)	146.64 (IQR 131.18 – 159.22)	147.73 (IQR 130.59 – 162.29)	β= -5.68 95% CI (-14.56 – 3.35) z= -1.23 p= 0.22	β= -7.12 95% CI (-16.17 – 1.93) z= -1.54 p= 0.12	β= 0.71 95% CI (-3.92 – 5.35) z= 0.3 p= 0.76
SBP 10 sec (absolute) in mmHg	127.99 (IQR 114.15 – 145.77)	122.12 (IQR 110.78 – 134.67)	126.52 (IQR 109.884 – 136.81)	β= -9.56 95% CI (-19.35 – 0.78) z= -1.88 p= 0.08	β= -11.36 95% CI (-21.34 – 1.45) z= -2.25 p= 0.07	β= 0.94 95% CI (-5.95 – 7.84) z= 0.27 p= 0.78
SBP 20 sec (absolute) in mmHg	130.33 (IQR 111.17 – 151.86)	128.03 (IQR 115.63 – 143.62)	127.72 (IQR 100.12 – 146.16)	β= -5.54 95% CI (-9.12 – 1.21) z= -1.06 p= 0.29	β= -9.12 95% CI (-19.46 – 1.21) z= -1.73 p= 0.09	β= 1.75 95% CI (-3.54 – 7.04) z= 0.65 p= 0.52
SBP 30 sec (absolute) in mmHg	132.37 (IQR 114.99 – 153.57)	130.31 (IQR 118.41 – 147.53)	128.64 (IQR 107.92 0 146.52)	β= -4.41 95% CI (-14.73 – 5.93) z= -0.85 p= 0.41	β= -6.63 95% CI (-16.99 – 3.71) z= -1.26 p= 0.21	β= 1.09 95% CI (-4.17 – 6.37) z= 0.41 p= 0.68
SBP 40 sec (absolute) in mmHg	134.07 (IQR 116.28 – 155.25)	129.55 (IQR 110.67 – 153.77)	132.91 (IQR 107.07 – 146.51)	β= -6.25 95% CI (-16.71 – 4.21) z= -1.17 p= 0.24	β= 0.09 95% CI (-5.26 – 5.44) z= 0.03 p= 0.97	β= -6.45 95% CI (-16.34 – 4.03) z= -1.21 p= 0.22
SBP 60 sec (absolute) in mmHg	136.09 (IQR 118.04 – 158.1)	131.62 (IQR 109.56 – 149.62)	134.21(IQR 115.32 – 146.51)	β= -9.83 95% CI (-20.89 – 1.21) z= -1.75 p= 0.08	β= -0.67 95% CI (-6.81 – 5.54) z= -0.22 p= 0.82	β= -8.54 95% CI (-19.63 – 2.55) z= -1.51 p= 0.13

SBP 120 sec (absolute) in mmHg	135.35 (IQR 125.3 – 161.79(	134.72 (IQR 111.54 – 152.07)	137.32 (IQR 115.32 – 152.35)	β= -3.81 95% CI -13.93 – 6.45) z= -0.73 p= 0.46	β= -1.31 95% CI (-11.53 – 8.92) z= -0.25 p= 0.81	β= -1.23 95% CI (-6.34 – 3.86) z= -0.48 p= 0.63
SBP steady state (120-180 sec) (absolute) in mmHg	136.47 (IQR 125.02 – 160.62)	132.83 (IQR 112.98 – 147.37)	135.83 (IQR 115.32 – 154.52)	β= -7.65 95% CI (-18.01 – 2.75) z= -1.44 p= 0.15	β= -6.22 95% CI (-16.65 – 4.19) z= -1.17 p= 0.24	β= -0.78 95% CI (-6.04 – 4.61) z= -0.26 p= 0.79
SBP nadir in mmHg (absolute)	117.26 (IQR 94.37 – 135.99)	110.99 (IQR 102.89 – 124.47)	118.72 (IQR 92.81 – 130.94)	β= -7.65 95% CI (-17.56 – 2.31) z= -1.5 p= 0.13	β= -6.44 95% CI (-16.32 – 3.43) z= -1.28 p= 0.21	β= -0.56 95% CI (-5.63 – 4.55) z= -0.21 p= 0.83
SBP recovery rate (seconds)	1.18 (IQR 0.81-2.15)	1.27 (IQR 0.66 – 1.87)	0.97 (IQR 0.58 – 1.32)	β= -0.19 95% CI (-0.59 – 0.21) z= -0.95 p= 0.34	β= -0.26 95% CI (-0.66 – 0.13) z= -1.32 p= 0.18	β= 0.04 95% CI (-0.16 – 0.24) z= 0.36 p= 0.72
SBP overshoot (absolute) in mmHg	145.97 (IQR 129.97 – 168.57)	142.12 (IQR 121.18 – 156.76)	142.36 (IQR 117.57 – 158.33)	β= -6.56 95% CI (-13.54 – 6.54) z= -2.1 p= 0.14	β= -3.56 95% CI (-7.54- 5.43) z= -1.74 p= 0.19	β= -0.97 95% CI (-7.92 – 6.01) z= -0.27 p= 0.78
SBP time to nadir (seconds)	11.36 (IQR 9.36 – 14.35)	10.42 (IQR 9.19 – 12.73)	10.67 (IQR 8.55 – 13.9)	β= -1.3 95% CI (-3.36 – 0.28) z= -2.23 p= 0.09	β= -1.1 95% CI (-3.33 – 0.72) z= -1.26 p= 0.21	β= -0.51 95% CI (-1.72 – 0.71) z= -0.82 p= 0.41

Supplementary Table 2: SBP values (absolute) under baseline, active and sham stimulation conditions, analysed via a mixed effects model, adjusted for baseline covariates

	Mean (SD) or median (IQR) of test results			*Mixed effects model - unadjusted*		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Diastolic Blood Pressure (DBP) ABSOLUTE VALUES						
DBP pre-stand mmHg	80.42 (IQR 72.65 – 96.87)	78.93 (IQR 72.32 – 88.53)	82.84 (IQR 71.82 – 89.87)	β= -3.73 95% CI (-11.23 – 3.82) z= -0.97 p= 0.33	β= -1.97 95% CI (-9.56 – 5.61) z= -0.51 p= 0.61	β= -0.89 95% CI z= p=
DBP 10 sec (absolute) in mmHg	72.33 (IQR 63.41 –83.53)	71.81 (IQR 63.41 – 77.53)	68.66 (IQR 61.91 – 77.49)	β= -2.74 95% CI (-9.57 – 4.04) z= -0.79 p= 0.43	β= -1.88 95% CI (-8.75 – 4.98) z= -0.54 p= 0.59	β= -0.44 95% CI (-3.92 – 3.04) z= -0.25 p= 0.81
DBP 20 sec (absolute) in mmHg	74.52 (IQR 62.27 – 91.61)	74.13 (IQR 59.67 – 84.87)	71.98 (IQR 61.71 – 85.12)	β= -1.84 95% CI (-9.37 – 5.64) z= -0.48 p= 0.63	β= -1.01 95% CI (-8.54 – 6.52) z= -0.26 p= 0.79	β= -0.42 95% CI (-4.29 – 3.42) z= -0.21 p= 0.83
DBP 30 sec (absolute) in mmHg	75.54 (IQR 67.53 – 92.36)	74.79 (IQR 63.31 – 90.39)	74.94 (IQR 64.39 – 87.57)	β= -1.45 95% CI (-9.15 – 6.29) z= -0.37 p= 0.71	β= 0.31 95% CI (-7.43 – 8.06) z= 0.08 p= 0.93	β= -0.89 95% CI (-4.81 – 3.03) z= -0.44 p= 0.65
DBP 40 sec (absolute) in mmHg	75.44 (IQR 68.31 – 94.68)	75.06 (IQR 66.01 – 88.21)	76.68 (IQR 66.48 – 89.66)	β= -2.01 95% CI (-9.71 – 5.73) z= -0.51 p= 0.61	β= 0.34 95% CI (-7.44 – 8.12) z= 0.09 p= 0.93	β= -1.17 95% CI (-5.12 – 2.76) z= -0.58 p= 0.56
DBP 60 sec (absolute) in mmHg	80.48 (IQR 69.89 – 92.76)	73.69 (IQR 64.86 – 82.69)	77.65 (IQR 67.06 – 87.65)	β= -5.41 95% CI (-13.09 – 2.28) z= -1.38 p= 0.16	β= -1.95 95% CI (-9.69 – 5.74) z= -0.49 p= 0.62	β= -1.74 95% CI (-5.65 – 2.17) z= -0.87 p= 0.38

DBP 120 sec (absolute) in mmHg	79.73 (IQR 70.4 – 88.57)	73.96 (IQR 66.01 – 84.26)	79.78 (IQR 68.84 – 88.35)	β= -2.49 95% CI (-10.56 – 5.54) z= -0.61 p= 0.54	β= 1.45 95% CI (-6.54 – 9.57) z= 0.36 p= 0.71	β= -1.99 95% CI (-6.01 – 2.02) z= -0.97 p= 0.33
DBP steady state (120-180) (absolute) in mmHg	82.41 (IQR 68.83 – 93.59)	74.39 (IQR 66.01 – 82.49)	79.39 (IQR 68.67 - 88.41)	β= -3.76 95% CI (-11.43 – 3.91) z= -0.96 p= 0.33	β= -0.32 95% CI (-8.05 – 7.39) z= -0.08 p= 0.93	β= -1.72 95% CI (-5.61 – 2.16) z= -0.87 p= 0.38
DBP nadir in mmHg (absolute)	63.05 (IQR 53.45 – 71.19)	61.9 (IQR 54.65 – 72.45)	66.5 (IQR 58.43 – 73.34)	β= -2.61 95% CI (-10.06 – 4.85) z= -0.68 p= 0.49	β= -0.61 95% CI (-8.07 – 6.85) z= -0.16 p= 0.87	β= -0.99 95% CI (-4.81 – 2.89) z= -0.51 p= 0.61
DBP recovery rate (seconds)	0.63 (IQR 0.46 – 1.24)	0.57 (IQR 0.37 – 1.07)	0.63 (IQR 0.42 – 1.01)	β= -0.07 95% CI (-0.35 – 0.19) z= -0.57 p= 0.57	β= -0.11 95% CI (-0.39 – 0.15) z= -0.86 p= 0.39	β= 0.02 95% CI (-0.11 – 0.16) z= 0.29 p= 0.77
DBP overshoot (absolute) in mmHg	82.4 (IQR 74.52 – 97.52)	80.52 (IQR 71.43 – 93.83)	81.01 (IQR 71.87 – 93.42)	β= -4.56 95% CI (-12.85 – 3.73) z= -1.08 p= 0.28	β= -1.65 95% CI (-9.95 – 6.63) z= -0.39 p= 0.69	β= -1.45 95% CI (-5.67 – 2.77) z= -0.67 p= 0.51
DBP time to nadir (seconds)	11.36 (IQR 9.36 – 14.35)	10.42 (IQR 9.19 – 12.73)	10.67 (IQR 8.55 – 13.9)	β= -2.54 95% CI (-4.64 – 0.41 z= -1.35 p= 0.21	β= -1.56 95% CI (-3.63 – 0.55) z= -1.45 p= 0.15	β= -0.48 95% CI (-1.85 – 0.89) z= -0.69 p= 0.49

Supplementary Table 3: DBP values (absolute) under baseline, active and sham stimulation conditions, analysed via an unadjusted mixed effects model

	Mean (SD) or median (IQR) of test results			*Mixed effects model adjusted for baseline HTN, age, sex, antihypertensive Rx, antidepressant Rx*		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Diastolic Blood Pressure (DBP) (ABSOLUTE VALUES)						
DBP pre-stand mmHg	80.42 (IQR 72.65 – 96.87)	78.93 (IQR 72.32 – 88.53)	82.84 (IQR 71.82 – 89.87)	β= -5.05 95% CI (-11.19 – 1.08) z= -1.61 p= 0.11	β= -4.37 95% CI (-10.51 – 1.81) z= -1.39 p= 0.16	β= -0.35 95% CI (-3.51 – 2.81) z= -0.22 p= 0.84
DBP 10 sec (absolute) in mmHg	72.33 (IQR 63.41 –83.53)	71.81 (IQR 63.41 – 77.53)	68.66 (IQR 61.91 – 77.49)	β= -4.09 95% CI (-10.1 – 1.91) z= -1.34 p= 0.18	β= -4.16 95% CI (-10.2 – 1.82) z= -1.35 p= 0.17	β= 0.01 95% CI (-3.06 – 3.1) z= 0.01 p= 0.99
DBP 20 sec (absolute) in mmHg	74.52 (IQR 62.27 – 91.61)	74.13 (IQR 59.67 – 84.87)	71.98 (IQR 61.71 – 85.12)	β= -3.44 95% CI (-9.61 – 2.72) z= -1.09 p= 0.27	β= -3.67 95% CI (-9.87 – 2.54) z= -1.16 p= 0.34	β= 0.11 95% CI (-3.05 – 3.54) z= 0.06 p= 0.91
DBP 30 sec (absolute) in mmHg	75.54 (IQR 67.53 – 92.36)	74.79 (IQR 63.31 – 90.39)	74.94 (IQR 64.39 – 87.57)	β= -3.23 95% CI (-9.51 – 3.03) z= -1.01 p= 0.31	β= -2.45 95% CI (-8.76 – 3.83) z= -0.76 p= 0.44	β= -0.39 95% CI (-3.65 – 2.81) z= -0.25 p= 0.81
DBP 40 sec (absolute) in mmHg	75.44 (IQR 68.31 – 94.68)	75.06 (IQR 66.01 – 88.21)	76.68 (IQR 66.48 – 89.66)	β= -3.78 95% CI (-10.11 – 2.54) z= -1.17 p= 0.24	β= -2.19 95% CI (-8.55 – 4.15) z= -0.68 p= 0.49	β= -0.81 95% CI (-4.05 – 2.42) z= -0.49 p= 0.62

DBP 60 sec (absolute) in mmHg	80.48 (IQR 69.89 – 92.76)	73.69 (IQR 64.86 – 82.69)	77.65 (IQR 67.06 – 87.65)	β= -5.92 95% CI (-12.45-0.69) z= -1.81 p= 0.09	β= -3.23 95% CI (-9.75 – 3.17) z= -1.00 p= 0.32	β= -1.34 95% CI (-4.65 – 1.96) z= -0.8 p= 0.42
DBP 120 sec (absolute) in mmHg	79.73 (IQR 70.4 – 88.57)	73.96 (IQR 66.01 – 84.26)	79.78 (IQR 68.84 – 88.35)	β= -3.45 95% CI (-10.34 – 3.42) z= -0.98 p= 0.32	β= 0.09 95% CI (-6.81 – 6.99) z= 0.03 p= 0.97	β= -1.77 95% CI (-5.22 – 1.67) z= -1.01 p= 0.31
DBP steady state (120-180) (absolute) in mmHg	82.41 (IQR 68.83 – 93.59)	74.39 (IQR 66.01 – 82.49)	79.39 (IQR 68.67 - 88.41)	β= -4.28 95% CI (-10.73 – 2.16) z= -1.3 p= 0.19	β= -1.55 95% CI (-8.03 – 4.92) z= -0.47 p= 0.63	β= -1.38 95% CI (-4.67 – 1.92) z= -0.82 p= 0.41
DBP nadir in mmHg (absolute)	63.05 (IQR 53.45 – 71.19)	61.9 (IQR 54.65 – 72.45)	66.5 (IQR 58.43 – 73.34)	β= -5.81 95% CI (-12.32 – 0.33) z= -1.85 p= 0.07	β= -3.63 95% CI (-9.83 – 2.52) z= -1.17 p= 0.24	β= -1.08 95% CI (-4.31 – 2.13) z= -0.66 p= 0.51
DBP recovery rate (seconds)	0.63 (IQR 0.46 – 1.24)	0.57 (IQR 0.37 – 1.07)	0.63 (IQR 0.42 – 1.01)	β= -0.08 95% CI (-0.35 – 0.18) z= -0.62 p=0.52	β= -0.14 95% CI (-0.41 – 0.12) z= -1.01 p= 0.31	β= 0.02 95% CI (-0.11 – 0.16) z= 0.38 p= 0.71
DBP overshoot (absolute) in mmHg	82.4 (IQR 74.52 – 97.52)	80.52 (IQR 71.43 – 93.83)	81.01 (IQR 71.87 – 93.42)	β= -7.62 95% CI (-14.28 – 0.94) z= -2.1 p= 0.13	β= -4.14 95% CI (-10.78 – 2.49) z= -1.22 p= 0.22	β= -1.71 95% CI (-5.62 – 2.21) z= -0.86 p= 0.39
DBP time to nadir (seconds)	11.36 (IQR 9.36 – 14.35)	10.42 (IQR 9.19 – 12.73)	10.67 (IQR 8.55 – 13.9)	β= -1.54 95% CI (-3.54- 0.05) z= -1.12 p= 0.34	β= -1.45 95% CI (-3.33 – 0.89) z= -0.45 p= 0.29	β= -0.49 95% CI (-1.72 – 0.72) z= -0.81 p= 0.42

Supplementary Table 4: DBP values (absolute) under baseline, active and sham stimulation conditions, analysed via a mixed effects model, adjusted for baseline covariates

	Mean (SD) or median (IQR) of test results			*Mixed effects model – unadjusted*		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Heart Rate (ABSOLUTE VALUES)						
HR pre-stand in bpm	69.89 (IQR 60.82 – 79.92)	70.29 (IQR 58.06 – 75.86)	69.08 (IQR 62.28 – 78.29)	β= -1.32 95% CI (-7.02 – 4.32) z= -0.45 p= 0.65	β= -1.11 95% CI (-6.85 – 4.62) z= -0.38 p= 0.7	β= -0.11 95% CI (-3.01 – 2.8) z= -0.07 p= 0.94
HR 10 sec (absolute) in bpm	75.27 (IQR 67.1 – 83.64)	76.05 (IQR 64.94 – 80.97)	77.19 (IQR 65.72 – 85.1)	β= -1.27 95% CI (-7.01 – 4.46) z= -0.44 p= 0.66	β= -1.35 95% CI (-7.13 – 4.43) z= -0.46 p= 0.64	β= 0.03 95% CI (-2.89 – 2.96) z= 0.02 p= 0.98
HR 20 sec (absolute) in bpm	74.22 (IQR 67.47 – 83.03)	76.79 (IQR 62.02 - 81.67)	77.07 (IQR 65.08 – 85.32)	β= -0.42 95% CI (-6.13 – 5.3) z= -0.14 p= 0.88	β= 0.59 95% CI (-5.17 – 6.35) z= 0.2 p= 0.84	β= -0.51 95% CI (-3.42 – 2.41) z= -0.34 p= 0.73
HR 30 sec (absolute) in bpm	75.09 (IQR 67.29 – 84.06)	76.75 (IQR 66.23 – 83.97)	77.8 (IQR 66.34 – 84.44)	β= -1.39 95% CI (-7.35 – 4.55) z= -0.46 p= 0.64	β= 1.39 95% CI (-4.59 – 7.39) z= 0.46 p= 0.65	β= -1.39 95% CI (-4.43 – 1.64) z= -0.9 p= 0.36
HR 40 sec (absolute) in bpm	75.77 (IQR 68.09 – 83.96)	77.65 (IQR 64.79 – 82.67)	77.58 (IQR 67.22 – 85.74)	β= -1.78 95% CI (-7.71 – 4.15) z= -0.59 p= 0.55	β= 0.89 95% CI (-5.07 – 6.86) z= 0.29 p= 0.76	β= -1.34 95% CI (4.34 – 1.66) z= -0.87 p= 0.38
HR 60 sec (absolute) in bpm	77.99 (IQR 69.31 – 86.86)	76.88 (IQR 65.92 – 83.54)	77.43 (IQR64.78 – 87.07)	β= -1.27 95% CI (-7.19 – 4.64) z= -0.42 p= 0.67	β= -0.07 95% CI (-6.02 – 5.89) z= -0.02 p= 0.98	β= -0.61 95% CI (-3.61 – 2.39) z= -0.4 p= 0.69

HR 120 sec (absolute) in bpm	75.41 (IQR 67.67 – 84.88)	74.96 (IQR 65.77 – 84.07)	76.74 (IQR 65.72 – 85.21)	β= -1.51 95% CI (-7.73 – 4.71) z= -0.48 p= 0.63	β= 0.09 95% CI (-6.16 – 6.35) z= 0.03 p= 0.97	β= -0.81 95% CI (-3.91 – 2.3) z= -0.51 p= 0.61
HR steady state (120-180) (absolute) in bpm	77.99 (IQR 67.14 – 84.5)	76.62 (IQR 66.11 – 83.52)	77.09 (IQR 64.93 – 85.11)	β= -1.68 95% CI (-7.62 – 4.23) z= -0.56 p= 0.57	β= -0.55 95% CI (-6.51 – 5.41) z= -0.18 p= 0.85	β= -0.57 95% CI (-3.51 – 2.42) z= -0.37 p= 0.71
HR min (absolute) in bpm	74.14 (IQR 65.2 – 83.1)	74.11 (IQR 59.07 – 84.02)	76.19 (IQR 65.55 – 83. 71)	β= -1.51 95% CI (-7.76 – 4.72) z= -0.48 p= 0.63	β= 2.27 95% CI (-4.01 – 8.55) z= 0.71 p= 0.47	β= -1.89 95% CI (-5.07 – 1.28) z= -1.17 p= 0.24
HR max (absolute) in bpm	76.26 (IQR 70.67 – 87.25)	80.99 (IQR 71.67 – 85.25)	80.99 (IQR 70.76 – 89.74)	β= -0.01 95% CI (-5.68 – 5.67) z= -0.01 p= 0.99	β= 0.51 95% CI (-5.21 – 6.23) z= 0.17 p= 0.86	β= -0.26 95% CI (-3.15 – 2.62) z= -0.17 p= 0.86
HR recovery rate (seconds)	-0.39 (IQR -0.66 to -0.15)	-0.36 (IQR -0.64 to -0.19)	-0.26 (IQR -0.43 to -0.14)	β= -0.14 95% CI (-0.4 – 0.18) z=-1.01 p= 0.31	β=0.06 95% CI (-0.24 – 0.36) z= 0.56 p= 0.57	β= -0.11 95% CI (-0.24 – 0.07) z= -1.51 p= 0.13
Time between min and max HR (seconds)	9.92 SD 6.65	11.72 SD 7.55	9.77 SD 8.52	β=1.6 95% CI (-1.58 – 4.9) z= 0.93 p= 0.35	β= -1.56 95% CI (-4.97 – 1.86) z=-0.9 p= 0.36	β= 1.58 95% CI (-0.14 – 3.31) z= 1.81 p=0.1

Supplementary Table 5: HR values (absolute) under baseline, active and sham stimulation conditions, analysed via an unadjusted mixed effects model

	Mean (SD) or median (IQR) of test results			*Mixed effects model adjusted for age, sex, baseline HR, polypharmacy, beta-blocker Rx*		
	Baseline (no stim)	Active stim	Sham stim	Baseline v Active	Baseline v Sham	Active v Sham
Heart Rate (ABSOLUTE VALUES)						
HR pre-stand in bpm	69.89 (IQR 60.82 – 79.92)	70.29 (IQR 58.06 – 75.86)	69.08 (IQR 62.28 – 78.29)	β= -1.18 95% CI (-6.42 – 4.05) z= -0.44 p= 0.65	β= -1.68 95% CI (-6.93 – 3.51) z= -0.63 p= 0.53	β= 0.24 95% CI (-2.41 – 2.91) z= 0.18 p= 0.85
HR 10 sec (absolute) in bpm	75.27 (IQR 67.1 – 83.64)	76.05 (IQR 64.94 – 80.97)	77.19 (IQR 65.72 – 85.1)	β= -1.17 95% CI (-6.63 – 4.27) z= -0.42 p= 0.67	β= -2.05 95% CI (-7.52 – 3.42) z= -0.73 p= 0.46	β= 0.43 95% CI (-2.34 – 3.21) z= 0.3 p= 0.76
HR 20 sec (absolute) in bpm	74.22 (IQR 67.47 – 83.03)	76.79 (IQR 62.02 - 81.67)	77.07 (IQR 65.08 – 85.32)	β= -0.72 95% CI (-6.01 – 4.57) z= -0.27 p= 0.79	β= -0.34 95% CI (-5.66 – 4.96) z= -0.13 p= 0.89	β= -0.18 95% CI (-2.87 – 2.51) z= -0.14 p= 0.89
HR 30 sec (absolute) in bpm	75.09 (IQR 67.29 – 84.06)	76.75 (IQR 66.23 – 83.97)	77.8 (IQR 66.34 – 84.44)	β= -1.69 95% CI (-7.23 – 3.84) z= -0.6 p= 0.54	β= 0.53 95% CI (-5.02 – 6.09) z= 0.19 p= 0.85	β= -1.12 95% CI (-3.93 – 1.69) z= -0.78 p= 0.43
HR 40 sec (absolute) in bpm	75.77 (IQR 68.09 – 83.96)	77.65 (IQR 64.79 – 82.67)	77.58 (IQR 67.22 – 85.74)	β= -1.66 95% CI (-7.24 – 3.91) z= -0.58 p= 0.56	β= 0.53 95% CI (-5.09 – 6.13) z= 0.19 p= 0.85	β= -1.09 95% CI (-3.93 – 1.73) z= -0.76 p= 0.44
HR 60 sec (absolute) in bpm	77.99 (IQR 69.31 – 86.86)	76.88 (IQR 65.92 – 83.54)	77.43 (IQR64.78 – 87.07)	β= -1.14 95% CI (-6.57 – 4.28) z= -0.41	β= -0.42 95% CI (-5.86 – 5.02) z= -0.15	β= -0.36 95% CI (-3.12 – 2.39) z= -0.26

				p= 0.67	p= 0.88	p= 0.79
HR 120 sec (absolute) in bpm	75.41 (IQR 67.67 – 84.88)	74.96 (IQR 65.77 – 84.07)	76.74 (IQR 65.72 – 85.21)	β= -1.42 95% CI (-7.11 – 4.26) z= -0.49 p= 0.64	β= -0.41 95% CI (-6.12 – 5.29) z= -0.14 p= 0.88	β= -0.51 95% CI (-3.36 – 2.35) z= -0.35 p= 0.73
HR steady state (120-180) (absolute) in bpm	77.99 (IQR 67.14 – 84.5)	76.62 (IQR 66.11 – 83.52)	77.09 (IQR 64.93 – 85.11)	β= -1.54 95% CI (-7.02 – 3.84) z= -0.57 p= 0.56	β= -1.03 95% CI (-6.48 – 4.42) z= -0.37 p= 0.71	β= -0.28 95% CI (-3.04 – 2.48) z= -0.2 p= 0.84
HR min (absolute) in bpm	74.14 (IQR 65.2 – 83.1)	74.11 (IQR 59.07 – 84.02)	76.19 (IQR 65.55 – 83. 71)	β= -1.93 95% CI (-7.72 – 3.82) z= -0.66 p= 0.51	β= 1.31 95% CI (-4.47 – 7.11) z= 0.45 p= 0.65	β= -1.62 95% CI (-4.52 – 1.31) z= -1.09 p= 0.28
HR max (absolute) in bpm	76.26 (IQR 70.67 – 87.25)	80.99 (IQR 71.67 – 85.25)	80.99 (IQR 70.76 – 89.74)	β= 0.14 95% CI (-5.15 – 5.44) z= 0.05 p= 0.96	β= 0.01 95% CI (-5.29 – 5.35) z= 0.01 p= 0.99	β= 0.06 95% CI (-2.62 – 2.75) z= p=
HR recovery rate (seconds)	-0.39 (IQR -0.66 to -0.15)	-0.36 (IQR -0.64 to -0.19)	-0.26 (IQR -0.43 to -0.14)	β= 0.07 95% CI (-0.21- 0.35) z= 0.5 p= 0.61	β= -0.21 95% CI (-0.48 – 0.05) z= -1.58 p= 0.11	β= -0.74 95% CI (-1.28 – 0.01) z= -0.58 p= 0.32
Time between min and max HR (seconds)	9.92 SD 6.65	11.72 SD 7.55	9.77 SD 8.52	β= 1.88 95% CI (-1.48 – 5.24) z= 1.1 p=0.27	β= -1.28 95% CI (-4.65 – 2.09) z= -0.74 p= 0.45	β= 1.52 95% CI (-0.18 – 3.29) z= 1.61 p= 0.12

Supplementary Table 6: HR values (absolute) under baseline, active and sham stimulation conditions, analysed via a mixed effects model, adjusted for baseline covariates