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Towards a patient-tailored approach of transcranial electrical stimulation as a treatment to post-comatose patients with disorders of consciousness

Alice Barra

Under the supervision of

Prof Steven Laureys and Dr Aurore Thibaut

Coma Science Group
GIGA Consciousness
University of Liège

Centre du Cerveau[2]
Centre Hospitalier
Universitaire de Liège

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Promotor:

Prof. Steven Laureys, MD, PhD, Université de Liège

Co-promotor:

Dr Aurore Thibaut, PhD, Université de Liège

Assessment Committee

Dr. Audrey Vanhaudenhuyse, PhD, Université de Liège

Dr. Jitka Annen, PhD, Université de Liège

Prof. Jean-François Kaux, MD, PhD, Université de Liège

Dr. Stephen Bornheim, PhD, Université de Liège

Prof. Riem El Tahry, MD, PhD, Université Catholique de Louvain

Prof. Anna Estraneo, MD, PhD, Scientific Institute for Research and Health Care Florence

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« Questo sentimento dell'origine delle cose è il punto da cui parto per guardare nel paesaggio, sapendo che non esistono risposte definitive, ma continuando a interrogarmi, perché nel gesto di pormi continuamente la domanda è contenuta la risposta »

This feeling of the origin of things is the starting point for me to look into the landscape, knowing there are no definite answers but keeping questioning myself, because the act of continuously asking myself the question contains the answer

Luigi Ghirri

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Scientific Publications

First and co-first author

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- Bodien, Y.G.*, **Barra, A.***, Temkin, N.R., Barber, J., Foreman, B., Vassar, M., Robertson, C., Taylor, S.R., Markowitz, A.J., Manley, G.T. and Giacino, J.T., 2021. Diagnosing level of consciousness: the limits of the Glasgow Coma Scale total score. *Journal of neurotrauma*, *38*(23), pp.3295-3305.

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Summary

In the past 50 years, resuscitation techniques have improved greatly, leading to a substantial increase in severe brain injury survivors. Following a coma resulting from a severe brain injury different scenarios are possible; a patient may die or, on the other hand recover rapidly, while, in some cases, post-coma patients may stay in a state that is called a Disorder of Consciousness (DoC). DoC are a rare disease that involve pathologically altered states of consciousness. They encompass the Unresponsive Wakefulness Syndrome (UWS) that is characterized by recovery of eye opening and presents only reflexive behaviours; the Minimally Conscious State (MCS) in which patients show reproducible signs of conscious awareness and, finally, the Minimally Conscious State STAR (MCS*) which is a category of patients behaviourally considered in UWS but with brain activity more compatible with the MCS. When patients recover functional communication and/or use of objects they are said to have emerged the MCS (eMCS).

Even though in the past years incredible progress has been made in the clinical diagnosis and care of patients with DoC, their communication deficiencies and motor disabilities continues to pose challenges for researchers and healthcare professionals. Undeniably, the rate of misdiagnosis of patients with DoC currently remains high (i.e., 38%) and treatment options are limited, thus haltering the prognosis of these patients. In this context, the research presented in this dissertation aims to solve some of the challenges mentioned above and to facilitate future studies aimed towards patient-tailored therapeutic approaches.

The first challenge pertains to the issues that arise with long experimental protocols, that can result in patients' fatigue, hence hindering the observed effectiveness of treatments in Randomized Controlled Trials (RCT). **Study 1** explores the possibility of replacing the currently most used behavioural scale (i.e., Coma Recovery Scale- Revised – CRS-R) with a shorter and easier to execute scale (i.e., Glasgow Coma Scle – GCS). We evaluated the accuracy of the GCS in assessing the level of consciousness in 2455 adults with Traumatic Brain Injury (TBI). We found that total GCS scores between 4 and 14 were associated with more than one DoC diagnosis and showed great variability. Moreover, a coma diagnosis was only observed in scores between 3 and 6 whereas most studies use 8 as a cutoff for coma stratification. Our results show that the GCS is not an appropriate tool to assess consciousness and other short scales (e.g., the Simplified Evaluation of CONsciousness Disorders – SECONDs) should be preferred to replace the CRS-R. Moreover, studies that stratify patients using the GCS total score should be interpreted with caution.

Another challenge in the care of patients with DoC is related to the paucity of available treatments. In **Study 2** we test a new Non Invasive Brain Stimulation (NIBS) technique as a potential treatment for patients with DoC. We conducted a RCT comparing transcranial Pulsed Current Stimulation (tPCS over mastoids at 6-10 Hz) and transcranial Direct Current Stimulation (tDCS over the left prefrontal cortex) to sham stimulation in a sample of 12 DoC patients. Our results are in line with previous studies regarding tDCS' effectiveness,

whereas tPCS did not produce significant behavioural improvement. The success of this double-blind RCT, simultaneously investigating two techniques, shows the feasibility of such multi-stimulations protocol that can be implemented in future studies to expand the research on NIBS treatments.

NIBS has showed promising results as a treatment for patients with DoC. However, the rates of patients responding to stimulations is still low (i.e., less than 50%). The characterization of tDCS responders' endotype is a crucial step towards individualized treatments and better more targeted inclusion criteria. **Study 3** investigates demographic features of responders and non-responders in a sample of 140 DoC patients. We found that the odds of responding to tDCS were four times higher for patients in a MCS at baseline compared to an UWS. Moreover, patients with a long time since injury and younger than 18 years old also had significantly higher odds of responding to the stimulation. Future tDCS studies should prefer enrolling DoC patients presenting these demographical variables.

The effectiveness of tDCS could also be improved by adapting the stimulation to individual factors such as vigilance levels. **Study 4** proposes a protocol for a RCT that applies tDCS when the patient shows high vigilance levels, low vigilance levels and at random vigilance levels. This is achieved through a closed-loop system that calculates in real-time the patients' vigilance levels and triggers the stimulation upon reaching pre-determined thresholds. We also present the preliminary behavioural results from the first 6 patients included in this RCT, suggesting that tDCS applied at high level of vigilance is the condition that results in highest and less variable improvements.

Finally, we explore the current challenges of NIBS therapeutic research for patients with DoC and provide recommendations for future studies. Since DoC is a relatively small research field compared to other biomedical sectors, we would like to highlight the incredible benefit that would result from increased collaboration between researchers. Therapeutic research on patients with DoC would achieve larger progress and faster results if a common conceptual framework was agreed upon by researchers and if centres united their efforts and data towards the common goal of improving the condition, prognosis and care of these patients.

Résumé

Au cours des 50 dernières années, les techniques de réanimation ont beaucoup progressé, ce qui a permis d'augmenter considérablement le nombre de survivants de lésions cérébrales graves. Après un coma résultant d'une lésion cérébrale grave, différents scénarios sont possibles. Le patient peut soit être déclaré en état de mort cérébrale soit se rétablir rapidement ou soit évoluer vers des états de conscience pathologiquement altérés (ECA). Ils englobent le syndrome d'éveil non-répondant (ENR), qui se caractérise par la récupération de l'ouverture des yeux et la présence de comportements réflexes ; l'état de conscience minimale (ECM), dans lequel les patients montrent des signes reproductibles de conscience et, enfin, l'ECM*, qui est une catégorie de patients diagnostiqués en ENR comportementalement mais dont l'activité cérébrale est plus compatible avec l'ECM, ce qui démontre une dissociation cognitivo-motrice (DCM). Lorsque les patients récupèrent une communication fonctionnelle et/ou une utilisation d'objets fonctionnelle, on dit qu'ils ont émergé de l'ECM (eECM) et donc de l'ECA. Même si, ces dernières années, des progrès incroyables ont été réalisés dans le diagnostic clinique et la prise en charge des patients en ECA, leurs problèmes de communication et leurs handicaps moteurs constituent un réel challenge pour les chercheurs et les professionnels de la santé. Il est indéniable que le taux de diagnostic erroné des patients en ECA reste actuellement élevé (38 %) et que les options thérapeutiques sont limitées, ce qui impact le pronostic de ces patients.

Les recherches présentées dans cette thèse visent à résoudre certains des défis mentionnés ci-dessus et à faciliter les études futures pour des approches plus adaptées aux patients.

Le premier défi concerne les problèmes qui se posent avec les longs protocoles expérimentaux, qui peuvent entraîner une fatigue chez les patients, ce qui entrave l'efficacité observée des traitements dans les essais contrôlés randomisés (ECR). L'étude 1 explore la possibilité de remplacer l'échelle comportementale la plus utilisée actuellement (c'est-à-dire l'échelle de récupération du coma révisée - CRS-R) par une échelle plus courte et plus facile à exécuter (c'est-à-dire le Glasgow Coma Scale - GCS). Nous avons évalué la précision de la GCS dans l'évaluation du niveau de conscience chez 2455 adultes souffrant de lésions cérébrales traumatiques (TBI). Nous avons constaté que les scores totaux du GCS compris entre 4 et 14 étaient associés à plus d'un diagnostic d'ECA et présentaient une grande variabilité. De plus, un diagnostic de coma n'a été observé que pour les scores entre 3 et 6 alors que la plupart des études utilisent 8 comme seuil pour la stratification du coma. Nos résultats montrent que le GCS n'est pas un outil approprié pour évaluer la conscience et que d'autres échelles courtes (par exemple, l'évaluation simplifiée des troubles de la conscience - SECONDs) devraient être privilégiées pour remplacer le CRS-R. En outre, les études qui stratifient les patients en utilisant le score total du GCS doivent être interprétées avec prudence.

Un autre défi dans la prise en charge des patients en ECA est lié à la rareté des traitements disponibles. Dans l'étude 2, nous testons une nouvelle technique de stimulation cérébrale

non invasive (NIBS) comme traitement potentiel pour les patients en ECA. Nous avons mené un ECR comparant la stimulation transcrânienne par courant pulsé (tPCS) et la stimulation transcrânienne par courant direct (tDCS) à un traitement fictif dans un échantillon de 12 patients en ECA. La tDCS a confirmé son efficacité, précédemment démontrée dans d'autres études, alors que la tPCS sur les mastoïdes à 6-10 Hz n'a pas produit d'amélioration significative du comportement. Le succès de cet ECR en double aveugle, qui étudie simultanément deux techniques, montre la faisabilité d'un protocole qui peut être mis en œuvre dans de futures études pour étendre la recherche sur les traitements NIBS.

Les NIBS ont montré des résultats prometteurs en tant que traitement pour les patients en ECA. Cependant, le taux de patients répondant aux stimulations est encore faible (moins de 50 %). La caractérisation de l'endotype des répondeurs à la tDCS est une étape cruciale vers des traitements individualisés et des critères d'inclusion plus ciblés. L'étude 3 examine les caractéristiques démographiques des répondeurs et des non-répondeurs dans un échantillon de 140 patients en ECA. Nous avons constaté que les chances de répondre à la tDCS étaient quatre fois plus élevées chez les patients en ECM au départ que chez ceux en ENR. De plus, les patients ayant une lésion depuis longtemps et âgés de moins de 18 ans avaient également des chances significativement plus élevées de répondre à la stimulation. Les futures études sur la tDCS devraient préférer le recrutement de patients en ECM présentant ces variables démographiques.

L'efficacité de la tDCS pourrait également être améliorée en adaptant la stimulation à des facteurs individuels tels que le niveau de vigilance. L'étude 4 propose un protocole pour un ECR qui applique la STCC lorsque le patient présente un niveau de vigilance élevé, un niveau de vigilance faible et un niveau de vigilance aléatoire. Ceci est réalisé grâce à un système en boucle fermée qui calcule en temps réel le niveau de vigilance des patients et déclenche la stimulation lorsque les seuils sont atteints. Nous présenterons également les résultats comportementaux préliminaires des 6 premiers patients inclus dans cet ECR.

Enfin, dans cette thèse, nous explorons les défis actuels de la recherche thérapeutique NIBS pour les patients atteints de ECA et fournissons des recommandations pour les études futures. Étant donné que les ECA sont un domaine de recherche relativement petit par rapport à d'autres secteurs biomédicaux, nous aimerions souligner l'incroyable avantage qui résulterait d'une collaboration accrue entre les chercheurs. La recherche thérapeutique sur les patients atteints de ECA progresserait davantage et obtiendrait des résultats plus rapides si les chercheurs s'accordaient sur un cadre conceptuel commun et si les centres unissaient leurs efforts et leurs données dans le but commun d'améliorer l'état de ces patients.

Abbreviations

Anterior Cingulate Cortex	ACC
Brain Computer Interfaces	BCI
Cerebro-Spinal Fluid	CSF
Cognitive Motor Dissociation	CMD
Coma Recovery Scale – Revised	CRS-R
Cortisol Awakening Response	CAR
CRS-R For Accelerated Standardized Testing	CRS-R FAST
Deep Brain Stimulation	DBS
Default Mode Network	DMN
Diffusion Tensor Imaging	DTI
Disability Rating Scale	DRS
Disorders of Consciousness	DoC
Dorsolateral Prefrontal Cortex	DLPFC
Electroencephalogram	EEG
Emergency Department	ED
emergent (from the) Minimally Conscious State	eMCS
Explainable Consciousness Indicator	ECI
Fronto-Parietal Network	FPN
Fluorodeoxyglucose Tracer	FDG-PET
Focused Shock Wave Therapy	F-SWT
Full Outline of Unresponsiveness Scale	FOUR
functional Locked-In-Syndrome	fLIS
Gamma-AminoBuytric Acid	GABA
Glasgow Coma Scale	GCS
Inferior Parietal Lobule	IPL
Intensive Care Unit	ICU
Lempel-Ziv Welch	LZW
Locked-In Syndrome	LIS
Long-Term Potentiation	LTP
Low-Intensity Focused Ultrasound	LIFU
Magnetic Resonance Imaging	MRI
Minimally Conscious State	MCS
Minimally Conscious State MINUS	MCS-
Minimally Conscious State PLUS	MCS+
Minimally Conscious State STAR	MCS*
Near-Infrared low-level Laser Therapy	N-LT
N-Methyl-D-Aspartate	NMDA
Non-Traumatic Brain Injury	Non-TBI
Non-Invasive Brain Stimulation	NIBS
Perturbational Complexity Index	PCI
Positron Emission Tomography	PET
Posterior Cingulate Cortex	PCC

Post-Traumatic Confusional State	PTCS
Primary Motor Cortex	M1
Quantitative EEG	qEEG
Randomized Controlled Trials	RCT
Rapid Eye Movement	REM
Recovery from Post-Traumatic Confusional State	rPTCS
Repetitive Transcranial Magnetic Stimulation	rTMS
Sensory Modality Assessment Rehabilitation Technique	SMART
Simplified Evaluation of CONsciousness	SECONDs
Spinal Cord Stimulation	SCS
Time Since Injury	TSI
transcranial Alternating Current Stimulation	tACS
transcranial Direct Current Stimulation	tDCS
transcranial Electrical Stimulation	tES
Transcranial Magnetic Stimulation	TMS
transcranial Pulsed Current Stimulation	tPCS
transcranial Random Noise Stimulation	tRNS
transcutaneous auricular Vagus Nerve Stimulation	tVNS
Traumatic Brain Injury	TBI
Unresponsive Wakefulness Syndrome	UWS
Vagal Nerve Stimulation	VNS
Vegetative State	VS
Wessex Head Injury Matrix	WHIM

1. Introduction

Based on the following publications:

- **Barra A***., Carrière M*., Laureys S., Martial C. From conscious to unconscious a spectrum of states. Chapter of book Beyond Neural Correlates of Consciousness Taylor & Francis (2020)

- **Barra A.***, Monti M. and Thibaut A. Non-Invasive Brain Stimulation Therapies to Promote Recovery of Consciousness: Where We Are and Where We Should Go?. *Seminars of Neurology.* Accepted

- Carrière, M., Mortaheb, S., Raimondo, F., Annen, J., **Barra, A.**, Binda Fossati, M.C., Chatelle, C., Hermann, B., Martens, G., Di Perri, C. and Laureys, S., 2020. Neurophysiological correlates of a single session of prefrontal tdcs in patients with prolonged disorders of consciousness: a pilot double-blind randomized controlled study. *Brain Sciences*, *10*(7), p.469.

- Lee, M., Sanz, L.R., **Barra, A.**, Wolff, A., Nieminen, J.O., Boly, M., Rosanova, M., Casarotto, S., Bodart, O., Annen, J. and Thibaut, A., 2022. Quantifying arousal and awareness in altered states of consciousness using interpretable deep learning. *Nature communications*, *13*(1), pp.1-14.

- Martens, G., Fregni, F., Carrière, M., **Barra, A.**, Laureys, S. and Thibaut, A., 2019. Single tDCS session of motor cortex in patients with disorders of consciousness: a pilot study. *Brain Injury*, *33*(13-14), pp.1679-1683.

- Sanz, L.R., Aubinet, C., Cassol, H., Bodart, O., Wannez, S., Bonin, E.A., **Barra, A.**, Lejeune, N., Martial, C., Chatelle, C. and Ledoux, D., 2021. SECONDs Administration Guidelines: a fast tool to assess consciousness in brain-injured patients. *JoVE (Journal of Visualized Experiments)*, (168), p.e61968.

To commence, we will provide an overview of the phenomenon of consciousness and describe several of the states in which it is altered. Following this, we will focus on disorders of consciousness, the specific category of pathological altered states of consciousness that represent the main focus of the research presented in this thesis.

In the second part of this introduction, we will present the state-of-the-art in clinical management and treatment of patients with disorders of consciousness, focusing on non-invasive brain stimulation, the central treatment approach of the following studies. Finally, we will outline the objectives of the present work.

1.1 What is Consciousness?

For millennia, consciousness has been one of the most controversial topics in science and philosophy: contemplating the enigmatic emergence of non-physical sentience from mere physical cells continues to occupy human minds today. The study of consciousness has historically concerned both religion and science, two of the main approaches to interpret the world. The study of consciousness does not only provide vast practical and clinical value, but it also contributes to a search for existential meaning. The topic of consciousness in scientific research has long been considered "taboo", likely due to these epistemological and philosophical connotations. However, we think that such connotations are essential to, and add provide additional perspectives to the research field.

One can approach consciousness scientifically, or rather the detection of presence of consciousness, by describing the broad spectrum of conditions that it entails, including those in which consciousness is altered. Within this spectrum, altered states of consciousness can be induced intentionally (e.g., anaesthesia, hypnosis), or as a result of pathology (e.g., disorders of consciousness, absence seizures).

It is essential to point out that consciousness can be described from two diametrically opposed points of view. The first, and most unequivocal, is the subjective one. Meaning that from an internal and phenomenological perspective, consciousness is the ability to be aware of one's self and/or its surroundings. This point of view is intuitive and does not give space to interpretation. This assumes that a conscious being will not likely have doubts about being conscious and it is related to "cogito ergo sum" philosophised by Descartes. The second, and more controversial point of view refers to the external third-person perspective. In other words, what are the signs that an external observer seeks when

searching for consciousness? When can we consider an entity as conscious? This latter perspective provides the starting point for the current most accepted definition of consciousness in neuroscience: a complex phenomenon that emerges from the presence of two components, arousal (or wakefulness) and awareness (Tononi et al. 2016). Indeed, primarily when trying to detect consciousness, one would search for signs of arousal or wakefulness. Despite the existence of cases in which a person can be asleep and conscious (e.g., while dreaming), consciousness mostly requires wakefulness to be present (Martial et al. 2020; Cicogna and Bosinelli 2001; Tononi 2005). However, the presence of wakefulness alone is not proof of consciousness (e.g., the Unresponsive Wakefulness Syndrome or Vegetative State – UWS/VS), awareness also needs to be present at some level. In neuroscience, arousal and awareness are considered to be the two main components of consciousness and are the two features that researchers try to measure when investigating the level of consciousness of subjects and patients.

While arousal or wakefulness manifests through eye opening, awareness pertains to the content of experience. Referring to "being aware of one's selves and/or its surroundings" (Tononi et al. 2016), awareness manifests internally through self-awareness and externally (i.e., third-person perspective) through interactions with the environment. Awareness can be further divided in two subcomponents: internal (e.g., awareness of self, metacognition, mind wandering) and external (i.e., awareness of the environment) (Vanhaudenhuyse et al. 2011; Christoff et al. 2016). It is important to note that internal and external awareness seem to be anti-correlated (meaning that when one is dominant the other one is less present) (Boly et al. 2007; Smallwood and Schooler 2006).

1.2 Altered States of consciousness

As consciousness is a phenomenon that emerges from the presence of arousal and awareness, it is not surprising that when these two are present at different levels they can result in different states of consciousness. When we think of someone fully conscious we envision a person being fully awake (arousal) and fully aware of themselves and their surroundings (awareness). However, if we think of a person that is completely unconscious (e.g., in a coma) we will envision someone that is not aware nor awake. Arousal and awareness can be present in many different ways and at different degrees, thus producing several states of consciousness.

Consciousness is not an all-or-nothing phenomenon, thus the boundaries between different states are not often clear-cut, and very often can overlap (Bates 2003). Altered states of consciousness may give us useful insight on what is consciousness and its proprieties. These states, involving different degrees of arousal and awareness are a unique opportunity for scientists to determine the necessary and sufficient conditions for consciousness to emerge and enrich our understanding of the conscious mind.

In Figure 1 we can see how altered states of consciousness can overlap and be placed in different "areas" of consciousness according to their degree of arousal and awareness.

1.3 Disorders of Consciousness

Resuscitation and neurosurgery techniques have advanced greatly in the past 50 years, leading to a considerable increase in survivors from severe brain injury. As a consequence, patients with Disorders of consciousness (DoC) resulting from severe brain injury after a period of coma have also increased. DoC refer to a pathologically altered state of consciousness that is considered to be a rare disease. However, the prevalence of patients with DoC (specifically Unresponsive Wakefulness Syndrome – UWS) in Europe, Asia and the USA was found to vary between 0.2 and 6.1 per 100 000 individuals (van Erp et al. 2014). To give another example, in Belgium, an average of 250 new cases of patients diagnosed as DoC are reported yearly according to a report from the Belgian Public Health Federal Service from 2004. It is important to note, however, that all these reports admit probable inaccuracies in the data, mostly due to the challenges in the accurate diagnosis of DoC, which will be discussed later in this dissertation. A recent longitudinal study (McCrea et al. 2021) reports that 52% of the patients with severe TBI and 75% of the patients with moderate TBI showed favourable outcomes one year after the injury.

Coma

Coma is defined by the absence of arousal and awareness, thus lack of consciousness (Laureys and Boly 2008). One important thing to highlight, is the difference between medically induced coma and injury-related coma. The first one is a choice of healthcare professionals in intensive care and emergency settings. It is induced by sedatives and allows the body and brain to rest after an injury and the patient to not experience discomfort and pain in the process. The second one is a direct biological consequence of the brain injury itself. It is generally caused by structural and/or metabolic lesions to the reticular

system or to widespread cerebral damage (Laureys, Owen, and Schiff 2004). Brain injury related coma lasts at least one hour and up to four weeks, differently from other transitory episodes of loss of consciousness such as syncope/fainting (Teasdale and Jennett 1976). Coma is characterized by the absence of spontaneous eye opening, despite stimulations (Plum 1983). It is also characterized by the absence of behavioural interactions with the environment and lack of sleep-wake cycles (Teasdale and Jennett 1974b). When a patient awakens from a coma, several scenarios are possible: the patient may recover rapidly or they may evolve into more dramatic scenarios such as disorders of consciousness or transition into a state of brain death and die. (See Figure 2 for a visual representation of motor and cognitive recovery trajectories after severe brain injury and coma).

Brain Death

Although the legislations differ between countries, there is a clear difference between severe brain injury and brain death. In 1959 Mollaret and colleagues introduced the term "irreversible coma" (Mollaret 1959) describing patients that showed a flat EEG, no brainstem reflexes, no spontaneous breathing and, of course, no consciousness. These are considered key characteristics of brain death. However, the criteria to determine brain death have evolved widely, also in accordance with law systems. Usually, brain death needs to be diagnosed following three steps (although the last step is optional in several countries) (Kondziella 2020). The first step is that all prerequisites to determine brain death are observed (i.e., the brain injury is known and irreversible; no confounding factors such as anaesthesia are present). The second step is the actual clinical assessment of brain death. The absence of the following signs is tested: brainstem reflexes, cortico-spinal- and spino-cortical transmission, spontaneous breathing. The third step is the confirmation of these results with laboratory investigations and, as mentioned before, they vary widely according to the local legislation. For instance, an electroencephalogram (EEG), evoked potential examination or angiographic computerised tomography might be mandatory to determine brain death in one country and not in another.

The Unresponsive Wakefulness Syndrome / Vegetative state (UWS/VS)

If and when the patient recovers spontaneous eye opening, but still does not show any signs of awareness of themselves or the environment, they are said to be in a UWS. The first known definition of UWS was coined in 1972 by Jennett and Plum (Jennett and Plum 1972) and it was back then called Persistent Vegetative State (PVS). The authors described

patients in a PVS as patients that had awaken from coma (thus showing spontaneous eye opening) but did not show behavioural responses to stimuli, nor any sign of social interaction, however their vagus nerve autonomic functions (e.g., respiratory functions, sleep-wake cycles, heart rate) remain preserved. Later on, in 1994, the US Multi-Society Task Force on PVS expanded the original definition with the following diagnostic criteria: "(1) no evidence of awareness of self or the environment and an inability to interact with others; (2) no evidence of sustained, reproducible, purposeful, or voluntary behavioural responses to visual, auditory, tactile, or noxious stimuli; (3) no evidence of language comprehension or expression; (4) intermittent wakefulness manifested by the presence of sleep-wake cycles; (5) sufficiently preserved hypothalamic and brain-stem autonomic functions to permit survival with medical and nursing care; (6) bowel and bladder incontinence; and (7) variably preserved cranial-nerve reflexes (pupillary, oculocephalic, corneal, vestibulo-ocular, and gag) and spinal reflexes" (The Multy-Society Task Force on PVS 1994). Along with these criteria, the Task Force also provided a first epidemiological report on prognosis, based on a retrospective data analysis which categorized as patients suffering from irreversible vegetative state, patients that were in PVS for more than one year after Traumatic Brain Injury (TBI) and more than three months after non-TBI (i.e., anoxic). Originally, the choice of the adjective "vegetative" (in the Oxford English Dictionary defined as "an organic body capable of growth and development but devoid of sensation and thought") was justified by the meaning of the verb "to vegetate" that refers to an organism that lives "a merely physical life, devoid of intellectual activity or social intercourse". In the thirty years following this first definition, however, the term vegetative has been associated to being "vegetable-like" which has a derogatory connotation and erroneously demotes patients from human beings to plants in the public conception. Moreover, it has been noted that the term "state" also has a chronicity implication that might be inaccurate in some cases. Some studies have showed that medical practitioners and healthcare workers felt uncomfortable using the term PVS (Kotchoubey 2005; Schoenle and Witzke 2004). The word "persistent" was also added by Jennett and Plum in contraposition to previously used terms such as "permanent" that carried more unfavourable prognostic meaning. To eliminate the above mentioned ambiguities and negative connotations, in 2010, Laureys and colleagues proposed a new name for this syndrome (Laureys et al. 2010), which was UWS. It is important to note that the name UWS has been adopted officially by the European Academy of Neurology, however their US counterpart, recommends the use of VS/UWS. Currently, the commonly accepted

diagnostic criteria and definition for UWS are: the presence of wakefulness (i.e., eye opening presented spontaneously or upon stimulation) but not responsiveness (i.e., absence of non-reflex behaviours and command following). Moreover, the UWS name is considered to be a neutral description including patients with various clinical symptoms, hence the term "syndrome". For instance, the term apallic syndrome had also been proposed to replace vegetative state, however, it was found that not all UWS patients lacked a preserved pallium (Laureys et al. 2010; Von Wild et al. 2007).

The Minimally Conscious State (MCS)

The Minimally Conscious State (MCS) was introduced by Giacino and colleagues in 1997 to distinguish patients incapable of showing any sign of consciousness (i.e., coma, UWS/VS) from those capable of showing some signs of awareness (Giacino et al. 1997). Nevertheless, it was not until 2002 that clear diagnostic criteria were defined during the Aspen Neurobehavioral Conference Workgroup (Giacino et al. 2002). There, a panel of experts reached consensus on the following behavioural signs of awareness required to assign a diagnosis of MCS: (1) reproducible responses to simple commands, (2) ability to answer yes/no questions (regardless of accuracy), showing purposeful behaviours towards external stimuli (e.g., holding objects, contextually appropriate emotional reactions, reaching for objects, visual pursuit or fixation of objects, localization of objects in space et cetera). Many authors have argued against the usefulness of distinguishing patients in UWS from patients in MCS, as they both could be considered severely brain damaged (Gosseries, Bruno, et al. 2011). However, several studies have showed that disentangling these categories has a high clinical value, as they do not have similar prognostic trajectories. Patients in MCS show more preserved brain structure and activity, they are more likely to improve in terms of independence of basic daily activities and have longer life expectancy (Claassen et al. 2019; Robinson et al. 2003; Estraneo and Trojano 2018). As for the debate around UWS/VS, the term "minimally" has also produced controversies in the last decade. Some thought it might be confusing and might carry a derogatory connotation. It has been suggested that it be replaced with the term "cortically-mediated state", based on the type of behavioural signs of awareness that we observe in these patients (Naccache 2018). However, to this day, both the most recent American and European guidelines on DoC still encourage the use of MCS (Kondziella et al. 2020; Giacino et al. 2018b).

Between 2002 and 2011, even if the diagnostic criteria for the MCS were clear and established, the array of behaviours that could be observed within this category was still wide and heterogeneous. For this reason, to facilitate the detection of signs of awareness and a correct diagnosis, a further sub-categorization of the MCS category was proposed in 2011(Bruno et al. 2011). With this new categorization, the Minimally Conscious State PLUS (MCS+) and the Minimally Conscious State MINUS (MCS-) were distinguished based on the complexity of observed behavioural signs of awareness. MCS+ patients notably show more complex behaviours that involve preservation of language functions to a certain level (e.g., response to commands, intelligible verbalization, ability to answer yes/no questions regardless of accuracy). On the other hand, MCS- show conscious behaviours (included in the MCS diagnostic criteria) that do not involve language understanding and/or production, such as automatic oriented movements, holding objects, contextually appropriate emotional responses and/or visual pursuit of objects. The differences between MCS- and MCS+ levels of behavioural complexity have also been supported by several neuroimaging studies that confirmed a significant higher brain preservation of language-related areas and networks in patients in MCS (e.g., Broca's and Wernicke's regions, left premotor) (Aubinet et al. 2020; Bruno et al. 2012).

Emergence from the Minimally Conscious State (eMCS)

When a patient recovers more complex abilities such as functional communication (i.e., correctly answering simple yes/no questions) or functional use of objects (i.e., appropriate use of two or more daily objects) they are considered to have emerged from the MCS (eMCS) (Giacino et al. 2002). As previously mentioned, consciousness is not an all-or-nothing phenomenon, and eMCS is a perfect example of this. Evidence shows that eMCS should be placed between the boundaries of DoC and full consciousness. However, the exact place it should occupy within this space is not clear-cut yet. For instance, Bodien and colleagues highlighted that eMCS and Post-Traumatic Confusional State (PTCS) (Sherer et al. 2020) share a lot of similarities in terms of behavioural endotype (Bodien et al. 2020) such as cognitive impairment, disorientation and disturbances in arousal and sleep-wake cycles. For these reasons, eMCS is considered to be the best end-point of DoC although not formally a DoC category.

The Minimally Conscious State (Star) (MCS*)

Covert consciousness, defines specific cases of DoC that show no behavioural response, probably due to severe motor impairments, but have brain activation patterns that are consistent with conscious DoC patients (Schiff 2015). These patients, are referred to as in MCS* (Thibaut et al. 2019, Schnakers et al. 2020) when their covert consciousness is hypothesized based on resting state neuroimaging data compatible with that of patients that are at least in a MCS. Patients with covert consciousness can sometimes also show response to commands with Brain Computer Interface (BCI) approaches and both active and passive functional Magnetic Resonange Imaging (fMRI) or EEG paradigms (Naci et al. 2012; Pistoia, Carolei, et al. 2015; Annen et al. 2018). When patients show signs of consciousness (e.g., P300 evoked potentials to EEG passive paradigms, response to commands during fMRI imaginery tasks) they can be labelled as suffering from a Cognitive Motor Dissociation (CMD). However, it is important to note that all these definitions have not been formally agreed on by experts of DoC research and are sometimes used intercheangebly or even replaced by other labels such as functional Locked-In-Syndrome (fLIS) and non-behavioural MCS. Thibaut and colleagues (Thibaut et al. 2021) have proposed the term MCS* to encompass all the conditions of covert consciousness, however, this has yet to be agreed on by specialized task forces. In this dissertation we will adopt MCS* to indicate covert consciousness as a general term, for practical reasons. Finally, some studies have also showed that patients in MCS* show better prognosis than patients in UWS, which is compatible with the prognostic trajectories of patients in MCS (Thibaut et al. 2021; Pan et al. 2020). (For a visual schematization of the motor and cognitive dissociation in recovery we can observe in MCS* see Figure 2).

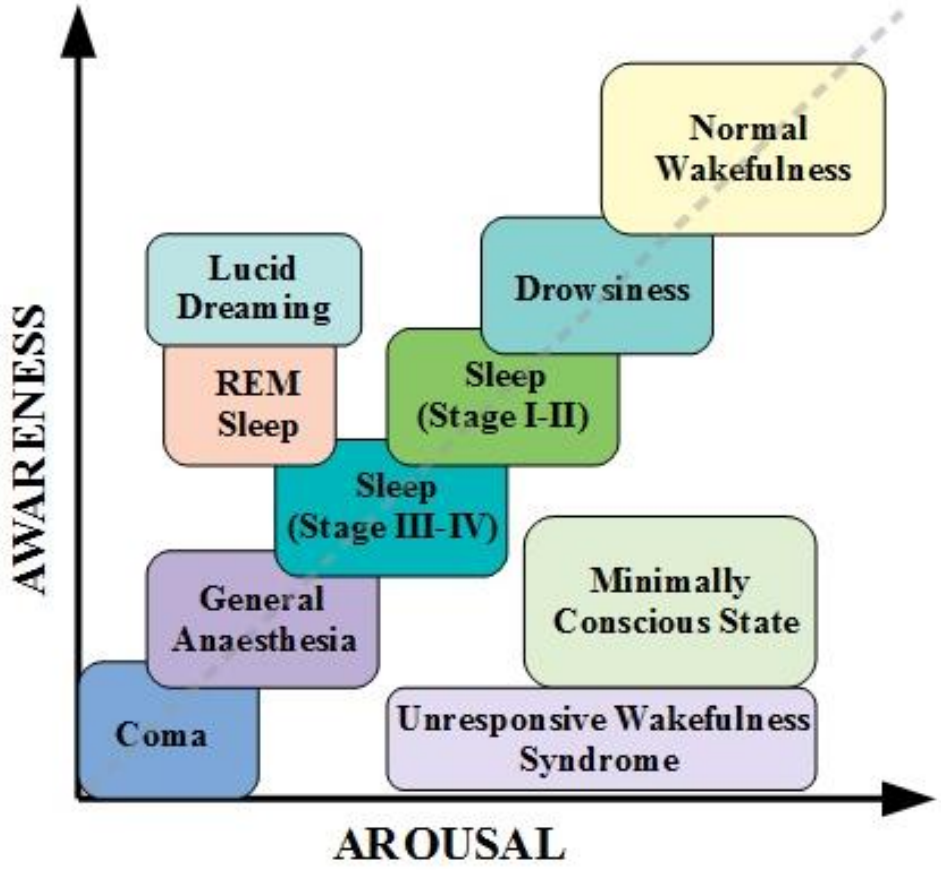

Fig. 1 Schema of Different States of Consciousness. Adapted from Barra, Carrière et al., 2020 with the authors permission illustrating different states of consciousness, mapped according to the level of arousal (x axis) and awareness (y axis). Compared to normal wakefulness, coma and general anaesthesia present very low levels of arousal and awareness. On the other hand, sleep stages I-IV however lower than normal, show higher arousal and awareness. During drowsiness both arousal and awareness are slightly diminished. REM sleep (characterized by the presence of dreams) shows low arousal and high awareness, as the content of consciousness is rich and can sometimes be encoded in memory. Lucid dreaming shows even higher content of awareness as subjects are aware of themselves and of dreaming while being asleep. Finally, the MCS and UWS show high arousal (i.e., the patient being fully awake) but low awareness, with MCS having higher content of consciousness (as demonstrated by interactions wih the environment) compared to UWS that, being unconscious, have levels of awareness similar to coma.

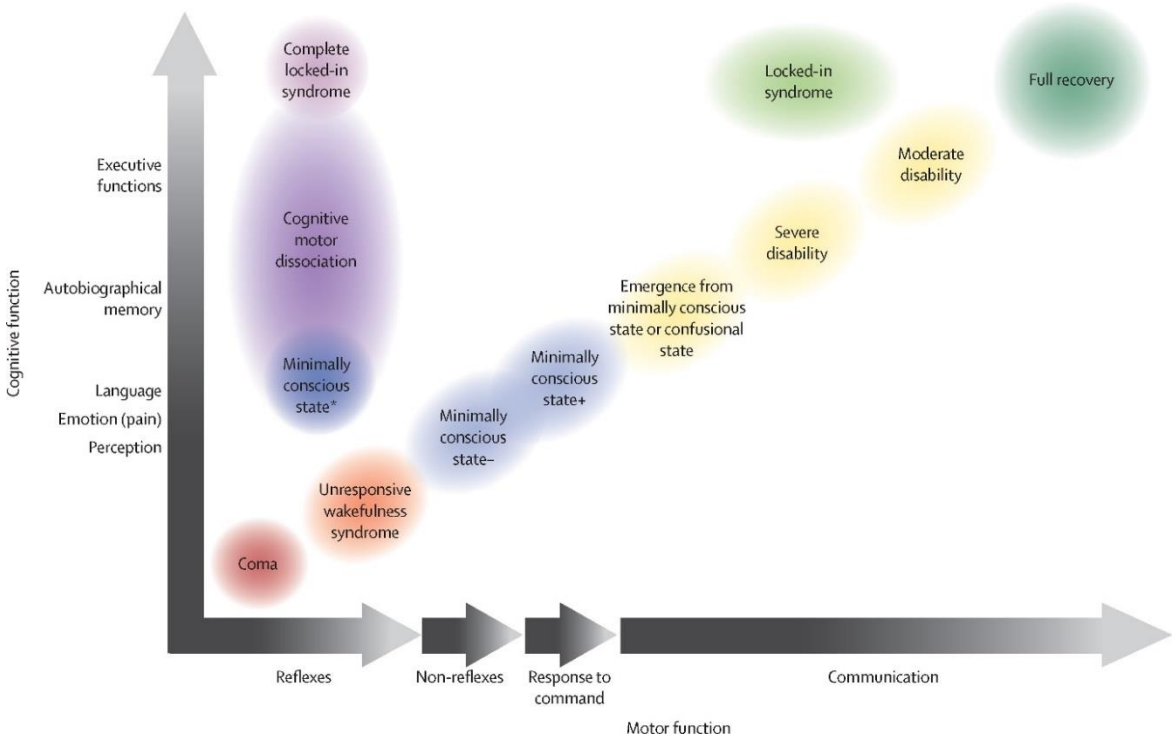

Fig. 2 Motor and cognitive evolution following a severe brain injury. Adapted from Thibaut et al. 2019 with authors permission. The different diagnoses after a severe brain injury can be best captured on a two-dimensional axis by comparing the degree of cognitive function against the degree of motor function. Red circles represent patients who are unconscious with only reflexive movements (coma and UWS). Blue circles represent patients in a MCS (MCS+ and MCS- depending on language preservation). When functional communication is detected (yellow circles), patients have emerged from the MCS and can evolve to a confusional state or severe or moderate disability, before a full recovery (dark green circle). Dissociations between motor and cognitive functions exist in locked-in syndrome (light green circle), cognitive motor dissociation (dark purple circle), and minimally conscious state* (dark blue circle). Brain Computer Interfaces can help detecting these challenging diagnosis. In rare cases, the diagnosis of complete locked-in syndrome (light purple circle) can be done through neuroimaging examinations. The black-to-white gradient represents the evolution from absence (black) to the recovery of a behaviour (white; eg, from no response to command to consistent response).

1.4 The main techniques to study consciousness

To study the neural correlates of cognitive phenomena of altered states of consciousness, three techniques are commonly used: Magnetic Resonance Imaging – MRI, Positron Emission Tomography – PET and Electroencephalogram - EEG. These techniques will briefly be described to give the readers the tools to navigate the results mentioned in the discussion.

PET allows us to highlight various metabolites in the body and brain, according to the type of radioisotope contrast that is used. This contrast is injected into the bloodstream and

reaches the body part of interest acting like a tracer. Among the many types of radioisotopes that exist, the fluorodeoxyglucose tracer (FDG-PET) is the main one used to visualize the brain's glucose uptake and thus, indirectly, brain activity. The brain (notably neurons and glia cells) uses glucose to function, thus, the radioactive glucose present in the tracer allows the PET scanner to detect the brain metabolism and create multi-dimensional images based on the distribution of the tracer in the tissues. These images, although directly associated to neural glucose consumption, are however limited by relatively poor temporal (up to one minute) and spatial resolution (up to one centimetre) (Vaquero and Kinahan 2015).

MRI, on the other hand, has better spatial and temporal resolution. Within this neuroimaging technique, two main modalities can be distinguished: structural and functional MRI. Structural MRI creates accurate images of brain morphology, by segmenting the brain into white matter, cerebrospinal fluid (CSF) and grey matter, and it is mostly used to detect brain lesions (Symms et al. 2004). Functional MRI (fMRI) generates dynamic representations of brain activity using the Blood Oxygenation Level Dependent (BOLD) signal, meaning that brain activity is measured by the amount of blood in the tissue (as neuronal activation is dependent on cerebral blood flow) (Bandettini 2012). This method can also be used to measure connectivity between brain regions. This can be achieved by functional connectivity analyses (i.e., temporal correlation of BOLD signal in separate brain areas) and effective connectivity analyses (i.e., the influence of one brain region activation over another brain region) (Friston et al. 1997). Finally, it might be interesting to note that a relatively novel technique called Diffusion Tensor Imaging (DTI), based on MRI, allows us to map specifically white matter fibres in the brain (Hulkower et al. 2013) investigating the integrity of connections between brain regions. The diffusion of water molecules in the brain is detected with this technique, which can be used to visualize the integrity of myelinated axons.

EEG is another widely used measure of brain activity, recording the electrical signal produced by a population of neurons through electrodes placed on the scalp. This technique is the most used in research and clinical settings because it is less expensive and more easily accessible than MRI and PET (Jackson and Bolger 2014). However, the spatial resolution of EEG is usually lower than that of PET and MRI. Generally, EEG is used in clinical settings for visual inspections of lateralization, epileptiform activity, encephalopathy and other valuable informations. The recording of EEG signal, coupled with numerical analyses such as wavelet analysis and Fourier transforms can give deeper

insights on the characteristics of the EEG signal such as quantify (i.e., quantitative EEG – qEEG) the amount of power of specific frequencies. The basic four frequencies that are most studied are: Delta (1-3 Hz), Theta (4-7 Hz), Alpha (8-13 Hz) and Beta (14-30 Hz). These frequencies are typically associated with different cognitive and consciousness states and levels of arousal of the cortex. For instance, Delta can be linked to states of deep sleep and subcortical lesions whereas Theta can be linked to light sleep, drowsiness and imagery states and it can be found in patients with brain injuries. Alpha power, that is more prominent over occipital regions, is usually associated with non-focused and relaxed awake states, whereas Beta power with attentive and alert awake states and is usually found in frontal regions (Barlow 1993). Apart from qEEG, electrophysiology can also be used to measure functional connectivity between brain regions and networks, using source localization algorithms, even if the timescale of EEG functional connectivity is in seconds, whereas for fMRI it is in milliseconds. EEG connectivity can be assessed through different methods that take into account the temporal correlation between distant areas (Miljevic et al. 2021). The covariation in amplitude and phase between signals and time series is called coherence. One example of such methods is to explore dynamics of connectivity by applying graph theory to the signal, to extract metrics such as the participation coefficient, which (in the alpha band) was proven to be able to predict diagnosis for patients with DoC (Thibaut et al. 2018). EEG signal can also be used in Brain Computer Interfaces (BCI) paradigms. BCIs can be used to communicate, as well as for diagnostic purposes. They rely on EEG signal that is recorded during a task, usually involving mental imagery or the presentation of a sensory stimuli (Abiri et al. 2019). The BCI detects differential brain region activations and provides an output on the screen that can be used to communicate. For instance, a subject (while wearing an EEG-BCI system) might be asked to focus on one perceptual stimulus (e.g., vibration applied to the left arm) if their intention is to answer "Yes" to a question or on another stimulus (e.g., vibration applied to right leg) to answer "No". The EEG signal, detected in different somatosensory regions of the cortex according to the body part the subject is focusing on, can be successfully distinguished by the BCI system to provide an answer on the screen (Guger et al. 2017).

EEG can measure the spontaneous electrical signal created by neurons, however it can also be used to probe the brain's response to an external stimulation, such as Transcranial Magnetic Stimulation (TMS). TMS is a non-invasive tool to stimulate the brain, it can send an electrical current in the brain (via strong changes in magnetic field) and elicit a response

in the brain that is called TMS- evoked potential. (See section on non-invasive brain stimulation for a detailed description of TMS).

The neural basis of consciousness can be explored with all of the aforementioned techniques, through different approaches. The first would be inferring, from our scientific knowledge of neurophysiology and neuroanatomy, the structures that are most likely to carry out the processes necessary for consciousness. The second is to explore, through brain imaging and neuropathological studies, the aforementioned structures when they are altered.

1.5 Neural correlates of consciousness

Thanks to the techniques described above much progress in identifying the neural correlates of consciousness has been made. In this section, we will describe the main brain structures and networks involved in the emergence of consciousness.

Key structures for consciousness

Overall, three structures have been identified as crucial for the presence of consciousness: the thalamus, the reticular formation nuclei and the posterior cingulate cortex.

The thalamus is composed of several nuclei, some of which are highly connected with sensory and motor cortices (Blumenfeld 2016). On the other hand, other nuclei of the thalamic system are involved in ascending regulation of arousal, as showed by temporary loss of consciousness when a stroke occurs in such regions (Guberman and Stuss 1983).

Another ensemble of nuclei that plays a big role in consciousness is the reticular formation (Parvizi and Damasio 2001). This formation is composed of four main groups: the reticular nuclei; the monoaminergic neurotransmitter system; the cholinergic nuclei and the autonomic nuclei (Moruzzi and Magoun 1949). Altogether, these formations have projections to the hypothalamus, the basal ganglia, the intralaminar nuclei of the thalamus, the cortex, the basal forebrain and many subcortical structures (Yeo, Chang, and Jang 2013; Blumenfeld 2016; Parvizi and Damasio 2001) and they have a role in modulating arousal and autonomic functions (Laureys, Owen, and Schiff 2004; Maldonato 2014).

Finally, another key region for consciousness is the Posterior Cingulate Cortex (PCC). This region includes the precuneus and the retrosplenial cortex (Leech and Sharp 2014) and it

is mainly involved in internal awareness (since it was found to be the most active at rest, for healthy subjects) (Gusnard and Raichle 2001).

Key networks for consciousness

Along with the brain regions mentioned above, three networks are considered as equally vital for the presence (and recovery) of consciousness. These networks are the Default Mode Network (DMN), the external fronto-parietal network (FPN) and the Salience Network (SN). Apart from these three networks, one "circuit" has also been proposed to be relevant, especially for the recovery of consciousness, the meso-circuit. The meso-circuit model will be briefly introduced in this section and then describe in more detail, later, how it is linked to consciousness recovery treatments.

The DMN includes brain regions such as the medial prefrontal cortex, the precuneus, the cingulate cortex (both anterior and posterior), the Inferior Parietal Lobule (IPL) and part of the temporal lobe and of the hippocampus. This network is mostly active when the brain is at rest and it was found to decrease its activity when the brain is engaged in a task (Raichle et al. 2001). Many neuroimaging studies have found activation of the DMN during mind wandering, day dreaming and resting inner thoughts (Mazoyer et al. 2001; Mason et al. 2007). For this reason, it is believed to be a critical network for the component of internal awareness of consciousness.

Another important network for consciousness is the FPN. Contrarily to the DMN, this network is associated with the external awareness component of consciousness (Steven Laureys 2005; A. Demertzi, Soddu, et al. 2011). The FPN is composed of lateral frontal cortices and superior parietal cortices that are connected bilaterally through the superior parietal fasciculus (Van Den Heuvel et al. 2009). Its role in external awareness is mainly linked to attentional processes and stimuli and action selection (Corbetta and Shulman 2002). Furthermore, it is widely connected with sensory systems (Brunetti et al. 2008; Dehaene and Changeux 2011).

As mentioned before, the DMN and the FPN are anti-correlated, in the sense that when one is most active the other one shows decreased activity (Fox et al. 2005; Tian et al. 2007). For instance, Boly and colleagues showed that if the DMN was dominating brain activity (thus the FPN being under-activated) a few seconds before presenting a stimulus, this would not be perceived (Boly et al. 2007). Moreover, Vanhaudenhuyse and colleagues

found that awake subjects at rest report a switch between internal (DMN) and external (FPN) awareness on average every 20 seconds (Vanhaudenhuyse et al 2011).

The salience network includes regions such as the ventro-lateral prefrontal cortex, the anterior insula and the anterior cingulate cortex (Critchley et al. 2004). It is important for the presence of consciousness as it is linked to prioritization of tasks, selection of relevant stimuli and allocation of working memory resources (Seeley et al. 2007). This network also plays a role in the anti-correlation between DMN and FPN supposedly through the von Economo cells. These cells, present only within this network, are bipolar neurons with one dentrite that allow rapid communication (i.e., task switching) between large brain areas (Allman et al. 2005; Sridharan, Levitin, and Menon 2008).

A model of consciousness pathways (that was then applied to consciousness recovery mechanisms) was developed by Schiff in 2010 (Schiff 2010). This model was inspired by neuroanatomy and results observed in the study by Laureys and colleagues (Laureys, Lemaire, et al. 1999). In healthy brains, thalamic activity (that prompts the activity of associative fronto-parietal cortices) is regulated by the feedback received and sent (i.e., bidirectional connectivity) to the cortex, mainly prefrontal. Thalamic activity is also inhibited by the globus pallidus, that in turn receives feedback (i.e., both thalamo-striatal and cortico-striatal pathways) and is inhibited by the striatum. The inhibition coming from the striatum to the globus pallidus and from the globus pallidus to the thalamus, creates excitation in this latter in non-pathological brains (due to a double inhibition that produces excitation). After severe brain injuries striatal neurons can experience a decrease in activity due to two main factors: one is that they are very sensitive to lack of oxygen and easily damaged (Grillner et al. 2005); and the other is the loss afferent signals from the thalamo-cortical and cortico-thalamic loop due to loss of neurons within the thalamus, caused by the injury. These two factors prevent the striatum from inhibiting the globus pallidus, thus, allowing it to abnormally inhibit the already damaged neurons in the thalamus. (See Figure 3 for visual representation of the meso-circuit model). This model is the rationale to explain the effectiveness of several of the therapeutic approaches to treat disorders of consciousness, as we will see more in detail later (Thibaut, Schiff, et al. 2019).

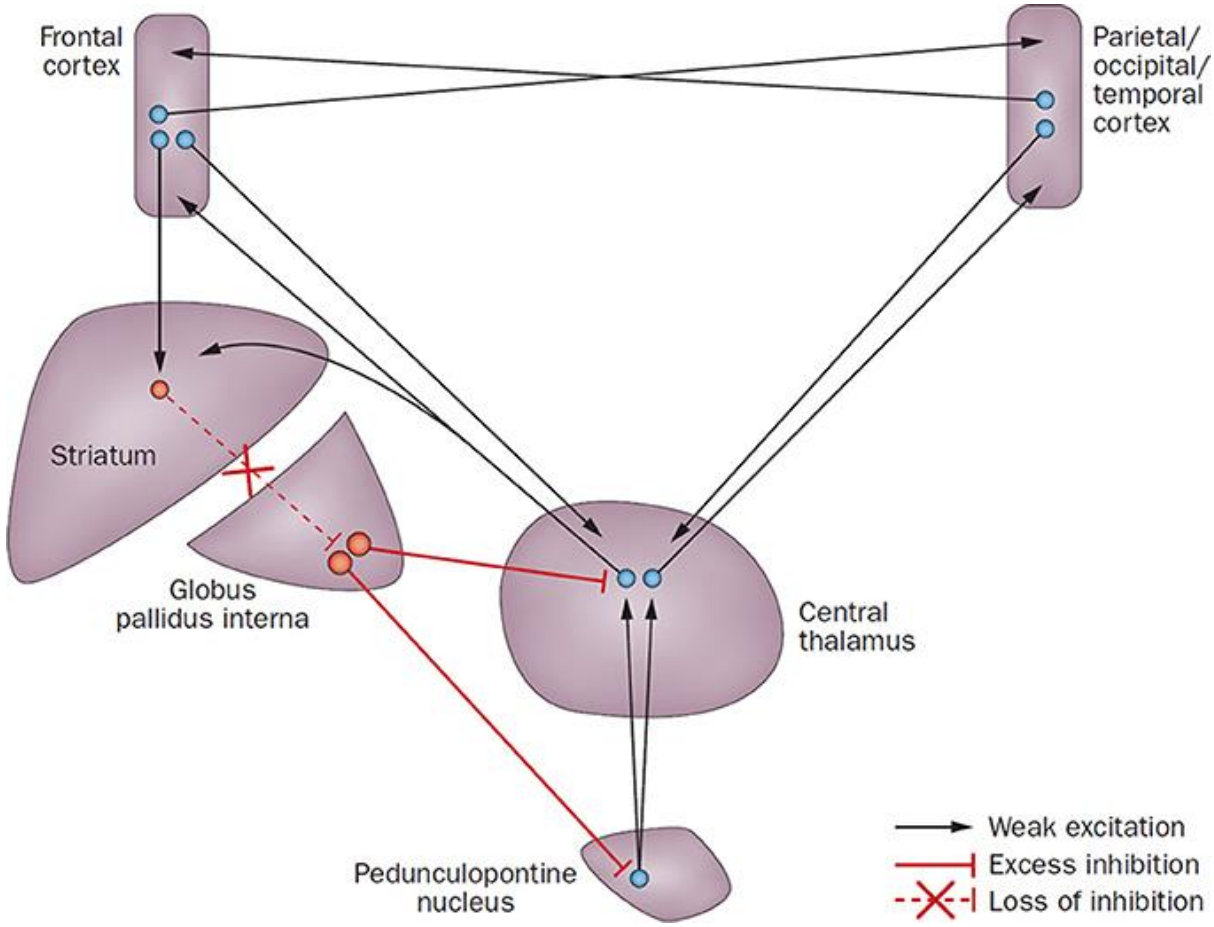

Fig.3 Visual representation of the meso-circuit model. From (J T Giacino et al. 2014) with authors permission. Reduced thalamo-cortical and thalamo-striatal signals caused by neuronal loss in the thalamus decreases the afferent signals to the striatum. Reduced inhibition from striatal neurons, in turn, allows the globus pallidus to actively inhibit both the thalamus and the pedunculopontine nucleus.

To summarize, the presence of consciousness does not rely on activity in one key region or one brain network alone. Preservation (or partial preservation) of key regions (i.e., PCC, thalamus and reticular formation) together with preservation of key brain networks (i.e., the DMN, the FPN, the FPN and the mesocircuit) is necessary for the presence of consciousness as well as for its recovery in pathological altered states of consciousness.

1.6 Neural correlates of DoC

In this section we will describe the main results from MRI, EEG, PET and TMS-EEG studies on the neural correlates of DoC as well as the differences between patients in MCS and UWS.

The study of neural correlates of DoC not only sheds light on which metabolic, electrophysiological and structural brain features are necessary for the presence of consciousness, but also contributes to increasing diagnostic accuracy of these patients, as it does not rely on motor interaction and communication contrarily to behavioural assessments. (See Figure 4 for neuroimaging results from different techniques compared between patients in UWS, MCS*, MCS and healthy controls).

fMRI studies have demonstrated that patients in MCS show activation in associative cortices similar to that of healthy subjects (Di et al. 2008; 2007). On the contrary, patients in UWS only present low-level activation in primary sensory areas. Notably, patients in UWS with activation in associative cortex have better recovery trajectories; highlighting the prognostic added value of neuroimaging tools (Owen et al. 2005). Resting state fMRI studies have also been used to explore functional connectivity in relevant brain networks. The connectivity within the DMN (previously cited for its important role in consciousness) has been shown to be a good indicator of consciousness, as it decreases linearly with consciousness levels (Vanhaudenhuyse et al. 2010). Specifically, the auditory network within the DMN, at rest was found to be the most sensitive to discriminate between UWS and MCS patients, with a rate of accuracy higher than 80% (Demertzi et al. 2015; Di Perri et al. 2016). Moreover, active fMRI paradigms have also been able to detect covert consciousness. The concept of covert consciousness, mentioned before, was hypothesized from the results of an fMRI study that found that one patient behaviourally in UWS could communicate with a mental imagery task (Owen et al. 2006). This elegantly designed study required the patient to imagine playing tennis or navigating their house in order to respond "yes" or "no" to questions, while they were in the MRI scanner. Owen and colleagues found that a few patients, who behaviourally showed no sign of consciousness and were considered in UWS, could not only perform the task but could also functionally communicate using this method. These results have been ground-breaking, as they have paved the way for several other neuroimaging studies and have highlighted the extreme

importance of cross-modal diagnosis. Many active fMRI paradigms (involving presenting the patient with stimuli while in the scanner) have been performed. For instance, visual stimuli (Monti, Pickard, and Owen 2013), auditory stimuli (Fernández-Espejo et al. 2008), noxious stimuli (Calabro et al. 2017) and emotionally relevant stimuli such as ones' own name (H. Di et al. 2007) or preferred music (Carrière, Larroque, et al. 2020) have been tested. All these studies have confirmed higher cortico-thalamic activity and connectivity in patients in MCS compared to UWS.

PET studies have also produced important results in terms of understanding the neural basis of consciousness, distinguishing between patients in UWS and MCS and detecting covert consciousness. For instance, it was found that up to 67% of patients in UWS showed preserved metabolic activity within the fronto-parietal network (key network for awareness) and could, thus, be considered as MCS* (Thibaut et al. 2021). At one-year follow up, these patients showed better outcome and better structural preservation and functional connectivity, confirming their compatibility with a diagnose of MCS*. Another main finding of PET studies, however less recent, lays the foundations for what is currently the most widely accepted theory of neural recovery of consciousness, that is the meso-circuit hypothesis, presented previously (Schiff 2010). In 1999, Laureys and colleagues found that when comparing patients before and after they recovered consciousness (i.e., from being in UWS to being in MCS), the recovery of connections between the thalamus and the cortex was the only significant change observed (Laureys, Lemaire, et al. 1999). This led to the hypothesis that rather than global glucose consumption of the brain, the regional distribution of consumption in the fronto-parietal network and connectivity between this latter and the thalamus was a crucial factor for the presence (and recovery) of consciousness (Laureys, Goldman, et al. 1999). The FPN's role in consciousness disorders is also supported by the fact that it is more preserved in patients in MCS (Laureys, Owen, and Schiff 2004). Brain metabolism in the fronto-parietal cortex has also been used to detect changes that could predict long-term recovery of patients in UWS (Stender et al. 2015). Finally, recent studies proved that FDG-PET is the most accurate neuroimaging tool to identify patients in MCS. When compared to fMRI, Stender and colleagues found that MCS detection was up to 93% accurate for FDG-PET, whereas FMRI showed only 43% accuracy (Stender et al. 2014). When FDG-PET data was coupled with EEG oddball paradigms the diagnostic sensitivity was still 94% and this method could also predict patients in UWS that would recover successful response to commands within 6 months (Hermann et al. 2021).

EEG can also assess cognitive functions and level of consciousness. It can also be used as a tool to communicate with patients without relying on motor output, through BCI (Naci et al. 2012; Guger et al. 2017; Annen et al. 2018). The electrophysiological pattern of DoC patients is characterized by diffuse slow wave activity (especially within the delta frequencies) (Lehembre et al. 2012). This frequency band is usually associated with slow wave sleep in healthy subjects. One study published in 2014 (Sitt et al. 2014) found that not only EEG complexity increased when patients in UWS transitioned to full consciousness (i.e., brain injured but fully conscious patients), but also that theta and alpha power could distinguish patients in MCS from patients in UWS whereas beta power could distinguish controls from patients with DoC. One study (Carboncini et al. 2014) using midazolam (i.e., a benzodiazepine GABA agonist) found that after infusion power spectra between 12 and 30 Hz were significantly increased. Another important EEG finding is that patients in MCS show spectral entropy fluctuations that resemble those of healthy awake subjects (Piarulli et al. 2016). These fluctuations complete a full cycle every 70 minutes on average (similar to healthy subjects) and support the hypothesis that patients in MCS have cyclical vigilance fluctuations. Chennu and colleagues (Chennu et al. 2017) found that high delta power was linked to poor prognosis and that it could also successfully distinguish patients in UWS, MCS and eMCS using quantitative EEG metrics. In their study, these metrics were also found to be highly correlated with brain metabolism (measured with FDG-PET), which we know to be a good prognostic and diagnostic tool. In opposition to high delta power, high alpha power at rest is linked to better prognosis (Bagnato et al. 2015). The ABCD model proposed by Drover and colleagues (Drover et al. 2011) and then explained within the mesocircuit model (Schiff 2016; Edlow, Claassen, et al. 2020), divides EEG patterns in four categories. The "A" category, usually found in general anaesthesia (Brown, Lydic, and Schiff 2010) presents frequencies below 1Hz and reflects almost complete cortico-thalamic deafferentiation. The category "B", characterized by 5-9 Hz oscillations, reflects severe deafferentiaion of cortico-thalamic pathways and is the most frequently observed in patients with DoC (Edlow, Claassen, et al. 2020). When oscillations between 5 and 9 Hz are coupled by higher frequencies (i.e., 20-35 Hz) this falls into the category "C". This reflects partial recovery of thalamic afferentiation and is usually observed in patients in MCS or severe brain injured but fully conscious patients (Drover and Schiff 2018). Finally, the category "D" is characterized by oscillations between 8 and 13 Hz coupled with higher frequencies oscillations (i.e., 20-35 Hz) and is typically observed in healthy subjects, reflecting normal cortico-thalamic

connectivity. Furthermore, Edlow and colleagues fit these categories into the meso-circuit model of recovery. They report that shifts between categories “A” and “B” to categories “C” or “D” are found in both acute spontaneous recovery (Claassen et al. 2016) and treatment-response recovery (Williams et al. 2013) in patients with DoC, thus likely being proof of thalamico-cortical al cortico-thalamic pathways recovery.

Another important diagnostic use of EEG arises when combined with Transcranial Magnetic Stimulation (TMS). TMS pulses provoke EEG evoked potentials that have specific characteristics (i.e., shape, timing and dynamic time course) in healthy subjects. Moreover, literature on how these TMS-EEG responses appear in other non-pathological altered states of consciousness such as anaesthesia and deep sleep allows to compare them to patients with DoC (Massimini et al. 2012). Patients in UWS present TMS-EEG responses remarkably similar to those observed during deep sleep (i.e., short, slow responses that remain localized under the stimulated site) suggesting impairment of brain connectivity (Ragazzoni et al. 2013). On the other hand, patients in MCS present complex responses that last longer and over time move across connected brain regions. These different responses have been the basis for the validation of a TMS-EEG diagnostic tool called the Perturbational Complexity Index (PCI). This index, obtained from compression of TMS-EEG responses and translated into a number that can distinguish conscious from unconscious participants using a cut-off of 0.32 (Casarotto et al. 2016). Finally, a very recent study developed a metric called Explainable Consciousness Indicator (ECI), that is based on TMS-EEG evoked potentials, and could successfully quantify arousal and awareness levels separately in both pathological and non-pathological altered states of consciousness, via machine learning algorithms (Lee et al. 2022).

To conclude, neuroimaging tools are a powerful supplement to behavioural assessments that, when available, should always be taken into account. They can provide valuable insight on the level of consciousness and prognosis of patients with DoC and they are the only way to detect covert consciousness. However, the overall applicability of these methods is relatively limited, as they require extensive training and the systems are not always easily accessible to all research structures.

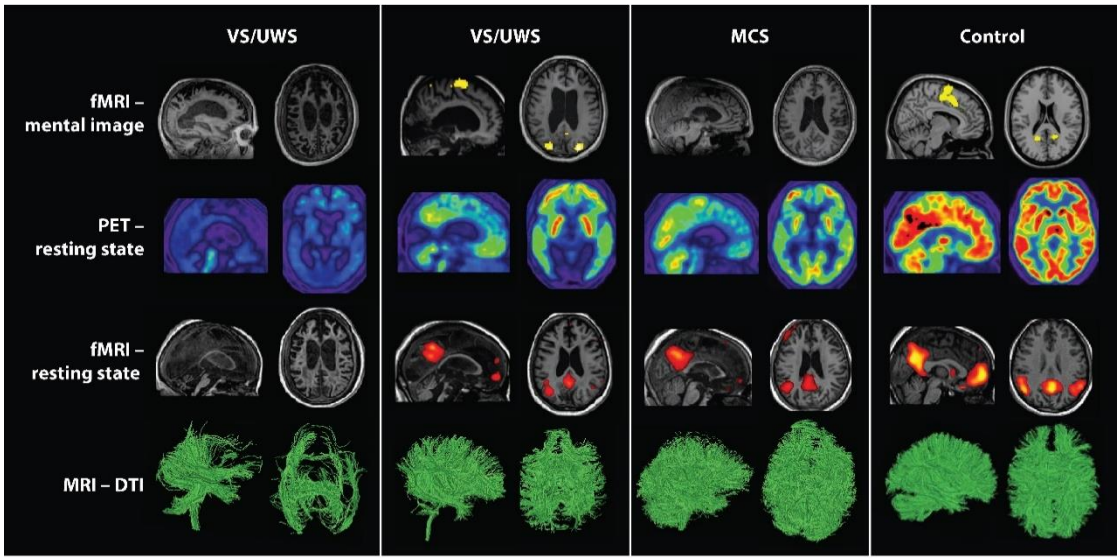

Fig. 4 Summary of neuroimaging results from different techniques and different states of consciousness. From Gosseries et al., 2014 with authors' permission. The first column shows results for a typical UWS patient, the second column shows results from a MCS* patient (behaviourally UWS), the third column a typical MCS patient and the last coloumn from a healthy subject. The first row shows results from the mental imagery fMRI task (Owen et al. 2008), showing activation in somatosensory cortices in the patient in MCS* and the control patient. The second row show results from FDG-PET brain metabolism, with a dramatic greater decrease in glucose metabolism in the patient in UWS compared to the patient in MCS and MCS* (that still show decreased metabolism compared to the control subject but not as dramatic). The third row shows results from fMRI resting state studies showing activation in the DMN (however smaller than control) for patients in MCS* and MCS. The last row results from structural DTI, showing greater white matter preservation for patients in MCS and MCS* compared to UWS.

1.7 Clinical care of patients with DoC

For an optimal care of patients with DoC, it is imperative to accurately evaluate the patient's level of consciousness. The reasons why diagnosis is so crucial to the treatment and prognosis of the patient are varied and most of them come from ethical issues. Firstly, several studies have showed that life expectancy and prognosis are different for patients in UWS compared to patients in MCS and this underlines why a correct diagnosis is crucial. Even if 78% of patients in UWS recovers consciousness within two years since the brain injury (McCrea et al. 2021) mortality rates between these patients and patients in MCS, as well as between aetiologies' categories, are very different. A recent review (Estraneo and Trojano 2018) showed that patients in UWS have slower recovery than patients in MCS and a higher proportion of mortality rates at 4, 6, 12, 18 and 24 months' post injury (See Figure 5 for details). Moreover, patients in UWS with traumatic aetiologies showed better

prognosis compared to patients in UWS resulting from anoxic and vascular injuries. Apart from diagnosis, other factors such as higher scores on behavioural scales and young age at baseline were found to be predictors of better prognosis (i.e., survival and/or clinical improvement at 2 years post-injury) (Estraneo et al. 2019).

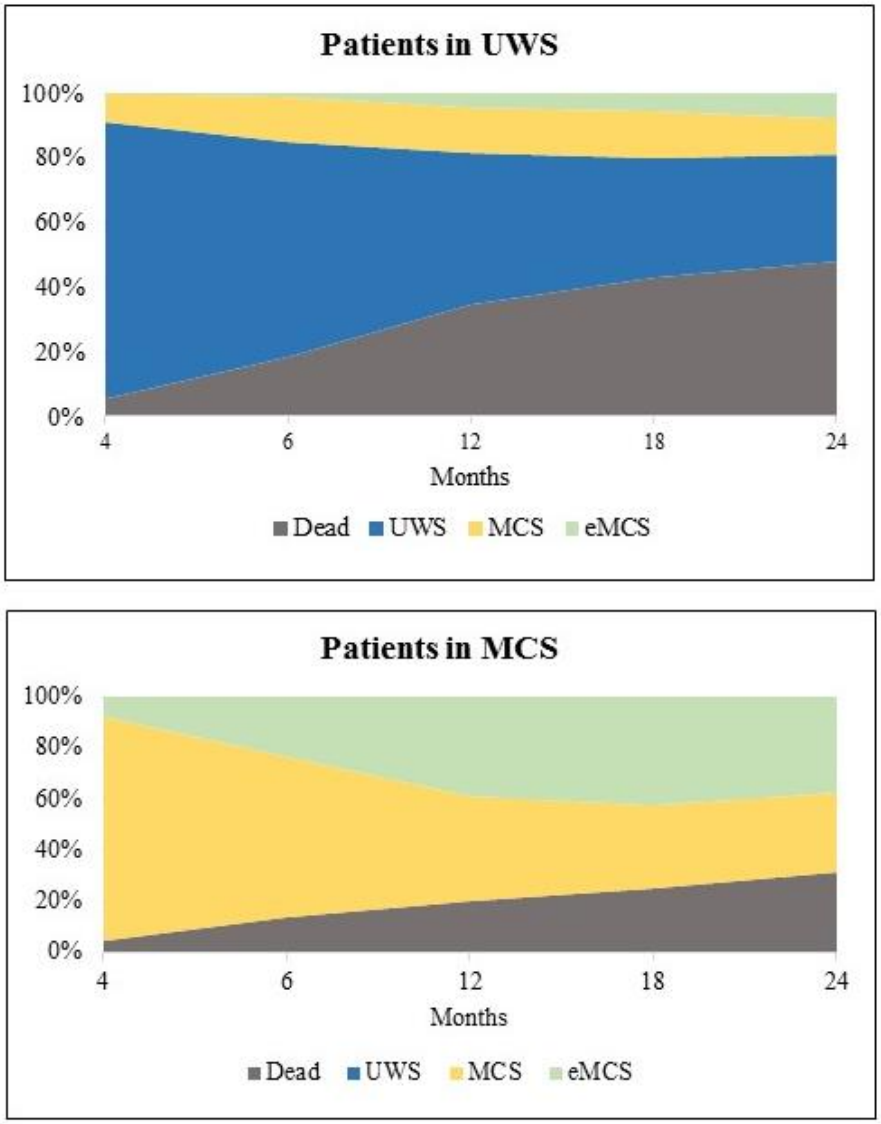

Fig. 5 Prognosis of UWS and MCS. Original figure created based on the data published by Estraneo and Trojano 2017, with the authors' permission. The two panels show different prognosis paths of 165 patients in UWS and 64 patients in MCS at 4, 6, 12, 18 and 24 months' post-injury. Note that differently from Estraneo and Trojano, here all patients are pooled together according to diagnosis, regardless of aetiology. Patients in MCS showed better outcome and faster recovery than patients in UWS. No patient in MCS regressed to a UWS and most emerged within the first year post injury. For patients in UWS patients with TBI showed overall better prognosis compared to anoxi and vascular. 78% of surviving patients in UWS recovered consciousness within 2 years but few emerged from MCS;

Furthermore, since the main difference between a patient in UWS and a patient in MCS is the presence of consciousness, this factor can change how healthcare workers perceive and interact with patients, as well as being crucial information for the patients' relatives and loved ones. In fact, studies have showed that clinicians have very different opinions regarding end-of-life decisions for patients in UWS compared to MCS. Demertzi and colleagues (Demertzi, et al. 2011) reported that two-thirds of the clinicians and researchers interviewed considered withdrawal of care for patients in UWS acceptable, whereas less than one-third considered it acceptable for patients in MCS. Moreover, when asked if they wished to be kept alive if they were in a UWS, 82% answered negatively while this was only 67% for being in an MCS. The discrepancy between the medical and the personal choices of the interviewees also gives a glimpse of how delicate it is for clinicians and researchers to navigate optimal care of patients with a DoC, from both a legal and an ethical perspective. Another reason for the importance of diagnosis of DoC that also pertains to the ethical sphere is pain perception. Some studies show that patients in MCS are likely to perceive pain, as suggested by brain activation of the pain matrix similar to that of healthy subjects. On the other hand, this activation seems to remain subcortical for patients in UWS (not reaching associative cortical areas) and we can, thus, hypothesize that the pain is not processed (Boly et al. 2005; Laureys et al. 2002). These results advocate for different analgesic treatments for these categories of patients. However, due to high misdiagnosis rates and other factors of uncertainty (such as the possibility of covert consciousness), it is still recommended to treat both patients in UWS and in MCS for pain (Fins and Bernat 2018).

Finally, another reason why accurate diagnosis of patients with DoC is important, is that recommended treatment options vary between patients in UWS and in MCS, especially when considering neuromodulation approaches (for more details, see the Section 1.8 of this introduction).

1.7.1 Behavioural assessment

The behavioural assessment of patients with neurological conditions relies heavily on communication with the patient and self-reported symptoms. However, patients with DoC are not capable of functional and reliable communication, except for patients in eMCS who are considered on the outside boundary of DoC. For this reason, an accurate diagnosis of patients with DoC is extremely challenging, as it relies solely on behavioural signs of

consciousness observed at the bedside and (when available) complementary information provided by neuroimaging exams. Even then, these behaviours can be easily missed due to many confounding factors such as fatigue, fluctuating arousal levels, aphasia, motor disabilities etc. A study performed in 2009 found that the rate of misdiagnosis of patients with DoC was up to 40% when patients were not evaluated with appropriate tools (Schnakers et al. 2009). A more recent similar study published in 2020 (Wang et al. 2020) confirmed the same percentage of misdiagnosis observed in 2009 (i.e., 38%). These numbers are still disturbingly high when taking into account the importance of accurate diagnosis. In the past years, several guidelines have been published to try to orient clinicians and researchers towards the best available practice to avoid misdiagnosis (Kondziella et al. 2020; Giacino et al. 2018). When all recommendations are followed, the rates of misdiagnosis are considerably lower, for instance, repetition of behavioural assessments can decrease diagnostic errors as low as 3% (Wannez et al. 2017).

The first necessary condition to establish accurate diagnoses of DoC is the use of a standardized scale validated for the assessment of levels of consciousness. Currently, there are several behavioural scales that are used for this purpose.

The Glasgow Coma Scale (GCS) has been validated in 1974 by Teasdale and Bennett (Teasdale and Jennett 1974a). It is still today the most used behavioural measure to assess the severity of brain injury and level of consciousness in acute-care settings (Teasdale et al. 2014). This scale is relatively simple and very rapid to perform, leading to its adoption into emergency and intensive care settings as well as daily clinical practice. The GCS includes three subscales: eye opening (evaluating arousal levels, with scores ranging from 1 to 4); motor functions (with scores ranging from 1 to 6) and verbal functions (ranging from 1 to 5). The total scores, thus, range from a minimum of 3 to a maximum of 15. Generally, scores up to 8 are used to define coma, scores from 9 to 12 indicate moderately severe brain injuries and scores from 13 to 15 mild brain injury (Jennett 1979). The relationship between GCS total scores and prognosis has been explored by many studies and, even though it appears there is indeed a prognostic value to the GCS, this behavioural tool presents several limitations (Bodart, Laureys, and Gosseries 2013). For instance, its inter-rater reliability is variable due to the administration and scoring of the scale not being procedurally standardized. Furthermore, the GCS does not assess certain behavioural signs of consciousness that we know now being among the first ones to be observed when patients recover consciousness (e.g., visual pursuit) (Martens et al. 2020).

The Full Outline of Unresponsiveness Scale (FOUR) was developed in 2005 by Wijdicks and colleagues (Wijdicks et al. 2005). The idea was to replace the GCS as the tool to assess level of consciousness in patients with severe brain injury. This scale measures eye response, motor response, brainstem reflexes and respiratory functions. Each subscale has scores ranging from 0 to 4, thus leading to total scores ranging from 0 to 16, where a score of 0 (implying absence of brainstem reflexes) corresponds to brain death. The FOUR has several advantages compared to the GCS. For instance, since it takes into account respiratory functions and does not assess verbal functions, it does not present confounding factors related to tracheostomy and intubation. Moreover, it measures visual pursuit (allowing to detect patients in a locked-in syndrome – LIS). It is also relatively rapid to administer, which is an important factor in intensive care settings.

The Wessex Head Injury Matrix (WHIM) was validated by Shiel and colleagues in 2000 (Shiel et al. 2000). It is composed of 62 items that are hierarchically ordered based on the recovery of behaviours observed in a sample of severely brain-injured patients. These items measure arousal level, attention, visual functions, memory, spatial orientation, temporal orientation, communication and social cognitive functions. The total score corresponds to the highest ranked behaviour observed in the patient. The WHIM was designed to detect small behavioural changes that denotes transition from UWS to MCS and EMCS. However, the administration of this scale is quite time-demanding and the diagnostic sensitivity is not very high (Seel et al. 2010).

The Sensory Modality Assessment Rehabilitation Technique (SMART) was developed in 1988 and then validated for patients with DoC (notably UWS) by Gill-Thwaites in 1997 (Gill-Thwaites 1997). It is a scale that measures all sensory functions (i.e., visual, tactile, auditory, olfactory, gustatory), communication, arousal levels and motor abilities. The idea was to provide a thorough evaluation of the patients' response to sensory stimulation allowing the assessor to detect subtle changes in patients' awareness. The scores assigned correspond to levels of responses that are hierarchical (ranging from level 1 – reflexive response, to level 5 - differentiating response). Even if the SMART has been assessed as a good tool to evaluate consciousness, it can take 1 hour or more to complete and is not free and available online but needs to be purchased following a specific training and application (Seel et al. 2010).

The Coma Recovery Scale – Revised (CRS-R) was developed, initially as the Coma Recovery Scale, by Giacino and colleagues in 1991. It was, then, revised in 2004 and validated as a standardized tool for the assessment of DoC (Giacino, Kalmar, and Whyte 2004). The CRS-R is currently recommended by both the European and the American guidelines for clinical practice of DoC patients. It is composed of 23 items, divided into 6 subscales. The subscales measure auditory functions (with scores ranging from 0 to 4), visual functions (with scores ranging from 0 to 5), motor functions (with scores ranging from 0 to 6), oromotor functions (with scores ranging from 0 to 3), communication abilities (with scores ranging from 0 to 2) and arousal levels (with scores ranging from 0 to 3). The scale also provides an additional guide for the assessment of brainstem reflexes. The items are organized hierarchically within the subscales, with the highest scores representing more complex behaviours that imply conscious awareness. Moreover, the presence of one item in a subscale is considered evidence of the presence of lower items in the same subscale. The biggest advantage of the CRS-R is its sensitivity to levels of consciousness, allowing accurate differential diagnosis of patients with DoC (Seel et al. 2010). All the items are clearly described in terms of administration and, when they are carried out by a trained professional, the CRS-R shows a high inter-rater reliability and stable scores across assessments. It is important to note that a recent study (Wannez et al. 2017) showed that repeated assessments with the CRS-R (i.e., at least 5) show a rate of diagnostic error as low as 3%, which is why all of the guidelines to clinical practice endorse at least five assessments for accurate diagnosis. One disadvantage of this scale is that it is highly time demanding and it requires training to be performed correctly. Another small limitation of the CRS-R is that its total score (ranging from 0 to 23) does not correspond to a specific diagnosis of DoC. The total score is calculated by adding the highest scores of each subscale and as such can be the result of multiple permutations. As an example, if a patient shows visual pursuit (score of 3 on the visual subscale, denoting at least an MCS-diagnosis) and only reflexes behaviours on the other subscales that sum up to 4 they will have a total score of 7. However, another patient showing only reflex behaviours and no sign of conscious awareness can also obtain a total score of 7. To overcome this issue a Modified Index of the CRS-R total score has been developed (Annen et al. 2019). This modified score, calculated translating each subscale score into a matrix, gives an output index between 0 and 99 and can accurately distinguish between UWS and MCS using the cutoff of 8.31.

The Simplified Evaluation of Consciousness (SECONDs) has been recently developed and validated by Aubinet and colleagues in 2021 (Aubinet et al. 2021). This scale was designed based on the CRS-R in order to overcome the CRS-R's issue of time of administration and need for training. The SECONDs is composed of 6 mandatory items (i.e., observation, command following, visual pursuit, visual fixation, oriented behaviours and arousal). Communication and localization to pain are performed conditionally, according to the type of response obtained in other items. This scale was validated on 57 patients and showed good reliability as well as consistency with the CRS-R. It is much faster to administer than the CRS-R (average of 7 minutes compared to average of 19 minutes) and it is accompanied by clear short instructions that can be consulted by the assessor on site without the need for training (Sanz et al. 2021). An additional index of the SECONDs, based on behaviour displayed, allows precise tracking of the patients' level of consciousness throughout time. All these features, make the SECONDs a valid competitor of the CRS-R, since it is tailored to be used in time-constrained settings such as intensive care units, thus ultimately reducing the risks of misdiagnosis. It is also a valid competitor to the GCS because, contrarily to this latter, it allows precise differential diagnosis of DoC.

All these behavioural tools to assess consciousness have a major drawback: that is relying on motor and language responses. However, as previously mentioned, patients with DoC often present comorbid disabilities such as aphasia, apraxia, spasticity, fluctuations in the level of arousal. For this reason, both the European and American guidelines on clinical practice of patients with DoC recommend complementing behavioural assessments with neuroimaging measures, in order to provide optimal diagnosis and care for patients. (For a summary of the behavioural scales presented see Table 1).

Scale	Structure	Total score	Advantages	Limitations
GCS	3 subscales (Eye opening, Motor, Verbal)	/15	• fast • well known • easy to administer	• not standardized • low inter-rater reliability • does not assess frequent MCS behaviours • low DoC diagnostic sensitivitiy • total score not linked to diagnosis
WHIM	62 items	/62	• hierarchical total score	• not tested inter/intra-rater reliability

			• assess MCS behaviours • standardized	• low DoC diagnostic sensitivitiy
SMART	7 domains, 29 modalities (items)	/5	• assesses every sensory modality • good DoC diagnostic sensitivity	• long • needs to be purchased after application and training
CRS-R	7 subscales, 23 items	/23	• standardized • good inter/intra-rater reliability • good DoC diagnostic sensitivity • most sensitive for MCS	• long • non-hierarchical total score • needs training • total score not linked to diagnosis
SECONDs	8 items	/8	• fast • standardized • good inter/intra-rater reliability • no training • total score linked to diagnosis	• no reflex assessment • less comprehensive

Table 1. Summary of the behavioural scales used to diagnose DoC. The table reports the structure of the scale (i.e., number of subscales and/or items); the total score range and the key advantages and limitations of each scale.

1.8 Treatments for patients with DoC

Patients with DoC represent a challenge for scientists and healthcare providers in terms of diagnosis, and consequently also in terms of prognosis and treatment. Indeed, even though specialists in this field have reached a better consensus on diagnosis in the last few years, therapeutic options remain limited and under-explored. Certainly, a key element to an optimal management of patients with DoC is therapy (both pharmacological and not) to improve the patients' condition and recovery. Several studies have been conducted in the past twenty years with the goal of testing therapies for DoC, both Randomized Controlled Trials (RCT) and open-label investigations. These studies, have not only found several promising treatment options for the treatment of patients with DoC, but have also given insight on the neural mechanisms underlying the recovery of consciousness. We will first describe the main pharmacological treatments explored in DoC research. Then we will focus on neuromodulation treatments (mainly on non-invasive stimulation techniques). Furthermore, we will provide a description of how the mesocircuit model succeeds in explaining the observed effectiveness of these treatments.

Pharmacological treatments

Several pharmacological trials have been successful in improving the functional recovery of patients with DoC. Dopaminergic drugs have been the main focus of research, since dopamine has an important role in the cortico-thalamic network, which is crucial for consciousness (Wisor et al. 2001). Specifically, dopamine seems to promote the recovery of consciousness by facilitating connections between the striatum and the globus pallidus (Schiff 2010). Among dopamine agonists drugs that have been tested in patients with DoC, the most effective, and also the only drug currently recommended by both the European and American guidelines, is amantadine. Amantadine was evaluated in a randomized two arms, placebo-controlled trial with a sample size of 184 sub-acute TBI patients (Giacino et al. 2013), unlike most other drugs that have been explored in mostly open-label studies. In this study, they found that the group of patients that received amantadine showed higher recovery rates (measured with the CRS-R and the Disability Rating Scale – DRS) compared to the patients that were in the placebo group. Another dopamine agonist that was reported to increase DoC patients' responsiveness in open label studies is apomorphine (Fridman et al. 2009). Since this drug has a relatively short half-life, better results have

been observed when administered continuously and subcutaneously (Fridman et al. 2010) and it is currently being tested in a randomized placebo-controlled trial with a larger sample size (Sanz et al. 2019). Interesting results also came from a double-blind crossover RCT, testing the effects of zolpidem in patients with DoC (Whyte et al. 2014). Zolpidem is a GABAergic agonist that is commonly used to facilitate sleep in healthy subjects, but can provoke paradoxical increases in arousal. In this study, they found that 5% of patients showed impressive behavioural improvements after taking zolpidem, such as the ability to walk, read and communicate. Other drugs have been tested in case studies and open-label studies but the lack of control and small sample sizes hinder our ability to understand their real effectiveness (Nardone et al. 2020; Carboncini et al. 2014; Matsuda et al. 2005; Martin and Whyte 2007). Finally, psychedelics have also been proposed as a potential treatment option for patients with DoC. Since it can increase EEG complexity in healthy subjects, it has been hypothesized that, with this mechanism, it could lead to enhanced responsiveness in patients with DoC (Scott and Carhart-Harris 2019).

Invasive neuromodulation treatments

Neuromodulation refers to a range of neuroscience techniques that apply electromagnetic and mechanical stimulations to a specific brain region in order to modify its spontaneous neural activation. These techniques can be sub-divided according to their level of invasiveness. Invasive brain stimulation techniques include Deep Brain Stimulation (DBS), Spinal Cord Stimulation (SCS) and Vagus Nerve Stimulation (VNS) among others.

DBS involves surgically implanting a small electrode that sends an electrical current in specific brain areas located usually deep inside the brain. In patients with DoC it commonly targets the intralaminar nuclei of the thalamus. DBS has been explored in several studies, but most of them were either single case or open-label and did not have a control condition, thereby limiting the conclusions that can be drawn. Most of these, reported behavioural improvements on the CRS-R with, sometimes, eMCS and a rate of 29% of patients responding to the stimulation (Chudy et al. 2018; Magrassi et al. 2016). One study, published by Schiff and colleagues, was the only double-blind crossover case study (Schiff et al. 2007). When the stimulator was on, the patient (who had been in a MCS for over 6 years) showed great behavioural improvement such as the ability to functionally communicate and oral feeding. These improvements disappeared when the stimulator was off and the patient reverted to his original state. DBS presents two major limitations. First,

the lack of proper control condition could inflate the rates of observed effectiveness. Second, the surgery required to implant the device is highly invasive and presents several hazards for patients with DoC, given their fragility.

Another invasive neuromodulation technique is SCS, that delivers a weak current to the spinal cord in order to reach the brain. A small electrode is implanted inside the spine, usually in the epidural space between the skin and the vertebrae. In patients with DoC the electrode is generally implanted between the C2 and C4 vertebrae. Most of the studies exploring SCS in patients with DoC so far, have not used any behavioural scale to assess improvement. A decrease in delta waves has been reported consistently in more than one study (Bai, Xia, Liang, et al. 2017; Bai, Xia, Li, et al. 2017). Gamma power modulation, an increase in alpha power and changes in prefrontal cerebral blood flow have also been reported (Zhang et al. 2018). One open-label study using the GCS report 7 out of 10 patients recovering from MCS and presenting a moderate disability after one year of SCS (Yamamoto et al. 2013). It is important to note that none of these studies had a control condition.

Finally, VNS targets the vagus nerve with a small electrode implanted subcutaneously near the neck, reaching the cervical part of the vagus nerve (for details on neuromodulation targeting the vagus nerve, see the following section). One case study using invasive VNS in a patient with UWS has been published in 2017 (Corazzol et al. 2017). This case report shows a significant behavioural improvement after appearing after the first month of stimulation, coupled with an increase in theta power.

Non-invasive Brain Stimulation treatments

Non-Invasive Brain Stimulation (NIBS) is a branch of neuromodulation that targets specific brain regions with electrical, magnetic or mechanical stimulation delivered on the skin, rather than directly on the nervous system, avoiding the need for invasive procedures. Within NIBS, some techniques have been investigated as treatments for patients with DoC: notably, rTMS, transcranial Electrical Stimulation (tES), transcutaneous auricular Vagus Nerve Stimulation (tVNS), Low-Intensity Focused Ultrasound (LIFU), Near-Infrared low-level Laser Therapy (N-LT) and Focused Shock Wave Therapy (F-SWT). We are going to first focus on the results obtained on rTMS and tES studies providing a detailed description of RCT studies and mechanisms of action. Then, we will summarize the findings of open-label studies using tVNS, LIFU, N-LT and F-WST. Note that tES is the primary focus of

the researches reported in this work. For this reason, we will describe its mechanisms of action and literature more thoroughly. (See Figure 6 for an overview of NIBS studies in DoC).

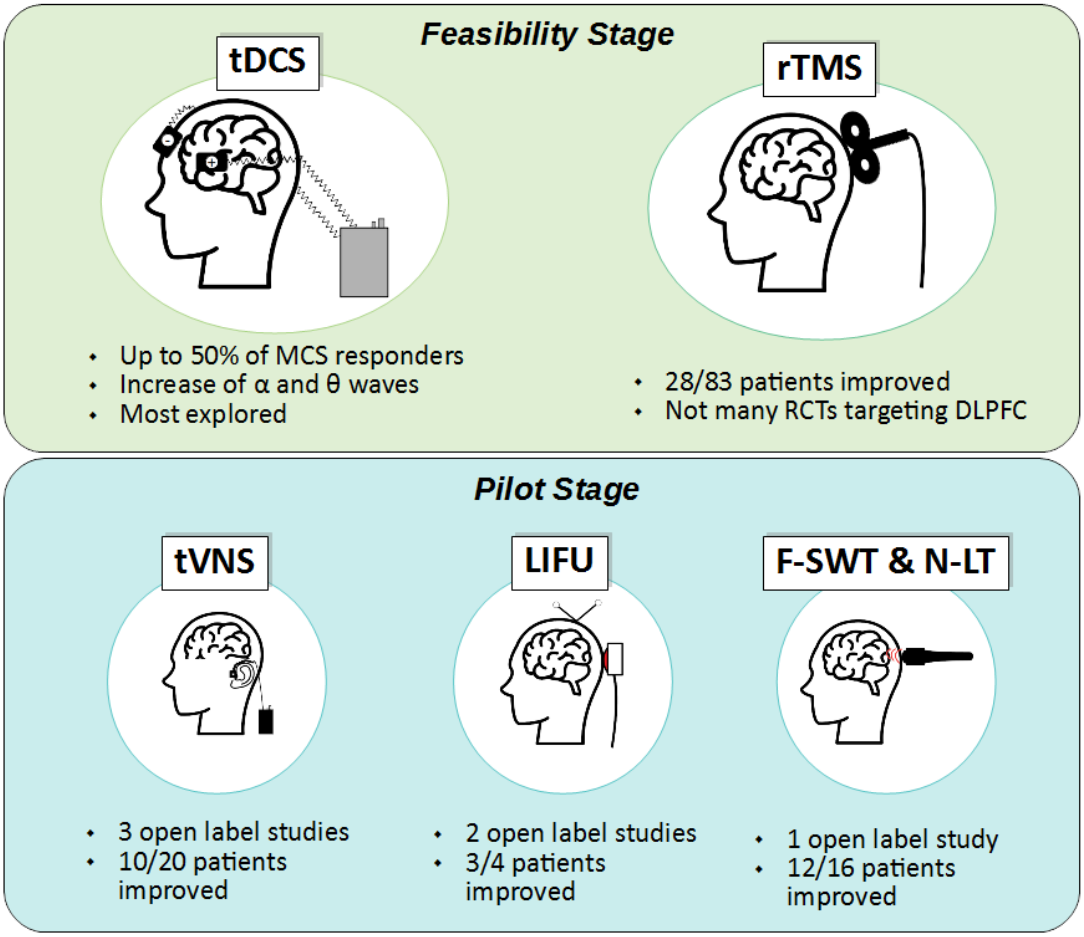

Fig. 6 Overview of NIBS studies in DoC. From Barra et al., 2022 with author's permission. This figure summarizes the existing literature on NIBS as a treatment for patients with DoC up to February 2022. tDCS and rTMS can be considered at the end of the feasibility stage of clinical evidence (C 1990) meaning that research is focused in confirming efficacy and assessing side-effects. For tVNS, LIFU, F-SWT and N-LT the sparsity of research makes them at the pilot stage of clinical evidence.

tES was the first NIBS technique to be studies in patients with DoC (Thibaut et al. 2014). This approach involves delivering a weak electrical current (usually between 1 and 2 mA) via electrodes placed on the scalp. tES can be further sub-categorized according to the type of current wave that is used (See Figure 7 for the different waveforms of tES). For instance, transcranial Direct Current Stimulation (tDCS) uses a squared continuous current; transcranial Random Noise Stimulation (tRNS) uses an alternate current with random amplitude and frequency; transcranial Alternating Current (tACS) uses a sinusoidal

alternate current with set frequency and amplitude and transcranial Pulsed Current Stimulation (tPCS) (a subcategory of tACS) uses a rectangular alternate current with set frequency and amplitude (Moreno-Duarte et al. 2014). Different types of current have different effects on brain tissues and slightly different mechanisms of action. One common denominator to the chemistry behind the effects of tES techniques is that they act on the neuronal membrane potential.

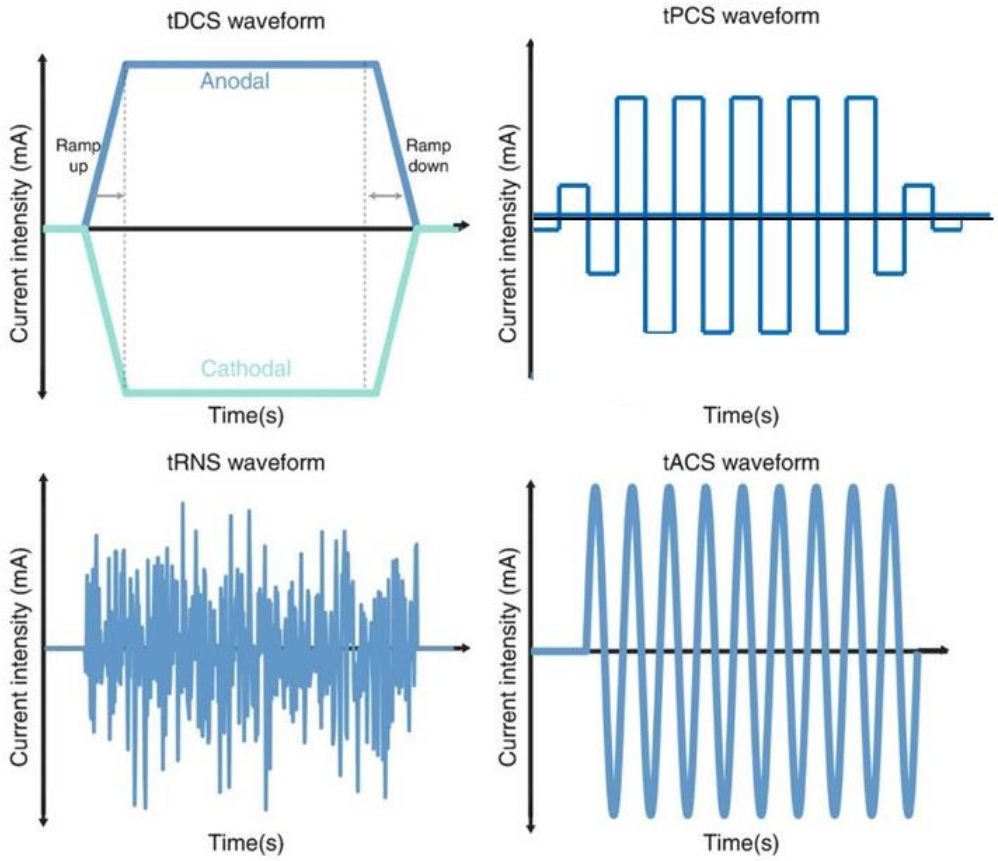

Fig.7 Waveforms of different types of tES. Adapted from Gebodh et al., 2019 with authors' permission. From left to right, top to bottom: Transcranial direct current stimulation, both anodal and cathodal, transcranial pulsed current stimulation, transcranial random noise stimulation and transcranial alternated current stimulation.

tDCS delivers a weak electrical current that is constant through at least two electrodes (i.e., anodal/positive electrode and cathodal/negative electrode). The current, passing through the anode and the cathode changes excitability of the neuronal membrane according to the direction of the current flow. If the current flows into the brain via the anode and exits it through the cathode the effect will be excitatory through membrane depolarization, thus lowering the threshold for action potential. On the other hand, if the direction is inverted it

will have inhibitory effects, through membrane hyperpolarization (Purpura and McMurtry 1965). These electrophysiological changes are immediate and act on the ion gated channels of the neurons that are involved in membrane polarization. The shift in membrane polarization, in turn, has effects on both firing rates (i.e., neuronal action potential threshold) and synaptic responsiveness to afferent stimuli (Nitsche et al. 2008). However, it is the long term effects of tDCS, lasting up to a few hours after the end of the stimulation, that are of interest for researchers that want to use tDCS as a therapeutic tool. These effects are associated with changes in the microenvironment between nerve cells, notably, by modifying synaptic strength of N-Methyl-D-Aspartate (NMDA) receptors or altering Gamma-AminoBuytric Acid (GABA) activity and modulating intra-cortical and cortico-spinal excitability in a way that resembles Long-Term Potentiation (LTP). The role of glutamatergic NMDA receptors in the after-effects of tDCS has been proved by the fact that, tDCS effects can be potentiated by adding NMDA agonists (Nitsche et al. 2004). Conversely, if NMDA antagonists were added, tDCS did not have any effect (Liebetanz et al. 2002). Note that most of the cited studies focused on anodal tDCS on the motor cortex and mechanisms might be different for other brain regions. To summarize, the effects of anodal tDCS depend on two separate but synergic mechanisms: the depolarization of ion gated synaptic channels and the facilitation of NMDA receptors induced probably by higher density of calcium within the intracellular space. Regarding the montages and parameters of tDCS, most set ups use two electrodes of 25 cm^2 on average, placed according to the target brain region. Current intensity varies between 1 and 2 mA and sessions do not last longer than 40 minutes usually. It has been shown that longer sessions can prolong tDCS effects (Stagg and Nitsche 2011), however stimulation duration of about 20 minutes is usually advised as longer stimulations may decrease and even reverse the observed effects (Hassanzahraee et al. 2020). A modelling study has found that most of the current delivered is absorbed by the skull and only up to 50% of it can reach the brain (Miranda, Lomarev, and Hallett 2006). Another modelling study (Ciechanski et al. 2018) showed that the current field of tDCS reaching the brain changed across ages, with higher concentration for younger patients, probably due to relatively lower skull thickness and amount of cerebrospinal fluid (CSF). Concerning the use of tDCS in healthy subjects and patients, several studies have showed that tDCS is a safe technique with little to no side effects (i.e., itching, skin redness, warm sensation) and can result in behavioural changes and improvements. For instance, Marshall and colleagues (Marshall et al. 2004) have showed that tDCS over DLPFC (anode on F3 or F4 according to the EEG 10-20 system

(Morley, Hill, and Kaditis 2016)) applied during sleep in healthy subjects, had positive effects on working memory. Always in healthy subjects prefrontal tDCS could also improve verbal fluency and procedural memory (Fregni et al. 2005; Iyer et al. 2005). In all these studies, effects disappeared after a few hours. To try to achieve longer lasting effects, some studies have tried daily stimulations for one or several weeks (Polanowska et al. 2013; Boggio et al. 2008; Antal et al. 2010). They have found that effects could be prolonged by increasing the number of sessions, to last up to 4 weeks after the end of the stimulation. The majority of studies on tDCS targeted either M1 or DLPFC. Usually, M1 is chosen when motor deficits want to be improved or when the objective is an improvement on motor tasks. The DLPFC, on the other hand, is the preferred target for more various tasks, as it is a region that controls executive functions, input from associative cortices, attention and arousal (Sturm and Willmes 2001; Heekeren et al. 2006; D'Esposito and Postle 2015). It comes as no surprise, thus, that the first tDCS target to be tested in patients with DoC was indeed the left DLPFC. Thibaut and colleagues have pioneered a whole field of research for patients with DoC, publishing a study on prefrontal tDCS in 2014 (Thibaut et al. 2014). Since then, the studies on this technique have multiplied and many montages and techniques have been tested. The most used target so far remains the DLPFC (Bai, Xia, Kang, et al. 2017; Bai et al. 2018; Carrière, Mortaheb, et al. 2020; Estraneo et al. 2017; Cavinato et al. 2019; Martens et al. 2018; Wu et al. 2019; Thibaut, Wannez, et al. 2017; Ye Zhang et al. 2017) and it appears that it produces the best behavioural results (Feng et al. 2020). Among the other brain targets, precuneus, M1 and the bilateral frontoparietal network have also been tested with tDCS (Huang et al. 2017; Thibaut, Piarulli, et al. 2019; Martens et al. 2020; Martens et al. 2019) but have showed minimal or no results. In tDCS studies to treat DoC, effectiveness is usually measured by an increase on the CRS-R total score after tDCS that is not observed neither before nor after the sham condition. Sometimes, however, authors report behavioural improvements referring to overall higher responsiveness, higher functional outcome or decreased spasticity, according to the study. In other cases, a patient is said to be a responder to tDCS when they demonstrate at least one new conscious behaviour after tDCS but not before nor after the sham (thus unrelated to total score but rather item-based). For instance, if a patient shows visual pursuit of objects after tDCS and visual pursuit was not observed before tDCS, nor before or after sham, they can be defined as responders to tDCS. The above mentioned studies have showed such behavioural improvements with effect sizes varying from 0.12 to 2.22 (i.e., Cohen's d). These effects, however, have been mostly observed at

the individual level and were not always significant at the group level. Also, it is important to note that most of the studies reporting behavioural improvement, observed it only on patients in MCS. However, two studies (Angelakis et al. 2014; Estraneo et al. 2017) found effects of tDCS in patients in UWS as well, even if in fewer patients compared to MCS. Regarding neurophysiological effects of tES in patients with DoC, changes in EEG occur most frequently when behavioural improvement is also observed. However, in two studies, EEG changes appeared in the absence of significant clinical improvement at the group level (Naro, Russo, et al. 2016; Carrière, Mortaheb, et al. 2020). It is important to note that effects of tES on EEG often differ between patients in MCS and patients in UWS, giving us insight on the reason behind the observed differences in rates of responders between these two categories (Xia, Liu, et al. 2017; Angelakis et al. 2014; Cavinato et al. 2019). EEG results have showed that tES decreases delta power in patients with DoC, regardless of diagnosis. Theta band power, on the contrary, was found to be increased after stimulation in several studies (Bai, Xia, Kang, et al. 2017; Naro, Russo, et al. 2016; Carrière, et al. 2020). Alpha power was also reported to increase after tES, in central (Carrière et al. 2020) and posterior sites (Cavinato et al. 2019). Bigger P300 frontal responses were found after tDCS, for patients in MCS (Ye Zhang et al. 2017). Apart from EEG, tDCS has also showed to increase fronto-parietal coherence, theta band coherence and inter-hemispheric connectivity (Bai et al. 2018; Cavinato et al. 2019; Carrière et al. 2020). Using TMS-EEG, Bai and colleagues found that tDCS increased global mean field potential within the 0-100 ms time window for both MCS and VS/UWS patients. However, while this increase in excitability was observed in all cerebral regions for MCS patients, it remained under the stimulation sites for VS/UWS patients (followed by a decrease in the 300-400 ms time window) (Bai, Xia, Kang, et al. 2017). This result is consistent with the differences in TMS-EEG evoked potentials between UWS and MCS patients mentioned before. Finally, another recent study exploring tDCS with TMS-EEG (Mensen et al. 2020) found that, although behavioural improvement was not observed, tDCS reduced slow-wave activity (i.e., delta power). When tDCS produced clinical improvements at the individual level, together with decreased delta power also an increase in high frequencies was observed in TMS-EEG evokes responses. Overall, tES has showed several positive results, evidencing its potential as a treatment for patients with DoC. So far, the subcategory of DoC patients in MCS seem to benefit more from the stimulation, with response rates as high as 50%.

tACS consists in delivering an alternating current to the brain. The current is pulsed and can be either rectangular (i.e., tPCS) or sinusoidal (i.e., tACS). In tPCS, the current is held at a certain amplitude for a short time, then interrupted and then the process is repeated againfor the whole duration of stimulation. On the other hand, in tACS, a sinusoidal wave that increases and decreases at repeated times (i.e., biphasic sine waves) is delivered (Zaghi et al. 2010). The results obtained in tACS studies have helped to develop some hypothesis on its mechanisms of action. However, these hypotheses have yet to be confirmed, as the use of this type of tES is even more recent than tDCS. For instance, Kanai and colleagues found that according to the frequency of tACS used, the effects of phosphene induction also changed (Kanai et al. 2008). To support this idea that the effects observed with tACS are dependent on the frequency and duration of the stimulation, Antal and colleagues (Chaieb et al. 2008), tested tACS at different frequencies (i.e., 1, 10, 15, 30, 40 Hz). They have found that stimulating at 10Hz improved implicit motor learning, even after only 5 minutes of stimulation, whereas the other frequencies did not produce this effect. In this study, the authors also report not finding any effect in cortical excitability as measured by TMS-evoked potentials. However, in another study using the same stimulation protocol they report finding increased TMS-evoked potentials' amplitude. The results on tACS are so far controversial and it is difficult to draw clear conclusions. However, using this technique, behavioural results together with electrophysiological results have been observed (Brittain et al. 2013; Alexander et al. 2019). It has been hypothesized that tACS effects are associated with endorphin release, changes in neurotransmitters such as urinary free catecholamines and an increase in released synaptic vesicles (Kirsch and Smith 2004; Briones and Rosenthal 1973). These results altogether suggest that tACS techniques play a role in neurotransmitter release (Moreno-Duarte et al. 2014; Zaghi et al. 2010). One study using tACS as a treatment for patients with DoC has been published in 2015 (Naro, Russo, et al. 2016). This study, targeting the cerebellum with tACS at 5Hz found an increase in theta power and coherence for patients in MCS after tACS, that was coupled with an increase in motor scores in the CRS-R.

tPCS is considered as part of tACS, thus supposedly relying on similar mechanisms, though not many studies on the chemistry behind its specific effects have been conducted. Several modeling studies have showed, however, that tPCS can reach subcortical structures when applied at intensities between 0.2-0.6 V/m (Datta et al. 2013; Guleyupoglu et al. 2013). Another hypothesis is that, since tPCS is usually applied over the mastoid bones,

the zygomatic arches etc., it might also rely on peripheral nerve stimulation, thus explaining the ability of tPCS to reach subcortical structures (Moreno-Duarte et al. 2014; Zaghi et al. 2010). Some studies have explored the effects of tPCS in neurological conditions and healthy subjects. For instance, it was found that in could enhance high-complexity cognitive functions, such as arithmetic tasks, speech comprehension and fine motor skills (Ruhnau et al. 2020; Morales-Quezada, Cosmo, et al. 2015; Miniussi, Harris, and Ruzzoli 2013). (Note that no study using tPCS as a treatment for patients with DoC has been published prior to Study 2 presented in this dissertation).

Another tES approach is tRNS, that uses an alternating current with random amplitude and frequency between 0.1 Hz and 640 Hz. The observed cortical excitability resulting from tRNS (Chaieb, Paulus, and Antal 2011) has been hypothesized to depend on the repeated opening of sodium channels (Paulus 2011). However, more studies are required to confirm this hypothesis. High frequency tRNS (i.e., between 100 and 640 Hz) has been found to enhance performance in visual learning tasks and to improve neuroplasticity. A long-term improvement in arithmetic learning was also observed after bilateral DLPFC tRNS, together with enhanced neural coupling efficiency that was hypothesized to facilitate neuroplasticity (Snowball et al. 2013). One study published in 2017 (Mancuso et al. 2017), explored tRNS targeting bilateral DLPFC in sub-acute patients in UWS, however did not result in any behavioural nor neurophysiological effect.

rTMS is another NIBS approach that has been widely explored as a treatment for patients with DoC. TMS involves the use of a coil placed on the scalp. The coil produces short magnetic pulses that generate an electrical current, perpendicular to the coil, that passes through the skull and reaches the brain in areas located beneath the coil. This technique can have varied parameters of stimulation. Pulsed TMS (single or paired) can induce neuronal action potential, as it delivers a current that is supra-threshold. rTMS, on the other hand, delivers below-threshold current that can induce changes in the excitability of the neuronal membranes. In this section we will describe rTMS as a treatment for patients with DoC, in opposition to the previously mentiond TMS-EEG technique, that uses single TMS pulses to probe the brain, mainly for diagnostic purposes. rTMS is already standard practice to treat some psychiatric and neurological conditions such as depression and schizophrenia (Dell'Osso et al. 2011; Fitzgerald and Daskalakis 2008). It is generally used when patients are resistant to pharmacological treatments or are allergic to certain drugs. rTMS is relatively accessible even though the device is not easily transportable and

requires trained professionals to be employed. It also allows for a good sham (i.e., placebo) condition that creates perceived effects for the subjects similar to those of the real stimulation. Furthermore, rTMS is also a safe technique, with side effects rarely observed and generally limited to headaches, discomfort (in very rare cases, generalized seizures have also been observed). For these reasons, rTMS is a very appealing technique for researchers. Compared to tES, not many studies have been performed using rTMS in patients with DoC, especially RCTs. However, literature on this technique is much more extended than for the NIBS techniques that we will describe later. Most RCT studies using rTMS, have targeted the primary motor cortex (M1) and recently, the left dorsolateral prefrontal cortex (DLPFC) has also been studied (Cincotta et al. 2015; He et al. 2018; Liu et al. 2016; Liu et al. 2018; Zhang et al. 2021; R. H. He et al. 2021). Open-label and case studies have also targeted the anterior cingulate cortex (ACC), the angular gyrus and the DLPFC (Xia, Liu, et al. 2017; Naro et al. 2015; Xie and Zhang 2012; Xia, Bai, et al. 2017). When taking into account rTMS studies targeting M1, only 2 out of 34 total patients were reported to show behavioural improvement. On the other hand, the two recent RCT studies targeting the DLPFC reported 28 out of 83 patients as responders. In open-label studies targeting the DLPFC, also some significant behavioural improvements were observed. Note that Xia and colleagues observed behavioural improvement not only on patients in MCS but also in UWS, which is not the case for tES studies, as we will see later. When targeting DLPFC, rTMS also produced a significant decrease in low frequency bands and an increase in alpha and beta power over frontoparietal regions (Xie and Zhang 2012; Xia, Liu, et al. 2017; F. He et al. 2018). Finally, Naro and colleagues, showed that targeting ACC resulted in restoration of cortico-cortical interactions and improvements in several TMS-evoked components. However, no behavioural measure was used in this study and we cannot make assumptions on its effectiveness. We recently conducted a review of the literature of NIBS as a treatment for patients with DoC and a summary of all tES and rTMS RCTs published between January 2002 and February 2022 can be found in Table 2.

Table 1. Details on RCTs using rTMS and tDCS

Authors	NIBS	Study design	N patients (etiology)	TSI	Procedure	Results	Study caveats
Bai et al., 2017	tDCS	Sham controlled, crossover	16 9 UWS 7 MCS	13.1 months (±9.2)	Single session (20min) active and sham stimulation over	tDCS increased global excitability for both MCS and UWS but different time	No blinding

			(4 TBI,12 non-TBI)	(mean, SD)	left DLPFC. CRS-R and TMS-EEG (to calculate GMFA- cortical excitability) measured before and after each session	windows: 0-100 ms for MCS in all regions. For UWS, after initial increase decreased at 300-400ms and changes remained localised	
Bai et al., 2017	tDCS	Sham controlled, crossover	17 9 UWS 8 MCS (5 TBI, 12 non-TBI)	12 months (± 8) (mean, SD)	Single session (20min) active and sham stimulation over left DLPFC. CRS-R and EEG measured before and after each session	No significant behavioral improvement. For MCS, increase in frontoparietal coherence, significant increase in theta band and significant decrease in delta band	No blinding
Carrière et al., 2020	tDCS	Double blind, sham controlled, crossover	11 10 MCS 1 eMCS (3 TBI, 8 non-TBI)	5 months (3-25) (median, IQR)	Single session (20min) active and sham stimulation over left DLPFC. CRS-R and EEG measured before and after each session	No significant behavioral effect (3/11 improved). EEG increased power in alpha central and theta frontal and posterior. Higher fronto-parietal and left to right parietal connectivity	Small sample
Cavinato et al., 2018	tDCS	Double blind, sham controlled, crossover	24 12 UWS 12 MCS 6 eMCS (9 TBI, 14 non-TBI)	25 months (8.7-65.5) (median, IQR)	10 sessions (1xday, 5xweek, 2 weeks, 20min) active and sham stimulation over left DLPFC. CRS-R, WNSSP and EEG measured before and after each session	Significant increase in WNSSP for MCS patients. no change in CRS-R. Increase in power and coherence for alpha and beta frontal and parietal for MCS. Higher delta for UWS	
Estraneo et al., 2017	tDCS	Double blind, sham controlled, crossover	13 7 UWS 6 MCS (1 TBI, 12 non-TBI)	> 3months	5 sessions (1xday, 5xweek, 20min) active and sham stimulation over left DLPFC. CRS-R and EEG measured at baseline, at day 5 and up to 3-month follow-up	No significant behavioral or EEG change. But 5/13 patients (3 in MCS and 2 in UWS) improved on CRS-R total score and EEG	Small sample, carry-over effect
Huang et al., 2017	tDCS	Double blind, sham controlled, crossover	33 33 MCS (20 TBI, 13 non-TBI)	6 months (± 5) (mean, SD)	5 sessions (1xday, 5xweek, 20min) active and sham stimulation over precuneus. CRS-R, measured at baseline, at day 5 of tDCS and 10 days follow-up	Significant behavioral improvement on the CRS_R (9/33 of the sample). The effects did not last at follow-up	
Mancuso et al., 2017	tRNS	Double blind, sham	9 9 UWS	1.4 months (±1)	5 sessions (1xday, 5xweek, 20min, 101-640 Hz) active and sham	No effect in CRS-R or CGI-I, one patient emerged from UWS at week 3 but no clear	Subacute small sample,

		controlled, crossover	(1 TBI, 8 non-TBI)	(mean, SD)	stimulation over bilateral DLPFC. CRS-R, EEG and CGI-I measured before cycle, each day of stimulation and at day 15 follow-up	parameter to explain recovery. Non-significant trend of increased arousal (based on CRS-R) at day 4 of stimulation	only UWS
Martens et al., 2018	tDCS	Double blind, sham controlled, crossover	27 12 MCS (12 TBI, 15 non-TBI)	10 months to 14 years	20 sessions (1xday, 5xweek, 20min) active and sham stimulation over left DLPFC separated by 8 weeks. CRS-R, measured at baseline, at week 4 and at week 8 follow-up	No significant behavioral improvement. subsample that received at least 80% of the stimulation sessions show an increase in CRS-R total scores. No difference between active and sham tDCS was found at 8-week follow-up	Heterogeneous TSI, drop-out, few assessments
Martens et al.,2019	tDCS	Double blind, sham controlled, crossover	10 4 UWS 6 MCS (5TBI, 5 non-TBI)	7 months (±13) (mean, SD)	Single session (20min) active and sham stimulation over left M1. CRS-R measured before and after each session	No significant behavioral improvement on the CRS-R, no improvement on the motor subscale. One patient 1/10 improved	Small sample
Martens et al., 2020	tDCS	Double blind, sham controlled, crossover	46 17 UWS 23 MCS 6 eMCS (22 TBI, 24 non-TBI)	12 months (5-47) (median, IQR)	Single session (20min) active and sham stimulation over bilateral frontoparietal network. CRS-R and EEG measured before and after each session	No significant behavioral improvement (5/46 responders). CRS-R improvement associated with lower baseline complexity in 1-8 Hz bands. Active tDCS significantly increased EEG complexity in 1-8 Hz	Heterogeneous TSI sample
Min Wu et al., 2019	tDCS	Double blind, sham controlled, between group	16 9 UWS 7 MCS (6 TBI, 10 non-TBI)	149 months (±144) (mean, SD)	3 arm design comparing 10 sessions (5xweek) of tDCS over left DLPFC, right DLPFC and sham. CRS-R, GOS-E and EEG measured at start and end of stimulation cycle and 3 months follow-up	No significant behavioral improvement, 1/16 responded to left DLPFC (improved GOS-E). Trend towards higher functional connectivity between DLPFC and other regions for left DLPFC tDCS. Right DLPFC only increased activation under stimulated sites	Very chronic, small sample, no blinding
Thibaut, et al., 2014	tDCS	Double blind, sham controlled, crossover	55 25 UWS 30 MCS	1 week to 19 years	Single session (20min) active and sham stimulation over left DLPFC. CRS-R measured	13/30 patients in MCS and 2/25 patients in UWS improved on CRS-R. At the group level, significant clinical improvement	No follow-up

			(25 TBI, 30 non-TBI)		before and after each session	(2 points on the CRS-R) for MCS patients	
Thibaut et al., 2017	tDCS	Double blind, sham controlled, crossover	16 16 MCS (11 TBI, 5 non-TBI)	> 3months	Five sessions (1xday, 5xweek, 20min) active and sham stimulation over left DLPFC. CRS-R, measured at baseline, at day 5 of tDCS and 1 week follow-up	9/16 responders. Significant clinical improvement (2 points on the CRS-R) maintained up to one week after the end of the stimulation	Short washout period
Thibaut et al., 2019	tDCS	Double blind, sham controlled, crossover	14 14 MCS (6 TBI, 8 non-TBI)	30 months (±32) (mean, SD)	Single session (20min) active and sham stimulation over bilateral DLPFC. CRS-R, MAS and EEG collected before and after tDCS	No significant behavioral improvement in CRS-R. Spasticity was reduced in finger flexors, 4/14 showed reduced hypertonicity. Responders showed higher theta and beta power	Single session, small sample
Zhang et al., 2017	tDCS	Double-blind, sham controlled, parallel	26 11 UWS 16 MCS (12 TBI, 14 non-TBI)	1 to 18 months	20 sessions (2xday, 5xweek, 20min) active and sham stimulation over left DLPFC. CRS-R, measured at baseline, at week 4 and at week 8 follow-up	Significant behavioral improvement (CRS-R) in MCS, together with increase in P300 amplitude	Heterogeneous population, lack of follow-up
Cincotta et al., 2015	rTMS	Double blind, sham controlled, crossover	11 11 UWS (2 TBI, 9 non-TBI)	35.4 month (mean)	5 sessions (1xday, 5xweek, 20min) active and sham stimulation over left DLPFC. CRS-R, CGI-I and EEG measured before, after the first session, the 5th session, 1 week later and 1 month later	No behavioral effect. No detectable EEG change.	Only UWS
He et al., 2018	rTMS	Double blind, sham controlled, crossover	6 3 UWS 2 MCS 1 eMCS (4 TBI, 2 non-TBI)	1 to 28 months	5 sessions (1xday, 5xweek, 20min) active rTMS and sham stimulation, 20-Hz over M1. CRS-R scores and EEG measured at baseline, at day 5 and 1-week follow-up	No significant behavioral or EEG change. 1/6 responder (from UWS to MCS-) improved on motor scale of CRS-R and showed increased alpha and beta power	Sample size, subacute patients
Liu et al., 2016	rTMS	Double blind, sham controlled, crossover	10 5 UWS 5 MCS (4 TBI, 6 non-TBI)	1 to 28 months	Single session (20min) active and sham stimulation, 20 Hz over M1. CRS-R and CBF velocity of the MCA	No behavioral CRS-R changes, increase in peak systolic velocity and mean flow velocity of the left Middle Cerebral Artery for MCS only, changes were temporally related to	One session of stimulation. Small sample

					measured before and after rTMS	the rTMS. No effects in UWS or in sham group	
Liu et al., 2018	rTMS	Double blind, sham controlled, crossover	7 2 UWS 5 MCS (5 TBI, 2 non-TBI)	3.1 months (±1.8) (mean, SD)	5 sessions (1xday, 5xweek) active and sham 20-Hz stimulation over M1. CRS-R scores, EEG and fMRI measured at day 0, day 5 and day 7 of the stimulation cycles	No significant behavioral improvement (1/7 responded), nor effect on functional connectivity but trend to enhance in nodes LPC, ITC and left DLPFC	Small sample, subacute patients
He RH et al., 2021	rTMS	sham controlled, two arms	50 36 UWS 14 MCS (14 TBI, 36 non-TBI)	86.1 days (±16.7)	10 sessions (1xday, 5xweek) active or sham 10-Hz stimulation over left DLPFC. CRS-R scores and plasma (for hormonal test) measured at day 0 and day 10 of stimulation	Significant change in CRS-R between groups 14/25 responders,significant changes in estradiol levels between groups (34 vs 23)	No blinding, no crossover
Zhang et al., 2021	rTMS	sham controlled, two arms	48 48 UWS	Not reported but longer than 3 months	Repeated sessions active or sham 5-Hz stimulation over left DLPFC. CRS-R scores, EEG and BAEP measured at day 0, day 30 and day 60 of stimulation	Significant changes in CRS-R between groups (at 60 days 12/24 responder, of which 5 became MCS), significant changes in EEG and BAEP	No blinding, no crossover, lack of protocol details

Table 2. Overview of all NIBS RCTs published between January 2002 and February 2022. From Barra et al., 2022 Accepted in Seminars of Neurology, with authors' permission. We searched PubMed for articles published in English using the following search terms: Persistent Vegetative State, Consciousness Disorders, UWS, MCS, minimally conscious state, Magnetic Field Therapy, Electric Stimulation Therapy, Ultrasonic Therapy, tDCS, otDCS, tRNS, tACS, tVNS, wave therapy, rTMS, transcranial current stimulation, transcranial magnetic stimulation, tAVNS, focused Ultrasound, vagus nerve stimulation, neuromodulation, non-invasive brain stimulation, shockwave therapy. Note that only tES and rTMS were explored in RCTs and are included in this table.

Regarding less explored NIBS approaches, tVNS uses a surface electrode placed on the auricular branch of cervical branch of the vagus nerve (i.e., generally on the outer ear cymba conchae). The electrode stimulates the nerve fibers of the vagus nerve with an electrical current and sends afferent vagal input to the brain, via the thick-myelinated auricular fibers of this nerve. This is a non-invasive technique to stimulate the vagus nerve that, contrarily to the surgical VNS, does not require implanting the electrode inside the skin (Ellrich 2019). The safety of tVNS has been explored in studies on healthy subjects and on patients with depression. Some very mild side effects have been observed (i.e.,

tingling, skin redness, itching) and less than 1% of study populations have reported symptoms like nausea and vomiting and dizziness (Busch et al. 2013; Schulz-Stübner and Kehl 2011; Silberstein et al. 2016). However, since research on tVNS is still quite recent it is hard to say if these side effects were a consequence of a specific dose of stimulation or the stimulation itself. tVNS is already used to treat patients with epilepsy and depression (Johnson and Wilson 2018), however, research on its effectiveness in patients with DoC is still recent and sparse. Three studies have explored tVNS in patients with DoC (i.e., one case report and two open label studies (Hakon et al. 2020; Noé et al. 2020; He et al. 2017)) and no RCT has been conducted yet. All three studies used repeated sessions, between 4 and 8 weeks of stimulation, from 1 hour to 4 hours per day. The single case study reported behavioural improvements on the motor and oromotor CRS-R subscales, observed at the end of 4 weeks of treatment. They also observed, with MRI, an increase in brain activity in the thalamus and precuneus and a decrease in functional connectivity between these regions and the cerebellum. However, the patient was 50 days post-injury at inclusion, thus, we cannot exclude that spontaneous recovery caused this behavioural improvement. Noé and colleagues (Noe et al. 2020) found that 5 out of 14 patients (i.e., N= 6 UWS, 8 MCS) showed behavioural improvements after 4 weeks of tVNS. All the responders were MCS patients. Finally, Hakon and colleagues (Hakon et al. 2020) using higher doses of stimulation, compared to the other studies, and found that 4 out of 5 patients improved on the CRS-R. Note that among these 4 patients, three transitioned from MCS to eMCS and one from UWS to MCS. Given these results, Briand and colleagues have proposed a pathway model for the mechanisms of action of tVNS (Briand et al. 2020). They hypothesized that stimulating the vagus nerve leads to activation of the lower brainstem (via the trigeminal nucleus and the nucleus solitaris), which in turn activates the upper brainstem (via the locus coeruleus and the raphe nuclei). Then, activation of these two regions in the upper brainstem cause the activation of other parts of the brain with neurotransmitters' modulation. Specifically, the locus coeruleus would activate globally the brain through norepinephrine modulation and the raphe nuclei would activate the limbic system (of which the thalamus is part of) and the frontal cortex via serotonin modulation.

Another technique, LIFU, uses low-intensity ultrasound waves (i.e., sonications) delivered to the brain via a transducer that can increase or decrease excitability of sodium and calcium voltage-gated channels. The brain target is generally calculated with the help of

MRI-guided neuro-navigation. LIFU can reach and stimulate deep brain regions, such as the thalamus, which is crucial for consciousness recovery as shown in the seminal paper by Schiff using DBS (Schiff et al. 2007). The exact reach depends on several factors such as settings, depth of focus, skull thickness and the device used (Cain, Visagan, et al. 2021). When aiming at the thalamus (which is the conventional target of this technique for patients with DoC) the maximal area of stimulation is approximately 0.5 cm radially. Mechanical agitation of particles is hypothesized to be the primary mechanism of action of LIFU, given the low intensities of the sonications of its use as a treatment (as opposed to non-invasive surgery that uses higher intensities and can create irreversible changes) (Pasquinelli et al. 2019). Consistent with this hypothesis of reversible mechanical agitation, LIFU does not appear to produce side effects in adults, this is why it is very appealing for researchers. Moreover, compatibility with neuroimaging techniques and ability to reach deeper brain structures add to its appeal. The study that paved the way for LIFU research as a treatment for DoC, was conducted on rodents and found that it could accelerate the process of responsiveness recovery from anaesthesia (Yooa et al. 2011). Since then, two open-label studies have been conducted in patients with DoC (Monti et al. 2016; Cain, Spivak, et al. 2021). Monti and colleagues applied a single session of sonication and found that the patients could functionally communicate and walk after the stimulation. However, since no control condition or blinding was present and the patient was only 19 days post-injury we cannot exclude spontaneous recovery. Cain and colleagues applied LIFU on 3 chronic patients with DoC and also found behavioural improvement in 2 out of 3 patients. This improvement increased linearly with the number of sessions and no side effects were observed. Since the patients in this study were suffering from more chronic DoC, we can hypothesize spontaneous recovery as less probable. However, lack of blinding and control condition are still an issue with this study.

One study (Cordula Werner, Byhahn 2016), used F-SWT and N-LT and compared them as treatments to DoC. These techniques are even less researched than LIFU and TVNS (that present already sparse literature). F-SWT delivers shockwaves on the scalp, targeting brain areas. The shockwaves are single, focused high intensity acoustic pulses that can reach the cellular level and produce energy changes within the stimulated area. The energy change is produced by both mechanical agitation and heating of the tissues, increasing (or decreasing) the excitability of the neurons. Prior to Cordula Werner and colleagues' study (Cordula Werner, Byhahn 2016), one open-label study had been conducted with F-SWT

on 5 patients with DoC (Lohse-Busch, Reime, and Falland 2013). They reported an improvement of 135% behavioural improvement on the CRS-R and, by the end of the treatment, all patients could intentionally communicate and be oral fed. However, not only did this study not use a control nor blinding, but the protocol used is also described very vaguely, thus, we do not know the exact parameters and length of stimulation. N-LT uses near-infrared laser light to reach the brain through the scalp. The type of laser can be red or infrared, continuous or pulsed and can increase or decrease neuronal membrane excitability with the interaction between the photon particles of the laser and the ionic particles in the neural channels. One case report has been published using N-LT on a patient with akinetic mutism and a patient with chronic DoC (Hesse, Schattat, and Werner 2014). Behavioural improvement was reported after 6 weeks of N-LT for the patient in UWS. However, not much detail about the improvement is given and the patient had to drop out of the study due to a seizure caused by pre-existing epilepsy. Finally, when comparing NLT and FSWT on 16 patients in a two arms design, Werner Cordula and colleagues (Cordula Werner, Byhahn 2016) found improvements on 12 patients on the CRS-R, SMART and FOUR. Note that one patient with no prior history of epilepsy experienced a seizure following F-SWT and had to drop out. Overall, these results are interesting and could be the start for exciting research. However, to make any assumption on these techniques' effectiveness, more RCTs are needed.

The meso-circuit model to explain treatments of DoC

The meso-circuit model has been the rationale for many researchers to explain the observed effects of both pharmacological and neuromodulation treatments of DoC. For instance, amantadine acts on dopaminergic modulation of associative cortices, and can facilitate the activity of both the frontal cortex and the striatum. In a case report PET study, it has been observed that the patients showed indeed higher metabolic activity in fronto-parietal regions (Schnakers, Hustinx, et al. 2008). This same mechanism can also be applied to other dopaminergic drugs such as apomorphine (Fridman et al. 2009). On the other hand, Zolpidem is a GABAergic agent, thus it is more likely to act on the globus pallidus (that is a brain hub for GABA release). By inhibiting the globus pallidus, according to the meso-circuit, it can increase thalamic activity. Regarding NIBS treatments, both tDCS and rTMS over the DLPFC might help stimulating cortico-thalamic pathways to the thalamus and to the striatum by directly increasing prefrontal activity. A model for the mechanisms of tVNS effectiveness has been already described before (Briand et al. 2020), overall tVNS

supposedly stimulates the thalamus via the numerous connections between the vagus nerve and the brainstem and via the connections of the latter with both the thalamus and the cortex. Deep brain stimulation's mechanisms of action are fairly intuitive as the electrode is implanted within the thalamus, stimulating it directly and reducing the deafferentiation of the cortico-thalamic loop. The same principle can be applied to LIFU techniques that stimulate the thalamus non-invasively.

To conclude, the mesocircuit model highlights possible targets for pharmacological and Neuromodulation therapeutic interventions. Prefrontal cortex, GABAergic and dopaminergic agents, the striatum, the globus pallidus, the vagus nerve and the thalamus are all possible candidates to stimulate in order to increase thalamico-cortical activity and result in consciousness recovery. (For an overview of the therapeutic targets within the meso-circuit model, see Figure 7).

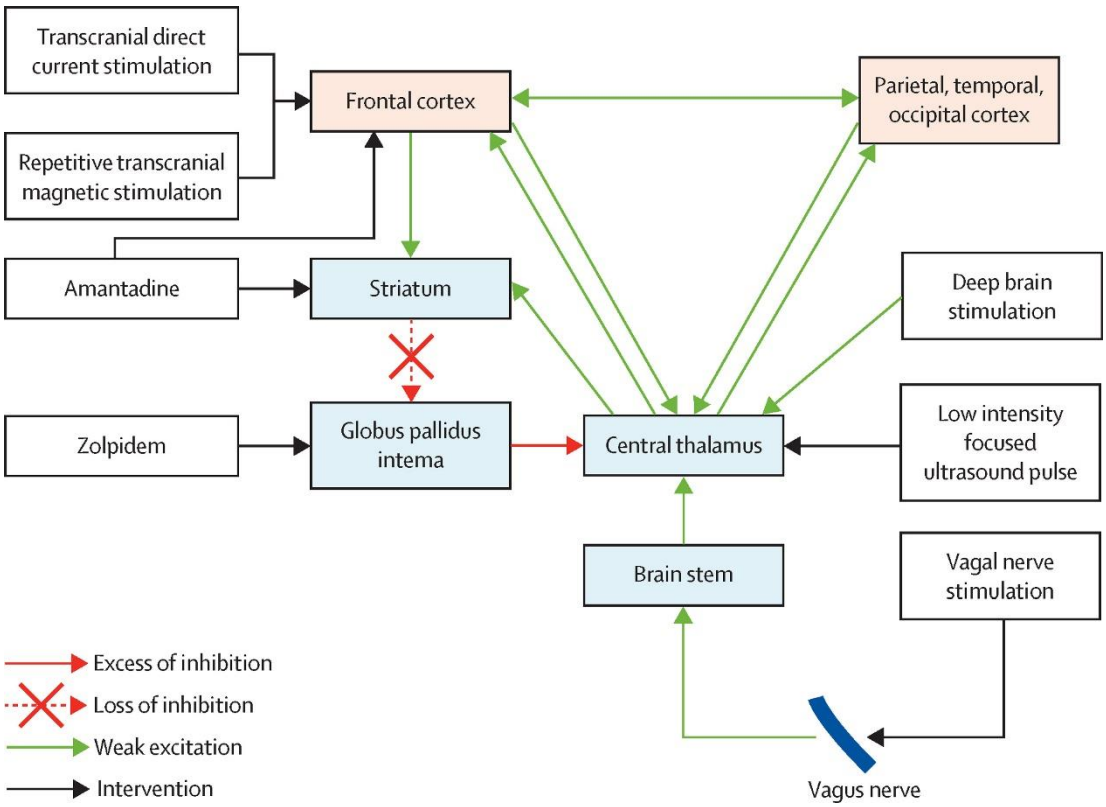

Fig. 7 Proposed mechanisms for therapies of DoC based on meso-circuit model. From Thibaut et al., 2019 with authors' permission.

These hypotheses have been supported by several neuroimaging studies conducted on treatments of DoC. (See Figure 8 for a visual representation of these results). For instance, amantadine (A) has been shown to result in fronto-parietal increased metabolism

(Schnakers, Hustinx, et al. 2008). On the other hand, zolpidem (B) resulted in increased prefrontal and mesiofrontal brain metabolism (Chatelle et al. 2014). A study comparing responders to non-responders to tDCS, found that a relative preservation of prefrontal areas was necessary for tDCS (C) to produce behavioural improvement (Thibaut, Di Perri, et al. 2015). rTMS over M1 (D) results in increased beta and alpha power, which as previously stated, should reflect recovery of cortico-thalamic pathways (Piccione et al. 2011; Edlow, Claassen, et al. 2020). Invasive VNS (E) resulted in increased brain connectivity patterns (Corazzol et al. 2017) whereas tVNS (F) resulted in increased functional connectivity between PCC, precuneus, thalamus, hypothalamus and prefrontal cortex (Yu et al. 2017).

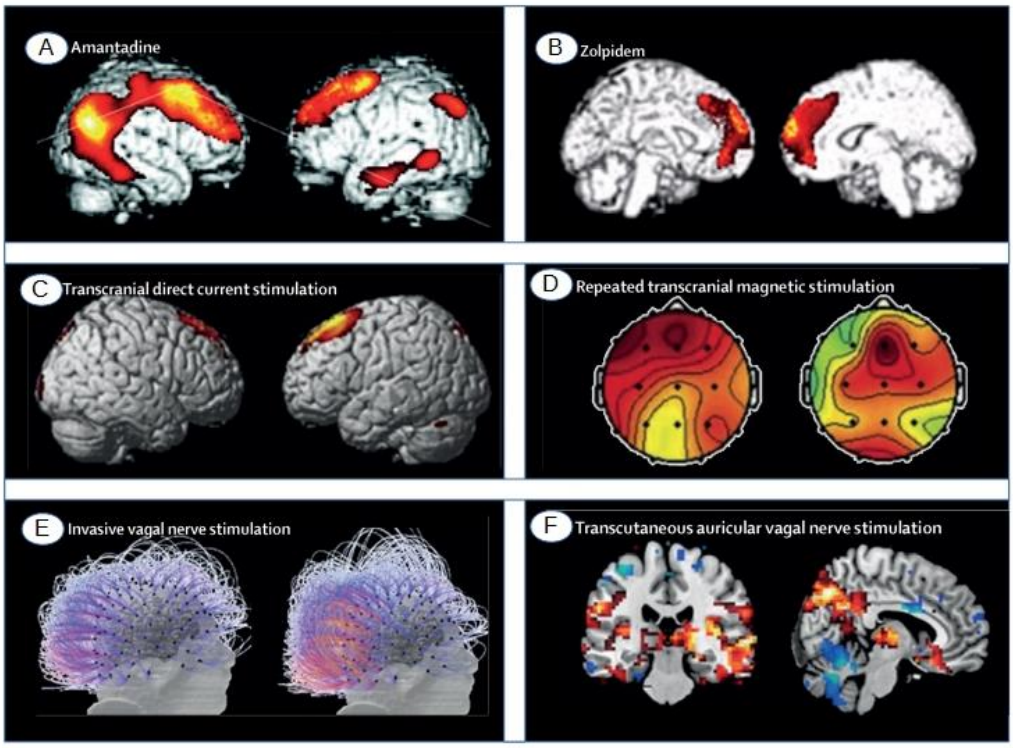

Fig. 8 Summary of Neuroimaging Findings on treatments to DoC. Adapted from Thibaut at al., 2019 with authors' permission. (A) Amantadine has been shown to increase brain metabolism in the fronto-parietal network in one patient in a MCS. Reproduced from Schnakers et al. 2008. (B) Zolpidem induced an increase in brain metabolism in the prefrontal and mesiofrontal cortex in three MCS responders. Reproduced from Chatelle et al. 2014. (C) Patients responding to tDCS had more preservation of brain metabolism in the DLPFC (stimulated area) compared with non-responders (n=13). Reproduced from Thibaut et al. 2015. (D) rTMS of 20 Hz on the M1 induced EEG increases in beta (shown), alpha, and delta band power in one MCS responder. Reproduced from Piccione et al. 2011. (E) Brain connectivity patterns before (left) and after (right) invasive VNS as measured with high-density EEG in one UWS patient who improved to a MCS after stimulation. Reproduced from Corazzol et al. 2017. (F) tVNS induced increases in connectivity between posterior cingulate, precuneus, hypothalamus thalamus and prefrontal and temporal cortex (red), and

decreases between the posterior cingulate, precuneus and cerebellum gyrus (blue) in one patient in UWS who transitioned to MCS after stimulation. Reproduced from Yu et al 2017.

In conclusion, there are several approaches that have been explored to treat patients with DoC. NIBS techniques have showed promising results and no severe side-effects. However, effectiveness and response rates of these stimulation techniques are still low and the works presented in this dissertation is aimed at exploring new ways to adapt treatment to the patients' needs in order to increase effectiveness and response rates.

1.9 Objectives

The research described in this dissertation has a main objective of improving the clinical management of patients with DoC. Fifty years have passed since the first official definition of patients with DoC, however, these patients remain a challenging population for researchers and healthcare professionals. The fact that they are not able to communicate affects both their diagnosis and their treatment, and thus their prognosis. Our work progresses towards solving some of the challenges that currently hinder research into the treatments of DoC.

To do so, the specific objectives of the research presented are the following:

a. Propose alternatives to the CRS-R to facilitate the execution of RCTs

b. Evaluate the effects of a novel tES approach in DoC

c. Define the characteristics of DoC patients that respond to tDCS

d. Proposing and testing a patient-tailored tDCS protocol in DoC patients

Objective a) To try to reduce the issues that arise with long experimental protocols (e.g., fatigue), **Study 1** explores the possibility of replacing the CRS-R with the GCS as the main behavioural scale in DoC research. This was investigated by evaluating the accuracy of the GCS in diagnosing the level of consciousness within a wide sample of patients with TBI. The results from this study not only can improve the design of RCT protocols in future studies but can also lead to a better stratification in intensive care settings and retrospective studies.

Objective b) In **Study 2** we tested the effectiveness of a new tES approach with a sham-controlled RCT, comparing tDCS and tPCS to sham. Several studies, have indeed showed that tPCS can potentially reach deeper structures in the brain. Based on the meso-circuit model, this technique was hypothesised to be an interesting candidate to treat DoC. We conducted an RCT to test this hypothesis with the hope of finding a new NIBS treatment for patients with DoC.

Studies 3 and 4 are more oriented towards the development of patient-tailored approaches in the research of NIBS treatments for DoC.

Objective c) **Study 3** explores the characteristics of DoC responders to tDCS with a retrospective analysis aiming at characterising their endophenotypic profiles. The results from this study could orient future RCTs towards more targeted subcategories of patients with DoC that might benefit more from NIBS (notably tDCS) treatments. In addition to improving patients' selection, these results could also help to increase the observed statistical effects of tDCS.

Objective d) **Study 4** – We will outline one protocol that uses a closed-loop system. This system was developed by acquiring data from patients with DoC and is based on the patients' vigilance levels. Since vigilance levels at the time of stimulation could influence the effectiveness of tDCS the implementation of this system could maximise the benefits of the stimulation. This closed-loop system performs a real-time, online calculation of the patients' individual vigilance thresholds, automatically triggering tDCS upon reaching them. This protocol is currently being tested in a RCT and we will also present its preliminary unpublished results.

Study 1: Diagnosing the level of consciousness: the limits of the Glasgow Coma Scale total score

Adapted from

Bodien, Y.G.*, **Barra, A.***, Temkin, N.R., Barber, J., Foreman, B., Vassar, M., Robertson, C., Taylor, S.R., Markowitz, A.J., Manley, G.T. and Giacino, J.T., 2021. Diagnosing level of consciousness: the limits of the Glasgow Coma Scale total score. *Journal of neurotrauma*, *38*(23), pp.3295-3305.

2.1 Summary

In nearly all clinical and research contexts, the initial severity of a traumatic brain injury (TBI) is measured using the Glasgow Coma Scale (GCS) total score. The GCS total score however, may not accurately reflect level of consciousness, a critical indicator of injury severity. We investigated the relationship between GCS total scores and level of consciousness in a consecutive sample of 2455 adult subjects assessed with the GCS 69,487 times as part of the multi-center Transforming Research and Clinical Knowledge in TBI (TRACK- TBI) study. We assigned each GCS subscale score combination a level of consciousness rating based on published criteria for the following disorders of consciousness (DoC) diagnoses: coma, unresponsive wakefulness syndrome/vegetative state, minimally conscious state, and post-traumatic confusional state, and present our findings using summary statistics and four illustrative cases. Participants had the following characteristics: mean (standard deviation) age 41.9 (17.6) years, 69% male, initial GCS 3–8=13%; 9–12=5%; 13–15=82%. All GCS total scores between 4–14 were associated with more than one DoC diagnosis; the greatest variability was observed for scores of 7–11. Furthermore, a wide range of total scores was associated with identical DoC diagnoses. Importantly, a diagnosis of coma was only possible with GCS total scores of 3–6. The GCS total score does not accurately reflect level of consciousness based on published DoC diagnostic criteria. To improve the classification of patients with TBI and to inform the design of future clinical trials, clinicians and investigators should consider individual subscale behaviors and more comprehensive assessments when evaluating TBI severity.

2.2 Introduction

The Glasgow Coma Scale (GCS), developed in 1974 by Teasdale and Jennett (Teasdale and Jennett 1974), is the most widely used behavioural measure to assess the severity of acute traumatic brain injury (TBI) (Teasdale et al. 2014; Reith et al. 2016). The scale's simplicity and rapid assessment approach has led to its international adoption for both diagnostic and prognostic (Marmarou et al. 2007) applications in pre-hospital, emergency department (ED), and intensive care unit (ICU) settings. The GCS has been designated a core TBI Common Data Element (Wilde et al. 2010) by the National Institute of Neurological Disease and Stroke and is commonly used in clinical trials both as a criterion for subject inclusion and as an approach for subject stratification (Maas, Roozenbeek, and Manley 2010). The GCS is scored based on behaviours observed across three subscale scores – eye-opening (score range 1–4), verbal (score range 1–5), and motor (score range 1–6) – that are summed to provide a total score ranging from 3–15.7 The total score is intended to reflect severity of injury, with scores of 3–8 indicating a severe injury, 9–12 a moderate injury, and 13–15 a mild injury. Moreover, GCS total scores of 3–8 are often used to define coma (Jennett 1979). Despite its widespread use, GCS administration and scoring is not standardized, and its psychometrical strength is moderate (Reith et al. 2016; Fischer et al. 2010). While there is a relationship between GCS total score and prognosis (Teasdale et al. 1979; Teasdale and Jennett 1976), this relationship is strongest for predicting death in large population studies (Healey et al. 2003) but not for predicting morbidity or functional outcome at the individual patient level (Steyerberg et al. 2008). Cognizant of its limitations, the original developers of the GCS wrote in 1978 that, although total scores may have prognostic utility, important information about current function and the potential for recovery is lost when reporting a GCS total score rather than individual component scores (Teasdale et al. 1979). In 1983 and again in 2014, Teasdale and colleagues (Teasdale et al. 2014; 1983) reaffirmed the importance of specifying the score for each of the three subscales and that failure to do so could limit the ability to detect changes in consciousness. Multiple studies have found that individual subscale scores are more diagnostically (Bhatty and Kapoor 1993) and prognostically (Reith et al. 2017) relevant than the total score. Moreover, different combinations of subscales that sum to the same total score are associated with variable mortality rates (Teoh et al. 2000). The implication of these findings spans across clinical and research settings where GCS scores are often further collapsed into three broad categories of mild, moderate, and severe TBI.

This coarse classification may mischaracterize individual patient prognosis, as evidenced by some ‘‘severe’’ patients having a favourable outcome (McCrea et al. 2021) and some ‘‘mild’’ patients having an unfavourable outcome (Nelson et al. 2019). From a clinical trial design perspective, reliance on GCS total scores may contribute to improper inclusion and stratification of subjects, leading to heterogeneous study groups, lack of statistical robustness, inaccurate interpretation of findings, and ultimately trial failure (Maas, Roozenbeek, and Manley 2010). Despite the evidence for using GCS subscale scores rather than a total score, GCS total scores remain the most common metric for establishing TBI severity and stratifying study subjects.6 Since the development and dissemination of the GCS, the diagnostic framework for classifying level of consciousness has evolved to be more precise. A definition of the minimally conscious state (MCS) was published in 2002 by the Aspen Neurobehavioral Conference Work- group (Joseph T. Giacino et al. 2002). MCS was further subdivided into MCS+ and MCS- based on presence or absence of language function, respectively (Thibaut et al. 2020). Distinguishing between the unresponsiveness wakefulness syndrome / vegetative state state (VS/UWS), MCS- and MCS+ diagnoses increases prognostic precision (Thibaut et al. 2020; Joseph T Giacino et al. 2020; 2018). The GCS was not designed, however, to differentiate these DoC states and lacks assessment of items such as visual pursuit and fixation that are crucial for detecting consciousness (Martens et al. 2020; Schnakers et al. 2006). In fact, the GCS has been estimated to have a false negative rate of 38% for detection of consciousness (Schnakers et al. 2006). It follows that, in some patients, neither GCS total scores nor subscale scores may characterize injury severity accurately. The association between GCS total scores and the current diagnostic criteria for level of consciousness is unknown. We identified all potential combinations of GCS total scores and used published diagnostic criteria for VS/UWS, MCS-, MCS+ (Giacino et al. 2002; Thibaut et al. 2020), and the post-traumatic confusional state (PTCS) (Sherer et al. 2020a) to match each GCS score combination with a level of consciousness. We then evaluated the observed incidence of each GCS combination in the Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) study. Finally, we provide case studies illustrating the diagnostic discrepancies that result from using GCS total scores.

2.3 Methods

TRACK-TBI is an 18-site observational study aimed at improving phenotypic accuracy and outcome precision for patients with TBI. Subjects at participating sites are enrolled within 24h of injury and followed up to 12 months if they meet the following criteria: documented TBI, computed tomography (CT) scan, and no significant polytrauma that would interfere with follow-up assessment (for full inclusion criteria, see the TRACK-TBI website - https://tracktbi.ucsf.edu/). The Institutional Review Board of each site approved the study protocol, and all subjects or surrogates provided written informed consent to participate. Acute hospitalization data elements, including GCS scores obtained in the field, ED, ICU, and on the hospital ward, were abstracted from medical records by research staff and documented in an electronic database. The GCS scores were available for the first five days of hospitalization and for the entire duration that intracranial pressure was monitored. Of the 2552 subjects (‡17 years old) enrolled in TRACK-TBI from 2014–2018, we excluded (1) subjects with no GCS scores (n = 28); (2) subjects for whom all GCS scores were confounded by periorbital swelling, intubation, and/or paralysis (n = 68); and (3) subjects for whom data errors (e.g., GCS date preceded injury date) could not be resolved (n = 1). The final sample included 2455 subjects with at least one unconfounded GCS score (Appendix Fig. 1). Collectively, these subjects were assessed with the GCS 69,487 times. Periorbital swelling, intubation, and/or paralysis were documented in 30,139 of the 69,487 GCS scores, leaving 39,348 unconfounded GCS scores for the primary analysis. Intubation was the most common confounding factor and was documented in 28,902 GCS scores. To reduce the potential for bias related to interpreting the verbal subscale in intubated patients, we excluded GCS scores confounded by intubation from the primary analysis. In a separate, secondary analysis, we analyzed scores confounded by intubation by imputing the verbal subscale score based on the Rutledge model, Equation 1.27

GCS combinations and associated level of consciousness

We identified the 120 possible GCS subscale score combinations (4 eye-opening · 5 verbal · 6 motor scores) by combining each subscale score with every other possible subscale score. Next, we assigned a diagnosis of coma, VS/UWS, MCS-, MCS+, PTCS, or recovery from PTCS (rPTCS) to each behaviour of each GCS subscale. We made these diagnostic determinations a priori based on: (1) the PTCS case definition,25 (2) published criteria for

coma (Posner et al. 2007), VS/UWS, MCS-, and MCS+ (Joseph T. Giacino et al. 2002), and (3) the Coma Recovery Scale-Revised (CRS-R) (Joseph T Giacino, Kalmar, and Whyte 2004) diagnostic distinctions between VS/UWS, MCS, and PTCS. We started by selecting the highest-level behaviour assessed by the GCS, ‘‘oriented’’ (verbal = 5), and assigning all GCS combinations with a verbal subscale score of 5 to the rPTCS group. Next, we assigned all GCS scores with the subsequent highest-level behaviour assessed by the GCS (i.e., ‘‘confused,’’ verbal = 4) to the ‘‘PTCS’’ group. We continued along this line of stepwise reasoning by identifying the subsequent highest-level GCS behaviour and assigning all GCS combinations with that behaviour the corresponding DoC diagnostic group (see Fig. 1, Panel B for a flow-diagram illustrating this procedure). We calculated the number of times each GCS subscale score combination was observed in the 39,348 unconfounded GCS scores and, separately, the 28,902 GCS total scores with documented intubation. Because the first GCS score is often used to determine eligibility for clinical trials, we repeated this analysis using each subject’s first GCS score, rather than all available GCS scores. Finally, we selected four subjects from the TRACK-TBI dataset to illustrate the challenges that arise when relying on GCS total scores for clinical management or research.

A

Subscale	Behavior	Score
Eye Opening Response	Eyes open spontaneously	4
	Eyes open to sound	3
	Eyes open to pressure	2
	None	1
Verbal Response	Oriented	5
	Confused	4
	Words	3
	Sounds	2
	None	1
Motor Response	Obey commands	6
	Localizing	5
	Normal flexion	4
	Abnormal flexion	3
	Extension	2
	None	1

B

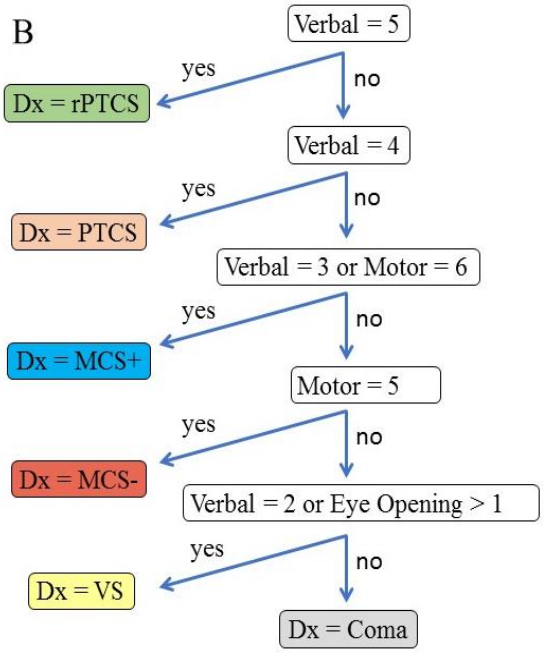

Fig. 1 Decision tree associating individual GCS subscale behaviours with DoC diagnoses. Panel A: Glasgow Coma Scale (GCS) subscales and behaviours, as described in: www.glasgowcomascale.org and associated scores. Panel B: The flow diagram illustrates our approach to assigning a disorder of

consciousness (DoC) diagnosis to GCS behaviors. We started by selecting the highest-level behavior on the GCS, "oriented" (verbal=5), and assigning all GCS combinations with a verbal subscale score of 5 to a *recovered from post-traumatic confusional state* (rPTCS) diagnostic group. Next, we assigned all GCS scores with the subsequent highest-level behavior on the GCS (ie., "confused", verbal=4) a diagnosis of PTCS. We continued along this stepwise line of reasoning by identifying the subsequent highest-level GCS behavior and assigning the corresponding DoC diagnosis to all GCS combinations with that behavior, until the only behaviors remaining were flexion and extension on the motor scale, reflective of coma. Because the GCS does not include all behaviors associated with DoC diagnoses, this decision tree is not meant to provide a clinical diagnosis. For example, patients with GCS behaviors consistent with vegetative state/unresponsive wakefulness syndrome (VS/UWS) were not assessed for MCS behaviors such as visual pursuit and automatic motor responses, which would have indicated a minimally conscious state (MCS). The color-coding in the DoC diagnostic categories in Panel B aligns with the color-coding of the behaviors in Panel A. For example, a GCS-based diagnosis of PTCS (shaded green in Panel B) is obtained by demonstrating orientation (GCS verbal=5, shaded green in Panel A). **Abbreviations:** MCS+ *minimally conscious state with evidence of language function* (best GCS subscale score is GCS verbal=3 or GCS motor=6); MCS- *MCS without evidence of language function* GCS (best GCS subscale score is GCS motor=5); PTCS *post-traumatic confusional state* (best GCS subscale score is GCS verbal=4); rPTCS *recovered from PTCS* (best GCS subscale score is GCS verbal=5); VS/UWS vegetative state/unresponsive wakefulness syndrome (best GCS subscale score is GCS eye opening >1 or verbal=2)

Data Analysis

For each GCS total score, we calculated: (1) the proportion of GCS subscale score combinations that could result in each DoC diagnosis; (2) the proportion of all GCS total scores that are associated with each DoC diagnostic category in the TRACK-TBI dataset; and (3) the proportion of TRACK-TBI subjects whose first valid GCS total score is associated with each DoC diagnostic category. Data summaries and descriptive statistics were compiled in Microsoft Access. TRACK-TBI data collection protocols, case report forms, and data sharing information can be found on the TRACK-TBI webpage.

2.4 Results

Participants

Demographic and clinical characteristics are provided in Table 1. Briefly, mean (standard deviation) age was 41.9 (17.6) years, and 69% were male. Using the traditional characterization of severity via ED GCS total scores, 13% (n = 314) had a severe, 5% (n = 119) had a moderate, and 82% (n = 1,975) had a mild TBI. For hospitalized participants,

GCS data were collected for mean (standard deviation) 2.4 (2.9) days, (median [interquartile range, IQR] = 1.5 [0.3–4.0] days, maximum 62 days). More than 90% of the GCS scores were obtained within the first seven days post-injury (Appendix Fig. 2), and each patient was assessed on average 16.0 (21.8) times (median [IQR] = 8 [2, 21.5] assessments, maximum= 263 assessments, Appendix Fig. 3).

Table 1. Patient Demographics and Injury Characterization

	All subjects	**All subjects included in analysis**[a]
	N=2552	*N*=2455
Age **Mean (SD)**	41.9 (17.6)	41.9 (17.6)
Sex		
Male	1767 (69%)	1699 (69%)
Female	785 (31%)	756 (31%)
Education years **Mean (SD)**	13.3 (2.9)	13.3 (2.9)
Unknown	175	162
Injury cause		
Road traffic	1456 (57%)	1395 (57%)
Fall	680 (27%)	660 (27%)
Other accident	133 (5%)	129 (5%)

Table 1. Patient Demographics and Injury Characterization		
	All subjects	**All subjects included in analysis**[a]
	N=2552	*N*=2455
Violence	169 (7%)	163 (7%)
Other	99 (4%)	94 (4%)
Unknown	15	14
ED GCS severity		
Mean (SD)	13.0 (3.8)	13.2 (3.6)
Severe (3-8)	361 (15%)	314 (13%)
Moderate (9-12)	123 (5%)	119 (5%)
Mild (13-15)	2000 (81%)	1975 (82%)
Unknown	68	47
Highest level of care		
ED	531 (21%)	508 (21%)
Ward	875 (34%)	870 (35%)
ICU	1146 (45%)	1077 (44%)

Table 1. Demographics and clinical characteristics of patients included. SD, standard deviation; ED, emergency department; GCS, Glasgow Coma Scale; IC, intensive care unit. [a]Subjects with at least one valid GCS score.

Potential GCS total score combinations and associated level of consciousness

Each GCS total score is associated with between one and 18 different combinations of GCS subscale scores. The 120 GCS combinations and associated DoC diagnoses are displayed in Appendix Table 1. Variability in DoC diagnoses is highest with GCS total scores of 7–10, each of which can result in a spectrum of DoC diagnoses (i.e., VS/UWS, MCS-, MCS+, PTCS, or rPTCS, [Fig. 2]). The only GCS total scores that invariably identify patients who are unconscious (coma or VS/UWS) are 3 and 4, and that invariably identify patients who have emerged from MCS (PTCS or rPTCS) are 14 and 15. Notably, a GCS total score of 7 or 8 cannot result in a diagnosis of coma, while a diagnosis of MCS is possible with any GCS total score in the range of 5–13. Interestingly, because the only GCS behaviour indicative of MCS- is localization (motor = 5), the lowest GCS total score associated with an MCS- diagnosis is 7 (eyes = 1, verbal = 1, motor = 5) while the lowest score associated with an MCS+ diagnosis is 5 (eyes = 1, verbal = 3, motor = 1).

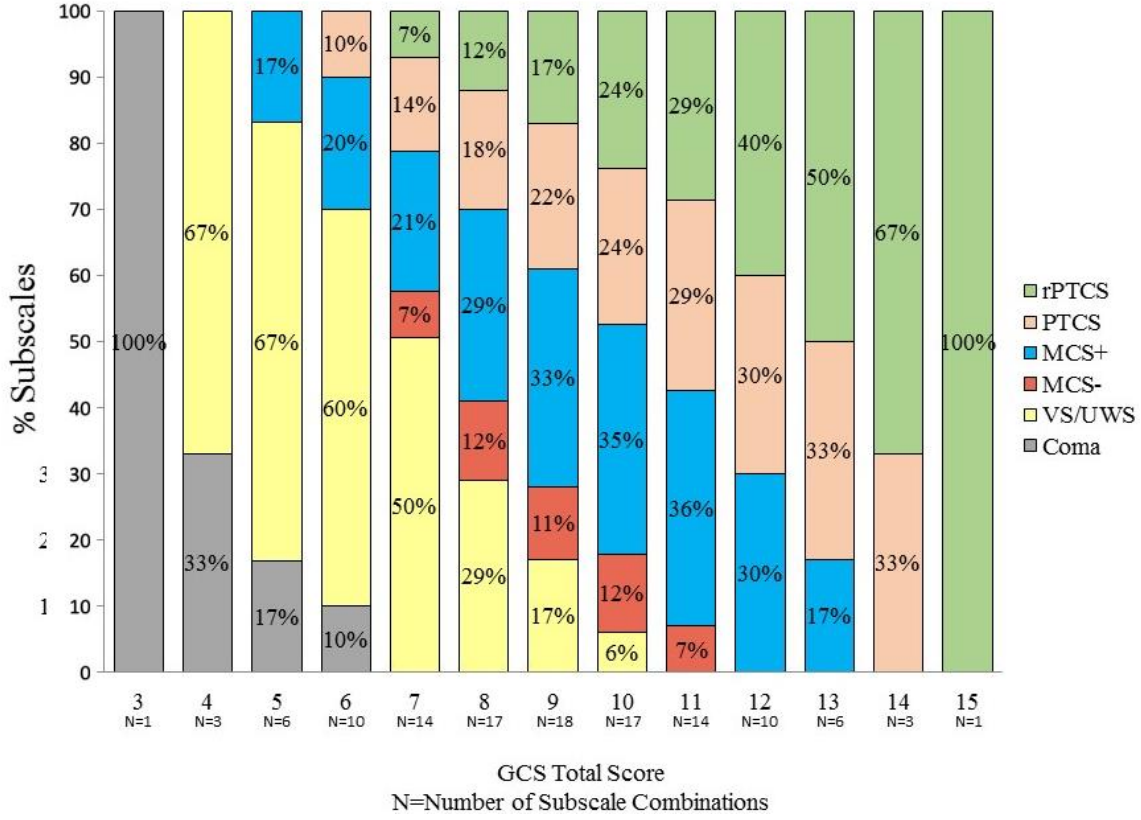

Fig. 2 Frequency of DoC diagnoses by GCS total score. The GCS is comprised of 3 subscales: eye opening, motor, and verbal responses. There are 120 possible combinations of subscale scores, each of which can be associated with a disorder of consciousness (DoC) diagnosis. All scores other than 3 and 15 are associated with multiple DoC diagnoses. Scores of 7-11 have the largest number of potential subscale combinations. The "n" below each GCS total score indicates the number of possible GCS subscale score combinations for each GCS total score. For example, there are 18 different ways a GCS total score of 9 could be achieved.

Analysis of GCS data in the TRACK-TBI study

The frequency with which each of the 120 GCS subscale score combinations occurs in the TRACK-TBI data- set is provided in Appendix Table 1. Not all GCS combinations and total scores are represented equally in this TRACK-TBI sample. Higher-level GCS behaviours are more prevalent, reflecting the characteristics of the TRACK-TBI dataset and the separate analysis of scores confounded by intubation (Appendix Fig. 4). Approximately 25% of the 120 GCS combinations were never observed across the entire TRACK-TBI dataset (Appendix Table 2). In the TRACK-TBI dataset, the only GCS total scores that were always associated with coma or VS/UWS were 3, 4, 5, and 6, and the only total scores that were always associated with recovery from MCS (i.e., PTCS, rPTCS) were

14 and 15. The variability of DoC diagnoses was highest for GCS scores of 7–12 (Fig. 3). For example, a GCS total score of 8, which, by convention, indicates a severe injury and is sometimes used to define coma, was observed 654 times and was associated with coma in 0%; VS/UWS in 5%; MCS- in 78%; and MCS+ in 17% of scores. Conversely, different total scores were associated with the same DoC diagnoses. For example, a diagnosis of MCS- was associated with 83% to 19% of GCS total scores of 7, 8, 9, 10 and 11. Results were similar when evaluating only the first valid GCS score across TRACK-TBI subjects (Supplementary Figure 5). Results of the secondary analysis imputing the GCS verbal score for intubated patients are in the Appendix Table 3.

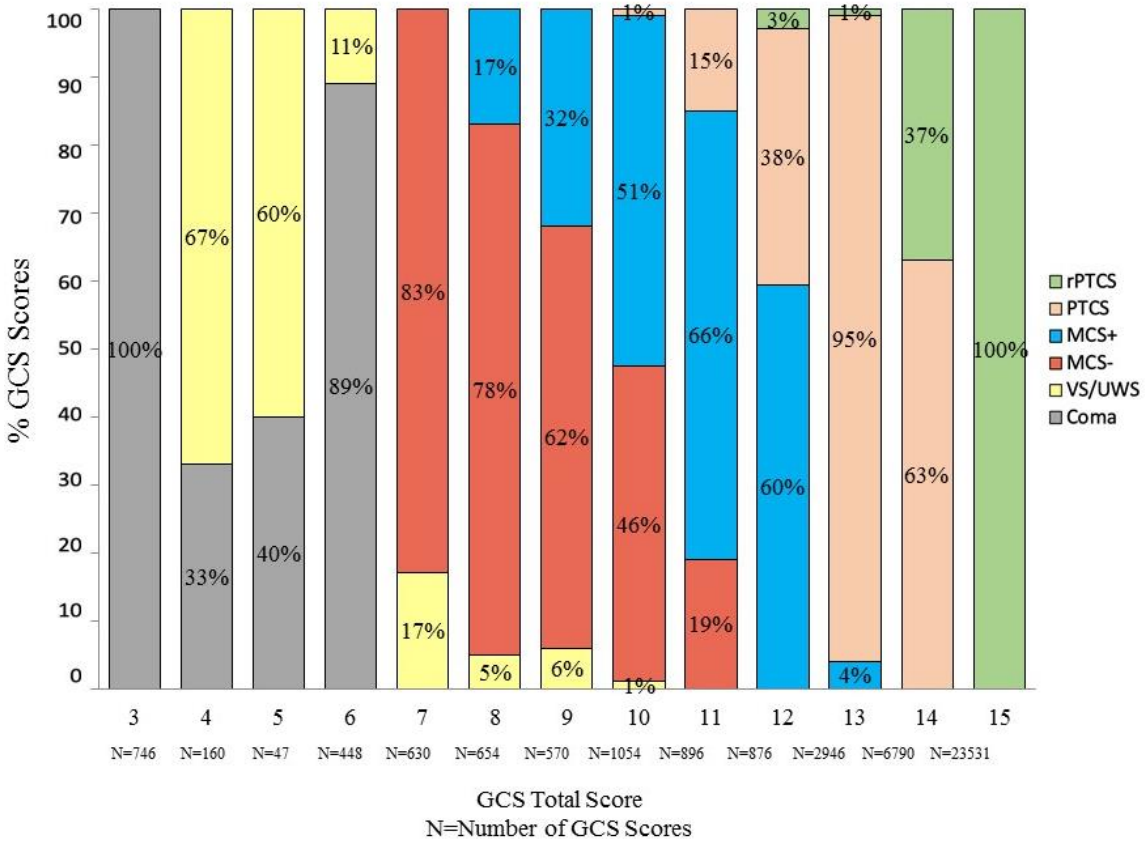

Fig. 3 Frequency of DoC diagnoses by GCS total score in the TRACK-TBI database. We assigned a disorder of consciousness (DoC) diagnosis to 39,348 GCS scores from 2,455 patients in the TRACK-TBI database based on the Decision Tree algorithm in Figure 1. Each color represents a different DoC diagnosis. Total scores ranging from 7-11 had the highest variability, such that a single GCS score could indicate multiple different DoC diagnoses, while different GCS total scores could indicate the same DoC diagnosis. The "n" below each GCS total score indicates the number of times each GCS total score occurs in the TRACK-TBI database.

Case Studies

We present 4 scenarios to illustrate how use of GCS total scores may result in misleading interpretation of level of consciousness (Figure 4). For Participants 1 and 2, GCS subscale behavioral profiles result in a total score of 8 and an injury categorization of "severe." However, for Participant 1, the profile is consistent with VS/UWS, while for Participant 2 the profile is consistent with MCS+. For Participants 3 and 4, the GCS total score differs by 3 points, total score=7 versus 10, corresponding to severe and moderate injury, respectively. Nevertheless, the behavioral profiles both reflect the same level of consciousness (ie., MCS-).

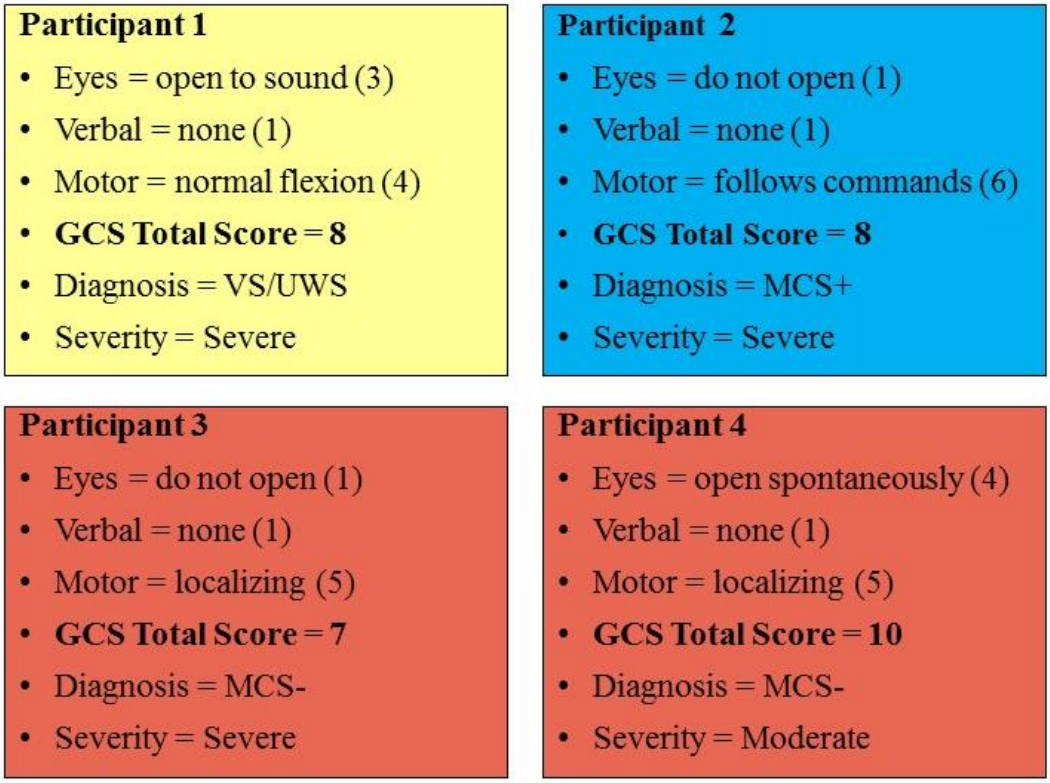

Fig. 4 Case illustrations of the variable association between GCS total scores and DoC diagnoses. For Participants 1 and 2, Glasgow Coma Scale (GCS) subscale score behavioral profiles result in a total score of 8 and a severe injury categorization. However, for Participant 1, the profile is consistent with a disorder of consciousness (DoC) diagnosis of VS/UWS, while for Participant 2 the profile is consistent with MCS+. Characterizing both patients as having a "severe" injury ignores the highly variable approach towards clinical management applied to patients who are unconscious versus those who are conscious and following commands. With regard to research, both Participants 1 and 2 would be allocated to the same study arm based on the total GCS score, despite having markedly different levels of consciousness. This would result in a heterogeneous group and make it more difficult to determine differences in treatment efficacy or outcome. For participants 3 and 4 the GCS total scores differ by 3 points and the total scores are consistent with severe versus moderate injury, respectively. Nevertheless, the behavioral profiles both reflect an MCS-

level of consciousness. In this case, ascribing different injury severity categories based on the total score would create an erroneous distinction between Participants 3 and 4 who are, in fact, functioning at the same level. In a research study, these two patients may be placed in separate severity groups, or one participant may be excluded, creating an artificial distinction based on GCS total scores that is not supported by the actual level of consciousness. Consequently, this may obscure the effect of treatment, reflecting instead heterogeneity across study groups.

2.5 Discussion

In this study of 2,455 participants with acute TBI, we found substantial heterogeneity in the level of consciousness associated with GCS total scores. While identical total scores reflected different levels of consciousness, different total scores reflected the same level of consciousness. Lower GCS total scores did not always indicate a more severe injury, and viceversa. Importantly, although a GCS total score of 8 is often used as the threshold to operationally define "coma" in clinical trials, no patients with GCS total scores of 7 or 8 had a diagnosis of coma. Given the prognostic relevance of precise assessment of level of consciousness (Giacino et al. 2020; Giacino, Kalmar, and Whyte 2004), and the importance of consistent subject stratification in clinical trials (Maas, Roozenbeek, and Manley 2010), the GCS total score may, therefore, be a suboptimal tool for defining TBI severity or monitoring recovery. Our results support prior studies suggesting that the behavioural subscale profile underlying the total GCS score may be more clinically meaningful than the total score itself (G. Teasdale et al. 1979; Healey et al. 2003; Bhatty and Kapoor 1993; Reith et al. 2017) and shed new light on the limitations of the GCS total score as a clinical and investigational tool.

The rationale of this study was to assess the diagnostic sensitivity of GCS total score for differential diagnosis of DoC. Hence, we showed that the GCS total score does not differentiate DoC diagnoses, therefore providing practical evidence that the GCS is not a suitable tool to diagnose DoCs. Moreover, as previously explained GCS subscales are also limited because they omit certain critical behaviours, such as visual pursuit (Martens et al. 2020), that are necessary to distinguish between VS/UWS, MCS- and MCS+. Failing to assess these behaviours could contribute to misdiagnosis, inaccurate prognosis, and heterogeneous clinical trial samples. It would have been interesting to calculate the percentage of patients that could have been misdiagnosed based on the lack in the GCS of visual pursuit assessment. Wannez and colleagues found that in chronic patients with DoC,

visual pursuit was the only sign of consciousness showed in 20% of the sample whereas visual fixation was the only sign of consciousness showed by 27% of the sample (Wannez et al. 2018). Based on these numbers, up to 57% of our sample might have been misdiagnosed due to the lack of assessment of these behaviours. However, due to the fact that almost 90% of our sample was acquired within 7 days post injury (i.e., in ICU settings), we cannot be sure the same percentages of Wannez and colleagues would apply in our sample. Moreover, because of this characteristic of our sample we did not have CRS-R scores for the patients that we could compare to the GCS scores to perform this analysis. GCS items also lack a standardized approach for subscale administration and a consistent way of documenting factors such as sedation and intoxication that influence performance (Reith et al. 2016). Guidance on how to assess behaviours such as command-following and what criteria must be met to document a localizing response are needed to ensure that changes in scores reflect the patient's true responsiveness rather than the examiner's approach towards administration of the measure. Recognizing these limitations of the GCS, the original authors have provided additional recommendations to aid in proper use of the subscale and total GCS scores (https://www.glasgowcomascale.org).

When one or more GCS subscales are confounded, the opportunity to detect conscious awareness is further reduced. In such cases, it is especially important to review individual behaviours rather than total scores and to conduct further comprehensive assessments aimed at differentiating VS/UWS from MCS. Approaches aimed at providing a score for confounded subscales, such applying imputation algorithms or assigning the lowest possible value of "1", may lead to misclassification of level of consciousness. In fact, we found that imputing verbal subscale scores drastically and artificially reduced the opportunity to observe the various DoC diagnoses associated with each GCS total score. Although there are alternate approaches to imputing confounded GCS verbal scores, none address the issue of reduced variability and there are no reliable approaches for imputing confounded eye and motor subscale scores. Therefore, when one or more GCS subscales are confounded, an accurate total score cannot be calculated. The Full Outline of UnResponsiveness (FOUR) score is designed to overcome some of the limitations of the GCS. However, the FOUR score also omits behaviours associated with MCS, has not been validated in TBI, and lacks strong psychometric properties (Fischer et al. 2010). Of the 120 combinations of the GCS total score, about 25% were not observed a single time across our sample of 39,348 GCS scores. This finding suggests that some GCS subscale

combinations are clinically unlikely to co-occur. Observation of these infrequent combinations clinically and in research may signal errors in administration or scoring or a failure to identify confounding factors.

Our mapping of GCS behaviours onto levels of consciousness was conducted objectively based on published diagnostic criteria. However, because the GCS does not test all behaviours associated with each DoC diagnosis, the DoC diagnoses we associated with GCS behaviours are approximations and not intended for clinical diagnostic purposes. Furthermore, because our study relies on a conceptual mapping of GCS behaviours onto DoC diagnoses and our sample is heavily weighted towards mild TBI, follow-up studies that prospectively evaluate level of consciousness using a comprehensive standardized bedside assessment in a large cohort of participants with severe TBI will be needed to confirm our findings. Interestingly, the GCS motor subscale has been shown to be more sensitive for classifying injury severity (Giacino et al. 2018b; Wijdicks et al. 2015) and predicting survival (Healey et al. 2003), compared to the GCS total score, though this may not be the case for older patients (Kupas, Melnychuk, and Young 2016). Given that the GCS eye-opening subscale does not include any behaviours differentiating VS from MCS and that the verbal subscale is often confounded by intubation, GCS motor subscale may also be the most likely to accurately characterize 6- and 12-month outcome. Finally, the utility of GCS scores for assessing consciousness may vary based on patient acuity and setting. For patients with GCS $\leq$ 8, regardless of the subscale composition of this score (e.g., E3, V2, M3 verses E2, V1T, M5) current guidelines from the Brain Trauma Foundation recommend neurosurgical intervention to place an intracranial pressure monitoring device (Ross et al. 1998). Whether outcome is affected by the GCS subscale composition of the total score that leads to the neurosurgical intervention, is unknown. However, our findings suggest that knowledge of the subscale scores may be important even in the setting of rapid triage for clinical procedures, to the extent that these decisions are informed by a patient's level of consciousness. Although more than 90% of GCS scores in this study were obtained within the first seven days post-injury, approximately 0.5% of GCS scores were obtained in what is considered prolonged DoC (more than 28 days post-injury). The generalizability of our findings to patients with prolonged DoC requires further investigation.

In the 45 years since its development, the GCS has continued to have a uniquely prominent role in assessment of TBI. The overwhelming adoption of the GCS is likely related to its

historical positioning as one of the first scales to quantify brain injury severity and create a common language around recovery from TBI-related coma. The GCS is also simple and fast to administer and is routinely identified as a statistically significant, if modest indicator of outcome at the group level (Teasdale et al. 2014; Marmarou et al. 2007; Teasdale and Jennett 1976; Deeb et al. 2021). However, despite the development of various revisions, the prognostic validity of the GCS, even at the subscale level, is insufficient for predicting outcome in individual patients. Nevertheless, early behavioural assessment of TBI, grounded in the GCS, continues to inform individual prognostic discussions that may lead to withdrawal of life-sustaining therapy. More refined methods for quantifying impairments in consciousness that maximize reliability, mitigate misdiagnosis, and hence reduce prognostic errors, are clearly needed. A standardized measure of level of consciousness with strong psychometric properties that assesses the full range of behaviours underlying DoC diagnosis should complement the GCS evaluation. The CRS-R, meets these criteria. However, the CRS-R is not validated in the ICU setting and is lengthy, making it difficult to administer in patients with acute TBI who are sedated, rapidly fluctuating, or clinically unstable. An abbreviated version of the CRS-R, the CRS-R FAST (CRS-R For Accelerated Standardized Testing) is currently being investigated (NCT #03549572). The FOUR score is designed for use in the ICU and can be administered in a few minutes, but has limitations for diagnosing DoC, as described above. Prior to assigning an injury severity classification based on GCS total scores, clinicians and investigators should, at a minimum, consider the information provided by the GCS behavioural profile and potential confounding factors. Subscale score analysis is especially important for GCS total scores of ≤ 8 due to the risk of incorrectly establishing a diagnosis of coma. Designing electronic medical record systems to prompt for GCS subscale scores and potential confounds may aid in ensuring routine documentation of this information. Accurate clinical assessment and successful clinical trials will ultimately require comprehensive behavioural measures that rigorously and reliably classify patients according to their level of consciousness, cognition, and function.

Study 2: Transcranial Pulsed-Current Stimulation versus Transcranial Direct Current Stimulation in Patients with Disorders of Consciousness: A Pilot, Sham-Controlled Cross-Over Double-Blind Study

Adapted from

Barra, A.*, Rosenfelder, M.*, Mortaheb, S., Carrière, M., Martens, G., Bodien, Y.G., Morales-Quezada, L., Bender, A., Laureys, S., Thibaut, A. and Fregni, F., 2022. Transcranial Pulsed-Current Stimulation versus Transcranial Direct Current Stimulation in Patients with Disorders of Consciousness: A Pilot, Sham-Controlled Cross-Over Double-Blind Study. *Brain Sciences*, *12*(4), p.429.

3.1 Summary

Transcranial direct-current stimulation (tDCS) over the prefrontal cortex can improve signs of consciousness in patients in a minimally conscious state. Transcranial pulsed-current stimulation (tPCS) over the mastoids can modulate brain activity and connectivity in healthy controls. This study investigated the feasibility of tPCS as a therapeutic tool in patients with disorders of consciousness (DoC) and compared its neurophysiological and behavioural effects with prefrontal tDCS. This pilot study was a randomized, double-blind sham-controlled clinical trial with three sessions: tPCS, tDCS, and sham. Electroencephalography (EEG) and behavioural assessments were collected before and after each stimulation. Post-pre differences were compared using Kruskal–Wallis and Wilcoxon signed-rank tests. Twelve patients with DoC were included in the study (eight females, four traumatic brain injury, 50.3 ± 14 y.o., 8.8 ± 10.5 months post-injury). We did not observe side-effects following tPCS, nor tDCS, and confirmed their feasibility and safety. We did not find a significant effect of the stimulation in EEG and behavioural outcomes. We demonstrated that tPCS is safe for patients with DoC. We did not observe neurophysiological or behavioural effects following tPCS. However, consistent with prior findings, our exploratory analyses suggest that tDCS induces behavioural improvements and an increase in theta frontal functional connectivity.

3.2 Introduction

DoC are comprised of a spectrum of states showing impaired arousal and awareness at variable levels. They include coma, UWS, and MCS.

To this day, even though significant progress has been made in understanding the neural correlates of DoC, the available treatments for DoC patients remain limited (Thibaut, Schiff, et al. 2019).

In the last decade, the scientific community has witnessed the development of neuromodulation treatment approaches. Neuromodulation refers to different brain stimulation techniques that can be either invasive or non-invasive and is used to treat several neuropsychological conditions (Gunn and Grobelna 2021; Rahimpour et al. 2021; Inagawa et al. 2019). Some neuromodulation approaches have proven to be such valid options that they are currently FDA approved in several countries (Lundstrom, Wharen, and Tatum 2021; Austelle et al. 2021; Cohen et al. 2022). Non-invasive brain stimulation such as tES is a branch of neuromodulation of particular interest for DoC as it is safe, inexpensive, easily integrated into rehabilitation and hospital environments, and does not require the patient's active participation. More specifically, tDCS uses a weak constant electrical current sent via, at least, one anode and one cathode (Giordano et al. 2017; Stagg and Nitsche 2011). TPCS is another type of tES that uses a rectangular alternate current with set frequency and amplitude (Jaberzadeh, Bastani, and Zoghi 2014)—as opposed to the continuous one of tDCS and has been hypothesized to induce effects not only by modulating the polarization of the membranes, but also by peripheral nerve stimulation (since it is usually placed over mastoids) and by carrying proteins and neurotransmitters (Moreno-Duarte et al. 2014).

tDCS over the left prefrontal cortex has previously showed to produce transient improvements in the behavioural responsiveness of DoC patients (Thibaut et al. 2014; Estraneo et al. 2017; Angelakis et al. 2014; Martens et al. 2018; Thibaut et al. 2017). More recently, tDCS has also been tested with different targets (Zhang et al. 2017; Martens et al. 2019; Huang et al. 2017; Martens et al. 2020) but so far, the brain region that has shown more consistently positive results seems to be left prefrontal cortex (Thibaut, Schiff, et al. 2019). Effects of tDCS have been most frequently observed in patients in MCS rather than UWS (Thibaut et al. 2014; Zhang et al. 2017; Cavinato et al. 2019). Neurophysiological

studies have found through EEG that tDCS could increase theta and alpha power and decrease delta power (Cavinato et al. 2019; Carrière et al. 2020).

tPCS has not yet been studied as a treatment for patients with DoC and only a few studies have explored it in patients with neurological conditions (e.g., Parkinson's disease) (Jensen et al. 2021; Alon et al. 2012). However, tPCS can enhance speech comprehension in healthy subjects (Ruhnau et al. 2020) as well as facilitate arithmetic tasks (Morales-Quezada et al. 2014) and enhance motor skills and cognitive functions (Miniussi, Harris, and Ruzzoli 2013), suggesting that this stimulation may also promote recovery in patients in MCS. Furthermore, it was shown, using computational modeling, that TPCS can modulate subcortical neural circuits (Miniussi, Harris, and Ruzzoli 2013; Datta et al. 2013). Previous modeling studies have found that tPCS can modify the electrical activity of cortical and subcortical structures (Datta et al. 2013), and improve frontal (Castillo-Saavedra et al. 2014) and interhemispheric neuronal connectivity (Morales-Quezada et al. 2014). For this reason, it has been hypothesized that tPCS could reach deeper brain structures compared to tDCS.

As this was a pilot study using both tPCS and tDCS in patients in DoC, our first objective was to evaluate the feasibility of a double-blind randomized controlled trial using these two different stimulation techniques, and assess the safety of tPCS in this population. In addition, we aimed to compare the neurophysiological and behavioural effects of a single session of tDCS and tPCS against the sham. To do so, we first evaluated the change in neurophysiological parameters (i.e., qEEG and functional connectivity), especially in the theta and alpha bands over the frontal and parietal areas. Based on the literature reported above, we hypothesize the following differences between the stimulation conditions: tPCS will enhance cortical activity in the frontal and parietal areas within the theta and alpha bands compared to the sham; tDCS will enhance cortical activity in the frontal areas within the theta band compared to the sham. Finally, we compared the Coma Recovery Scale-Revised (CRS-R) (Giacino, Kalmar, and Whyte 2004) total scores and CRS-R index between conditions. We hypothesized that both tPCS and tDCS would promote recovery of signs of consciousness on the CRS-R compared to the sham.

3.3 Methods

This was a pilot double-blind sham-controlled randomized crossover clinical trial exploring the effects of two different stimulation techniques (tDCS and tPCS) against the sham stimulation on behavioural and neurophysiological outcomes in patients with at least one sign of consciousness (i.e., CRS-R diagnosis of MCS or eMCS).

Study design: Randomized placebo-controlled, multi-center double-blind crossover pilot study.

Participants

Written informed consent in accordance with the Declaration of Helsinki was obtained by the legal representative of each patient. The ethics committee approved this study of the University Hospital of Liège, and by each local ethic committee as needed (Clinicaltrial.gov: NCT03115021). All patients were included while in rehabilitation centers (Wallonia, Belgium); the amount of rehabilitation they received remained unchanged during the study protocol. Participants were pre-screened by their physicians and, if they met the inclusion criteria, they were assessed for eligibility. We planned a priori to include 12 patients in the sample as this was a pilot feasibility study.

The inclusion criteria were the following: (a) age between 18 and 75; (b) history of acquired traumatic or non-traumatic severe brain injury leading to a DoC; (c) a minimum of two CRS-R assessments, performed by a trained clinician or research staff before inclusion, detecting at least one, but not all, signs of consciousness (MCS/eMCS); and (d) in stable medical condition (e.g., no infection, intubation, recent hospitalization, escalating or de-escalating medications).

Participants were excluded in the following cases: (a) history of major neurologic or psychiatric comorbidity present at the time of enrollment; (b) evidence or report of untreated seizure; (c) presence of a metallic implant or implanted electronic brain medical devices (e.g., pacemaker); and (d) history of cranioplasty without healed cranial flap, based on medical records. Note that the reason why we excluded patients with untreated epilepsy is that previous tES trials on patients with DoC have enrolled patients treated for epilepsy and have not found any side effects.

Blinding

Patients received all three stimulation sessions (tPCS, tDCS, sham) in random order in a 1:1:1 ratio. The patient, the researcher administering the treatment, and the researcher

evaluating the response to the treatment were all blinded to the condition. A researcher not involved in data collection created sealed randomization envelopes and assigned them to each patient in a chronological order. To ensure blinding of both the researchers and the patient, both systems (i.e., electrodes for tPCS and for tDCS) were mounted for each session; one researcher launched the stimulation (according to the coded order in the sealed envelope) while the researcher involved in behavioural assessment remained away from the patient's room to ensure full blinding. Moreover, the EEG analysis was performed with a blinding code: conditions (i.e., tPCS, tDCS and sham) were renamed with a numerical code that was revealed to the researcher only when the analysis was finished.

Study protocol

Each patient was evaluated by a trained professional at least twice with the CRS-R before being enrolled in the study to ensure that the patient met the diagnostic inclusion criteria. The protocol included three sessions and each patient completed all three sessions in a random order: (i) tPCS, (ii) tDCS, and (iii) sham. Each session was separated by a minimum of five days to avoid any carryover effect. This washout period was established based on similar studies and on the fact that the effects of a single session of tDCS should not last more than a few hours after the end of stimulation (Nitsche and Paulus 2001). Each session was structured as follows: the CRS-R was performed, and 10 min of resting EEG were recorded. Twenty minutes of stimulation were launched. After the stimulation, the CRS-R and the EEG were collected again. For a schematic view of the protocol, see Figure 1.

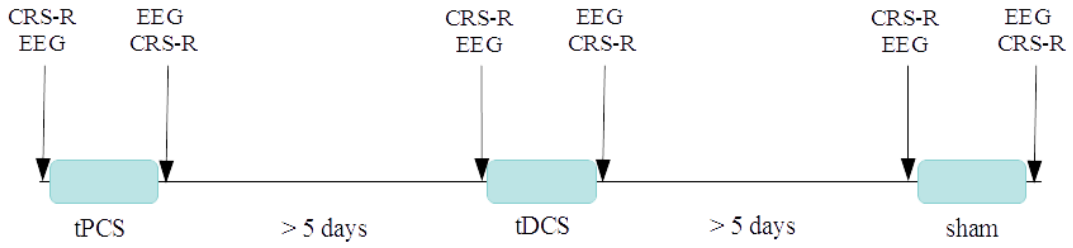

Fig. 2. Schematic representation of the protocol. Note that the order of the sessions was randomized for each patient (the order of sessions for each patient can be found in Table 1). During each session both devices were mounted together with the EEG cap, to keep the same montage across sessions and allow blinding.

Interventions

For prefrontal tDCS, we used a NeuroConn DC-Stimulator PLUS device (Ilmenau, Germany) delivering a maximum of 2 mA of current for 20 min. The anode was placed over F3 (International 10/20 EEG System), corresponding approximately to the left prefrontal cortex. The cathode was placed over the contralateral orbitofrontal area. Both anode and cathode were round rubber electrodes with a diameter of 4 cm (12 cm^2). For bi-mastoid tPCS, we used the NeuroConn DC Stimulator MOBILE (Ilmenau, Germany), which delivered a random frequency between 6–10 Hz with a biphasic current of 2 mA peak to peak for 20 min. The rubber electrodes were placed over the mastoids. These squared electrodes covered an area of 16 cm^2 (4 × 4 cm). Both tPCS and tDCS electrodes were fixed using a conducting paste (Ten20, Weaver and Company, Aurora, CO, USA) to reduce impedances and were placed under the EEG cap. For sham, the same parameters were set as for the active condition (both tDCS and tPCS montages), but the device automatically turned off after 30 s to simulate the initial tingling sensation of the active current.

Assessments

As this was a pilot study, our primary goal was to evaluate the feasibility of a randomized double-blind clinical trial by comparing two different types of tCS. We defined feasibility as successfully carrying out the pilot study on the sample (i.e., successful mounting of the systems, double blinding, execution of the sessions, no adverse events during the stimulations). We assessed the side-effects of the stimulation by observing the patients before, during, and after each session of stimulation (both sham and active) with a particular attention to redness of the skin, irritation of skin, seizures, facial and verbal signs of pain and/or discomfort, which were reported on the patient's case report form. Caregivers and families were asked to report to the researchers if they noticed any side-effect in the days following the stimulation. Moreover, to further investigate the safety of the stimulations, a trained professional checked the EEG recordings post-stimulation for ictal and irritative abnormal activities.

Our secondary goal was to evaluate the effects of bi-mastoid tPCS and left prefrontal tDCS on neurophysiology (EEG) and behavioural measures (CRS-R). To record EEG, we used a portable device developed by NeuroConn (Ilmenau, Germany). The NeuroPrax-Tes

2.6.17 system is a full-band DC-EEG system that is compatible with tCS stimulation and allows for the recording of EEG at high sampling rates. This system is composed of a cap with 27 gel-based (25 recording electrodes and two references, the maximum number of electrodes available for NeuroConn systems) electrodes, one amplifier, and one portable computer. The EEG was mounted at the beginning of the session for every patient by the researchers and the stimulation electrodes were placed under the EEG cap. The recording lasted 10 min, and the sampling rate of the recordings was kept as default at 4000 Hz. The reference electrodes (one reference and one ground) were placed behind the patients' ears with adhesive electrodes. For the behavioural assessment, we used the CRS-R (Giacino, Kalmar, and Whyte 2004). A trained researcher performed the CRS-R, before and after each stimulation, in the patient's room, which was kept quiet, and the patient was made as comfortable as possible, either in bed or in a wheelchair.

Data analyses

Analysis of the crossover effect: First, we performed a Kruskal–Wallis test on the baselines (i.e., pre-stimulation) to compare sham, tPCS, and tDCS and make sure that they did not differ at the baseline. We performed this analysis on both the EEG data and behavioural data.

EEG preprocessing: EEG data were preprocessed using custom scripts in MNE-Python (Gramfort et al. 2013). Data were filtered with a high-pass filter and low-pass filter set at 1 Hz and 30 Hz, respectively. The sampling rate was downsized from 4000 to 500 Hz. The signal was further divided into 2 s epochs. Epochs of 2 seconds were chosen to allow for better selection of epochs without high frequency noise and motor artefacts. Visual inspection was used to identify and remove artifactual signal epochs and noisy channels. An independent component analysis (ICA) based on the Infomax algorithm (Bell and Sejnowski 1995) was used to remove eyeblink and muscle artifacts (based on their temporal, frequency and spatial distribution). Afterwards, bad channels were interpolated using spherical interpolation, and data were re-referenced to the average of all electrodes (i.e., average referencing). If the extracted clean epochs were not sufficient (i.e., <90 epochs of 2 s) to perform the statistical analyses, the patient was discarded from the EEG analyses. One patient was discarded from EEG analyses for this reason (see Figure 2 for details).

EEG power: Power spectral density (PSD) was calculated using custom scripts in MNE-Python (Gramfort et al. 2013) for each band (i.e., delta 1–4 Hz, theta 4–8 Hz, alpha 8–13 Hz, and beta 12–30 Hz), for each electrode, and averaged over the epochs with a trimmed mean that allowed us to exclude outliers. Welch's method was used to extract the resulting averaged power bands with a 1 s window overlapped at 50% and a frequency resolution of ½ Hz. We focused the analyses on frontal electrodes (i.e., F3, F4, F7, F8, Fc1, Fc2, Fc5, Fc6, Fp1, Fp2, Fz) and parietal electrodes (i.e., Cp5, Cp6, P3, P4, Pz) as this follows the meso-circuit hypothesis on the recovery from DoC (Schiff 2010; Edlow, Claassen, et al. 2020).

EEG connectivity: The connectivity between pairs of channels was calculated for each band, in channels encompassing the frontal and parietal region, using the debiased weighted phase lag index (dwPLI). First, data were analyzed in the time-frequency domain regarding power and current spectral density, using the spectral transfer function. Then, the dwPLI was computed from the peak frequency in any pair of electrodes. For an in-depth description of the analyses, see (Chennu et al. 2017). The dwPLI, first introduced by Vinck (Vinck et al. 2011) and then implemented by Chennu for patients with DoC (Chennu et al. 2017), is supposedly more stable across noise and sensitivity to volume-conduction compared to the wPLI and less positively biased to sample sizes as it is an estimation of the squared wPLI. It is based on the following equation:

$$\hat{\Omega}^{w} \equiv \frac{\sum_{j=1}^{N}\sum_{k\neq j}\Im\left\{X_j\right\}\Im\left\{X_k\right\}}{\sum_{j=1}^{N}\sum_{k\neq j}\left|\Im\left\{X_j\right\}\Im\left\{X_k\right\}\right|}.$$

The resulting connectivity matrix was collapsed to each frequency band (i.e., delta, theta, alpha, beta) and the median voltage was calculated for each frequency band. We focused the analyses on the frontal and parietal regions using the same electrodes as for power, based on the literature regarding the mesocircuit model (Schiff 2010; Edlow, Claassen, et al. 2020).

Statistical analyses

The feasibility of the use of tPCS in a randomized double-blind clinical trial was inferred from the success of the present pilot study. The safety of the stimulation techniques (with a focus on tPCS, as the safety of tDCS has already been demonstrated (Thibaut et al. 2014)) was monitored by reporting any side-effects observed by the researchers, caregivers, or family before and after each session and by screening post-stimulation EEG recordings for the presence of abnormal (ictal/irritative) activity.

EEG data were analyzed using RStudio (version 1.2.5001; RStudio, Inc., Boston, MA, USA). We performed Kruskal–Wallis analyses on the pre and post differences (i.e., delta—Δ) of the three conditions. The power differences pre and post for each electrode were averaged for each patient to account for the subject's variability. For instance, when performing the analysis for frontal regions, we took the power values for each frontal electrode (i.e., FP1, F3, F7, Fc1, Fc5, FP2, F4, F8, Fc6, Fc2, FZ) post-stimulation, subtracted them from the pre-stimulation values, and then calculated the average of those differences for each patient. We extracted the power data for each power band, but we focused the statistical analyses on alpha and theta over the frontal and parietal regions, based on our a priori hypothesis. The same analysis was performed for connectivity data. *p* values were considered significant after correcting for multiple comparisons for the three conditions, three regions, and two power bands using a Bonferroni correction ($p < 0{,}003$). Behavioural data was analyzed using RStudio. We performed a Kruskal–Wallis test to assess the main effect of the intervention on the three conditions' pre- and post-differences (delta) using CRS-R total scores. We also transformed CRS-R total scores in the CRS-R index using the method described by Annen and colleagues (Annen et al. 2019), since this index is more sensitive to changes and directly linked to diagnosis (with a cutoff at 8.315 that distinguishes patients in UWS from patients in MCS). With this method, the CRS-R index is calculated by combining scores for reflexes and cognitive behaviors of every CRS-R subscale (only the highest observed behavior for each subscale) and computes the score from a transposition matrix. We then performed the same analyses on CRS-R index deltas. We conducted post-hoc tests to determine whether the main effect of treatment was significant with the Wilcoxon signed-rank test. *p* values were considered significant after correcting for multiple comparisons for the three conditions using a Bonferroni correction ($p < 0.0125$).

Further exploratory analysis: Given the results we obtained on both behavioural and EEG data, we performed a posteriori exploratory analysis investigating whether tPCS was similar to the sham and different from tDCS. We therefore compared the sham and tPCS combined [tPCS + sham] to tDCS. This was based on the similarities we observed between tPCS and the sham and we performed this analysis on RStudio for both the behavioural data (CRS-R total scores and CRS-R modified index) and the EEG data (qEEG and connectivity of alpha and theta, frontal and parietal) with an unpaired Wilcoxon signed-rank test.

3.4 Results

We enrolled 13 patients, but one patient died right after enrollment; therefore, 12 patients were included in the analyses (10 MCS, 2 EMCS, 8 F, 4 TBI, 50.3 ± 14 y.o., 8.8 ± 10.5 months post-injury). Eleven of these patients received all three sessions, whereas one received only tPCS and sham stimulations due to a decompressive craniectomy without flap replacement under the stimulation site that took place briefly after enrolling the patient. Details on the patients' enrollment can be found in the flow chart in Figure 1. Demographic details of the patients can be found in Table 1.

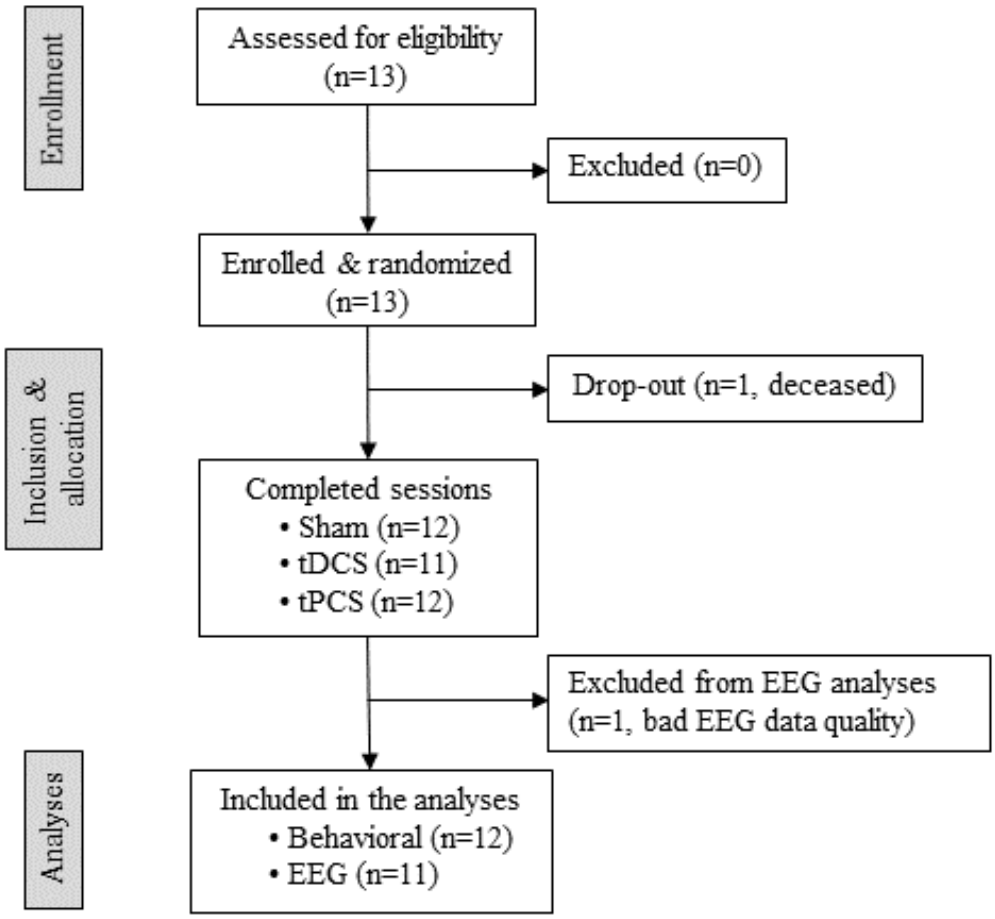

Fig. 2 Flow chart of patient enrolment. The specifics on patients' enrolment, allocation, and analysis are reported. We were in contact with the collaborating centres, and when a patient matched the inclusion criteria, we were contacted by their health care team to go screen the patient and confirm eligibility. This is why this could be considered as a convenience sample (i.e., non-probability sampling, typical of pilot studies). Thirteen patients were screened for eligibility. All patients were randomly allocated to a specific order of the three sessions (see Table 1 for details on patients' scores and characteristics; See Table 2-4 for details on the CRS-R scores and subscores). One patient died after the randomization for medical reasons external to the study. Eleven patients received all three sessions, one patient received tPCS and sham, but no tDCS due to decompressive craniectomy that took place after the first two sessions. One patient was not included in the EEG analysis due to the poor quality of the EEG data. Transcranial direct current stimulation (tDCS), transcranial pulsed current stimulation (tPCS), electroencephalography (EEG)

ID	Age	Sex	Diagnosis	TSI	Aetiology	Order of Sessions	CRS-R Measure	Pre Sham	Post Sham	Pre tDCS	Post tDCS	Pre tPCS	Post tPCS
ID01	71	F	MCS+	12.8	Stroke	sham tPCS tDCS	Total Score	15	13	10	15	14	14
							Index	49.6	47.5	3.5	64.9	55.9	49.3
ID02	31	F	MCS+	5	Anoxic	tDCS tPCS	Total Score	8	10	9	9	9	9
							Index	14.9	31.5	16	15.9	23.2	15.2

						sham							
ID03	50	F	MCS-	4	Stroke	tPCS tDCS sham	Total Score	4	2	2	4	7	5
							Index	3.4	1.37	1.37	1.3	21.1	11.8
ID04	63	M	MCS-	2.5	TBI	sham tPCS /	Total Score	5	6	N/A	N/A	3	3
							Index	19.8	6.5	N/A	N/A	3.1	3.1
ID05	47	F	MCS-	3	Stroke	sham tDCS tPCS	Total Score	7	5	9	6	7	8
							Index	5.8	3.8	15.2	4.8	6.9	7
ID06	65	F	MCS+	5.8	Stroke	tPCS sham tDCS	Total Score	5	4	5	10	4	7
							Index	4.5	3.5	4.5	29.8	3.4	13.1
ID07	60	F	eMCS	33	TBI	tDCS sham tPCS	Total Score	14	15	18	19	17	16
							Index	55.8	57.6	82	90	73.6	65.2
ID08	34	M	MCS-	4.5	TBI	tPCS tDCS sham	Total Score	8	10	11	10	10	10
							Index	22.2	23.6	24.6	23.6	23.6	23.6
ID09	51	F	MCS-	4	Stroke	tDCS sham tPCS	Total Score	10	10	9	8	9	11
							Index	23.5	23.6	22.5	21.5	23.2	32.6
ID10	23	M	MCS+	2	TBI	sham tPCS tDCS	Total Score	13	15	17	18	15	15
							Index	4.2	57	66.3	74.6	49.6	49.6
ID11	53	F	MCS-	3	Stroke	sham tDCS tPCS	Total Score	7	6	7	8	6	5
							Index	14.5	13.5	5.9	7	6.2	4.5
ID12	56	M	eMCS	3	Stroke	tDCS tPCS sham	Total Score	20	19	17	18	20	20
							Index	91.3	82.3	66.3	74.6	91.3	91.3

Table 1. Clinical characteristics of the sample and behavioural results. Age is reported in years. Diagnosis refers to the two screening CRS-R before enrolment (minimally conscious state—MCS, emergence from minimally conscious state—eMCS. Aetiology reported (traumatic brain injury—TBI). Time since injury (TSI) reported in months. Total CRS-R scores pre- and post-stimulation are reported for each condition; the index of the CRS-R is reported on the second row for each patient. N/A—Not assessed.

ID	**Sham Pre**							**Sham Post**						
	CRS-R Total Score (Index)	A	V	M	O	C	Ar	CRS-R Total Score (Index)	A	V	M	O	C	Ar
ID01	15 (49.6)	3	3	5	2	0	2	13 (47.5)	3	2	6	0	0	2

ID02	8 (14.9)	3	0	2	2	0	1	10 (31.5)	4	0	2	2	1	1
ID03	4 (3.4)	0	1	2	0	0	1	2 (1.37)	0	1	0	0	0	1
ID04	5 (19.8)	1	3	0	1	0	0	6 (6.5)	2	1	1	2	0	0
ID05	7 (5.8)	1	1	2	1	0	2	5 (3.8)	1	1	0	1	0	2
ID06	5 (4.5)	0	0	2	2	0	1	4 (3.5)	0	0	1	2	0	1
ID07	14 (55.8)	3	4	5	0	0	2	15 (57.6)	3	4	5	2	0	1
ID08	8 (22.2)	2	3	2	0	0	1	10 (23.6)	1	3	2	2	0	2
ID09	10 (23.5)	1	3	2	2	0	2	10 (23.6)	1	3	2	2	0	2
ID10	13 (4.2)	3	3	4	1	0	2	15 (57)	4	4	4	1	0	2
ID11	7 (14.5)	1	2	2	2	0	0	6 (13.5)	0	2	2	2	0	0
ID12	20 (91.3)	4	5	5	2	2	2	19 (82.3)	4	5	5	2	1	2

Table 2. Details on subscores of patients before and after sham stimulation. The Table shows the ID of each patient before (Pre) and after (Post) sham stimulation, the CRS-R total scores and CRS-R index between parentheses and the subscale scores for each subscale: A – Auditory, V – Visual, M – Motor, O – Oromotor, C – Communication, Ar – Arousal.

ID	**tDCS Pre**							**tDCS Post**						
	CRS-R Total Score (Index)	A	V	M	O	C	Ar	CRS-R Total Score (Index)	A	V	M	O	C	Ar
ID01	10 (3.5)	3	N/A	5	1	0	1	15 (64.9)	4	4	5	1	0	1
ID02	9 (16)	3	1	2	2	0	1	9 (15.9)	3	1	2	2	0	1
ID03	2 (1.37)	0	1	0	0	0	1	4 (1.3)	0	3	0	0	0	1
ID04	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ID05	9 (15.2)	3	1	2	1	0	2	6 (4.8)	1	1	1	1	0	2
ID06	5 (4.5)	0	0	2	2	0	1	10 (29.8)	0	0	5	3	0	2
ID07	18 (82)	4	5	6	1	0	2	19 (90)	4	5	6	1	1	1
ID08	11 (24.6)	2	3	2	2	0	2	10 (23.6)	1	3	2	2	0	2
ID09	9 (22.5)	1	3	2	1	0	2	8 (21.5)	1	3	2	0	0	2
ID10	17 (66.3)	4	4	4	2	1	2	18 (74.6)	3	4	6	2	1	2
ID11	7 (5.9)	0	1	2	2	0	2	8 (7)	1	1	2	2	0	2
ID12	17 (66.3)	3	5	4	2	1	2	18 (74.6)	3	4	5	2	2	2

Table 3. Details on subscores of patients before and after tDCS stimulation. The Table shows the ID of each patient before (Pre) and after (Post) tDCS stimulation, the CRS-R total scores and CRS-R index between parentheses and the subscale scores for each subscale: A – Auditory, V – Visual, M – Motor, O – Oromotor, C – Communication, Ar – Arousal.

ID	tPCS Pre							tPCS Post						
	CRS-R Total Score (Index)	A	V	M	O	C	Ar	CRS-R Total Score (Index)	A	V	M	O	C	Ar
ID01	14 55.9	3	3	5	0	1	2	14 49.3	4	3	4	2	0	1
ID02	9 23.2	4	0	2	2	0	1	9 15.2	3	0	2	2	0	2
ID03	7 21.1	2	3	0	1	0	1	5 11.8	1	2	0	1	0	1
ID04	3 3.1	1	0	0	2	0	0	3 3.1	1	1	0	1	0	0
ID05	7 6.9	2	1	2	1	0	2	8 7	2	1	2	1	0	2
ID06	4 3.4	0	0	2	1	0	1	7 13.1	0	0	2	3	0	2
ID07	17 73.6	3	4	6	1	1	2	16 65.2	3	4	6	1	0	2
ID08	10 23.6	1	3	2	2	0	2	10 23.6	1	3	2	2	0	2
ID09	9 23.2	1	3	2	2	0	1	11 32.6	3	3	2	2	0	1
ID10	15 49.6	3	4	4	2	0	2	15 49.6	4	3	4	2	0	2
ID11	6 6.2	1	1	2	2	0	0	5 4.5	1	1	2	0	0	1
ID12	20 91.3	4	5	5	2	2	2	20 91.3	4	5	5	2	2	2

Table 3. Details on subscores of patients before and after tPCS stimulation. The Table shows the ID of each patient before (Pre) and after (Post) tPCS stimulation, the CRS-R total scores and CRS-R index between parentheses and the subscale scores for each subscale: A – Auditory, V – Visual, M – Motor, O – Oromotor, C – Communication, Ar – Arousal.

Feasibility and Safety

The feasibility of performing a randomized clinical trial in a double-blind design, comparing two stimulations to the sham, was demonstrated with this pilot, in which patients completed the assigned sessions without any side-effects. Blinding was achieved by mounting both devices every time, regardless of the condition. Safety of tPCS in patients with DoC was demonstrated as we did not observe any side-effects in any of the patients during the stimulation sessions. Moreover, when EEG recordings post stimulation were inspected, no abnormal activity was detected. At the individual level, for one subject

(i.e., ID01), the family reported trouble falling asleep and longer wakefulness for one night after receiving active tPCS. No other change after stimulation was reported for any of the subjects.

Crossover effect

We did not find any significant difference between groups at the baselines before each session for EEG nor for the behavioural data.

EEG

The Kruskal–Wallis test showed no significant difference between the stimulation conditions for EEG power for the alpha and theta bands (see Annexes for details). Frontal and parietal theta band connectivity did not show any significant differences. See Table 5 and Figure 3a, b for details.

	Sham	**tDCS**	**tPCS**	**Chi-Squared (*p*-Value)**
Alpha frontal	−0.08 (−0.12; 0.01)	0.10 (−0.016; 0.15)	0.02 (−01; 0.09)	4.90 (0.08)
Alpha parietal	0.000 (−0.02; 0.06)	0.045 (0.001; 0.12)	0.011 (−0.04; 0.13)	0.673 (0.71)
Theta frontal	−0.037 (−0.09; 0.09)	0.142 (−0.05; 0.19)	−0.122 (−0.20; 0.01)	5.697 (0.05)
Theta parietal	−0.12 (−0.20; 0.00)	−0.04 (−0.19; 0.03)	−0.01 (−0.09; 0.00)	0.08 (0.64)
CRS-R total score	−0.5 (−1; 1.25)	1 (−0.5; 1.5)	0 (−0.25; 0.25)	1.47 (0.49)
CRS-R modified index	−1.04 (−2.09; 1.47)	4.52 (−0.26; 10.42)	0 (−6.93; 0)	4.11 (0.13)

Table 5. **Details on EEG Connectivity data and behavioural data.** Cells report median and (IQR25—IQR75) of post- and pre-differences of EEG data (each band and region for each condition; for example, alpha frontal median for sham condition was −0.08 and IQR25 −0.12, IQR75 0.01) and behavioural data (CRS-R total scores and modified index for each condition. For example, the median of CRS-R total scores

for sham condition was −0.5 and IQR25 −1, IQR75 1.25). The last column reports the Chi-squared and *p*-value (between parentheses) for the Kruskal–Wallis comparing that band and region between conditions for the EEG data and the Chi-squared and *p*-value for the Kruskal–Wallis between conditions for behavioural data.

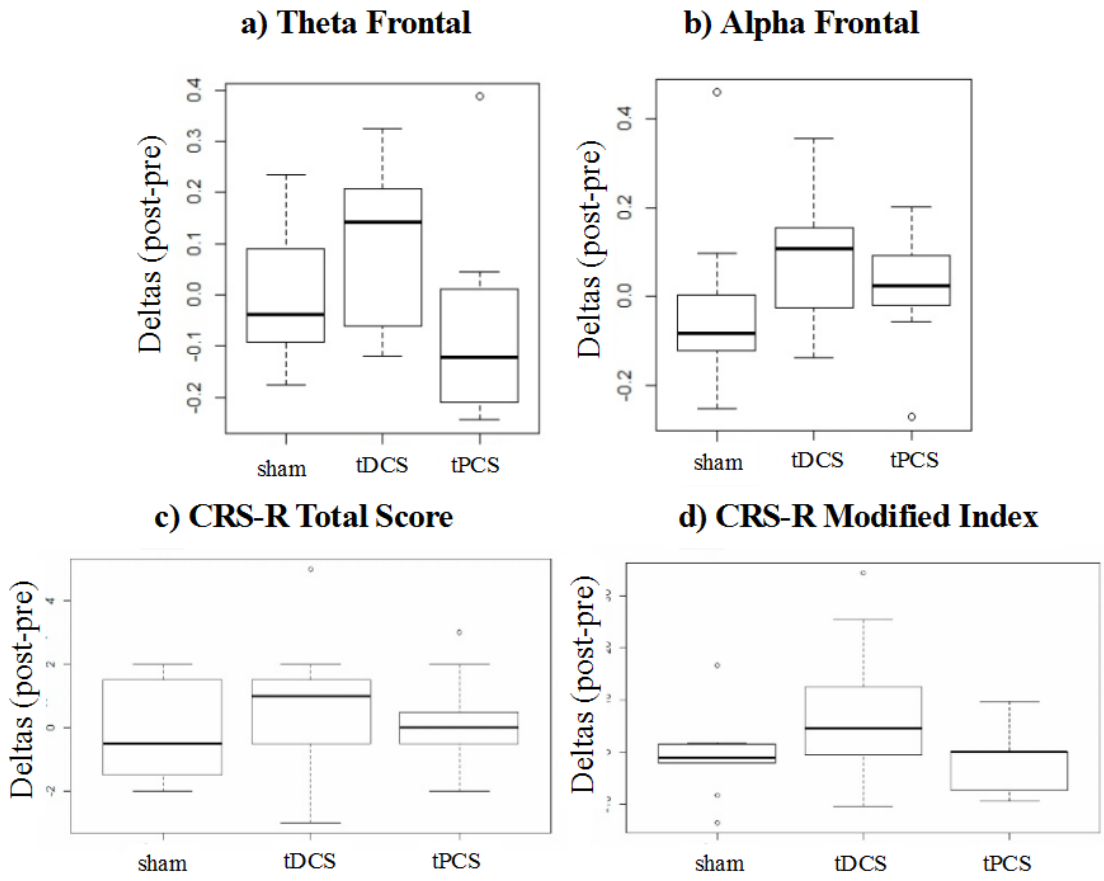

Figure 3. (**a**–**d**) **Boxplot group differences between treatment conditions.** (**a**) Functional connectivity in the frontal theta band, (**b**) in the frontal alpha band, (**c**) CRS-R total score, and (**d**) CRS-R index. The rectangles represent the interquartile range 25 and 75; the bold horizontal lines inside the rectangles are the medians of each group, empty circles indicate potential outliers (minimum or maximum value in the data) whereas the whiskers are calculated by the default boxplot R function and represent 1.5 distance from IQR 25 and IQR 75. CRS-R = Coma Recovery Scale-Revised, Sham = sham treatment, tDCS = transcranial direct current stimulation treatment, tPCS = transcranial pulsed current stimulation treatment, IQR = interquartile range.

Behavioural

For the behavioural data, we did not find any significant changes in CRS-R scores at the group level for any of the conditions. This analysis was performed with the Kruskal–Wallis test on both the CRS-R total score (Chi squared= 1.47, df = 2, *p*-value = 0.47) and on the

CRS-R index (Chi-squared= 4.11, df = 2, *p*-value = 0.12). See Table 2 and Figure 3c, d for details.

Exploratory analysis

When comparing each technique separately with the Wilcoxon signed-rank test, we found that for both the CRS-R total score and for the modified index tPCS and sham were very similar, almost reaching *p*-values of 1. Therefore, to increase statistical power and better investigate the differences between tPCS and tDCS, we conducted an exploratory analysis comparing [tPCS + sham] to tDCS with an unpaired Wilcoxon signed-rank test. We found a significant difference between tDCS vs. [tPCS + sham] conditions for theta connectivity in the frontal regions (W = 59, p = 0.04) (for individual results, see Table 6; for visual schematization of theta frontal data, see Figure 4a). This exploratory analysis also showed a significant difference between the two groups when looking at the CRS-R index (W=384, p= .041). When performing this analysis on CRS-R total score, however, the difference did not reach significance (W= 111, p= .267). For a visual representation, see Fig 3b-c.

	(tPCS + sham)	**tDCS**	**W (*p*-Value)**
Alpha frontal	−0.00 (−0.10; 0.05)	0.10 (−0.01; 0.15)	71 (0.11)
Alpha parietal	0.00 (−0.02; 0.11)	0.04 (0.00; 0.12)	96 (0.58)
Theta frontal	−0.060 (−0.16; 0.02)	0.14 (−0.05; 0.20)	59 (0.04)*
Theta parietal	−0.046 (−0.15; 0.00)	−0.03 (−0.19; 0.03)	104 (0.82)

Table 6. Detailed results from the exploratory analysis testing [tPCS + sham] against tDCS condition. Cells report the median and (IQR25—IQR75) of each band and region for each condition. For example, the alpha frontal median for the [tPCS + sham] condition was −0.002 and IQR25 −0.107, IQR75 0.051. The last column reports the Wilcoxon index and *p*-value (between parentheses) for the Wilcoxon signed-rank statistical tests. tPCS = transcranial pulsed current stimulation, sham = sham, tDCS = transcranial direct current stimulation. The * indicates significance of p-value

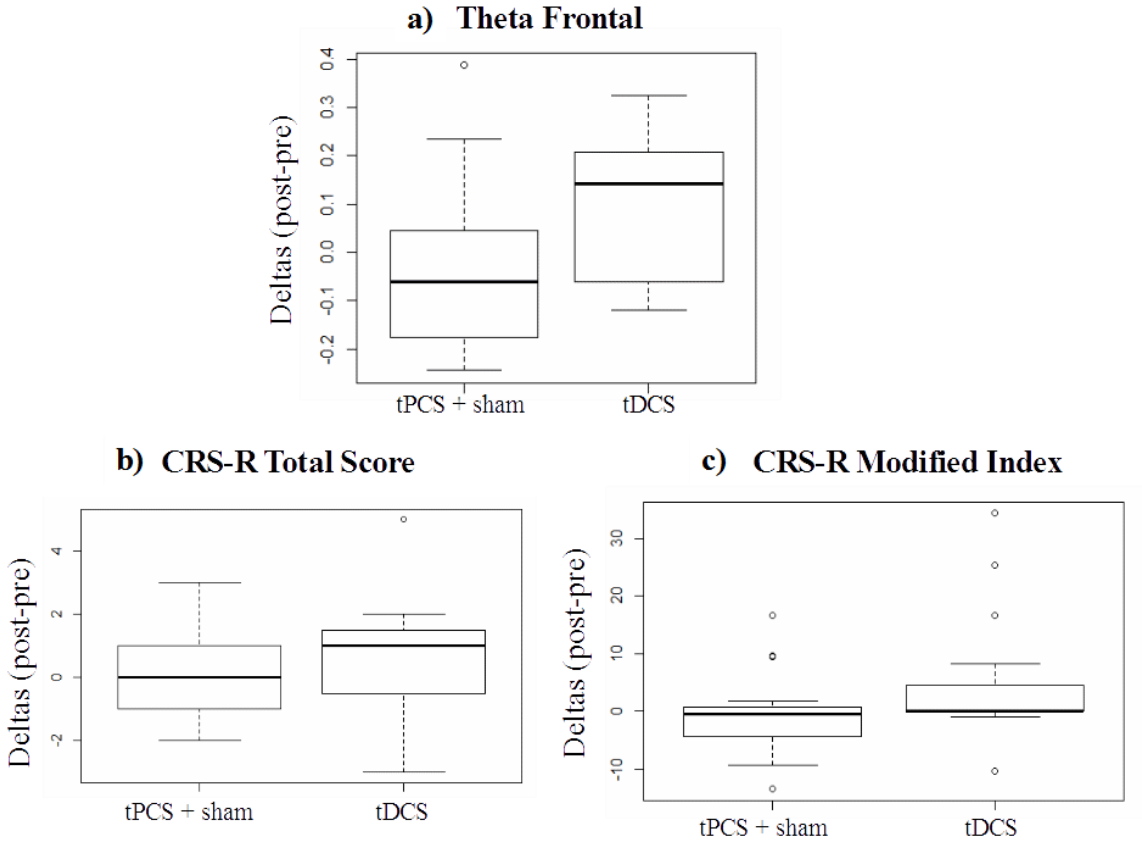

Fig. 4 Group differences between treatment conditions on ([tPCS + sham]) and tDCS: (a) on theta frontal functional connectivity, (b) on CRS-R total score, and (c) on CRS-R index. Calculated with unpaired Wilcoxon signed-rank test. tPCS = transcranial pulsed current stimulation, sham = sham, tDCS = transcranial direct current stimulation.

3.5 Discussion

To our knowledge, this is the first clinical trial on the use of tPCS stimulation in patients with DoC. Altogether, this work demonstrated the safety of tPCS in patients with DoC and the feasibility of combining two tES techniques in randomized controlled double-blind clinical trials.

The successful completion of this pilot study, without major issues nor side-effects to the stimulations, opens the door for future studies to investigate the use of tPCS as a therapeutic intervention for patients with DoC. Based on our outcome measures, we did not find any significant main effect of one session of bi-mastoid tPCS compared to left prefrontal tDCS or to sham on the behavioural or electrophysiological outcomes.

When comparing all three types of stimulation, we noticed that tPCS seemed to be similar to the sham. Therefore, in an exploratory analysis, we merged the two groups [tPCS + sham] and compared them to tDCS to increase the statistical power. Doing so, we observed an improvement following tDCS at the neurophysiological and behavioural levels compared to [tPCS + sham]. These analyses were exploratory and must be interpreted with caution.

According to our functional connectivity analyses, there was no significant effect of bi-mastoid tPCS stimulation on theta and alpha bands, as predicted. Alpha functional connectivity in the frontal and central regions has been reported to be boosted following tPCS in a study on healthy controls using the same frequency as in this study (Morales-Quezada, Castillo-Saavedra, et al. 2015). Although we used the same stimulation bandwidth (6-10 Hz), we did not observe a significant increase in frontal alpha functional connectivity compared to the other stimulation groups. It is important to note that Morales-Quezada and colleagues obtained these results on healthy participants, while it is well known that patients with prolonged DoC likely suffer from altered EEG patterns which could have limited the effect of 6-10 Hz tPCS. On the other hand, tPCS applied with a frequency between 1–5 Hz was shown to modulate theta power and fronto-temporal connectivity in healthy individuals (Morales-Quezada et al. 2014). Therefore, to increase theta band power in DoC patients, targeting this frequency range (1–5 Hz) could be more effective (Dissanayaka et al. 2020). Another parameter that could explain the absence of effects is that the number of sessions of tPCS was too small to induce any significant behavioural and neurophysiological changes. This may also apply to tDCS given its relatively small effect size (Castillo-Saavedra et al. 2016).

Regarding tDCS, results from our exploratory EEG analyses tend to confirm recent studies. We found an increase after left prefrontal tDCS for theta frontal connectivity. This result not only replicates those of previously mentioned studies (Cavinato et al. 2019; Carrière, Mortaheb, et al. 2020), but also supports the idea that theta band connectivity in EEG might serve as a biomarker of responders to non-invasive brain stimulation with tDCS, as proposed in previous studies (Thibaut et al. 2018; Hermann et al. 2020).

In the current literature, most studies have also generally found an effect of tDCS on alpha band connectivity (Cavinato et al. 2019; Carrière, Mortaheb, et al. 2020; Hermann et al. 2020) and we could not reproduce this result in the present study. It should be noted that Hermann and colleagues did not find effects on isolated alpha connectivity but on cross-

frequency theta-alpha functional connectivity (e.g., 4–10 Hz), which was pronounced in responders parieto-occipitally when compared to non-responders. Thus, investigating combined frequency bands and intra- as well as inter- hemispheric functional connectivity seems promising for further tDCS/tPCS trials in DoC patients.

Regarding the behavioural effects, we did not find an effect of tPCS stimulation on the CRS-R total score and CRS-R index. Although we know from healthy subject studies that tPCS can increase performance in attention-switching tasks (Morales-Quezada et al. 2016) we did not observe similar effects in DoC patients. Regarding arousal, in a study targeting patients with chronic visceral pain, tPCS or combined tDCS-tPCS stimulation did not influence self-reported sleepiness (Thibaut, Russo, et al. 2017). Similarly, wakefulness was not significantly changed by the stimulation in our study. Arousal was reported to be higher in one patient (caregivers reports), however, this was only observed at the individual level. The absence of behavioural effects could be explained by several factors. The first one is the small sample size, which might have led to underpowered statistical tests. As we did not use a neuronavigation system, electrode location might have been imprecise. In addition, patients received a single session of tES, when we know that repeated sessions are to be preferred, to induce significant and long-lasting behavioural effects. Finally, the overall length of each session (with a duration of about 3 hours) is likely to have caused an increase in the patients' fatigue. Further speculations regarding the effectiveness of tPCS in DoC patients cannot be made yet, as this is, the first study of tPCS application in this field.

In the exploratory analysis of this study, we found that tDCS slightly improved the performance of patients on the CRS-R index compared to the [tPCS + sham]. We addressed this finding to the fact that there is already evidence for the efficacy of left prefrontal tDCS as a tool to enhance the responsiveness of patients with DoC (Thibaut, Schiff, et al. 2019). Other studies showed positive behavioural effects of tDCS when stimulation was applied for at least one week: Estraneo and colleagues found clinical and electrophysiological improvements in five out of 13 patients; however, these changes lasted over several months (Estraneo et al. 2017). Another study applying 5 days of prefrontal tDCS also found a significant long-lasting clinical improvements one week after treatment (Thibaut, Wannez, et al. 2017). Finally, tDCS applied for 4 weeks at home had a significant effect on 27 patients with DoC, demonstrating the stability of its effect even if applied in non-clinical settings (Martens et al. 2018).

Study limitations

The biggest limitation of our study was the sample size of twelve patients, which decreased our statistical power to detect a treatment effect. The reason behind this choice of sample size was that this combination of stimulation montages is technically challenging for researchers and very demanding for patients, as the protocol lasts about 3 hours including EEG/tES placement and two CRS-R assessments. For this reason, this was planned to be a pilot study to test safety and feasibility.

Regarding the lack of neuronavigation, montages tailored to the patients' specific brain lesions might have more probabilities of success (Martens et al. 2020), since the structural preservation of brain areas targeted by the stimulation seem to be a crucial factor to respond to stimulation (Thibaut, Di Perri, et al. 2015). In the present study, we could not use this approach as not all patients underwent recent neuroimaging investigation that could have helped us to adapt tES montages to each patient. However, this should definitely be considered for future studies.

Regarding the stimulation parameters, in our case, although the frequency that was chosen for tPCS was based on modeling studies, it might not have been optimal for DoC patients. Based on the neural noise hypothesis, spontaneous frequencies need to be present in the brain at the baseline in order to be entrained by neuromodulation (McDonnell and Ward 2011). A frequency-range between 6 and 10 Hz (as the one used in our study) was able to produce EEG changes in healthy participants (Vasquez et al. 2017). We can thus hypothesize that stimulation within this range in patients with DoC, which demonstrate predominant activities within the delta-theta range, failed to produce neurophysiological results. In this context, targeting lower frequencies (e.g., 1–5 Hz) could be more effective for patients with DoC. In addition, the placement of the electrodes might have influenced the level of cortical activation (Datta et al. 2013). Future studies might look at different modalities of stimulation targeting, for instance, the prefrontal cortex instead of the mastoids.

Regarding the EEG functional connectivity results, another drawback to mention is that the Mohawk method pipeline (Chennu et al. 2017) for the extraction of functional connectivity values has not yet been validated with a 27 electrodes system and was initially developed for a 265 channel high-density EEG. For this study, the pipeline has been adapted according to the Neuroconn EEG system, so there could have been unpredicted weaknesses in the pipeline derived from the number of electrodes. However, Chennu and

colleagues reported that the median dwPLI connectivity was a relatively stable index against reducing the number of electrodes down to at least 11 electrodes. Another study on multichannel tDCS to reduce hypertonia in DoC patients found an effect of the treatment on beta connectivity with only eight EEG recording channels (Thibaut, Piarulli, et al. 2019).

Another limit to our study is probably the choice of applying only one session of stimulation and not repeated sessions. As mentioned before, repeated session studies have shown stronger and longer behavioural effects. However, they take longer to complete and the dropout rates are higher than those of single session studies. As this was planned to be a feasibility study, we decided to start with a single stimulation session. However, future studies should explore the effects of repeated sessions of tPCS.

Finally, fluctuating arousal and fatigue could have induced a bias during the behavioural and electrophysiological measurements. Each session lasted around 3 hours, and for patients with DoC, whether in bed or in a wheelchair, such a long protocol is likely to induce fatigue. This, in turn, can impact both neurophysiological and behavioural outcomes. Maintaining arousal is a key element to attention and awareness (Chennu and Bekinschtein 2012), which is known to be impaired in DoC patients (Olivia Gosseries, Vanhaudenhuyse, et al. 2011). The behavioural assessments were always preceded by an arousal protocol, as specified in the guidelines of the CRS-R (Giacino, Kalmar, and Whyte 2004). Nevertheless, in the future, short protocols should be preferred to avoid fatigue.

4. Study 3: Characterization of the endotype of responders

Adapted from:

Barra A.*, Huerta R.*, Martens G., Gosseries O., Laureys S. and Thibaut A. 2022. Characterization of the endotype of DoC responders to tDCS. *Brain Stimulation.* Submitted as Letter to the Editor.

4.1 Summary

The treatment for patients with disorders of consciousness (DoC) still presents many challenges. So far, more than ten clinical trials on transcranial direct current stimulation (tDCS) in DoC have been published with a rate of responders varying from 20% to 50%. However, many questions on the effects of tDCS at the individual level (endotype of responders) remain unanswered. Our goal was to explore the characteristics of responders through a retrospective analysis of a pooled sample, obtained from seven tDCS double-blind randomized controlled trials, targeting several brain regions, conducted between 2014 and 2021 in patients with DoC. We created a database including: date of birth, sex, aetiology, diagnosis, time since injury (TSI), date of stimulation, tDCS response. We explored these predictors with a logistic regression model. 140 patients were included (43 Females, 65 TBI, 95 patients in MCS, mean age 45.18 ± 16.72 y.o., mean time since injury – TSI 34.42 ± 55.67 months) of which 34 were responders (24.28%). Three variables significantly influenced the probability of being a responder: diagnosis (in favour of MCS, [OR = 3.51 (CI: 1.10 14); p-value = 0.017]), age (in favour of under 18 yo, [OR = 0.07 (CI: 0.00 0.86); p-value = 0.04]) and TSI (in favour of longer TSI, [OR = 1.12 (CI: 1.02, 1.25); p-value = 0.010]). This study is a first step towards a characterization of the endotype of tDCS responders. Since the first study on tDCS in patients with DoC in 2014, rates of responders are still low. With these results, we would like to orient future studies to favour enrolment of patients with prolonged MCS.

4.2 Introduction

The diagnosis of patients with DoC has reached a good consensus among the research community. However, Nowadays, patients with DoC have access to a limited array of treatment options to improve their condition. TDCS has been successfully tested for patients with DoC, it is currently the most explored NIBS technique and has showed effects in up to 50% of patients in MCS (Thibaut, Schiff, et al. 2019). However, many questions on the effects of tDCS as a treatment for patients with DoC remain unanswered. For

instance, why do only half of patients in MCS benefit from the stimulation? Why does it seem that patients in UWS do not have any effect from tDCS? What are the demographic characteristics and endotype that distinguish patients that respond to tDCS from those that do not? The objective of this study is to plunge into these questions about responders through a retrospective analysis of a pooled sample obtained from all the published tDCS studies performed at the Coma Science Group.

4.3 Methods

We pooled together the data from the totality of tDCS published studies that had been performed between 2014 and May 2021 by the Coma Science Group (GIGA Consciousness, University of Liège). These studies (Thibaut et al. 2014; Thibaut, Wannez, et al. 2017; Martens et al. 2018; 2019; Thibaut, Piarulli, et al. 2019; Carrière et al. 2020; Martens et al. 2020) explored tDCS as a treatment for patients with DoC and were performed in a randomized double-blind design with a sham control condition. In all these studies, responders were defined as patients that showed a new conscious behaviour (measured by the CRS-R) after the real tDCS that was never showed before nor after sham. We created a database including the following information about the subjects: sex, aetiology (TBI vs non-TBI), diagnosis at baseline (UWS vs MCS), TSI (in years) at inclusion, age at inclusion (categorized as: < 18, 18 to 60 and > 60 years old), in which protocol they were included (Thibaut et al. 2014; Thibaut et al. 2017; Martens et al. 2018; 2019; Thibaut et al. 2019; Carrière et al. 2020; Martens et al. 2020) and if they were responding to tDCS. We then proceeded to find the predictors of treatment response with logistic regression. We tested the goodness of fit of our model with a likelihood ratio test (significance set at 0.05). We considered non-linear associations between the continuous variables and the logit of being a responder by adding quadratic terms. Indeed, one of the assumptions of the logit equation is that the relation between the logit (tDCS response) and the continuous variables is linear, thus, to test if this was the case, we added a quadratic term to the model and tested for changes in the model fit. We tested for multiple collinearity with the variance inflation factor. We plotted the predicted probabilities from our logistic regression model against TSI at inclusion to graphically display our results.

4.4 Results

140 patients were included in this retrospective study (43 Females, 65 TBI, 95 patients in MCS, mean age 45.18 ± 16.72 y.o., mean time since injury – TSI 34.42 ± 55.67 months) of which 34 responded to tDCS (see Table 1 for complete database). Three variables resulted significant from our analysis on predicted probability of being a responder: diagnosis, age and time since injury at the time of stimulation (for details see figure 1). Patients in MCS showed four times the odds of being a responder compared to UWS [OR = 3.51 (CI: 1.10 14); p-value = 0.017]. TSI also increases the probability of being a responder, as the months since injury increase [OR = 1.12 (CI: 1.02, 1.25); p-value = 0.010]. Note that the interaction between TSI and diagnosis (i.e., MCS and UWS) results in very different predicted probabilities of being a responder (see figure 2 for details). Being between the age of 18 and 60 seems to decrease the probabilities of responding to tDCS compared to being younger than 18 (age, in favour of under 18 yo, [OR = 0.07 (CI: 0.00 0.86); p-value = 0.04]. This result is difficult to interpret; we know the age variable has a significant influence in the model prediction but we do not know in detail how different age categories are involved in this influence. We do know, however, that we do not have many patients under the age of 18 in our sample, thus this could have skewed the results.

ID	Responder	Diagnosis	Sex	TSI (months)	Aetiology	DLPFC	Age (years)
1	No	UWS	M	1.4	anoxic	Yes	75
2	No	MCS	M	13.1	TBI	Yes	25
3	No	MCS	M	44.5	TBI	Yes	35
4	No	MCS	F	133.3	TBI	Yes	56
5	No	MCS	F	1.7	TBI	Yes	52
6	No	MCS	F	21.3	TBI	Yes	25
7	No	MCS	M	15.7	TBI	Yes	25
8	No	MCS	M	104.4	anoxic	Yes	39
9	No	MCS	M	106.1	hem	Yes	74
10	No	MCS	M	92.6	anoxic	Yes	57
11	No	MCS	M	29.3	anoxic	Yes	30
12	No	MCS	M	47.5	other	Yes	70
13	No	MCS	M	20.2	TBI	Yes	31
14	No	MCS	M	15.3	TBI	Yes	47
15	No	MCS	M	4.0	hem	Yes	64

16	No	MCS	M	89.3	anoxic	Yes	25
17	No	MCS	M	33.3	TBI	Yes	35
18	No	MCS	M	2.6	TBI	Yes	23
19	No	MCS	F	6.0	TBI	Yes	56
20	No	MCS	M	25.0	TBI	Yes	25
21	No	MCS	M	70.0	TBI	Yes	24
22	No	MCS	M	110.0	TBI	Yes	31
23	No	MCS	F	17.2	TBI	Yes	39
24	No	MCS	M	3.0	anoxic	Yes	56
25	No	UWS	F	26.8	TBI	Yes	27
26	No	UWS	M	1.9	TBI	Yes	18
27	No	UWS	M	1.0	anoxic	Yes	70
28	No	UWS	M	1.0	hem	Yes	57
29	No	UWS	F	4.2	anoxic	Yes	49
30	No	UWS	M	231.6	anoxic	Yes	36
31	No	UWS	M	7.5	anoxic	Yes	49
32	No	UWS	M	15.2	anoxic	Yes	43
33	No	UWS	M	24.7	anoxic	Yes	31
34	No	UWS	F	58.3	anoxic	Yes	42
35	No	MCS	M	28.3	TBI	Yes	31
36	No	UWS	M	6.7	TBI	Yes	22
37	No	UWS	F	16.2	anoxic	Yes	50
38	No	UWS	M	12.8	anoxic	Yes	39
39	No	UWS	M	96.3	anoxic	Yes	49
40	No	UWS	M	16.2	TBI	Yes	26
41	No	UWS	M	11.2	TBI	Yes	25
42	No	UWS	M	51.5	anoxic	Yes	28
43	No	UWS	F	1.2	anoxic	Yes	30
44	No	UWS	M	3.0	anoxic	Yes	75
45	No	MCS	F	26.4	anoxic	Yes	51
46	No	MCS	M	17.9	anoxic	Yes	60
47	No	MCS	M	38.3	TBI	Yes	34
48	No	MCS	M	157.5	TBI	Yes	32
49	No	MCS	M	25.1	other	Yes	45
50	No	MCS	M	17.8	TBI	Yes	23
51	No	MCS	M	87.8	TBI	Yes	27
52	No	MCS	M	158.5	hem	Yes	63
53	No	MCS	M	14.4	other	Yes	55
54	No	MCS	M	28.4	other	Yes	59

55	No	MCS	M	14.6	anoxic	Yes	43
56	No	MCS	M	5.0	anoxic	No	33
57	No	MCS	M	1.5	TBI	No	69
58	No	UWS	F	1.3	hem	No	70
59	No	MCS	M	46.6	TBI	No	27
60	No	MCS	M	10.5	TBI	No	41
61	No	MCS	F	3.8	hem	No	28
62	No	UWS	F	7.6	TBI	No	51
63	No	MCS	F	33.7	TBI	No	27
64	No	UWS	M	8.1	TBI	No	26
65	No	UWS	F	5.7	anoxic	No	48
66	No	UWS	F	6.3	anoxic	No	51
67	No	MCS	M	10.9	TBI	No	38
68	No	UWS	M	1.1	anoxic	No	47
69	No	UWS	F	11.3	anoxic	No	40
70	No	eMCS	F	70.3	TBI	No	37
71	No	UWS	M	1.4	anoxic	No	44
72	No	UWS	M	2.8	anoxic	No	57
73	No	UWS	F	1.4	anoxic	No	46
74	No	MCS	F	20.7	anoxic	No	21
75	No	MCS	M	46.5	TBI	No	47
76	No	MCS	M	5.7	hem	No	64
77	No	MCS	M	4.3	anoxic	No	66
78	No	MCS	M	8.2	anoxic	No	58
79	No	UWS	F	4.9	anoxic	No	48
80	No	UWS	M	1.0	anoxic	No	60
81	No	eMCS	F	3.9	TBI	No	27
82	No	MCS	F	6.4	hem	No	49
83	No	MCS	M	3.3	anoxic	No	61
84	No	UWS	F	26.9	anoxic	No	42
85	No	MCS	M	18.6	TBI	No	32
86	No	MCS	M	12.9	TBI	No	20
87	No	UWS	M	12.4	TBI	No	33
88	No	UWS	F	10.1	anoxic	No	61
89	No	UWS	F	9.4	TBI	No	68
90	No	UWS	M	1.6	anoxic	No	75
91	No	UWS	F	19.5	anoxic	No	45
92	No	MCS	F	15.9	TBI	No	50
93	No	UWS	F	1.2	anoxic	No	60

94	No	MCS	F	69.0	TBI	No	79
95	No	eMCS	M	11.5	TBI	No	61
96	No	UWS	M	12.0	TBI	No	32
97	Yes	MCS	F	83.0	TBI	Yes	31
98	Yes	MCS	M	1.3	anoxic	Yes	71
99	Yes	MCS	M	1.2	hem	Yes	65
100	Yes	MCS	M	13.3	TBI	Yes	15
101	Yes	MCS	M	36.5	TBI	Yes	28
102	Yes	MCS	M	141.4	anoxic	Yes	35
103	Yes	MCS	M	158.3	TBI	Yes	30
104	Yes	MCS	M	378.6	TBI	Yes	43
105	Yes	MCS	M	81.3	TBI	Yes	26
106	Yes	MCS	F	10.4	anoxic	Yes	39
107	Yes	MCS	M	44.4	TBI	No	21
108	Yes	MCS	F	101.0	TBI	Yes	35
109	Yes	MCS	M	316.7	TBI	Yes	47
110	Yes	MCS	M	1.0	TBI	Yes	49
111	Yes	MCS	M	2.6	anoxic	Yes	86
112	Yes	MCS	F	47.7	hem	Yes	69
113	Yes	MCS	M	48.7	anoxic	Yes	11
114	Yes	UWS	F	2.8	anoxic	Yes	44
115	Yes	UWS	M	0.9	hem	Yes	68
116	Yes	MCS	F	10.9	hem	Yes	76
117	Yes	UWS	M	15.7	hem	Yes	67
118	Yes	MCS	M	143.4	TBI	Yes	34
119	Yes	MCS	F	41.3	TBI	Yes	30
120	Yes	MCS	M	93.3	anoxic	Yes	56
121	Yes	UWS	M	10.6	anoxic	No	64
122	Yes	eMCS	F	13.6	hem	No	63
123	Yes	eMCS	M	46.7	TBI	No	58
124	Yes	MCS	M	3.5	TBI	No	60
125	Yes	MCS	M	71.5	TBI	No	61
126	Yes	eMCS	F	12.0	TBI	No	62
127	Yes	eMCS	F	5.6	TBI	No	21
128	No	MCS	M	3.6	other	Yes	31
129	No	UWS	M	3.4	TBI	Yes	24
130	No	MCS	M	9.5	anoxic	Yes	64
131	No	MCS	F	14.0	hem	Yes	72
132	No	MCS	M	3.3	hem	Yes	51

133	Yes	MCS	M	25.7	TBI	Yes	41
134	No	eMCS	M	9.0	anoxic	Yes	46
135	Yes	MCS	M	11.6	anoxic	Yes	61
136	No	MCS	F	4.4	anoxic	Yes	48
137	Yes	MCS	M	5.7	TBI	Yes	64
138	No	MCS	M	5.6	TBI	Yes	35
139	No	MCS	M	11.8	TBI	Yes	24
140	No	MCS	M	10.3	anoxic	Yes	53

Table 1. Database with informations about the patients. List of columns: ID; Responder (Yes or No); Diagnosis (unresponsive wakefulness syndrome – UWS, minimally conscious state – MCS, emergent from the minimally conscious state – eMCS); Sex (male – M, female – F); Time Since Injury at inclusion (in months); Aetiology (traumatic brain injury – TBI, anoxic, haemorrhagic, other – meningitis/mixed/infective); received stimulation on DLPFC (Yes or No); Age (in years).

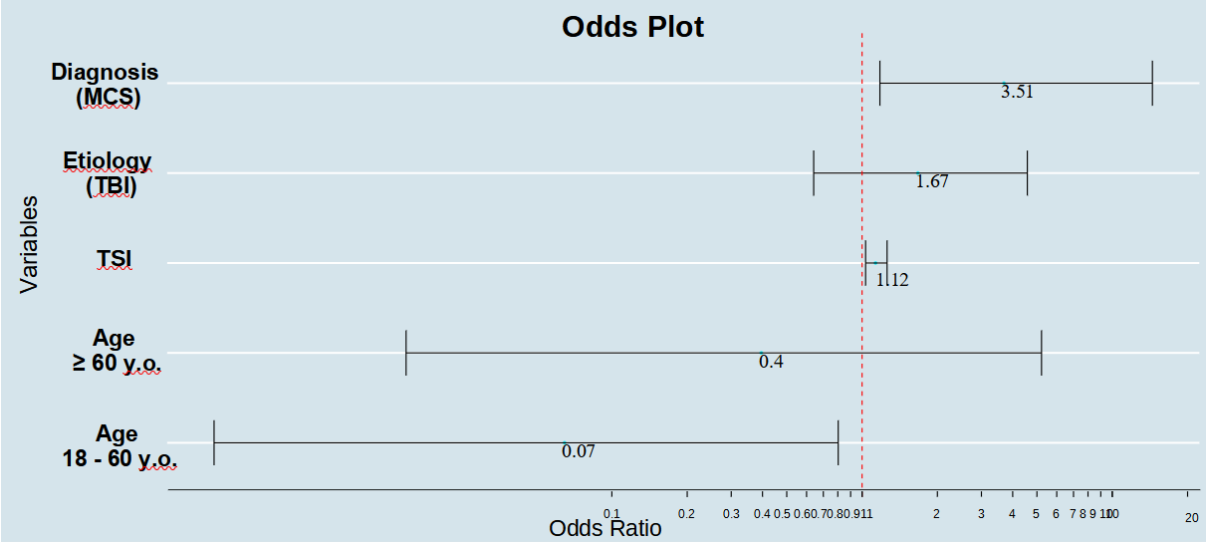

Fig. 1 Odds Plot of being a responder for each variable. The variables' names are presented on the left. For Diagnosis, MCS was the reference and for aetiology TBI was the reference.

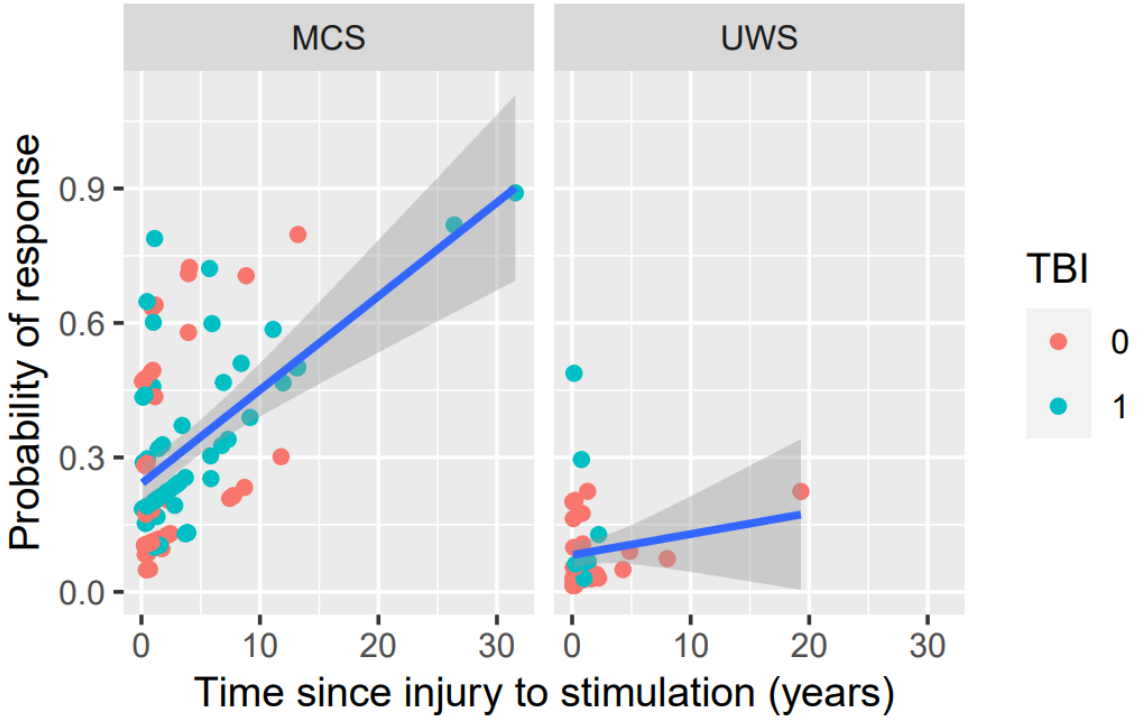

Fig. 2 Odds plotted according to interaction between TSI and diagnosis. The odds of being a responder are plotted according to the months (X axis), they increase with the number of months since injury for patients in MCS. We analysed the significance of aethiology in predicting the odds of being a responder both for the whole sample and for MCS and UWS patients separately and it was not a significant, however we added the information for TBI (blue) and non-TBI (pink) in our graph to provide this information. In our MCS subsample 54 were TBI, 24 anoxic, 12 hemorragic and 5 presented other aethiologies (e.g., infective, mixed). In our UWS subsample 11 patients were TBI, 30 anoxic and 4 hemorragic.

4.5 Discussion

The fact that MCS patients were significantly more often responding to tDCS compared to UWS has been demonstrated in a recent meta-analysis (Feng et al. 2020). Indeed, past studies have showed that MCS patients responded to tDCS more frequently than UWS. However, being able to prove that diagnosis has a significant role in the odds of being a responder, regardless of the targeted area, nicely confirms what researchers observed. Regarding TSI, it might seem counterintuitive that the more time passes between the injury and the stimulation and the more are the chances of responding to tDCS. However, several factors may play a role in this result. For instance, we know that after TBI the brain re-organizes itself throughout time (Ramanathan et al. 2021), especially in the cortex and in the motor tracts (Batschelett et al. 2022; Moreau-Debord et al. 2021). We could thus hypothesize that tDCS, to enhance neuronal firing after a severe brain injury, needs to

target brain areas that are more stable and already reorganized. This is also in line with our previous finding that responders present less atrophy in the stimulated area compared to non-responders (Thibaut, Di Perri, et al. 2015). Regarding the impact of age, as there are only 2 patients under 18 years old, we cannot draw any conclusion about age. However, as it seems that younger patients might respond better, more studies in children and adolescents should be performed as tDCS has been demonstrated to be a safe technique. To conclude, we think that the characterization of the endotype of responders to tDCS is a first step towards the development of targeted therapies. Thanks to these results we recommend to favour the enrolment of (young) MCS patients in prolonged DoC and to continue to recruit these patients even years after their injury.

5. Study 4: A novel closed-loop EEG-tDCS approach to promote responsiveness of patients in minimally conscious state: a study protocol

Adapted from:

Martens, G.*, Ibáñez-Soria, D.*, **Barra, A.***, Soria-Frisch, A., Piarulli, A., Gosseries, O., Salvador, R., Rojas, A., Nitsche, M.A., Kroupi, E. and Laureys, S., 2021. A novel closed-loop EEG-tDCS approach to promote responsiveness of patients in minimally conscious state: A study protocol. *Behavioural Brain Research*, *409*, p.113311.

5.1 Summary

tDCS applied over the prefrontal cortex has been shown to improve behavioural responsiveness in patients with DoC following severe brain injury, especially those in MCS. However, one potential barrier of clinical response to tDCS is the timing of stimulation with regard to the fluctuations of vigilance that characterize this population. Indeed, a previous study showed that the vigilance of MCS patients has periodic average cycles of 70 min (range 57 – 80 min), potentially preventing them to be in an optimal neural state to benefit from tDCS when applied randomly. To tackle this issue, we propose a new protocol to optimize the application of tDCS by selectively stimulating at high and low vigilance states. EEG real-time spectral entropy is used as a marker of vigilance and to trigger tDCS, in a closed-loop fashion. A software developed for this purpose (the Closed-Loop Manager - CLM) works in concomitance with the NIC stimulation software, calculating spectral entropy to then detect vigilance thresholds. When the threshold is detected the CLM triggers NIC to launch the tDCS without the researcher. We are conducing a randomized controlled crossover clinical trial on 16 patients in prolonged MCS who undergo three EEG-tDCS sessions 5 days apart (1. tDCS applied at high vigilance; 2. tDCS applied at low vigilance; 3. tDCS applied at a random moment). Behavioural effects are assessed using the Coma Recovery Scale-Revised at baseline and right after the stimulations. EEG is recorded throughout the session and for 30 min after the end of the stimulation. This unique and novel approach provides a patient-tailored treatment option, currently lacking in the field of disorders of consciousness.

5.2 Introduction

A severe injury to the head causing relevant brain damage can lead to an episode of coma, evolving either to brain death, full recovery or partial recovery with prolonged DoC. Although significant progress has been made in understanding the neural correlates of DoC (Giacino et al. 2014; Giacino et al. 2018b), current treatment options for DoC patients remain very limited (Thibaut, Schiff, et al. 2019). During the last 20 years, research on neuromodulation methods have flourished, as they represent a safe, cost-effective and straightforward technique that can be easily integrated in rehabilitation programs. However, the clinical effects seem inconsistent as several trials showed no relevant behavioural improvements using the same type of montage of Thibaut and colleagues in 2014 (Thibaut et al. 2014; Carrière, Mortaheb, et al. 2020; Mensen et al. 2020) or multichannel montages (Martens et al. 2020). A major challenge in the application of tDCS to DoC patients is the variability in the underlying brain state of individuals, impacting their behavioural response. Indeed, DoC patients typically present fluctuations in vigilance impacting their responsiveness to external stimuli (Giacino et al. 2018b; Candelieri et al. 2011; Schiff 2005; Cruse et al. 2013). Piarulli and colleagues used spectral entropy as measured in resting EEG to highlight the periodicity of fluctuations and suggested that spectral entropy variability in MCS could mirror the aforementioned behavioural fluctuations (Piarulli et al. 2016). The 6 MCS patients explored in their study presented a periodicity of 70 min (range 57 - 80 min), which could be comparable to the fluctuations in attention observed in healthy controls, while patients in UWS did not present this type of periodicity. Other studies supported the hypothesis that EEG spectral entropy may reflect the level of consciousness as well as the level of vigilance (Gosseries et al. 2011; Mateos et al. 2018). Even if spectral entropy has not been proved to be a direct proxy of vigilance levels for patients with DoC, it has been used as a measure of vigilance for healthy subjects (Shi et al. 2013). Moreover, generally conditions of low or absent consciousness are characterized by low spectral entropy whereas full consciousness is usually associated with high complexity EEGs and higher spectral entropy (Piarulli et al. 2016). A key component to tDCS responsiveness might thus be the timing of interventions with respect to the vigilance level, which could explain the inconsistent rate of responders reported in previous trials. The administration of tDCS during specific time windows (i.e., periods of low or high vigilance) could influence its clinical efficacy in MCS patients, since it is known that the positive effects of tDCS are dependent on the brain state (Nitsche

et al. 2003; Kuo et al. 2008). To this end, recent advances in tDCS software and hardware enable the implementation of a closed-loop set-up by complex computations performed in real-time. Proof-of-concept studies showed the efficiency of such approaches using EEG patterns to trigger tDCS in both animal models of epilepsy (Berényi et al. 2012) and healthy subjects (Leite et al. 2017). Using this technology to target specific levels of vigilance when applying tDCS in DoC patients could provide insight in the patterns of responsiveness and optimize future applications. This type of approach has not been reported in the literature for DoC patients yet. The proposed research tests a closed-loop system using EEG-vigilance measures (i.e., spectral entropy) to define the best moment for the application of tDCS in MCS patients, on an individual basis. The aim is to investigate whether tDCS applied during high or low vigilance states is more effective in increasing the level of consciousness than intervention during random vigilance states, as measured by behavioural and electrophysiological metrics. The random vigilance state serves the purpose of the control condition, as it is launched independently of the vigilance state, similar to what was usually done in previous clinical trials not taking into account patients' level of vigilance when applying tDCS. Thus, we would expect it to provoke less changes compared to the vigilance-based conditions (low or high), at the behavioural and neurophysiological levels. Our primary outcome parameter are the changes in behavioural responsiveness as measured with the CRS-R before and after stimulation. We hypothesize a greater increase in the CRS-R total score as well as a larger number of responders in patients receiving tDCS during high vigilance states, than for the other two conditions. Our secondary outcome parameters are the changes in power spectra and brain connectivity in the alpha and theta bands. We hypothesize to observe enhanced theta and alpha activity following active tDCS applied at high vigilance, compared to tDCS applied at low or random vigilance. We also aim to investigate in an explorative manner whether these EEG changes correlate with behavioural outcomes.

5.3 Methods

Study Design

This is a prospective multicentric randomized crossover trial. Ethical approval has been obtained by the local ethics committee. The study is registered on ClinicalTrials.gov (NCT03810079) and is being conducted in accordance with the Declaration of Helsinki

and with the Consolidated Standards of Reporting Trials (CONSORT) guidelines. All included patients' next of kin or legal representative have provided written informed consent to participate after the procedures, risks, and benefits of the study have been explained.

Population

Patients are screened through our institutional hospital and university databases. Inclusion criteria are as follows: 1) stable diagnosis of MCS provided by the inpatient specialized facility medical staff or by the patient's physician. The stable diagnosis of MCS confirmed by the investigators on at least 2 additional CRS-R performed within 1 week prior to tentative inclusion. The MCS condition must be due to acquired brain injury; 2) adult (18 years old – 65 years old); 3) French, English or Dutch-speaking; 4) at least 28 days post injury and; 5) central nervous system-active medication stable for at least a week. Exclusion criteria are as follows: 1) open craniectomy; 2) ventriculo-peritoneal shunt under the stimulated area (prefrontal cortex); 3) pacemaker; 4) ion channel blockers medication, 5) metallic cerebral implants and; 6) severe medical condition(s) that might influence clinical diagnosis or EEG activity (e.g., severe hepatic insufficiency, renal failure, or sub-continuous or abundant epileptiform discharges on standard EEG recordings). Note that since we know benzodiazepines and similar drugs could alter the power spectra (Carboncini et al. 2014) and thus, likely also the spectral entropy, we only include patients that have stable medications. In this way we can control the possible confounding factor of the drugs as they would alter the EEG in a consistent way across sessions. Indeed, if the medications are stable across sessions and the sessions are performed at the same time of the day each time we can ensure that the alteration will be the same for all the conditions. We conducted an a priori sample size estimation based on the individual CRS-R data relative to the chronic (i.e., >28 days post injury) MCS patients included in our previously published randomized clinical trial testing the effect of a single prefrontal tDCS session (Thibaut et al. 2014). The effect size in favour of the active tDCS treatment for this subsample of 21 patients was 1.03 (mean ± SD of the CRS-R total score difference for the active group: 1.048 ± 1.244; for the sham group: -0.095 ± 0.889). Based on this effect size and a power of 0.90 with an alpha error probability of 0.05, the sample size was estimated at 13 patients. To compensate for the potential amount of dropouts (20 % based on our previous experience), we will include 16 MCS patients.

Materials

The tDCS closed-loop system used, is the Starstim in its 20 channels version (Neuroelectrics, Barcelona). Starstim hybrid tES-EEG device enables the sensing of neural activity through EEG and is capable of simultaneously delivering non-invasive tES stimulation. Starstim's software streams in real-time the sensed EEG using Lab Streaming Layer (LSL) communication protocol (Kothe et al. 2019). LSL technology provides fast transmission and accurate synchronization and time-stamping of EEG samples. The Closed-Loop Manager (CLM), a software specifically designed for this closed loop application, receives the EEG streaming and analyzes vigilance levels in real-time. Vigilance is assessed by studying spectral entropy fluctuations over the frontal cortex. The CLM was designed to analyse spectral entropy online, based on the signal received from Fz, this electrode was chosen because of its relative stability of the signal despite motor high frequency noise. Moreover, Piarulli and colleagues found that the spectral entropy calculated based on Fz, Cz and Pz was comparable. This analysis, presented in the Analyses Section, is an adaptation of the one introduced by Piarulli et al. (Piarulli et al. 2016) to the real-time and experimental constraints (i.e., nursing interventions, prolonged recording duration, stimulation triggering) of the present study. CLM monitors vigilance levels at a 1-minute rate and analyzes its fluctuations detecting slope changes in the spectral entropy curves. The high and low vigilance thresholds are detected based on a local extrema observation designed to reduce the false positives such as: the local maximum must be preceded by a positive slope within the 5 minutes before the detection and it must me followed by a negative slope within 5 minutes from detection. This was mainly based on the observed time-courses of spectral entropy wavelet analyses performed by Piarulli and colleagues and the resulting oscillatory components contributing to this time-evolution. CLM is also in charge of remotely interfacing the device to start the tDCS onset at 1) high vigilance states (i.e., increasing positive slope), 2) low vigilance states (i.e., increasing negative slope) or 3) random vigilance states (i.e., states in which the stimulation is launched at a random moment, excluding positive and negative slopes). In order to ensure patient's safety and comfort, the total stimulation dose is limited and the impedance levels at stimulation channels are continuously monitored, aborting stimulation if impedances that do not allow the current to stay constant at its configured value are detected. The system architecture is presented in Fig. 1.

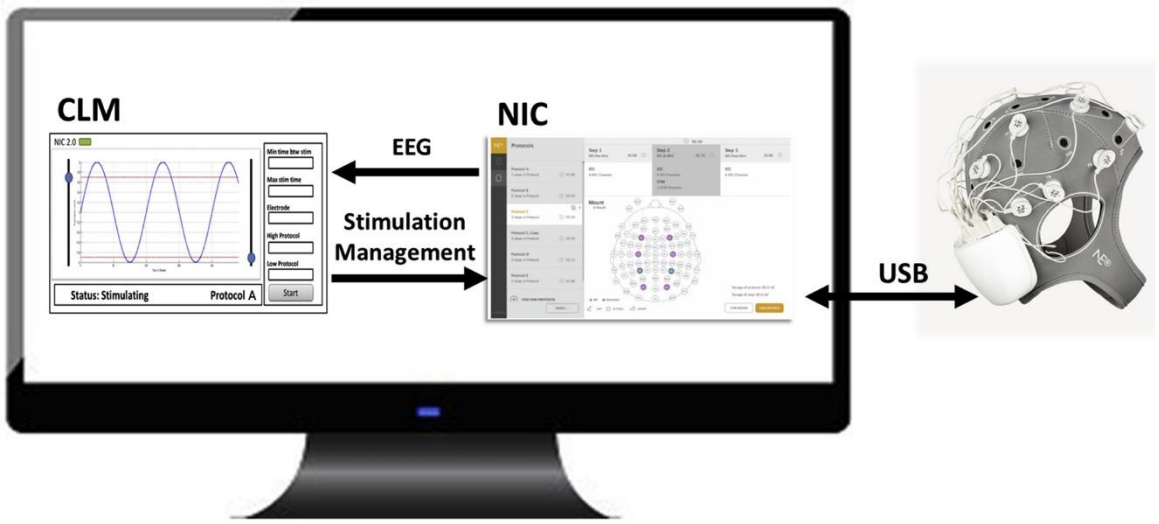

Fig. 3 Closed-loop hardware software and setting. The Closed-loop manager application is connected to the NIC software that is connected to the tDCS device via USB. The NIC software records the EEG while th CLM feeds the EEG into the algorithm online and dtects the spectral entropy. Upon detection of the chosen threshold (i.e., protocol A, B or C) the CLM triggers NIC to initiate the stimulation.

Procedures

This study is a randomized controlled study, using a crossover design with three sessions performed on three different days at similar times of the day spaced by at least 5 days to allow for an appropriate washout period while constraining the total duration of the whole protocol. The three sessions are as follows: 1. tDCS applied at high vigilance level; 2. tDCS applied at low vigilance level and; 3. tDCS applied at random vigilance level. The investigators are blinded to the session allocation as the screen depicts the same information for each session. The randomization of the three sessions order is conducted by a third party not involved in data collection or analysis, in a 1:1:1 allocation ratio. Each session consists of an initial behavioural assessment performed by experienced and trained clinicians using the CRS-R (Giacino, Kalmar, and Whyte 2004). The CRS-R is administered in the patient's native language as validated French and translated Dutch versions are available (Schnakers, Majerus, et al. 2008; Lovstad et al. 2010a) in addition to the original English one. After a screening EEG stage, that lasts between 30 min to 120 minutes, to monitor the fluctuations in vigilance levels used to trigger the stimulation, a 20-minutes tDCS stimulation session is performed. Right after the stimulation, post-intervention assessments of behaviour (CRS-R) and neural activity (30-minute EEG) is conducted to evaluate the effects of the neural stimulation. In the case of the random vigilance stimulation, the 20-min tDCS stimulation session starts randomly (within a 30 to

120 minutes, time-window), to ensure that the EEG monitoring phase is similar across all three stimulation conditions. The experimental protocol is described in Fig. 2.

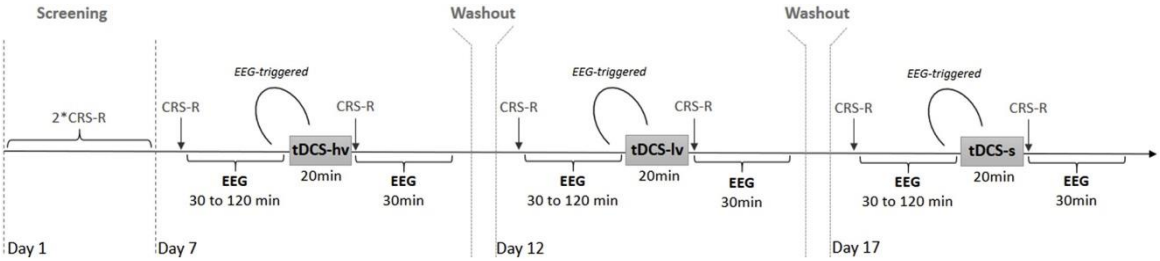

Fig. 2. Study protocol timeline. CRS-R = Coma Recovery Scale-Revised; tDCS-hv = high vigilance tDCS session; tDCS-lv = low vigilance tDCS session; tDCS-s = random vigilance tDCS session. The patient is assessed twice with the CRS-R to confirm at least a MCS state, then the three sessions are performed in a randomized order. Each session is structured as follows: a CRS-R is performed, the EEG cap is mounted and the NIC and CLM are launched, the CLM calculates online the SE for period of time that can go between 30 and 120 minutes and is not controlled by the experimenter. When the CLM detects the vigilance treshold (with the local maximum principle) planned it triggers the NIC to launch the tDCS. The tDCS lasts 20 minutes and after it the CRS-R is performed again and another 30 minutes of EEG are recorded.

Stimulation and EEG assessment

The multi-channel stimulation targets the prefrontal cortex bilaterally and thereby brain executive functions. The stimulation set-up has been optimized to target this area using the Stimweaver algorithm [40]. The optimized montage delivered uses F3, Fz and F4 as anodes, and P7, Cz, P8 as cathodes. Fig. 3 shows the placement of the stimulation electrodes (left) along with the induced electric field in the cortex, anodes are represented in red and cathodes in blue. In the figure on the right, both stimulation electrodes (blue) and EEG electrodes (magenta) are represented. This montage corresponds to a standard 19 electrodes in the 10–20 system. During non-stimulation intervals, all 19 electrodes are used for recording EEG. Stimulations are applied for 20 min using six 3.14 cm2 Ag/AgCl gelled electrodes, each one delivering a constant current as shown in Fig. 3 with a 15 s ramp up/down period for a smooth current transmission and somatosensory perception. The patients is kept awake during the stimulation through auditory and/or tactile stimulation.

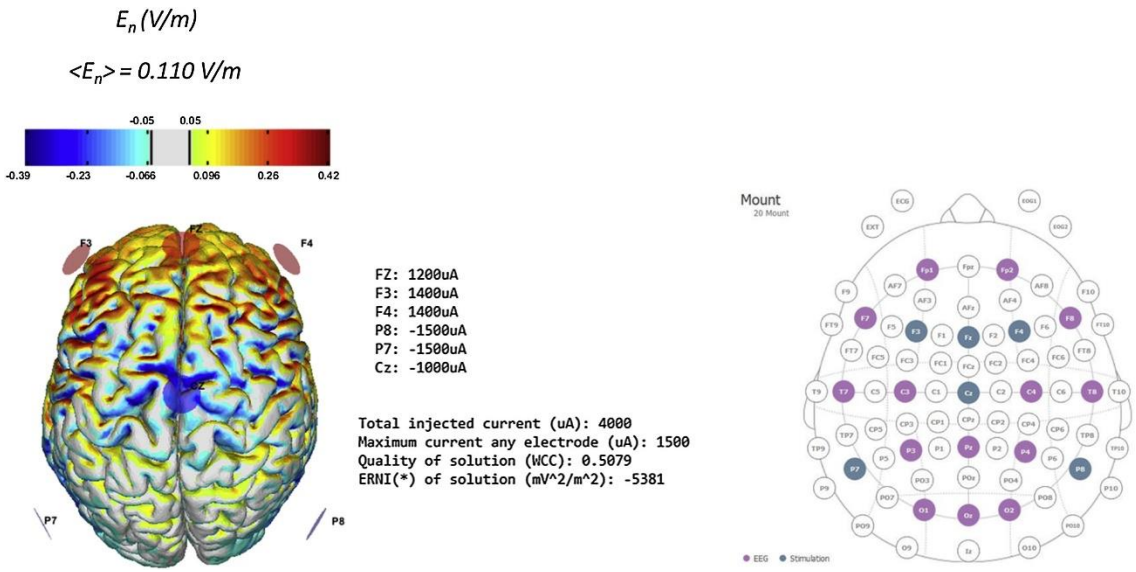

Fig. 3. Optimized stimulation montage. Current modelling built with Stimweaver (left) and montage configuration that will be used for both EEG recordings and stimulation (right). Fz, F3, F4 are the anodes whereas Cz, P7, P8 are the cathodes. The total amount of current injected is 4 mA.

Data collection and management

Demographic and clinical data relating to the past and current medical history are collected either via review of the medical records or discussion with family members and clinicians familiar with the case to supplement the data acquired from the medical chart. All information collected during this study is confidential. Data management complies with the General Data Protection Regulation (EU 2016/679) and a specific information sheet informs patients' legal representative of the nature of collected data and their rights regarding this data.

Analyses

Behavioural Data

Statistical analyses on the behavioural data will be performed on R software when the data acquisition will be completed, using Kruskal Wallis rank sum tests to calculate the differences in delta CRS-R total scores between the three conditions (high vigilance, low vigilance and random vigilance). In case of significant results ($p < 0.05$), post hoc Dunn tests will be performed for pairwise comparisons. Bonferroni correction will be applied for multiple comparisons. In an exploratory manner, we will also investigate the impact of

aetiology (i.e., TBI or non-TBI) and time since injury on intervention effects using Fisher's test and Spearman correlation, respectively.

EEG data

The spectral entropy feature calculation algorithm has been implemented from (Piarulli et al. 2016) to integrate the CLM real-time platform. First, the raw spectral entropy is averaged, using a moving window of 5 minutes (75 epochs) with 4 seconds shifts. In the averaging process, the median is used instead of the mean (compared to Piarulli et al. 2016) in order to reduce the influence of abnormally high or low values. Epochs containing artifacts are rejected before the averaging stage. Allowing the algorithm to calculate SE instant values without dramatic influences of artefacts. The window to evaluate the spectral entropy time-course is then limited to a maximum duration of one hour. Then the averaged SE is standardized to zero mean and standard deviation one. The resulting signal is then smoothed by computing its fitting to a polynomial of order 5. Local maximum and minimum peaks are then detected. Within the first 30 minutes local extrema cannot be detected, as this time frame has been established as the minimal baseline time interval needed for the correct calculation of the polynomial fitting. Local extrema are found when positive or negative deflections are observed in the spectral entropy time-course. One limitation of the proposed methodology for the detection of high and low SE levels, is that SE is computed in a 5-minute time window. Thus, in the stimulation is launched approximately within 5 minutes after the SE maximum or minimum occurs. However, given that the SE time-course is estimated to be in the range of 50-70 minutes this delay should be acceptable. Depending on the protocol, a maximum or a minimum threshold is applied over the smoothed signal to trigger the tDCS. Stimulation at random vigilance levels is achieved by randomly selecting the stimulation starting point between the 30–120 min time interval. For the statistical analysis on the EEG effects of tDCS, the relative power of delta (1–4 Hz), theta (4.5–8 Hz), alpha (8.5–12 Hz) and beta (12.5–30 Hz) will be calculated by dividing each frequency band power by the total power of the broadband signal (0.5–45 Hz) for each electrode. Changes in power and connectivity values after high, low and random vigilance stimulations will be analysed at the group level using t-tests with related cluster permutation tests.

5.4 Discussion

Patients with DOC need targeted individual interventions to optimize their responsiveness to interventions such as tDCS. This study will be the first of its kind to use a closed-loop EEG-tDCS approach to determine the optimal time window to apply stimulation, based on the patient's own vigilance level. Fluctuations in vigilance represent a well-known challenge for clinicians and researchers working with this clinical population which may prevent optimal diagnostic assessments and therapeutic interventions. Online EEG provides a relatively affordable tool to tackle this issue and to sharpen the use of non-invasive brain stimulation techniques. Several teams already used EEG as an outcome measure to evaluate the effects of tDCS. Schestatsky and colleagues (Schestatsky, Morales-Quezada, and Fregni 2013) showed that the effects of tDCS, whereas mostly occurring on the underlying cortex, could also be detected in distant connected brain regions. They strongly supported the importance of EEG markers as a trigger for a closed-loop tDCS stimulation protocol. So far, many studies have found consistent EEG changes accompanying tDCS. For instance, Luna et al. found a modulation of alpha power that was different according to the region stimulated and was not correlated with changes in performance (Luna et al. 2020). In another recent pilot study, Carriere and colleagues identified a tendency for higher power in the alpha and theta band at the group level after active tDCS and higher connectivity in the alpha band between left and right parietal regions, and in fronto-parietal regions (Carrière, Mortaheb, et al. 2020). In a study investigating tDCS with TMS-EEG (Mensen et al. 2020), reduced slow-wave activity and response slope were found following tDCS; both these measures are proxies of cortical bistability and correlated with high-frequency suppression. This latter component, however, was not found to be modulated by tDCS and the authors hypothesized that reducing slow activity might not be sufficient to translate in a behavioural improvement in consciousness. Bai and colleagues also explored tDCS effects with TMS-EEG and found a global cerebral excitability increase in early time windows and that these neuromodulatory effects significantly changed in spatial and temporal features between patients in MCS and UWS (Bai, Xia, Kang, et al. 2017). Neuroimaging and neurophysiological approaches have therefore a relevant role in identifying different response patterns. Regarding EEG brain connectivity, tDCS responders (compared to non-responders) showed higher theta centrality, using graph theory analysis, indicating that this new feature can be used to predict tDCS response in DOC patients (Thibaut et al. 2018).

Metabolic, structural and electrophysiological patterns of responsiveness have thus been investigated in DOC, but the literature is scarce regarding brain-state dependent application of tDCS, although the effects of tDCS are dependent on ongoing cortical activity (Ohn et al. 2008). Selectively administering tDCS during specific EEG states, in a closed-loop fashion, is therefore a promising approach. For DOC patients, these patterns should reflect the level of vigilance, as these patients are prone to arousal fluctuations, biasing the levels of responsiveness observed (Giacino et al. 2018b; Schnakers et al. 2009). The spectral entropy of the frontal EEG signals has been shown to reliably indicate the level of consciousness (Gosseries et al. 2011), while periodic fluctuations have only been observed in MCS patients (Piarulli et al. 2016). Applying tDCS at selected vigilance moments can lead to several scenarios which we aim to investigate here. Even though our hypothesis is that tDCS applied at high vigilance is more effective (as measured with the CRS-R), we might also consider the hypothesis that a period of high vigilance represents a plateau in the cognitive abilities of patients in MCS and that no additional effects of tDCS will be observed. Conversely, while our hypothesis is that tDCS applied at a low vigilance state will not lead to any relevant effect as the patient is less responsive, it might be that tDCS applied during low vigilance will increase their level of vigilance sufficiently enough to benefit from the stimulation effects translated into relevant behavioural changes. As these scenarios will need to be confirmed or not, we need an additional reference point within our study with this random control condition independent of the patient's vigilance level. From a feasibility perspective, recent works show that scalp-recorded low-density EEG is able to detect patterns of interest based on a pre-defined algorithm and to trigger tDCS within a milliseconds time frame (Leite et al. 2017). The implication of feasibility of brain state-triggered interventions hereby goes beyond the application of tDCS. A couple of studies on transcranial alternating current stimulation (tACS) employed a closed-loop system to modulate specific brain oscillations. During sleep for instance, tACS was triggered during fast spindles to enhance motor memory consolidation (Lustenberger et al. 2016). The effects of closed-loop tACS in memory consolidation have been confirmed in other studies (Jones et al. 2018; Ketz et al. 2018) and this setup has shown therapeutic benefits in sleep quality (Robinson et al. 2018), and tremor suppression (Brittain et al. 2013). The field of closed-loop application of non-invasive brain stimulation is still subject to important challenges such as development of accurate and optimized triggering algorithms, translation to clinical use and correct identification of inputs to feed the system

with. Nevertheless, the potential clinical applications are multiple, and could enrich the therapeutic options for DoC patients. The present study will be the first proof of concept towards this application. It is difficult for DoC patients to actively engage in conventional rehabilitation tasks and thereby prompt their brain to be in an "active state". Using a closed-loop EEG-tDCS approach to monitor patients' vigilance and determine the most appropriate moment to trigger the stimulation represents an important step forward in the management and care of this population.

5.5 Preliminary results of the RCT

This study protocol has been developed in collaboration with the partners of the LUMINOUS Project (H2020-FETOPEN-2014-2015-RIA N. 686764) between 2018 and 2020. In order to develop the Closed-Loop Manager software, we acquired 3 resting state EEG pilot recordings of 6 hours in patients in MCS (see Table 1 for details on the pilot EEG patients). These pilots were used to improve the algorithm and the usability of the software and the resulting EEG analyses confirmed the compatibility of spectral entropy of the patients with the results observed by Piarulli and colleagues (see Figure 4). Moreover, the algorithm successfully predicted the spectral entropy patterns and consequent vigilance threshold from the pilot acquisitions.

Patient ID	Diagnosis	Sex	Age	TSI	Aetiology	PET results (preserved metabolism)
Pilot_01	MCS – (visual fixation)	M	63 y.o.	9 months	anoxic	73%
Pilot_02	MCS+ (response to commands)	F	71 y.o.	10 months	anoxic	64%
Pilot_03	MCS- (visual pursuit)	M	60 y.o.	12 months	anoxic	49%

Table 1. Demographic informations on patients included in pilot acquisitions. Patient ID, Diagnosis (higher observed behaviour), Sex, Age, Time since injury (in months), aethiology, FDG-PET reduction in global brain metabolism compared to control subjects.

Thanks to these pilot data acquisitions, we were able to start the data acquisition for the RCT that this protocol was designed for.

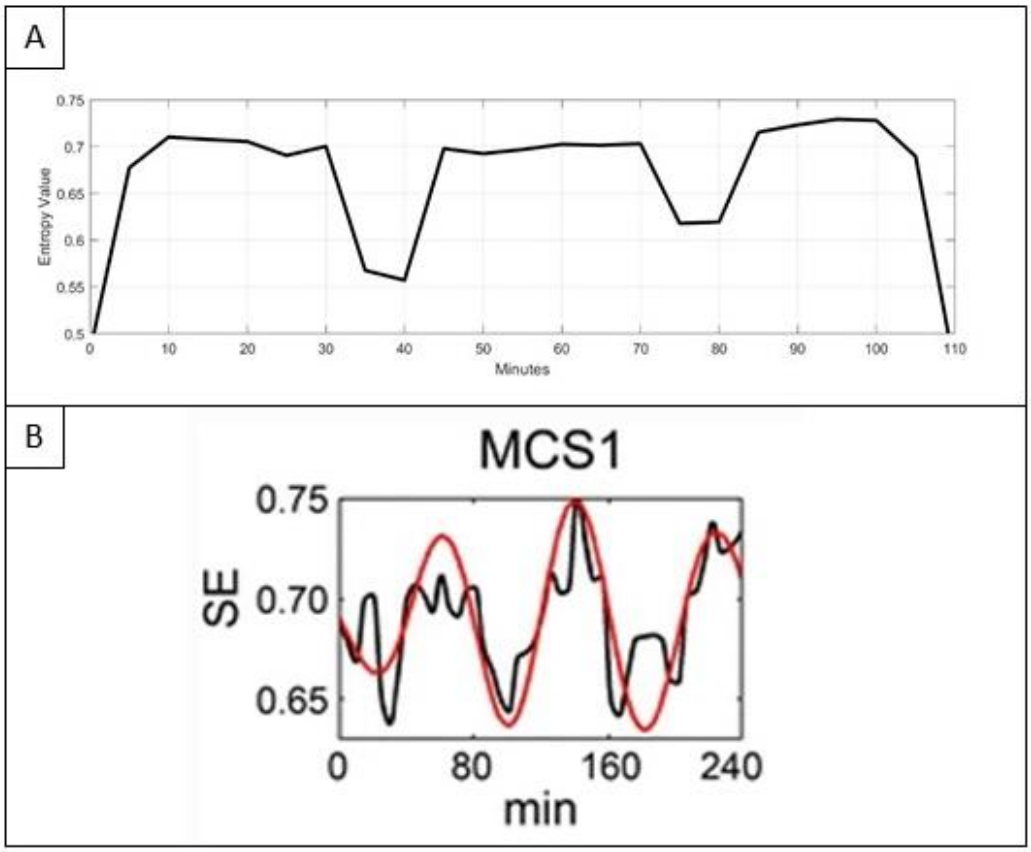

Fig.4 Comparison of spectral entropy from Pilot_01 and MCS1 in Piarulli et al. In panel A is is showed the spectral entropy recorded and calculated by the Closed Loop Manager for the first patient included in pilot acquisition. In panel B is is showed the spectral entropy observed by Piarulli et al., 2016 for patient MCS1. The two patterns are compatible.

Preliminary results

Seven patients have been enrolled, so far, in this randomized double-blind controlled trial, however one had to be excluded due to technical issues (N= 6, 2 females, mean age 44,5 ±18 years old, mean TSI 112 months ±158, 4 TBI). (For the current flowchart of screened, enrolled and excluded patients, see Figure 5. For details of the patients' demographics see Table 2). In Table 3 there are the behavioural results from the six patients enrolled so far.

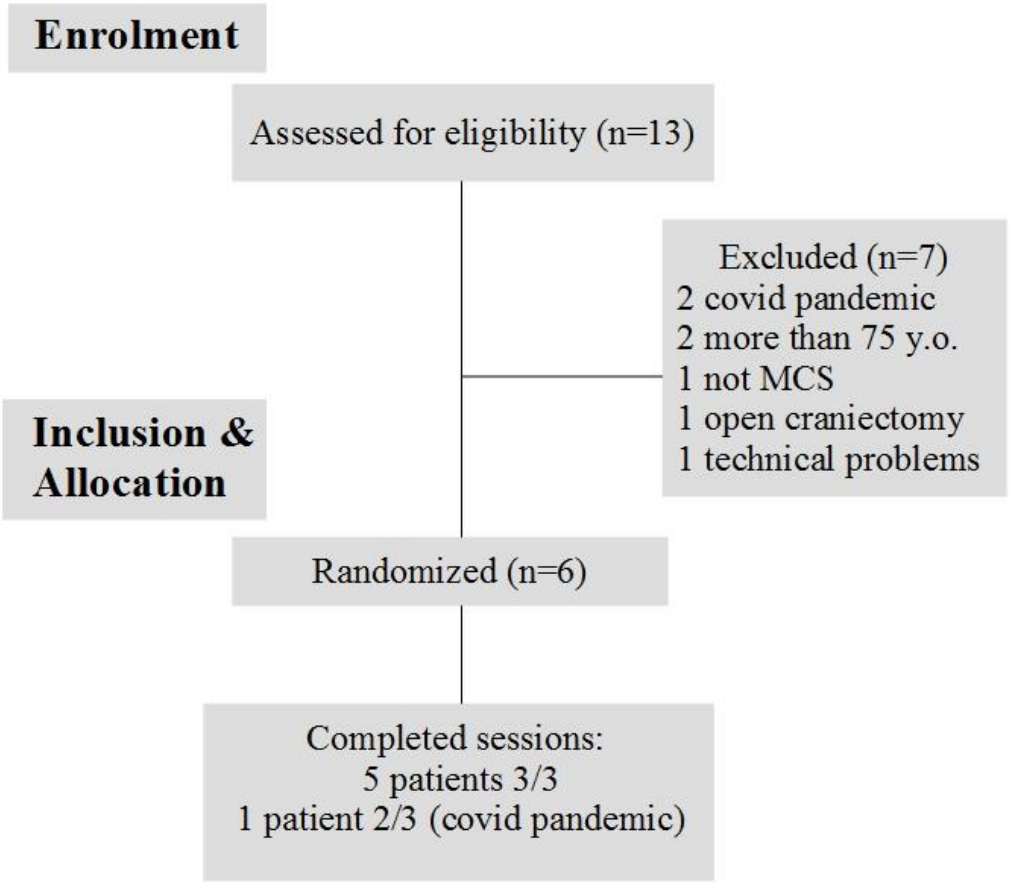

Fig. 5 Flowchart of patients inclusion. 13 patients were assessed for eligibility. 2 patients could not be included because they were assessed for eligibility in February 2020 and then the lockdown due to COVID-19 blocked the data acquisition. 4 patients did not match inclusion criteria. 1 patient was included but after performing the first session had to be excluded, due to technical issues with the closed-loop system.

ID	Diagnosis	Age (years)	TSI (months)	Aetiology	Sex	Sessions completed
CL_ID01	MCS-	74	92	Meningioma	F	3/3
CL_ID02	MCS+	31	161	TBI	M	3/3
CL_ID03	MCS+	42	54	TBI	M	2/3
CL_ID04	MCS+	18	3	Stroke	F	3/3
CL_ID05	MCS+	37	5	TBI	M	3/3
CL_ID06	MCS-	58	450	TBI	M	3/3

Table 2. Demographic informations of the 6 patients enrolled so far. List of coloumns: ID, Diagnosis, Age in years, Time since injury in months, aetiology, sex, number of completed sessions. The reason why CL_ID03 received only 2/3 sessions, is that the patient was enrolled in February 2020 and data acquisition had to be stopped due to the COVID-19 pandemic emergency.

Preliminary behavioural results

ID	Pre High	Post High	Pre Low	Post Low	Pre Random	Post Random
CL_ID01	12 0/3/5/2/0/2	12 0/3/5/2/0/2	14 2/3/5/2/0/2	14 2/3/5/2/0/2	13 1/3/5/2/0/2	14 2/3/5/2/0/2
CL_ID02	11 3/3/2/1/0/2	11 3/3/2/1/0/2	9 2/3/1/2/0/1	8 2/3/1/1/0/1	8 1/3/2/1/0/1	3 0/0/1/1/0/1
CL_ID03	11 1/4/2/2/0/2	12 2/4/2/2/0/2	NA	NA	10 2/3/2/1/0/2	8 2/1/2/1/0/2
CL_ID04	7 3/0/1/1/0/2	7 3/0/1/1/0/2	7 3/1/1/1/0/1	5 1/1/1/1/0/1	7 3/1/1/1/0/1	6 1/1/1/1/0/2
CL_ID05	21 4/5/6/2/2/2	21 4/5/6/2/2/2	19 3/5/6/2/0/2	21 3/5/6/2/2/2	20 4/5/6/2/1/2	20 4/5/6/2/1/2
CL_ID06	8 1/3/2/1/0/1	10 2/3/2/1/0/2	7 1/1/2/2/0/1	9 1/3/2/2/0/1	10 2/3/2/2/0/1	13 3/5/2/2/0/1

Table 2. Behavioural results of the preliminary data acquisitions. In the table the CRS-R total scores for each pre and post session and between parenthesis the subscale scores (e.g., CL_ID01 showed for pre tDCS at high vigilance a CRS-R total score of 12: auditory subscale 0, visual subscale 3, motor subscale 5, oromotor subscale 2, communication subscale 0, arousal subscale 2). The colour green indicates increase in the total score and the colour yellow a decrease.

tDCS with bilateral prefrontal stimulation at high vigilance threshold resulted in behavioural improvements in two cases (i.e., in both cases the improvement consisted in localisation to sounds). Localisation to sound was not showed before the stimulation but it was showed in other sessions, thus no new conscious behaviour was observed. Furthermore, localisation to sounds is not considered to be a sign of consciousness according to the guidelines of the CRS-R, however, a recent study by Carriere and colleagues (Carrière, Cassol, et al. 2020) suggests that it could and should be considered as such. When taking into account median of pre and post scores of high vigilance tDCS we found that there was a slight increase (median of pre-stimulation at 11 and median of post-stimulation at 11.5) after the stimulation. Interquartile ranges 25 and 75 also show a decrease post stimulation, with overall higher results (IQR 25 and 75 pre-stimulation 8.75 and 11.7 respectively; IQR 25 and 75 post-stimulation 10.25 and 12 respectively). To summarize, we can observe a slight improvement following tDCS at high vigilance threshold and less scattered results (See Figure 2 for details).

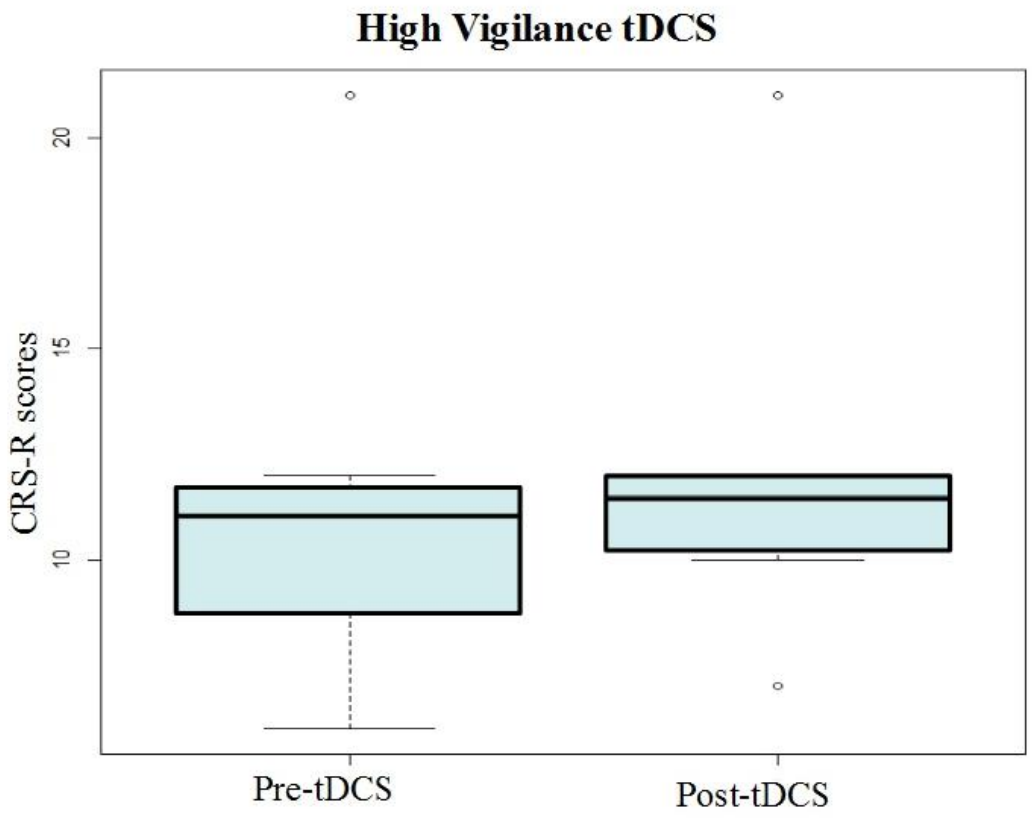

Fig. 2 Results of the medians and interquartile ranges 25 and 75 for tDCS at high vigilance threshold. The line in the center of the box represents the median, the vertical lines represent the interquartile ranges 25 (below) and 75 (above), the box represents the standard deviation regrouping 50% of the results.

Regarding tDCS stimulation at low vigilance thresholds we observe two cases of decrease in behavioural performance (i.e., in one case oromotor vocalizations and in one case response to commands were not observed after the stimulation). We also observe two cases of behavioural improvements (i.e., one case of functional communication, one case of visual pursuit). Note that all these improvements were behaviours that were observed in other instances during data acquisitions, that is to say no new conscious behaviour was observed after tDCS at low vigilance threshold. When calculating medians and interquartile ranges of tDCS stimulation at low vigilance levels we do not observe strong differences between pre- and post-stimulation results (pre-stimulation median: 9.5; post-stimulation median: 8.5). We note a slight decrease in the 50% central values as well as a slight decrease in the dispersion of the results (IQR 25 and 75 pre-stimulation 7.5 and 13 respectively; IQR 25 and 75 post-stimulation 8 and 12.75 respectively) (See Figure 3 for details). To summarize, tDCS at low vigilance did result in some behavioural changes but

these were not consistent and the ranges of observed behaviours is wide, not allowing us to advance any hypothesis.

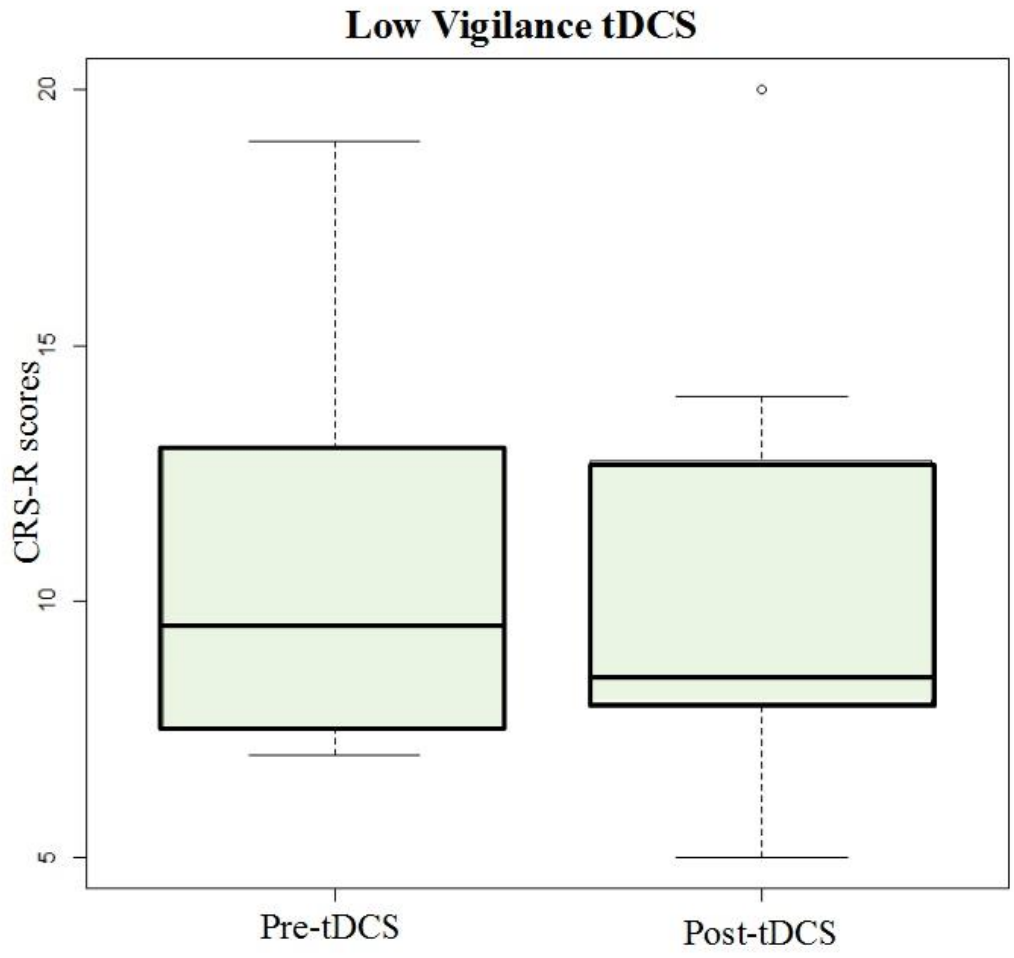

Fig. 3 Results of the medians and interquartile ranges 25 and 75 for tDCS at low vigilance threshold. The line in the center of the box represents the median, the vertical lines represent the interquartile ranges 25 (below) and 75 (above), the box represents the standard deviation regrouping 50% of the results.

Finally, tDCS at random vigilance thresholds (our control condition) resulted in three cases of decrease in total CRS-R score (i.e., one case of auditory reflexes and visual pursuit, one case of visual pursuit and one response to commands not observed after the stimulation). Moreover, one instance of behavioural improvement was observed and this represented not only an increase in total score but two conscious behaviours observed that were never observed before not after any other session (i.e., response to commands and object recognition). However, it might be important to note that this patient (i.e., CL_ID06) showed behavioural improvement after all three sessions of stimulation. Regarding medians and interquartile ranges of tDCS at random vigilance levels before and after the stimulation we observe a higher median value (i.e., pre-stimulation median: 10; post-

stimulation median: 13) as well as an enlarged gap between the interquartile ranges 25 and 75 (pre-stimulation IQR: 8 and 13; post-stimulation IQR: 6 and 14). In addition, there was a wider standard deviation, showing a greater dispersion of the results of the CRS-R assessments.

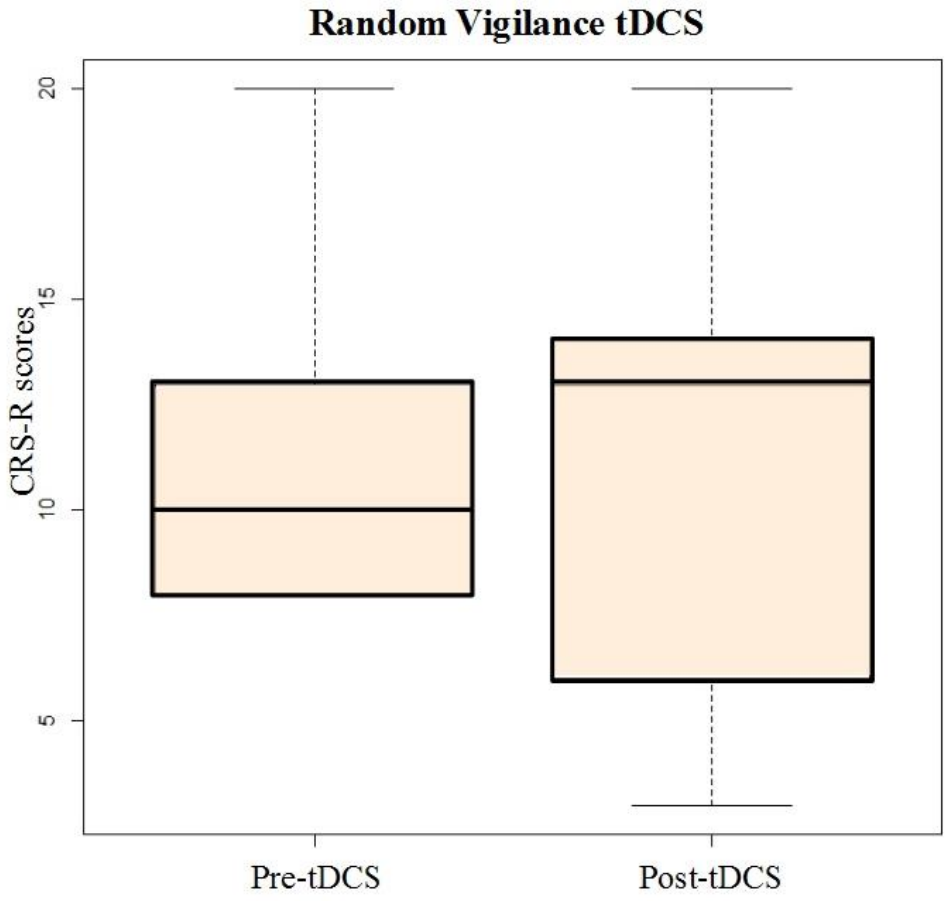

Fig. X Results of the medians and interquartile ranges 25 and 75 for tDCS at random vigilance levels. The line in the center of the box represents the median, the vertical lines represent the interquartile ranges 25 (below) and 75 (above), the box represents the standard deviation regrouping 50% of the results.

Finally, even if these findings are only preliminary they carry important informations. However, to make assumptions on the real effectiveness of tDCS at specific vigilance thresholds we need to complete the data acquisition and perform statistical analyses on significance of the effects.

6. Discussion

6.1 Challenges of NIBS research in patients with DoC

In this section, we will describe the challenges and recommendations for future research that pertain to NIBS techniques.

The current challenges in therapeutic options for patients with DoC have been described by the Curing Coma Campaign task force (Edlow et al. 2021). Based on this gap analysis, neuromodulation as a therapy for DoC currently presents three main research gaps that need to be solved: 1) large scale multicentre RCTs based on a unified framework need to be performed, and this is a necessity that applies to all types of therapy research; 2) it is crucial to identify and define the endotypes of responders of each technique of NIBS; 3) we need to optimize and individualize stimulation montages and parameters, with the help of neuroimaging and modelling studies. In this section, we will discuss each of these above-mentioned gaps.

Conducting large scale RCTs

Sample sizes in the studies that have been published so far, using double-blind sham controlled RCT designs, are relatively small. For instance, studies using tES vary in sample size between 9 and 55 (median 16) whereas for studies using rTMS sample sizes vary between 6 and 50 patients (median 18), see Table 1 in Introduction for details. These small numbers cause underpowered statistics and a consequent increase in the type II error risk (i.e., false negative). The reasons behind the difficulty in screening and enrolling patients with DoC in NIBS trials are multiple. Firstly, as previously mentioned, DoC are considered a rare disease and the epidemiological rates of patients presenting this condition are relatively low, making it hard to find patients to include in studies. Secondly, patients with DoC commonly present several comorbid factors and illnesses (Estraneo et al. 2018; Pistoia, Sacco, et al. 2015) that are usually exclusion criteria for NIBS treatments, further limiting the already small number of eligible patients. For instance, many patients with DoC have undergone a craniectomy with or without cranioplasty or have been implanted with shunt devices to relieve intracranial pressure, at some point during the time-course of their injury. Craniectomy (usually under the stimulated area) is an exclusion criterion of most NIBS treatments because, as we have seen, most of the current is dispersed in the scalp before entering the brain. Epilepsy and seizures are also a common comorbid factors of DoC (Briand et al. 2022) and, when untreated, most researchers prefer not to apply

NIBS, to avoid unexpected complications. However, when applied following the guidelines these techniques are safe and appear to not reduce the epileptogenic threshold (Haffen et al. 2011; Machii et al. 2006; Matsumoto and Ugawa 2017; Bikson, Datta, and Elwassif 2009). Interestingly, cathodal tDCS has showed to successfully decrease epileptic seizures and epileptiform activity when applied to epileptogenic brain areas in patients with epilepsy that are drug-resistant (San Juan et al. 2018).

Finally, the extreme fragility of patients with DoC makes them prone to fatigue, additional infections and inflammatory syndromes, which causes high rates of drop-outs from studies for logistical reasons (e.g., the patient is moved from the ward to intensive care due to a viral infection) and for pharmacological reasons (e.g., if the patient is given anaesthetics or medications that are not dosage-stable while enrolled in a NIBS study usually they have to be excluded). One example of the challenges encountered by researchers in data acquisitions and patients' enrollment in RCTs can be described from a situation that occurred during the data acquisitions of Study 4. One patient (i.e., CL_ID05) was included in the study and after a few days before the third session of data acquisition, the patient started showing signs of illness and was finally diagnosed with infectious pneumonia. He was then transferred to a hospital (from the rehabilitation center he was staying in) in order to receive care for this illness. By the time he was back in the rehabilitation center, with a health condition stable enough to be considered safe enough to perform the experiment, more than a month had passed. For this reason, we could not perform the third session of tDCS with the closed-loop system, as originally planned, and compare it to the other two that had been acquired before a severe illness (almost two months before). We, thus, had to start from the first session again and perform all three stimulation protocols. This example depicts the typical challenges that researchers have to overcome in order to acquire data with patients with DoC in RCT studies. A way to overcome these issues would be to conduct multicentre studies in which research centres pool their data together in order to achieve higher sample sizes and statistical power. DoC experience a lack of funding from private companies and government healthcare institutions due to their label of rare condition, that translates to limited financial interest, and this obstructs such large multicentre RCT studies. Fatigue in patients is also a frequent issue that is encountered when performing RCTs and can make the completion of experimental sessions more difficult. Another reason why RCTs are needed (that does not pertain to statistical power and sample sizes) is the fact that some NIBS techniques (e.g., LIFU, tVNS etc.) have yet

to be explored with proper control and blinding conditions. Even if current results allow us to be optimistic on their effectiveness, we cannot overlook these study limitations and thus we cannot make any inference on their actual effectiveness so far.

Identifying and defining endotypes and biomarkers of responders

The small sample sizes and low statistical power of studies hinder the possibility of conducting stratification and subgroup analyses (both behavioural and from neuroimaging data) to investigate the features that distinguish patients that benefit from NIBS (i.e., responders) from those that do not. The low rates of significant results observed in NIBS studies might be linked to this issue. Indeed, as long as the features that characterize responders and the biological factors that allow them to benefit from the stimulation are unknown, targeting the right patients to include in NIBS trials is difficult and this translates to reduced likelihood of observing positive and significant results. For instance, since tVNS stimulates the thalamus and the cortex via the thalamus, we can assume that patients with cortical rather than subcortical lesions might be a more adequate population for this therapy. However, as long as no RCT study has been conducted on tVNS this hypothesis cannot be confirmed or confuted.

An important detail provided by current results that seems to be crucial to the identification of responders' endotype is the difference in rates of responders between patients in MCS and patients in UWS. So far, tDCS has not shown significant behavioural improvement in patients in UWS and, at the individual level, very few cases of clinical recovery have been reported (Thibaut et al. 2014; Estraneo et al. 2017). On the other hand, RCTs using rTMS have demonstrated slightly higher response rates in patients in UWS (He et al. 2021; Zhang et al. 2021). This interesting difference might shed new light on the different mechanisms of action between the two techniques. A way to explore this would be to first investigate demographic and neurophysiological differences between responders and non-responders for each technique. Then, a logical next step would be to compare these characteristics in responders to tDCS and responders to rTMS. Metabolic, structural and electrophysiological data from responders should be further investigated in order to reach a consensus on biomarkers that can potentially predict response to stimulation. tDCS studies have more extensive literature on this regard, compared to rTMS. For instance, Thibaut and colleagues, as mentioned before, have found that grey matter atrophy and

brain metabolism in consciousness-related regions (ie., the precuneus and the thalamus) and regions below stimulation electrodes (i.e., left prefrontal cortex) were significantly different between responders and non-responders (Thibaut et al. 2015). Theta power and connectivity have also been found to differ in these two categories in several studies (Thibaut et al. 2018; Carrière et al. 2020; Hermann et al. 2020). In opposition to these results, the decrease in delta power that has been observed in several studies using tDCS, does not seem to be a determining factor in the effectiveness of the stimulation. One study exploring tDCS effects with TMS-EEG (Mensen et al. 2020) has, indeed, found that tDCS reduced slow-wave activity (i.e., delta power) but this was not followed by an observable behavioural improvement. When a behavioural improvement was observed, not only did show slow-wave activity decreased but high frequencies power increased too, suggesting it might be the combination of these two factors that produces the clinical result and not the decrease in delta power alone. All this information enriches our knowledge on differences between responders and non-responders and takes us a step further in their characterization. However, other studies need to confirm (or confute) if the presence of these markers alone is sufficient to predict tDCS responsiveness. One ongoing project is trying to identify the patients' connectome to anticipate amenability of neuromodulation approaches (Edlow, Barra, et al. 2020) using neuroimaging techniques to predict endotypes that are more likely to respond to certain stimulations.

Finally, patients with covert consciousness (i.e., MCS*) represent a challenge as well as an opportunity for the characterization of responders. It has been showed in a case report that tDCS could unveil covert consciousness and the patient could show behavioural improvement (Thibaut et al. 2018). This suggests that patients in MCS* might benefit from the stimulation. In this study, the patient received prefrontal tDCS and only after the stimulation (across 10 behavioural examinations) was able to show response to commands. This suggests that stimulating the DLPFC facilitated motor pathways and the patient's ability to execute the commands. This result not only deepens our understanding of the important role that the DLPFC plays in many cognitive and motor processes, but also on the nature of the limitations that patients in MCS* might experience. Indeed, this case report proved that some patients in UWS might actually suffer from covert consciousness and that this latter can be facilitated by stimulating high-level cortical areas such as the DLPFC. At the same time, given the possibility that up to 67% of patients in UWS could be actually in MCS* (Thibaut et al. 2021), if NIBS studies will focus on patients in MCS

as target population (as suggested before) MCS* patients could miss the opportunity of benefitting from the stimulation due to an inclusion bias. Finally, another way to improve the outcomes of NIBS therapies would be to test their effects when coupled to other interventions such as behavioural and pharmacological therapies.

Optimizing parameters of NIBS and maximizing the benefits

The most important goal for future neuromodulation research should be to further optimize stimulation parameters in order to maximize the effects of the treatment. For instance, even though tDCS studies have showed that repeated stimulation sessions produce higher effect sizes compared to single sessions (Estraneo et al. 2017; Thibaut, Wannez, et al. 2017; Martens et al. 2018), not many repeated tDCS session studies have been published in the last few years. Some studies have showed that the number of responders increase along with the number of sessions, suggesting that some patients might need more than one stimulation to show behavioural improvement (Thibaut, Wannez, et al. 2017; Martens et al. 2018). Increasing the number of sessions is thus likely to increase both the number of responders and the probability of significant results and could also facilitate the identification of responders. Indeed, if more responders and stronger statistical effects are observed, the database on responders from which researchers can investigate the endotype of responders would be bigger. It is important to note that, due to reasons mentioned before on why enrolling patients with DoC is very challenging and lack of funding, repeated sessions studies are even harder to conduct since they require additional infrastructure and resources and have higher rates of drop-out.

Apart from the number of sessions, the brain target of the stimulation is also a big factor in the optimization of NIBS therapies. So far, the DLPFC is not only the most explored target in tDCS, but also shows significantly stronger results (Feng et al. 2020). rTMS studies, on the other hand, have not yet extensively explored the DLPFC as a target in RCT studies and this is a major drawback. Only two very recent studies have tested it and, so far, have showed significant results. The reasons behind DLPFC effectiveness as a stimulation target are not yet clear, however we can have at least two hypotheses. The first, is based on the DLPFC's key role in consciousness networks such as the DMN (Demertzi et al. 2015; Di Perri et al. 2016) and in many cognitive tasks measured by most behavioural assessments (Goldman – Rakic 1995). The second, pertains to the role of the DLPFC in

the meso-circuit hypothesis that has been mentioned before (Schiff 2010; Thibaut, Schiff, et al. 2019). However, it would be interesting to conduct a neuroimaging or *in vitro* study to confirm this model. Two studies, so far, have found proof from neuroimaging of a feedback system between the prefrontal cortex and the striato-pallidal fibers (Li et al. 2021; Fridman et al. 2014). However, to optimize NIBS approaches, it would be better to replicate these results in similar protocols that also involve NIBS stimulation.

Another important objective for future research to optimize NIBS effectiveness is the individualization of stimulation parameters to the patients' specific profiles. Some approaches tailoring therapies to the patients' needs have been launched (Li et al. 2021; Mencarelli et al. 2020), but they have yet to be implemented in NIBS RCTs. Study 4 (Martens et al. 2021), presented in this dissertation is a first attempt at this implementation. Future NIBS studies should use more adaptive approaches that tailor stimulation parameters to the specific subject. For instance, the parameters and location of stimulation could be adapted according to the patients' specific lesions through neuroimaging examinations. Regarding the location of stimulation, one study on stroke patients (Baker, Rorden, and Fridriksson 2010) showed that the patients showing greatest behavioural improvements to tDCS had surviving brain tissues under stimulating electrode. Moreover, the exact location of electrode positioning is usually calculated on the scalp with EEG 10-20 parameters instead of neuroimaging data on patients' lesions. Thus the successful targeting of the chosen region cannot be confirmed with complete confidence (e.g., targeting F3 for left DLPFC stimulation).

Another factor that could maximise NIBS effects is coupling with other therapies. Both tDCS and rTMS have showed, in other populations, stronger effects when coupled to other interventions such as physical therapy or pharmacological treatments (Ilić et al. 2011; Tsagaris, Labar, and Edwards 2016; Vedeniapin, Cheng L 2010). This suggests that NIBS might act as a catalyst for the other intervention and thus speed up the recovery. However, it has been suggested that tDCS should be targeting the same brain region and network of the therapies it is coupled with, in order to show synergistic effects (Thibaut, Zafonte, et al. 2017). For instance, one study found that when comparing tDCS targeting DLPFC to tDCS targeting M1 to reduce pain and sleep complications in patients suffering from fibromyalgia, only M1 resulted in the desired effect (Roizenblatt et al. 2007). One case study coupling tDCS targeting the hand region in M1 and robotic upper limb therapy (Friel et al. 2017) also found enhanced effects of the behavioural therapy in the stimulation

condition compared to sham. To give an example with another brain target, one RCT (Jung et al. 2011) using tDCS over Brodmann areas 45 (involved in speech production) coupled with speech therapy, found that aphasic patients showed greater benefits of the therapy in the active tDCS condition. Given these results, we can hypothesize that tDCS over M1 might enhance the effects of physical therapy as they both target the motor networks. On the other hand, the effects of tDCS over M1 might be small or absent if coupled to speech therapy (that targets language-related networks).

It might be also interesting to compare prefrontal rTMS to prefrontal tDCS in the same sample, to understand which of the two shows better outcomes and to identify if the responder's endotype of this brain target is the same for both techniques or not. On a similar assumption, it could also be informative to compare NIBS techniques and more established pharmacological treatments such as amantadine, to sham in a "non-inferiority trial" (Brunoni et al. 2015). In this way, we would have more information about the differences and similarities on the mechanisms of action of these treatments. For instance, we could confirm or confute the hypothesis derived from the meso-circuit model on the mechanisms of consciousness recovery (Schiff 2010). Based on this model, amantadine and prefrontal tDCS should have similar mechanisms of action (both involving an increased excitability of prefrontal cortex) (Edlow et al. 2021; Thibaut, Schiff, et al. 2019). However, amantadine is also hypothesized to act on the striatum. If patients responded in the same way to these two treatments, we could hypothesize that the prefrontal cortex is the main cause of the observed behavioural improvement. However, if the patients responded differently, for instance with higher rates of responders to amantadine compared to tDCS, we could assume that the striatal stimulation of amantadine caused the difference in observed effectiveness in consciousness recovery.

6.2 How our results can orient future studies

Study 1. Propose alternatives to the CRS-R to facilitate execution of RCTs

To optimize the care of patients with DoC, their correct diagnosis is essential for a multitude of reasons. As we have thoroughly described before, a diagnosis of UWS has a different impact on how the patient is perceived by healthcare professionals and families, compared to MCS (Demertzi, Ledoux, et al. 2011). The life-expectancy and prognosis are not the same. Indeed, patients in MCS show higher percentages of recovery (i.e., emergence from the MCS) within one and two years post injury, whereas patients in UWS

have higher mortality rates in the same time frame (Estraneo and Trojano 2018). Moreover, the daily care and treatment options between these two categories differ greatly. For instance, patients in MCS can perceive pain and more attention should be paid to any sign of discomfort (Demertzi et al. 2013). We also know that patients in MCS usually respond more to NIBS treatments (Feng et al. 2020) and, for this reason, many therapeutic trials do not enrol patients in UWS. These differences have a lot of consequences on the care of individual patients. Furthermore, prognosis and care managements of patients in UWS are even more dramatically different from those of patients in eMCS and PTCS (Nakase-Richardson, Yablon, and Sherer 2007; Sherer et al. 2020b).

For all these reasons, an accurate diagnosis of the level of consciousness of patients with DoC is of vital importance both in intensive care settings and in retrospective studies and RCTs. The goal of this study was to understand if the GCS is a reliable tool to assess the level of consciousness. The rationales to conduct this study were the following: the first one is that the CRS-R is currently the most widely used and only recommended scale to assess patients with DoC but it has several limitations that affect its clinical translation. The second reason, directly linked to the first one, is that the GCS is the most known scale of brain injury in the healthcare and rehabilitation world, it is used in most ICU settings and could have been a good substitute of the CRS-R.

As previously mentioned, the GCS is very popular and most healthcare workers understand the clinical meaning and relevance of its total scores and subscores, thus creating a commonly understood language based on the scale (Wilde et al. 2010; Marmarou et al. 2007). Moreover, it is fast to administer and does not require extensive training to be performed correctly.

On the other hand, the CRS-R was developed specifically to assess DoC (Giacino, Kalmar, and Whyte 2004), thus allowing for an accurate differential diagnosis (Schnakers et al. 2009). This scale is, however, limited by various factors. The first drawback of the CRS-R is that it requires training to be assessed (Lovstad et al. 2010b) and it is not well known nor adopted outside of structures that have an expertise in patients with DoC. One study showed that only 33% of the 96 physicians interviewed with an online survey had knowledge of the CRS-R (Chaturvedi et al. 2021). Moreover, the CRS-R is very time-demanding and this causes a series of disadvantages. The time required to perform the CRS-R is on average 17 minutes as showed by Aubinet and colleagues (Aubinet et al.

2021) and this adds difficulties for both clinicians and researchers that wish to use the CRS-R in experimental protocols. It is indeed harder to complete complex experimental designs that involve more than one measure (e.g., EEG and CRS-R) or repeated measures, if the behavioural measure alone requires on average 20 minutes of patients' concentration and no interruption. Moreover, patients often show signs of fatigue throughout the evaluation and sometimes even annoyance and non-compliance due to the repetitive nature of the exam. The CRS-R indeed involves 4 repetitions of most of the items and this can create frustration and de-motivation in patients, as showed by forced eye closure with resistance to eye opening (van Ommen et al. 2018) and other behavioural signs of distress. Furthermore, the length of the exam also hinders the possibility of observing the best possible response from patient and, thus, also the real effects of the stimulation, if it happens during a RCT. It has been showed that tDCS after-effects, for instance, do not last more than a few hours after one stimulation session (Nitsche et al. 2004). In a RCT exploring tDCS effects on patients with DoC involving also other measures, such as EEG, the EEG recording is usually performed right after the stimulation to capture the electrophysiological changes. This would involve between 10 and 20 minutes according to the improvement of impedances and the recording time. After the EEG recording the researcher would perform the behavioural assessment and the CRS-R would involve between 12 and 45 minutes to complete, leading to a total time of maximum 70 minutes after the end of the stimulation. Such long assessments increase the risk of behavioural assessments being performed outside the window of tDCS after-effects, thus decreasing the probability of observing any improvement. Finally, another drawback of the CRS-R is the non-linear association between the total score and the diagnosis (Sattin et al. 2015). This would not create an issue if researchers and clinicians systematically reported the subscale scores (Giacino et al. 2009) or if they provided the CRS-R total score modified index proposed by Annen and colleagues (Annen et al. 2019). However, this does not happen very often.

For all the reasons mentioned, we wanted to orient future studies to replace the CRS-R with the GCS, as the main measure in RCTs. The GCS is already widely used in most clinical and research settings; it is a lot faster to administer than the CRS-R and its total score is linked to brain injury severity. However, the results of our study reject this possibility. The fact that the translation from the GCS total score to the diagnostic criteria of DoC show such low levels of accuracy, make it unadvisable to adopt this scale as the

main behavioural tool to assess DoC. This is true not only for ICU settings, but especially in therapeutic trials that aim at detecting subtle behavioural improvements. On another note, as mentioned in our study, the subscales of the GCS seem to be a more accurate measure than the total score when assessing consciousness (Bhatty and Kapoor 1993; Reith et al. 2017; Teoh et al. 2000). The motor subscale, especially, is correlated to consciousness levels and prognosis (Giacino et al. 2018a; Healey et al. 2003). However, if we consider the severe motor impairments that are often observed in patients with DoC, such as spasticity or plegia (Thibaut, Chatelle, et al. 2015; Martens, Laureys, and Thibaut 2017), it is not advisable to rely on solely the motor responses to evaluate patients with DoC.

We would also like to stress that the results of this study have an impact on all studies that use the GCS as a stratification tool to group categories of patients (Maas, Roozenbeek, and Manley 2010; G. Teasdale et al. 1979). Systematic reviews, retrospective studies and RCTs should not rely on GCS total scores to stratify patients. As we have seen, the GCS total scores from 3 to 8 are usually considered "coma", however 0% of patients in our sample were compatible with "coma" when they had a total score of 7 and 8. On the other hand, 83% of patients with a total GCS score of 7 were compatible with being in MCS- and, for patients with a total score of 8, 73% were compatible with MCS- and 17% with MCS+. These results are unsettling in the sense that a great number of studies are likely to have erroneously pooled in the "coma" category patients that were not only minimally conscious but might have presented language-related signs of awareness (Bruno et al. 2011). Since we have previously highlighted the need for a better characterization of responders to treatments in DoC, we can hypothesize that a retrospective study aiming to explore neural correlates of responders to a treatment in acute settings is likely to use GCS total scores to stratify patients (since it is the most used scale in ICUs). If in this hypothetic scenario the researchers would take into account GCS total scores to categorize patients' levels of severity and consciousness levels, we know that their results would be likely to include a wide array of patients endotypes, thus being misleading.

For this reason, we would like to highlight the necessity of a shift in the "gold standard" scales used in studies involving patients with DoC (Giacino et al. 2009), in favour of other short standardized and sensitive scales to facilitate their clinical use and research implementation. Since the GCS total score cannot reliably assess levels of consciousness, the most favourable option is currently the SECONDs scale, presented earlier in this work (Aubinet et al. 2021). The SECONDs scale, bypassing most of the issues related to the

CRS-R, is the only standardized short scale that has high inter- and intra-rater reliability and provides a score that is directly related to the level of consciousness (Sanz et al. 2021). Research in the field of DoC cannot rely on a scale that has been proven to not be able to accurately measure DoC. Instead, a more accurate tool that was designed specifically for it (i.e., the SECONDs and potentially the CRS-R FAST, if the validation study confirms the preliminary good reliability) should be preferred. Moreover, we think that intensive care settings should also prefer more sensitive scales that allow not only the assessment of injury severity but also of the level of consciousness. For this reason, the CRS-R FAST is being validated in the ICU, and the SECONDs (already validated in sub-acute patients with DoC) is also currently being validated in ICU settings.

Study 2. Evaluate the effects of a novel tES approach

Beside the use of appropriate assessment tools, in order to improve care of patients with DoC, it is also crucial to investigate novel tES techniques and parameters and compare them to conventional ones in feasibility studies to expand the array of possible treatments of DoC. In Study 2 we showed, for the first time, that tPCS is a feasible and safe technique for the treatment of patients with DoC. Although, using these stimulation parameters, we did not find a significant effect of tPCS compared to sham on the level of consciousness or on electrophysiological outcomes. However, our findings provide a foundation for future studies to continue investigating the efficacy of tDCS and tPCS stimulation to promote the recovery of consciousness after a severe brain injury. Indeed, the lack of side-effects and irritative/ictal activity in EEG post-stimulation in this sample prove that tPCS is a safe technique. Our complex within subjects protocol, with montage of both tES systems, resulted in successful completion of the sessions and blinding of the conditions. This allows for future studies to implement such protocol to explore two stimulation techniques at the same time and investigate the differences in the observed effects. This is important as treatments for patients with DoC are still limited and new techniques and types of stimulations should be explored in order to expand the therapeutic options for patients with DoC (Edlow et al. 2021; Thibaut, Schiff, et al. 2019).

It is interesting to note that although no significant behavioural improvement was observed after tPCS at the group level, one patient did improve following the stimulation (i.e., ID09) with a change in diagnosis. The patient had been consistently diagnosed as in MCS- before and after both sham and tDCS, however, only after tPCS the patient showed reproducible

response to commands, denoting a MCS+ (Bruno et al. 2011). Moreover, another patient (i.e., ID01) showed increased arousal and agitation as reported by relatives after tPCS, with no behavioural improvement measured by the CRS-R after tPCS. Both of the patients mentioned above had severe brain injury of anoxic aetiologies. It has been hypothesized that tPCS applied over mastoids (as in our study), could reach subcortical structures by acting also on peripheral nerve stimulation (Zaghi et al. 2010; Moreno-Duarte et al. 2014). Following this hypothesis and the meso-circuit model for tVNS mechanisms of action (Briand et al. 2020; Thibaut, Schiff, et al. 2019), the behavioural improvement observed in ID09 and the increased arousal levels reported for ID01, both having anoxic aetiologies, might support the idea that tPCS effects result from both the frequency of stimulation applied and the stimulation of peripheral afferent nerves reaching the brainstem. According to the meso-circuit hypothesis, indeed, tVNS stimulates consciousness recovery through the vagus nerve that from the brainstem reaches the thalamus and the cortico-thalamic pathways (Schiff 2010; Briand et al. 2020). Since we know that to respond to transcranial stimulation preservation of the underlying grey matter is needed (Thibaut, Di Perri, et al. 2015), we can hypothesize that the stimulation was facilitated by the presence of preserved nerve fibers under the stimulating electrodes. For this reason, it might be interesting to test tPCS in anoxic populations with DoC to shed new light on the mechanisms of action of tPCS. Moreover, it could be informative to re-test tPCS with the same parameters in these two patients using also neuroimaging techniques, such as fMRI and EEG, before and after the stimulation and investigate what are the observed differences in brain activity and electrophysiology. Indeed, another hypothesis on the cause of these behavioural changes might be that these patients presented high theta rhythm at baseline compared to the other patients in our study, since we know theta power and connectivity is a key feature of responders to tES (Thibaut et al. 2018). These results, even if observed at the individual level, might suggest that, with adjustments to the stimulation parameters of tPCS, we might observe in the future better behavioural effects. Performing the proposed fMRI and EEG studies in anoxic patients with DoC might allow us to identify characteristics that are necessary for a patient to respond to tPCS and better orient future inclusion criteria in clinical trials. Moreover, if these analyses (especially MRI) were to find that our patients presented lesions under stimulating electrodes, this could also explain the lack of strong behavioural effects (Thibaut, Di Perri, et al. 2015). Neuroimaging-guided neuronavigation is already widely used in rTMS studies to avoid lesions and find better positioning of the stimulating coil (Schoenfeldt-Lecuona et al. 2010; Langguth et al. 2010; Herwig et al.

2001). This approach helps increasing the effects of the stimulation and should be applied also in tDCS studies, given our knowledge on the importance of grey matter preservation (Thibaut, Di Perri, et al. 2015). As previously mentioned, the sole estimation of the brain target with surface EEG 10-20 parameters might not be enough to ensure the success of targeting the actual brain region (De Witte et al. 2018) and with severe brain injured patients this is more relevant than in healthy subjects.

Another finding from this study, that is relevant for the optimization of future clinical trials, is that alpha power was not significantly increased after tDCS, nor tPCS. This might be explained as previously stated by the fact that patients in DoC do not often show this frequency band in their spontaneous EEG pattern (Schiff, Nauvel, and Victor 2014; Bai, Lin, and Ziemann 2021). We plan to perform a further analysis specifically on alpha power at baseline of our sample to explore the hypothesis that the lack of such frequency might have hindered the entrainment of alpha power. The neural noise hypothesis (McDonnell and Ward 2011) and the stochastic resonance model (Fertonani and Miniussi 2017a) state that in order to entrain a certain frequency, the same frequency should be present at baseline and that the effects of tES result from both the frequency and intensity applied and the state of the brain. For this reason, the prior lack of alpha rhythm in our patients' EEG patterns might have caused the lack of alpha rhythm entrainment (as showed by lack of significant EEG results in the alpha band). The lack of alpha entrainment might also explain the lack of observed behavioural improvement, since we know that alpha and theta increases are usually observed after tDCS when behavioural improvements occur (Thibaut et al. 2018; Carrière, Mortaheb, et al. 2020; Hermann et al. 2020). Furthermore, Thibaut and colleagues hypothesized that prefrontal tDCS could be a powerful tool to stimulate under-activated theta-band network connections, resulting in clinical improvements at the same time (Thibaut et al. 2018). At the group level, this assumption is consistent with our results. The tDCS treatment, in our exploratory analysis, was associated with an increase in frontal theta connectivity and an improved behavioural responsiveness (i.e., CRS-R index) compared to the [tPCS + sham] treatment. Our analysis was only exploratory, thus we cannot be sure of its statistical strength, but is however consistent with previous results. Given all this information, testing for the presence at baseline of theta band power and connectivity could be a good predictor of the patient's likelihood of benefitting from the stimulation. Future studies on characterization of EEG biomarkers of responders to tDCS should explore the presence of this EEG frequency at baseline and if this hypothesis is confirmed, it might be worth adding this test to the inclusion criteria for tDCS RCTs.

Furthermore, we hypothesize that theta enhancement post stimulation might be a more important mechanism for consciousness recovery than alpha enhancement, based on our results and those of previous studies such as (Carrière, Mortaheb, et al. 2020; Thibaut et al. 2018).

The reason why we performed our exploratory analysis pooling together tPCS and tDCS, was dictated by statistical factors. Indeed, since our sample size was small for all the reasons described before, our statistical power was low and tPCS and sham showed similar indexes of effectiveness in our sample. However, given the one case of diagnosis improvement and the case of increased arousal after tPCS, we think it might be worth continuing exploring tPCS in patients with DoC adapting the stimulation parameters. For instance, since we confirmed previous results on the importance of theta power for behavioural improvement, testing tPCS using theta frequency might be a worthy alternative to the protocol used here. Another investigation, that might be worth performing, would be to reproduce the modelling study by Morales-Quezada and colleagues (Morales-Quezada, Castillo-Saavedra, et al. 2015) using theta frequency tPCS but with frontal positioning of the electrodes. This tPCS montage and parameters could potentially produce better results given the importance of theta rhythm in behavioural improvement (Thibaut et al. 2018) and the effectiveness of prefrontal stimulation compared to other targets (Feng et al. 2020). We would also like to point out that in the exploratory analysis the behavioural improvement was not significant when considering the CRS-R total score and it was significant only when taking into account the CRS-R Index. As previously mentioned, the CRS-R Index (Annen et al. 2019) is directly linked to differential diagnosis and thus a more sensitive score to analyse. The difference in significance of our results, further stresses how the CRS-R total score is a major drawback and limitation of this scale. We therefore, recommend using the CRS-R Index as the main outcome of RCTs in patients with DoC, when the CRS-R is the behavioural measure. However, the SECONDs and its additional index (Aubinet et al. 2021; Sanz et al. 2021) should still be preferred, in order to shorten the length of the assessment. Overall, we would like to orient future studies towards shorter protocols, such as crossover trials in which one group is assessed with EEG and one group with the CRS-R might lead to better results since the length of the session would be dramatically reduced. Repeated session studies also show overall bigger effect sizes and tDCS effects that last longer in time (Thibaut, Wannez, et al. 2017; Estraneo et al. 2017; Martens et al. 2018) and should be preferred. However, in order to perform repeated session studies or crossover trials the sample size

usually needs to be bigger (due to statistical power and drop-out rates) and this is very difficult for patients with DoC. The information in the flowchart of our study is a good example of why patients' enrolment is challenging in this population. Of a total of 13 patients screened, one received a craniectomy after enrolment and could not receive tDCS, one deceased due to an infection and one had to be excluded from EEG analysis due to motor artefacts that could not be eliminated from the signal. These dropout rates and issues encountered are not rare in patients with DoC. For this reason, a better way to perform the suggested studies would be to rely on multi-centre studies that unite forces and sample sizes under the same strict RCT protocol in order to achieve stronger statistical power and, hopefully, a more reliable measure of the effectiveness of tES treatments.

To conclude, given the feasibility proved by our study in comparing simultaneously two tES techniques we think it might be worth exploring tACS in patients with DoC. First, modelling studies should establish the correct frequency to apply to stimulate theta and theta-alpha connectivity in the prefrontal cortex (since we know that the effects of tACS highly depend on the frequency of stimulation (Chaieb et al. 2008)). Then, a RCT could be performed comparing tACS and tPCS. These two techniques are hypothesized to have similar mechanisms of action (Moreno-Duarte et al. 2014). However, tACS has been more extensively explored, compared to tPCS, in other disorders such as depression (Alexander et al. 2019) and schizophrenia (Mellin et al. 2018) with promising results. Comparing these two techniques might also expand our understanding of the differences between their mechanisms of action.

Define the characteristics of tDCS responders

To increase the observed effectiveness of tDCS and other NIBS techniques, beside exploring new techniques and new parameters of stimulation, a characterization of the endotype of patients with DoC that respond to the stimulation is a crucial step towards individualized treatments and targeted inclusion criteria. The meta-analysis published by Feng and colleagues (Feng et al. 2020) proved that the difference between patients in MCS and UWS in the numbers of responders was significant. However, in this study we showed that being in MCS at baseline increases the odds of being a responder by four times compared to patients that are UWS at baseline. This is an important information for future studies and the fact that our results confirmed those of Feng and colleagues (i.e., 140

patients with 24% responders) highlights how diagnosis at baseline is crucial for tDCS response.

One reason to why patients in MCS respond better to tDCS than patients in UWS might be found in the underlying brain preservation and metabolism (Thibaut, Di Perri, et al. 2015). Indeed, patients in MCS higher connectivity and brain metabolism in regions that are crucial for consciousness such as the DMN and precuneus (Demertzi et al. 2015; Di Perri et al. 2016). Given this information, we can hypothesize that tDCS enhances the activation of these regions and the connectivity in their pathways because the baseline functionality of these is somehow already preserved, which might not be the case for patients in UWS (Demertzi et al. 2015). To support this hypothesis, several studies on stroke populations have showed that patients with larger brain lesions (Bolognini et al. 2015) and white matter deterioration (Bradnam et al. 2012) experienced less benefit from tDCS. It is interesting to note that the first RCT using amantadine in patients with DoC (Joseph T. Giacino et al. 2013) did not observe different rates of response to the drug between patients in UWS and patients in MCS (even if recovery rates were faster for patients in MCS). According to the meso-circuit model of treatments to DoC (Thibaut, Schiff, et al. 2019) amantadine and prefrontal tDCS should act on the same areas (i.e., prefrontal cortex), however, the former being a dopamine agonist might also activate the striatal neurons of the basal ganglia. This difference, might be a key point in the observed differential effects between the two techniques according to diagnosis. It is also important to note, also, that some studies (Estraneo et al. 2017; Thibaut et al. 2014) have observed clinical response to tDCS in patients with UWS, however more rarely than for patients in MCS. We know that up to 67% of patients that are behaviourally in UWS might actually show brain patterns compatible with MCS (Thibaut et al. 2021), thus being considered in MCS*. For this reason, these rare cases of patients in UWS showing behavioural improvement after tDCS, might be caused by covert consciousness (Thibaut et al. 2018).

The fact that long TSI seems to increase the odds of being a responder significantly is also an important piece of the puzzle of characterization of responders and is compatible with late recovery studies on DoC (Estraneo et al. 2010; Estraneo et al. 2019). Our sample was composed of acute, subacute and chronic patients (i.e., ranging between 0.9 to 251 months post injury). Apart from the possible explanation of the re-organization of the brain after severe brain injury (Milosevic et al. 2021; Chen, Cohen, and Hallett 2002), another factor that could play a role in this result is that patients with very chronic DoC do not usually have several hours of rehabilitation (e.g., speech therapy, physiotherapy) per day.

Compared to more extensive rehabilitation programs, it might be easier to detect tDCS effects in settings where the patients are less stimulated. Indeed, if multiple therapies are applied every day, the patients might be more tired at the moment of the stimulation, as well as overstimulated. However, we are not suggesting that patients should have less rehabilitation hours but rather that for tDCS' effects to be detected, calmer living situations might be beneficial. Several studies (Estraneo et al. 2010; Estraneo et al. 2019) have showed that late recovery is indeed possible for patients in UWS, especially those with TBI aetiologies (Estraneo and Trojano 2018; Estraneo et al. 2010). Future studies should make use of this information on recovery and envision recruiting patients that have been many years in DoC into tDCS trials. Furthermore, the fact that patients with very chronic DoC can respond to tDCS comes as a surprising and promising result for their prognosis. Clinicians in general, know that the more time has passed since the appearance of an illness or a disorder with no improvement, the less likely it is that treatments will be effective, as showed by results in stroke and TBI patients on neuroplasticity (Puderbaugh et al. 2021). However, our results go against this and give rise to hope for more chronic patients.

The increased odds of being a responder for young patients might have resulted as significant from our analysis because of the very small number of patients we have in our sample. Only two patients were under the age of 18 and both responded to tDCS so this might have biased the model towards the observed significance. The age categories we used for this analyses were not equally distributed and this is a limitation of our study. The reason we divided our sample in these age groups is that: we first explored age as a continuous variable with a quadratic term to relax the linearity assumption, this increased model performance, however quadratic terms are not easily interpretable. To preserve the non-linear effects of age while leaving interpretable results, we needed at least three categories. We selected the categories based on two criteria: physiology and clinical importance. We selected the category of non-adults because we thought it would be more relevant for the scientific discussion (since most inclusion criteria exclude <18). But having only two patients in that category is indeed a great limitation. However, several studies (Estraneo et al. 2013; Estraneo et al. 2019; Estraneo et al. 2010) do report that young DoC patients show better prognosis and that patients that showed late recovery of consciousness tended to be younger than those who did not. For this reason, even if our data might have resulted by our sample characteristics, they are still supporting stronger results from other studies and it might be interesting to perform a RCT on patients with DoC under 18 years old, to investigate the possibility of increased effectiveness of tDCS.

We do not know how tDCS mechanisms might change in brains that are still developing and that is the case for teenager patients (Giedd et al. 1999), especially in prefrontal brain regions (Sowell et al. 1999). However, Heise and colleagues found that anodal tDCS (targeting M1) led to stronger effects in young adults compared to elderly adults (Heise et al. 2014). One modelling study (Ciechanski et al. 2018) found that the electric fields vary between age ranges, probably due to skull thickness and grey matter volume, but this study also used M1 as a modelling target, thus we cannot be sure the same results would appear with prefrontal tDCS stimulation. It has been hypothesized (L. M. Li, Uehara, and Hanakawa 2015) that, since aging results in increased skull thickness and proportion of CSF (Beauchamp et al. 2011), this could cause the tDCS current to be shunted from the high conductivity of the CSF and decreased by the skull thickness in older brains. Moreover, it is also known that neuroplasticity is higher in children (Mundkur et al. 2005), thus tDCS might have a greater impact in this population due to higher proneness to structural and dynamic changes.

Finally, we would like to highlight that, even if not reported in our study, due to editorial limitations, we repeated the analysis on the predicted odds of being a responder on the sub-sample of patients that had prefrontal stimulation alone, thus excluding all the other targets (i.e., N=91 of which 26 responders – 28%). The results did not change, meaning that the same variables were significantly increasing the odds of being a responder also for prefrontal stimulation alone, which we know is currently considered the most effective tDCS target for patients with DoC (Feng et al. 2020). Moreover, when analysing the variable of brain target, although the odds of response were 1.67 times higher for DLPFC stimulation compared to other montages [OR= 1.67 (CI: 0.61, 4.97); p-value = 0.33], this variable did not improve the goodness of fit of the model (p-value from LRT = 0.32) and was not significant.

We would like to highlight how important it is to better understand what characterizes responders to tDCS. A better insight on which patients (in terms of age, TSI, brain lesions and structure, aetiology, EEG patterns, sex, diagnosis) are more likely to respond to tDCS could not only optimize targeting of future studies but also the observed effectiveness of tDCS itself. This study was only a first step in this direction, focusing mostly on demographic information, but has produced informative results. Other factors that should be investigated as possible characterization of tDCS responders would be neuroimaging data such as baseline EEGs, structural brain features of responders and baseline behavioural factors such as baseline CRS-R total scores, presence of specific behaviours

at baseline and others. However, it is important to note that a balance between targeting the most responsive population and a big sample size might be difficult to reach. Indeeed, for all the reasons mentioned above, enrolling patients with DoC in RCTs is challenging and drop-out rates are high. Optimizing inclusion criteria to target patients that are likely to be responders might increase observed effects of stimulations but might also make it more and more difficult to enrol patients. However, we think that if multicentre studies with common protocol and conceptual frameworks are conducted throughout all DoC expertise research centres, we could achieve sample sizes large enough to have strong statistical power and simultaneously targeting specific endotypes populations.

Finally, we think it is of vital importance researchers build a common definition of what can be considered a responder and what cannot, in order to allow a correct characterization of responders. So far, every DoC research institution has defined response to tES in a slightly different way. As explained before, some studies define it as an improvement in the total score of the behavioural scale, some studies define it as a new behaviour seen following the stimulation, others define it as a new behaviour that was not present in any other session. This may lead to confusion and also an impossibility to create a common database of responders that could help us to investigate their characterizing features. We think that, even if restrictive, the most conservatory (thus reliable) definition of responder should be adopted, that is to say, patients should be considered as responders to tES when they show at least one new conscious behaviour after tES (or other NIBS) that had not been observed before nor after any other session (i.e., sham and/or other stimulation conditions). The reason behind this suggestion is that behavioural fluctuations are a hallmark feature of patients with DoC (Cortese et al. 2015; Candelieri et al. 2011). This presents a challenge when attempting to establish a clinically significant difference (e.g., pre-post stimulation) and determining the effectiveness of a treatment. We performed an exploratory study on a database of 243 subjects (60.3% male; age 62.6 ±25; 48.9% TBI) with the goal of understanding the degree of normal variability of patients with DoC. Our objective was to determine the frequency of fluctuation in CRS-R subscale scores (as the total score does not bring any information about consciousness levels) in adult patients with DoC. We analyzed the percentage of cases showing behavioural fluctuations across the sample in 6 consecutive CRS-R assessments performed over a maximum of 21 days. We defined as fluctuation any change in CRS-R subscale score between consecutive exams and we also

reported the magnitude of these fluctuations. (See Figure 1 for the unpublished results on frequency of fluctuations).

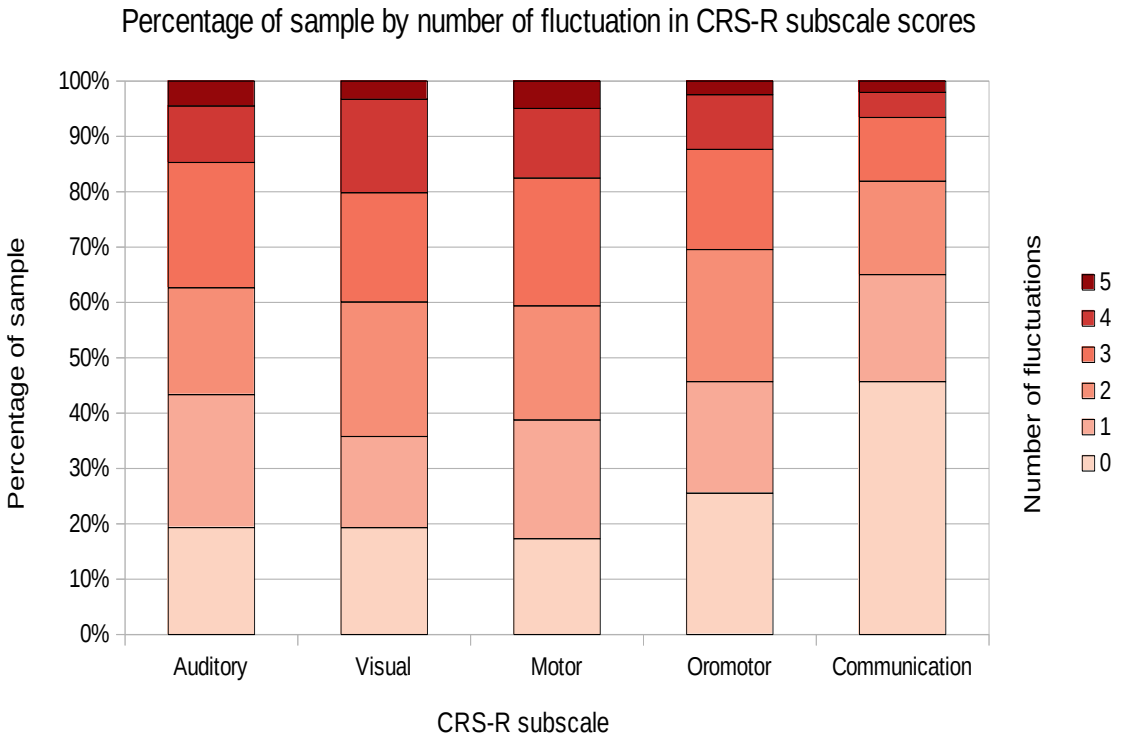

Fig. 4 Frequency of behavioural fluctuations in CRS-R subscale scores in patients with DoC between 28 and 90 days post injury. Original figure from unpublished material (Barra A, Tan C, Martens G, Dana C, Bodien YG, Giacino JT). N= 243 subjects. Each column represents the percentage of the sample demonstrating the number of total fluctuations observed over the 6 evaluations, specified by color (i.e. 0; 1; 2; 3; 4; 5). Most of the sample demonstrated fluctuations on all 5 subscales. Approximately 60% of the sample experienced between 1 and 3 fluctuations. Fluctuation was observed less frequently in the communication subscale.

Our results show that across 6 examinations 60% of the sample showed between 1 and 3 behavioural fluctuations on at least one of the subscales of CRS-R. Moreover, when investigating the magnitude (i.e., point change in subscale score when a fluctuation occurred) we found that most of the fluctuations involved a 1-point change in subscale score, however changes as high as 5 points were observed, depending on the subscale. (See Fig. 2 for details on unpublished results on the magnitude of observed fluctuations)

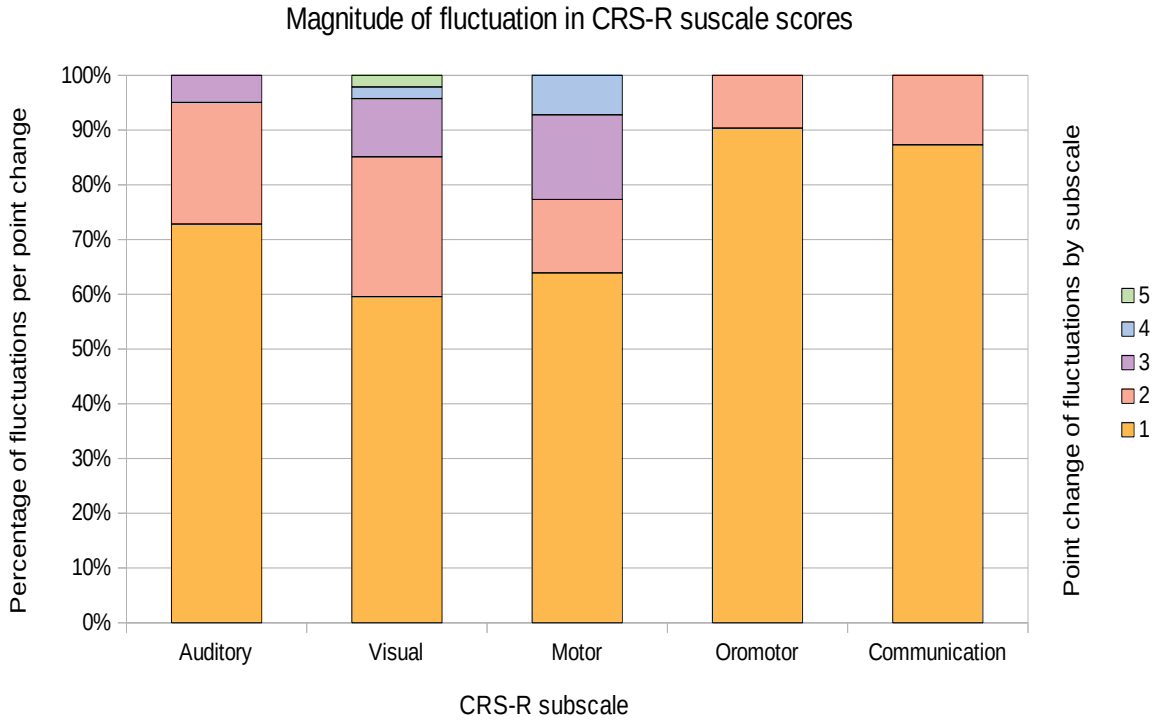

Fig. 5 Magnitude of fluctuations in CRS-R subscale scores in patients with DoC between 28 and 90 days post injury. Original figure from unpublished material (Barra A, Tan C, Martens G, Dana C, Bodien YG, Giacino JT). N= 243 subjects. Each column represents the frequency with which the CRS-R subscale scores changed by 1, 2, 3, 4 or 5 points between examinations. While 1-point fluctuations occurred most often, we observed changes as high as 5 points, depending on subscale.

These results highlight the importance of a common definition of responders for all RCTs exploring treatments for patients with DoC. Moreover, they suggest that a slight 1-point improvement in one of the subscales might be the result of a behavioural fluctuation and not the real effects of the stimulation. Given all this information, we would like to orient researchers towards a definition of responders that is less likely to erroneously identify as effects of stimulation spontaneous fluctuations. In addition, having a large database of tES trials using the same definition of clinical response, will allow us to perform subgroup analyses on features of responders with higher statistical power.

Proposing and testing a patient-tailored tDCS protocol

Apart from characterizing the features of tDCS responders, other individual factors could influence the observed effectiveness of the stimulation, such as the patients' level of vigilance. In Study 4 we proposed a protocol that could trigger the stimulation according

to these vigilance levels with a closed-loop system. However, we have to highlight that the development and execution of Study 4 encountered several obstacles. During the development phase of this study we found that the best way to deliver a 2 mA current would have been to stimulate bilaterally the prefrontal cortex. This choice of target, resulted in limitations regarding patients' enrolment. Indeed, many patients with DoC have shunts and/or open craniectomies and since one of tDCS exclusion criteria is usually the presence of such factors under the stimulating electrodes, having bilateral prefrontal stimulation electrodes decreased our chances of encountering eligible patients. Another limitation to this protocol is caused by the complex set up needed to carry out the experiment. The NIC software and Closed Loop Manager software work in concomitance in order to record EEG, calculate the patients' spectral entropy and vigilance threshold and trigger the stimulation. To do so, the two applications need impeccable impedances and this is already a major challenge in patients with DoC. As we know, these patients do not communicate and often cannot consciously contain their spontaneous motor reflexive movements, resulting in artefacts in the EEG signal. For this reason, obtaining perfect impedance levels in patients with DoC is generally very challenging. While this does not cause a major issue in most studies involving EEG, since motor artefacts can be removed in the preprocessing phase; in this specific protocol it is a considerable obstacle, as the EEG data are analyzed online. The Closed Loop Manager, indeed, aborts the tDCS if the impedances levels are not optimal during the stimulation time and this cannot be controlled by the researchers. During the data acquisition for this study, it has occurred several times that, while the tDCS was being delivered, the impedances levels were detected as not optimal by the software and the stimulation was aborted. This change in impedances were caused by the patient moving in some cases, but in other cases by signal corruption between the two softwares, due to computing issues. When the stimulation is aborted the experiment needs to be re-launched, as the vigilance threshold needs to be recalculated from the start. As described before, to calculate vigilance thresholds the software needs between 30 and 120 minutes. In this context, when the experiment is aborted, it is impossible to relaunch the entire procedure again on the same day. In addition, for patients with DoC, the length of this effort is very tiring and can cause uncomfortableness and distress, without considering that during this time they might have other therapies or medical examinations scheduled. All these factors, plus the fact that this study involved three sessions per patient, caused the data acquisition for this study to be very challenging and increased the likelihood of drop-outs.

Regarding the preliminary results presented here, they should be interpreted with extreme caution. In this dissertation, they have been reported in a descriptive way and no statistical analysis has been conducted on the group level, thus we cannot infer that the changes described will be generalized when the entire sample will be collected. Despite being only preliminary, some of the results of stimulating according to vigilance thresholds observed so far, are quite interesting.

One intriguing observation we can make from these preliminary results is that tDCS at high vigilance was the only condition that never resulted in a decrease in behavioural performance, so far. The absence of behavioural worsening was also associated with higher post-stimulation median and smaller variance of results. We could interpret this as a preliminary proof that the effects of tDCS applied at high vigilance thresholds are less likely to be contaminated by confounding factors. On the other hand, no patient has showed a new sign of consciousness or a diagnostic improvement and the only positive effects we observed were slight improvements in the CRS-R total scores for two patients, which we have seen, should not be counted as a response to tDCS. Stimulation at low vigilance thresholds also resulted in some small behavioural improvements measured by the CRS-R total score. Moreover, this tDCS condition also resulted in two cases where we observed decreased behavioural improvement. For this reason, the variance of tDCS at low vigilance is very wide and the median post-stimulation slightly lower than the pre-stimulation. Finally, regarding tDCS applied at random vigilance levels (which in our conceptualization of the protocol is supposed to reflect normal – non-closed loop application of tDCS) resulted in the highest rates of observed decrease in performances. Even though the median of the post-stimulation scores is higher than the pre-stimulation ones, the variance is very wide. This seems to replicate the disparate results observed with non-closed loop tDCS in small sample studies that can produce some increases, as well as decreases, in behavioural performances and are often difficult to interpret. It might also be informative to assess when the random condition triggered the stimulation in terms of vigilance level. Indeed, since tDCS is triggered at random levels of vigilance there is a possibility that it might have been prompted during low vigilance levels.

One interesting case we would like to highlight, is CL_ID06. This patient showed behavioural improvement after all tDCS conditions, suggesting that he might be very responsive to tDCS and benefit from multiple sessions of stimulation. Moreover, CL_ID06 showed the only relevant clinical response according to the definition of responder proposed before (i.e., object recognition showed after stimulation at random vigilance

levels and not before nor after any other condition). One interesting characteristic of this patient is that he presents the longest TSI in our sample. Patient CL_ID06 has been in a chronic DoC for more than 37 years. This long TSI and good response to tDCS fits nicely with what we report in Study 3 and supports the idea that very chronic patients can still benefit from tDCS.

We reported here our protocol and preliminary results from a tDCS study applying the stimulation at the patients' specific high, low and random vigilance thresholds. Even if these findings are only preliminary, they carry valuable preliminary information. However, to make assumptions on the real effectiveness of tDCS at specific vigilance thresholds we need to complete the data acquisition and perform statistical analyses on the significance of the effects.

Overall the results are promising: stimulating at specific vigilance levels seems to produce less varied effects compared to stimulating at random vigilance levels. The importance of vigilance levels in modulating tDCS effectiveness might be partly explained by the activity-dependent model of tDCS mechanisms of action proposed by Fertonani and Miniussi (Fertonani and Miniussi 2017b), which states that are engaged in the execution of a task. Supporting this hypothesis, one study using prefrontal tDCS (Esposito et al. 2022a) found that the observed effects changed according to arousal levels, with the strongest effects showed with moderate arousal levels. Another study (Clow et al. 2014a) using rTMS, reports finding greater response to the protocol with higher cortisol levels. Cortisol is a steroid hormone (Edwards et al. 2001) that underlies circadian rhythm and notably Cortisol Awakening Response (CAR) is considered a biomarker of awakening (Clow et al. 2010). Based on this information, coming both from a neurophysiological and a behavioural perspective, we can assume that tDCS effects might be greater when the patient is engaged and vigilant. Interestingly, one study (Neuling, Rach, and Herrmann 2013a) has showed that tACS could increase alpha rhythm in healthy subjects only during low vigilance levels (i.e., eyes closed). However, in healthy subjects, low vigilance levels and relaxed states are linked to increased alpha power in the EEG (Travis, Kondo, and Knott 1974), which is not the case for patients with DoC (Bai, Lin, and Ziemann 2021). On the contrary, higher alpha power is a marker of higher levels of consciousness in patients with DoC and unconscious patients do not usually present it (Naro, Bramanti, et al. 2016; Piarulli et al. 2016). For this reason, we can assume high alpha rhythm corresponds to high vigilance levels in patients with DoC and, thus, the results from

(Neuling, Rach, and Herrmann 2013b) support the hypothesis that applying tDCS in patients with DoC at high vigilance thresholds can produce better results.

Nevertheless, to better understand which vigilance thresholds produces the strongest behavioural effects, the data acquisition needs to be completed. However, the set up needed to detect vigilance thresholds and trigger the stimulation is far from being ready to be implemented in daily care and still presents several limitations. Future studies should explore faster and easier ways to detect vigilance levels in patients with DoC (e.g., biological and/or hormonal markers of arousal) that could replace the current system. For instance, in a study (Clow et al. 2014b) cortisol level and CAR, that is measured usually about 15 minutes after waking up and then at regular intervals (Stalder et al. 2016), could be a good alternative to online EEG. Furthermore, continuous pupil dilation assessment (i.e., pupillometric imaging) is also used to measure physiological arousal levels (Wingate et al. 1998; Esposito et al. 2022b). However, these measures have to first be tested in patients with DoC and compared to literature in healthy subjects to be validated. Another approach, to stimulate at high vigilance levels and maximize the effects of tDCS, might be finding a way to actively manipulate the patients' vigilance levels. For instance, if our RCT were to find that low vigilance thresholds produce the best results, we could use relaxation techniques to induce low vigilance in the patient before applying the stimulation. On the other hand, if we were to confirm our hypothesis that high vigilance thresholds are the optimal stimulation moment, we could increase the patients' arousal level with stimulations such as preferred music or pharmacological components such as caffeine. Regarding music, since intense emotions are supposedly linked to increased physiological arousal (Schachter and Singer 1962) and it has been shown that emotionally powerful music can increase skin conductance (Rickard 2004) which is a typical measure of physiological arousal (Bach, Friston, and Dolan 2010). Moreover, exposing patients with DoC to their preferred music was reported to increase activation of the frontoparietal network and precuneus (Carrière, Larroque, et al. 2020; Okumura et al. 2014). These results make preferred music a good candidate to actively increase vigilance levels in patients with DoC and helping applying tDCS during high vigilance. Another alternative to produce this change in arousal could be the use of a more classical arousal-enhancer such as caffeine, that has been proven to be a successful tool in increasing resting state arousal in several studies (Barry et al. 2005; Clubley et al. 1979).

Future perspectives for a tES patient-tailored approach

In order to maximize the effects of tES as a treatment for patients with DoC we need to orient research towards a patient-tailored approach. However, in order to do so, scientists need to reach consensus on a common conceptual framework for RCT studies. Research should focus on short and easily implemented protocols that will allow for lower drop-out rates and better observed results. The first change to implement in RCT protocols would be to replace the CRS-R (Giacino, Kalmar, and Whyte 2004) with the SECONDs (Aubinet et al. 2021). In this way we would have an accurate measure of diagnosis, with an index that is directly linked to diagnosis (Sanz et al. 2021), without the time-demanding limitations of the CRS-R. Another important and necessary issue to tackle is the characterization of responders.

For instance, based on our results from Study 2 and preliminary results from Study 4 (as well as several other studies previously cited), theta power and connectivity, especially over frontal regions seems to be an important predictor of behavioural responsiveness to tDCS and consciousness recovery. From a prognostic perspective, recently, Bareham and colleagues (Bareham et al. 2020) found that theta (and alpha) connectivity could successfully predict behavioural scores and recovery of patients with DoC. Modeling studies should further investigate the role of theta frequency in consciousness recovery and especially fitting this frequency band into the meso-circuit model. One study using levodopa dopaminergic stimulation (Eckart et al. 2014), confirmed the role of prefrontal dopaminergic activity in maintenance of working memory. Moreover, the behavioural effects of dopaminergic stimulation were coupled with an increase in low theta frequencies (i.e., 2-4 Hz) over prefrontal regions. Since theta frequencies are mostly increased in responders (after tDCS) over prefrontal regions, we can hypothesize that there is a connection between this frequency and dopaminergic neurotransmitters. This would fit with the meso-circuit model that highlights the role of dopaminergic increase within cortico-thalamic pathways in the recovery of consciousness (Schiff 2010; Thibaut et al. 2019). Furthermore, theta oscillations in the prefrontal cortex are believed to be a sign of interaction between the DLPFC and the hippocampus (Buzsáki 2002; Benchenane, Tiesinga, and Battaglia 2011), as well as between the hippocampus and the striatum (DeCoteau et al. 2007), thus being a possible marker of functional connectivity between attentional and memory processes. Finally, one study (Ishii et al. 1999) also showed that

during mental arithmetic tasks midline theta rhythm was enhanced in the prefrontal cortex, suggesting this frequency was linked to focused attention.

Apart from theta frequency, diagnosis, TSI and age might be predictors of responsiveness to tES. The goal of future studies should be to characterize responders not only to tES but also other NIBS and pharmacological treatments in order to have such precise information about the endotypes that each one would be relevant to a specific treatment option. For instance, given the patient's sex, age, aetiology, TSI, diagnosis, specific brain lesions, resting state neuroimaging data, electrophysiology data and behavioural profile we would be able to assign them a patient-tailored plan of treatment that was previously agreed on by the whole research field, as it resulted from a common database of responders.

Another important objective, that research should aim towards, would be the implementation of longitudinal studies. Not only would this be facilitated by a common framework and definition of responders, but we could also investigate the effects of tES and other techniques long term. As of yet, no study has explored the effects of more than a few months of tES, whereas it would be very informative to test what applying tES for several years would produce. One case study using at-home tDCS (Pilloni et al. 2019) reported good results on cerebellar ataxia symptoms after 60 sessions. Not many studies have explored the effects of more than 20 sessions of tDCS, but tDCS at-home seems to be safe (Charvet et al. 2020). Some longitudinal pharmacological trials have been conducted in patients with DoC. For instance, Fridman and colleagues showed the benefits of continuous intravenous apomorphine treatment for over 6 months (Fridman et al. 2010). In this study, the effects of the drug continue to increase (even if less substantially compared to the first month of treatment) throughout the whole administration phase. Another study using bromocriptine also found positive effects after 6 months of treatment (Passler and Riggs 2001). No study using pharmacological treatments has tested more than 6 months of treatment to our knowledge. Regarding longitudinal treatments using tES, the repeated application of tES could be allowed by ad-hoc devices such as the one used by Martens and colleagues (Martens et al. 2018). This device does not allow to change the parameters of stimulation (thus the stimulation intensity and length being set prior by the researchers) and records the amount and dates of sessions delivered. A good idea would be to try to implement this device in nursing homes, before patients are transferred to their residence, as patients would probably be more surveyed. The feasibility of this implementation has been proved by Martens and colleagues and there is no obstacle

stopping researchers from launching such longitudinal study in nursing homes. Especially given the importance of long TSI observed in our Study 3, this could lead to stronger effectiveness of tDCS.

7. Conclusions

Patients with DoC suffer from a tragic condition. As a result of severe brain injuries and subsequent communication deficiencies, providing them with appropriate care and treatment poses significant challenges to researchers and healthcare professionals. Fifty years after the first definition of patients with DoC, the research field has advanced considerably and has reached a consensus on various aspects of DoC such as diagnosis and ethical considerations. On the other hand, treatment studies on this condition are not yet performed under a unified framework, which hinders progression within the research field.

The research presented here has allowed us to make the following conclusions:

1) A shift in the behavioural scale used to assess consciousness in RCT is needed, to favour shorter protocols. The GCS does not, however, fullfill all the prerequisites to replace the CRS-R. So far, the most amenable alternative to the CRS-R seems to be the SECONDs.

2) New tES techniques as treatment for patients with DoC need to be explored. Our first RCT investigating tPCS and tDCS provides a successful example of double-blind simultaneous investigation of two tES techniques for future studies. However, tPCS at 6-10 Hz over mastoids has not resulted in meaningful clinical improvement in our sample.

3) Future studies should target patients that are in MCS at baseline and with longer time since injury, since these factors increase the odds of responding to tDCS. Moreover, patients younger than 18 years old also seem to respond better to tDCS.

4) Individual factors such as fluctuating vigilance could impact the effectiveness of tDCS. Our preliminary results might support the hypothesis that high vigilance thresholds create less dispersed results. Given the lack of statistical analyses and size of the sample it is impossible to make inferences yet. However, the difficulties encountered implementing an EEG-based closed-loop system in an RCT suggest that other ways of manipulating vigilance levels might be more easily applied in the daily clinical care of patients.

The works presented in this dissertation orient researchers towards a more unified conceptual framework that is necessary to embark on the first steps towards a patient-tailored approach. Therapeutic research of patients with DoC will benefit from an

established consensus of the optimal tools to assess the behavioural effects of a treatment. Moreover, a common definition of which cases should be considered as clinically meaningful (i.e., responder to treatment) is paramount. Since patients with DoC are fragile, few in number, non-communicative and greatly heterogeneous, their treatment would benefit from adaptive endophenotypic and individualised approaches. However, envisioning advances in the development of such approaches without the collaboration and compliance (on the framework) of the expert research centres is difficult.

Here, we would like to stress that DoC is a relatively small research field compared to other neuroscientific and biomedical fields. Recent gatherings of the experts of DoC, (Kondziella et al. 2020; Giacino et al. 2018) has led to great advances in the clinical care of these patients. However, in the therapeutic research sector, this collaboration and common ground has yet to be achieved, thus halting progress. Sharing the common goal of improving the condition of these patients, we prospect that the field would greatly benefit from increased collaboration between researchers, uniting their efforts (and data) in spite of the economic and political convolutions it would entail. This will allow the field to implement larger, more powerful clinical trials that will lead to faster results and will facilitate a path towards the ultimate goal of individualized therapies for patients with DoC.

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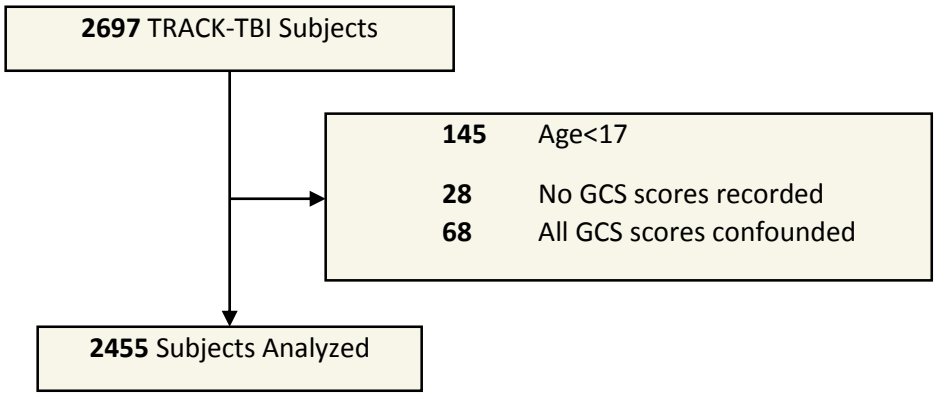
Appendix

Appendix

(From Study 1) Diagnosing Level of Consciousness: The Limits of the Glasgow Coma Scale Total Score

Supplementary Results

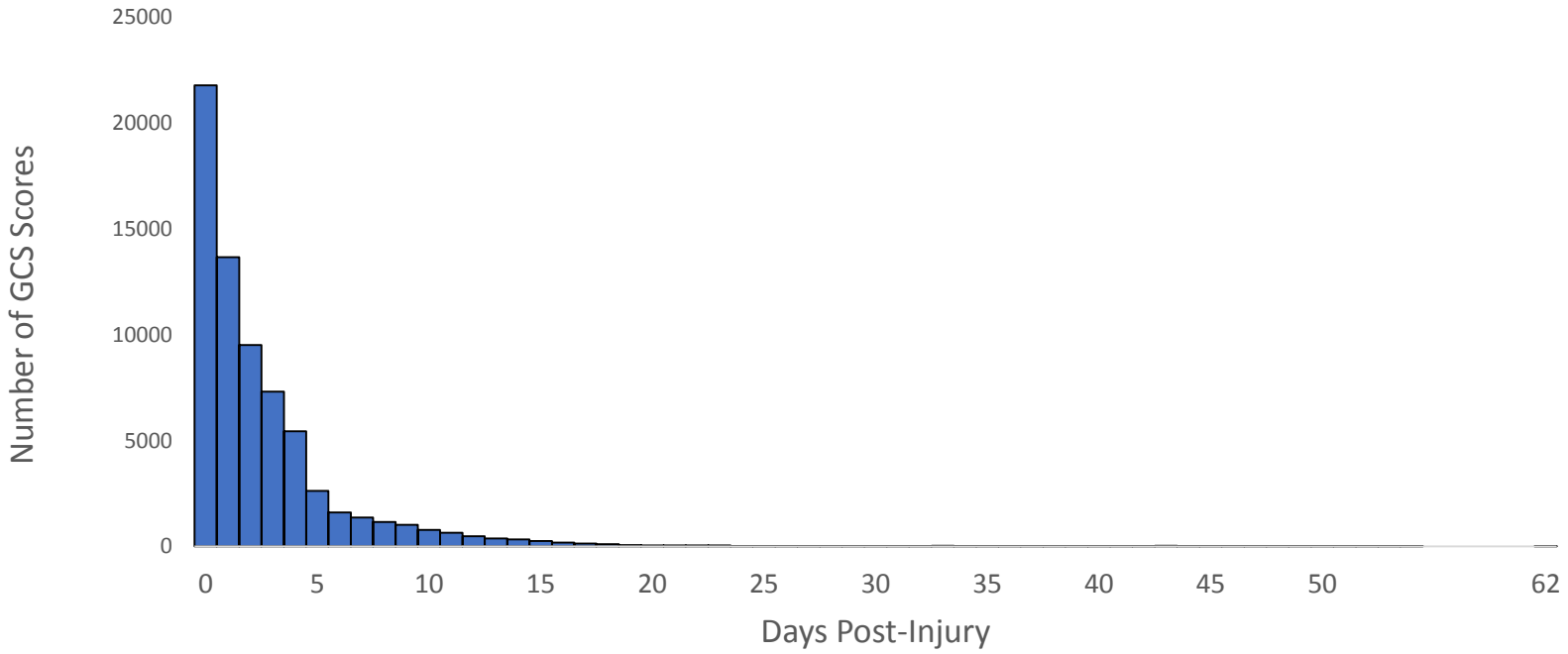
Appendix Figure 1



Appendix Figure 1: Consort Diagram. Subjects were enrolled into the Transforming Research and Clinical Knowledge in Traumatic Brain Injury (TRACK-TBI) study from 2014 to 2018. We excluded subjects younger than 18 years of age, subjects who did not have any Glasgow Coma Scale (GCS) scores, subjects for whom all GCS scores were confounded, and subjects for whom all GCS scores contained data errors (e.g., date of GCS preceded date of injury) that could not be reconciled.

Appendix

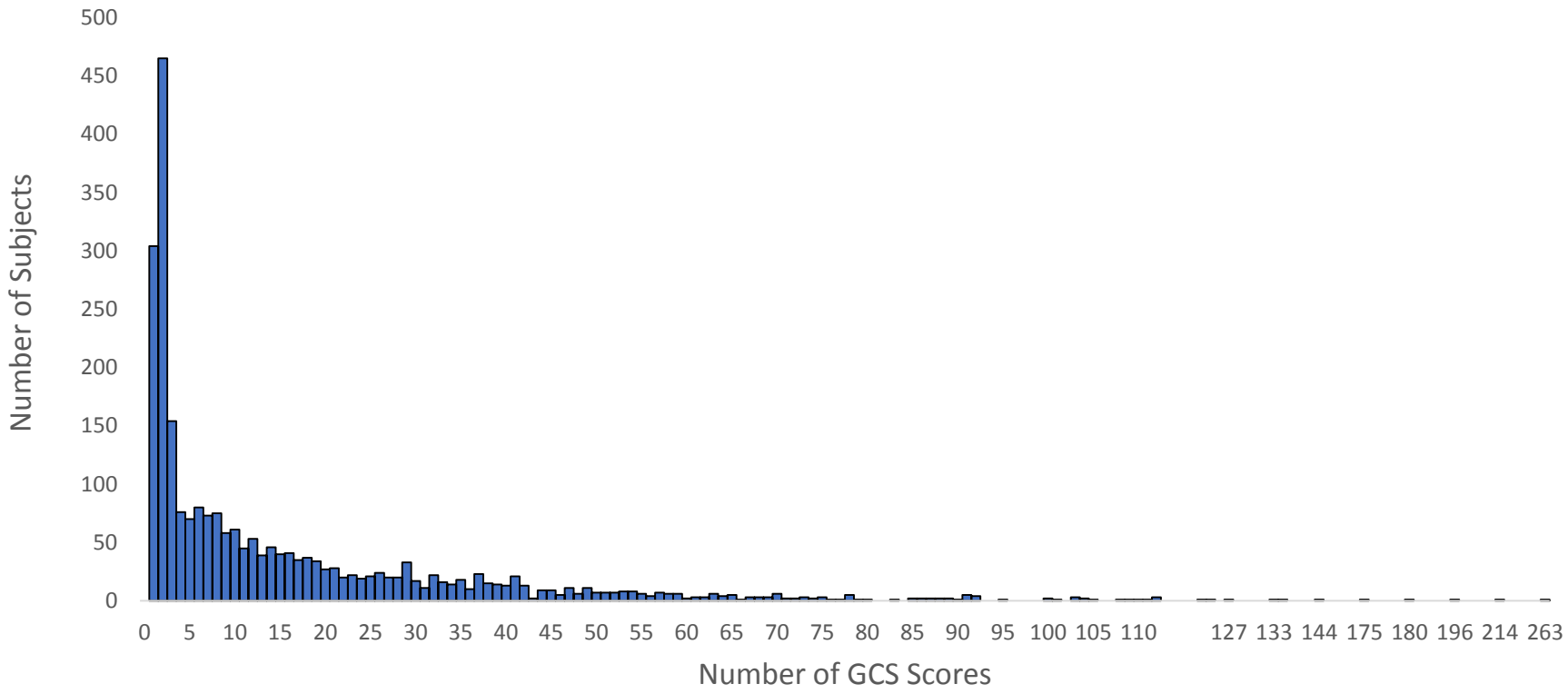
Appendix Figure 2: Number of GCS scores obtained on each day post-injury



Appendix Figure 2: Number of GCS scores obtained each day post-injury. GCS data were collected for mean (standard deviation) 2.4 (2.9) days (median [IQR] = 1.5 [0.3-4.0] days, maximum=62 days). More than 90% of the GCS scores were obtained within the first seven days post-injury

Appendix

Appendix Figure 3: Frequency of GCS assessment across subjects



Appendix Figure 3: Frequency of GCS assessment across subjects. Each patient was assessed, on average, 16.0 (21.8) times (median [IQR] = 8 [2, 21.5] assessments, maximum = 263 assessments)

Appendix

Appendix Table 1

Combination #	GCS Subscale Score			GCS Total Score	Associated DoC Diagnosis	Subjects with at Least One Instance of Each GCS Combination	Subjects with Each Combination as the First Post-Injury GCS	Total Number of Times Each GCS Combination Occurs
	Eye	Verbal	Motor					
1	1	1	1	3	Coma	222 (9.0%)	164	746
2	1	1	2	4	Coma	23 (0.9%)	12	53
3	1	2	1	4	VS	11 (0.4%)	5	65
4	2	1	1	4	VS	10 (0.4%)	3	42
5	1	1	3	5	Coma	19 (0.8%)	11	19
6	1	2	2	5	VS	3 (0.1%)	1	5
7	1	3	1	5	MCS+	0	0	0
8	2	1	2	5	VS	2 (0.1%)	1	2
9	2	2	1	5	VS	1 (0.0%)	0	11
10	3	1	1	5	VS	4 (0.2%)	1	10
11	1	1	4	6	Coma	63 (2.6%)	22	398
12	1	2	3	6	VS	8 (0.3%)	4	8
13	1	3	2	6	MCS+	0	0	0
14	1	4	1	6	PTCS	1 (0.0%)	1	1
15	2	1	3	6	VS	3 (0.1%)	0	7
16	2	2	2	6	VS	5 (0.2%)	5	5
17	2	3	1	6	MCS+	1 (0.0%)	0	1
18	3	1	2	6	VS	0	0	0

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19	3	2	1	6	VS	1 (0.0%)	1	1
20	4	1	1	6	VS	4 (0.2%)	0	27
21	1	1	5	7	MCS-	76 (3.1%)	22	526
22	1	2	4	7	VS	13 (0.5%)	9	15
23	1	3	3	7	MCS+	0	0	0
24	1	4	2	7	PTCS	0	0	0
25	1	5	1	7	rPTCS	0	0	0
26	2	1	4	7	VS	28 (1.1%)	11	79
27	2	2	3	7	VS	2 (0.1%)	0	5
28	2	3	2	7	MCS+	0	0	0
29	2	4	1	7	PTCS	0	0	0
30	3	1	3	7	VS	1 (0.0%)	1	2
31	3	2	2	7	VS	0	0	0
32	3	3	1	7	MCS+	0	0	0
33	4	1	2	7	VS	2 (0.1%)	2	2
34	4	2	1	7	VS	1 (0.0%)	1	1
Combination #	**GCS Subscale Score**			**GCS Total Score**	**Associated DoC Diagnosis**	**Subjects with at Least One Instance of Each GCS Combination**	**Subjects with Each Combination as the First Post-Injury GCS**	**Total Number of Times Each GCS Combination Occurs**
	Eye	**Verbal**	**Motor**					
35	1	1	6	8	MCS+	22 (0.9%)	3	112
36	1	2	5	8	MCS-	28 (1.1%)	6	126
37	1	3	4	8	MCS+	1 (0.0%)	1	1

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38	1	4	3	8	PTCS	0	0	0
39	1	5	2	8	rPTCS	0	0	0
40	2	1	5	8	MCS-	53 (2.2%)	7	382
41	2	2	4	8	VS	10 (0.4%)	10	10
42	2	3	3	8	MCS+	1 (0.0%)	0	1
43	2	4	2	8	PTCS	1 (0.0%)	1	1
44	2	5	1	8	rPTCS	0	0	0
45	3	1	4	8	VS	10 (0.4%)	2	13
46	3	2	3	8	VS	0	0	0
47	3	3	2	8	MCS+	0	0	0
48	3	4	1	8	PTCS	0	0	0
49	4	1	3	8	VS	2 (0.1%)	0	7
50	4	2	2	8	VS	1 (0.0%)	1	1
51	4	3	1	8	MCS+	0	0	0
52	1	2	6	9	MCS+	9 (0.4%)	2	62
53	1	3	5	9	MCS+	11 (0.4%)	2	23
54	1	4	4	9	PTCS	0	0	0
55	1	5	3	9	rPTCS	0	0	0
56	2	1	6	9	MCS+	27 (1.1%)	2	89
57	2	2	5	9	MCS-	38 (1.5%)	12	193
58	2	3	4	9	MCS+	7 (0.3%)	2	9
59	2	4	3	9	PTCS	0	0	0
60	2	5	2	9	rPTCS	1 (0.0%)	0	1

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61	3	1	5	9	MCS-	37 (1.5%)	5	158
62	3	2	4	9	VS	10 (0.4%)	6	12
63	3	3	3	9	MCS+	0	0	0
64	3	4	2	9	PTCS	0	0	0
65	3	5	1	9	rPTCS	0	0	0
66	4	1	4	9	VS	7 (0.3%)	1	21
67	4	2	3	9	VS	1 (0.0%)	0	1
68	4	3	2	9	MCS+	1 (0.0%)	1	1
69	4	4	1	9	PTCS	0	0	0

Combination #	**GCS Subscale Score**			**GCS Total Score**	**Associated DoC Diagnosis**	**Subjects with at Least One Instance of Each GCS Combination**	**Subjects with Each Combination as the First Post-Injury GCS**	**Total Number of Times Each GCS Combination Occurs**
	Eye	**Verbal**	**Motor**					
70	1	3	6	10	MCS+	9 (0.4%)	0	23
71	1	4	5	10	PTCS	4 (0.2%)	2	4
72	1	5	4	10	rPTCS	1 (0.0%)	0	1
73	2	2	6	10	MCS+	18 (0.7%)	4	61
74	2	3	5	10	MCS+	25 (1.0%)	8	68
75	2	4	4	10	PTCS	3 (0.1%)	0	3
76	2	5	3	10	rPTCS	1 (0.0%)	0	1
77	3	1	6	10	MCS+	80 (3.3%)	10	382
78	3	2	5	10	MCS-	36 (1.5%)	6	143
79	3	3	4	10	MCS+	3 (0.1%)	2	6

Appendix

80	3	4	3	10	PTCS	0	0	0
81	3	5	2	10	rPTCS	0	0	0
82	4	1	5	10	MCS-	38 (1.5%)	8	347
83	4	2	4	10	VS	6 (0.2%)	4	11
84	4	3	3	10	MCS+	0	0	0
85	4	4	2	10	PTCS	2 (0.1%)	2	2
86	4	5	1	10	rPTCS	2 (0.1%)	0	2
87	1	4	6	11	PTCS	18 (0.7%)	1	65
88	1	5	5	11	rPTCS	0	0	0
89	2	3	6	11	MCS+	11 (0.4%)	0	29
90	2	4	5	11	PTCS	20 (0.8%)	6	60
91	2	5	4	11	rPTCS	1 (0.0%)	0	1
92	3	2	6	11	MCS+	41 (1.7%)	0	182
93	3	3	5	11	MCS+	28 (1.1%)	2	102
94	3	4	4	11	PTCS	6 (0.2%)	3	7
95	3	5	3	11	rPTCS	0	0	0
96	4	1	6	11	MCS+	59 (2.4%)	12	280
97	4	2	5	11	MCS-	31 (1.3%)	6	167
98	4	3	4	11	MCS+	1 (0.0%)	1	1
99	4	4	3	11	PTCS	0	0	0
100	4	5	2	11	rPTCS	2 (0.1%)	1	2

Appendix

Combination #	GCS Subscale Score			GCS Total Score	Associated DoC Diagnosis	Subjects with at Least One Instance of Each GCS Combination	Subjects with Each Combination as the First Post-Injury GCS	Total Number of Times Each GCS Combination Occurs
	Eye	Verbal	Motor					
101	1	5	6	12	rPTCS	6 (0.2%)	0	21
102	2	4	6	12	PTCS	50 (2.0%)	1	163
103	2	5	5	12	rPTCS	1 (0.0%)	0	1
104	3	3	6	12	MCS+	46 (1.9%)	5	224
105	3	4	5	12	PTCS	50 (2.0%)	15	153
106	3	5	4	12	rPTCS	1 (0.0%)	1	1
107	4	2	6	12	MCS+	29 (1.2%)	2	152
108	4	3	5	12	MCS+	38 (1.5%)	8	147
109	4	4	4	12	PTCS	7 (0.3%)	4	13
110	4	5	3	12	rPTCS	1 (0.0%)	0	1
111	2	5	6	13	rPTCS	10 (0.4%)	2	13
112	3	4	6	13	PTCS	298 (12.1%)	64	2640
113	3	5	5	13	rPTCS	8 (0.3%)	3	11
114	4	3	6	13	MCS+	45 (1.8%)	7	123
115	4	4	5	13	PTCS	64 (2.6%)	33	159
116	4	5	4	13	rPTCS	0	0	0
117	3	5	6	14	rPTCS	463 (18.9%)	51	2455
118	4	4	6	14	PTCS	679 (27.7%)	382	4306
119	4	5	5	14	rPTCS	24 (1.0%)	7	29

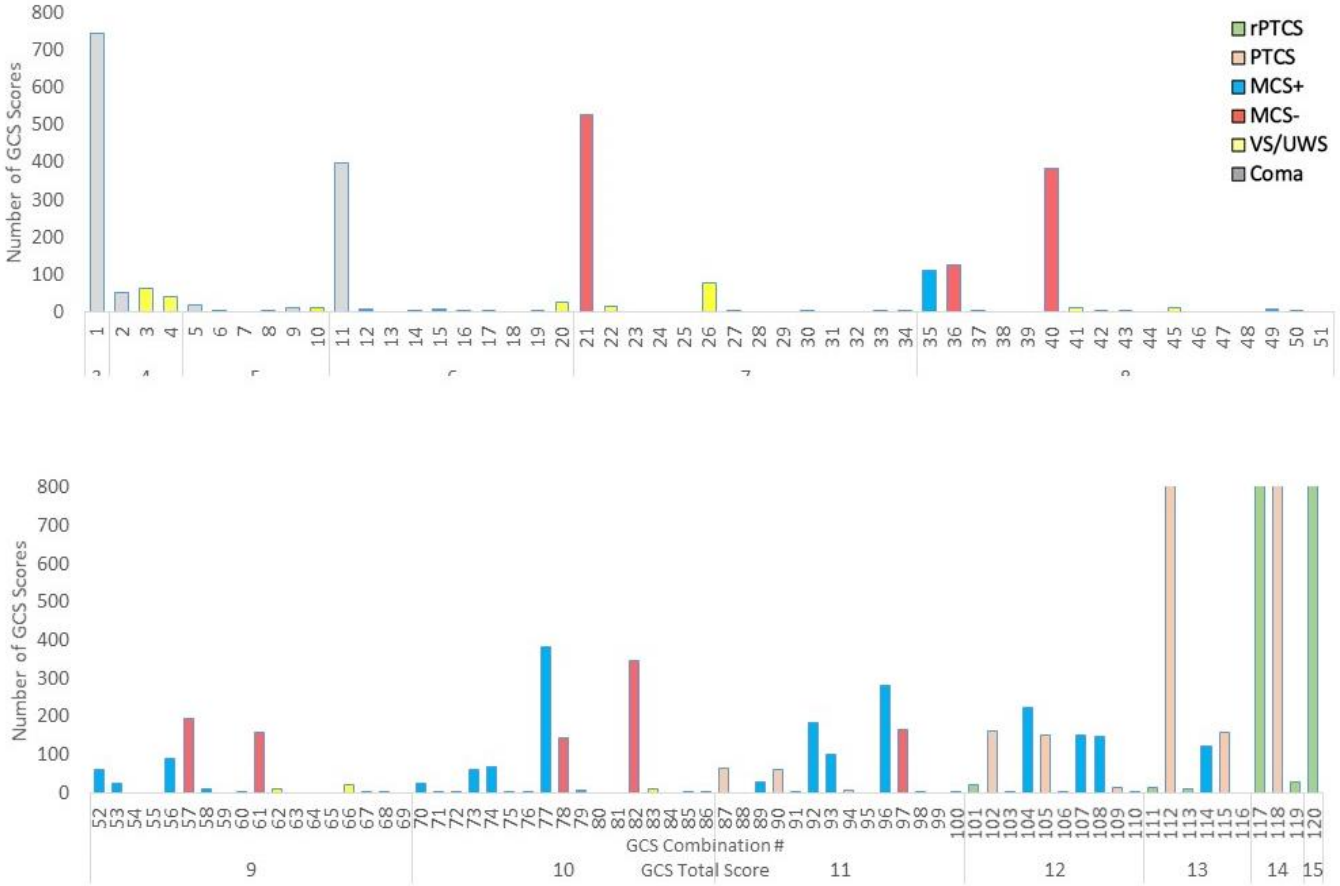
Appendix

120	4	5	6	15	rPTCS	2078 (84.6%)	1451	23531
Total							2455	39348

Appendix Table 1: DoC Diagnosis associated with each GCS combination. We assigned a disorder of consciousness (DoC) diagnosis to each possible combination of Glasgow Coma Scale (GCS) subscales. The DoC diagnosis was based on criteria published by the Aspen Multisociety Taskforce[2] and the decision-tree flow diagram in main manuscript Figure 2. The highest-level GCS behavior observed determined the DoC diagnosis. The DoC diagnoses are approximations because the GCS does not assess all behaviors that could contribute to an MCS diagnosis (e.g. the GCS does not assess visual pursuit).

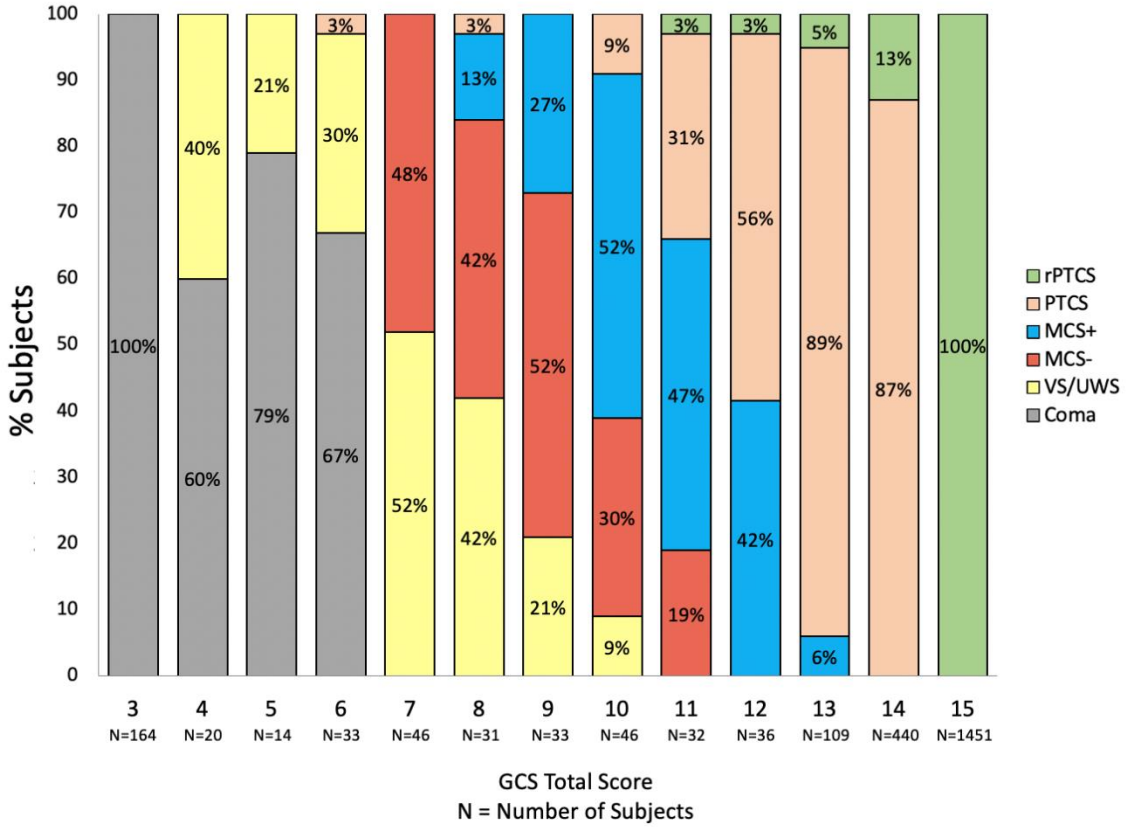
Appendix

Appendix Figure 4



Appendix Figure 4: Number of times GCS combination occurs in 39,348 TRACK-TBI GCS scores. GCS Total Scores 3-8 (top) and 9-15 (bottom) Although the GCS has 120 possible subscale score combination, some combinations occurred infrequently or never. Bars are shaded to reflect the associated disorder of consciousness (DoC) diagnosis. The double x-axis indicates each of the 120 GCS total score combinations organized by the total score associated with each combination. The y-axis displays the number of times each of the 120 GCS combinations occurs in the TRACK-TBI. A diagnosis of coma is evident only for total scores 3-6. A diagnosis of MCS- is possible with total scores ranging from 7-11, while a diagnosis of MCS+ is possible with total scores ranging from 8-13.

Appendix Figure 5



Appendix Figure 5: Proportion of subjects whose first valid GCS score is associated with each DoC diagnosis. First valid GCS total score and its associated disorder of consciousness (DoC) diagnosis in 2,455 subjects in the TRACK-TBI database. Each color represents a different DoC diagnosis. Total scores ranging from 7-11 had the highest variability such that a single score could indicate multiple different DoC diagnoses, different GCS total scores could represent the same DoC diagnosis. The "N" below each GCS total score indicates the number of subjects with which each total score as the first GCS total score in the TRACK-TBI database.

Appendix Table 2

Combination #	GCS Subscale Score			GCS Total Score	Associated DoC Diagnosis
	Eye	Verbal	Motor		
7	1	3	1	5	MCS+
13	1	3	2	6	MCS+
18	3	1	2	6	VS
23	1	3	3	7	MCS+
24	1	4	2	7	PTCS
25	1	5	1	7	rPTCS
28	2	3	2	7	MCS+
29	2	4	1	7	PTCS
31	3	2	2	7	VS
32	3	3	1	7	MCS+
38	1	4	3	8	PTCS
39	1	5	2	8	rPTCS
44	2	5	1	8	rPTCS
46	3	2	3	8	VS
47	3	3	2	8	MCS+
48	3	4	1	8	PTCS
51	4	3	1	8	MCS+
54	1	4	4	9	PTCS
55	1	5	3	9	rPTCS
59	2	4	3	9	PTCS
63	3	3	3	9	MCS+
64	3	4	2	9	PTCS
65	3	5	1	9	rPTCS
69	4	4	1	9	PTCS
80	3	4	3	10	PTCS
81	3	5	2	10	rPTCS
84	4	3	3	10	MCS+
88	1	5	5	11	rPTCS
95	3	5	3	11	rPTCS

99	4	4	3	11	PTCS
116	4	5	4	13	rPTCS

Appendix Table 2: **GCS combinations that never occur in TRACK-TBI**. Among 39,348 GCS scores in the TRACK-TBI database, 31 GCS combinations were never observed.

Appendix Table 3

Combination #	GCS Subscale Score			Imputed GCS Total Score	Number of Occurrences in TRACK-TBI	Associated DoC Diagnosis
	Eye	Imputed Verbal	Motor			
1	1	1	1	3	5511	Coma
2	1	1	2	4	2037	Coma
4	2	1	1	4	252	VS
10	3	1	1	5	13	VS
12	1	2	3	6	1424	VS
16	2	2	2	6	269	VS
22	1	2	4	7	2426	VS
27	2	2	3	7	432	VS
31	3	2	2	7	32	VS
34	4	2	1	7	59	VS
50	4	2	2	8	227	VS
53	1	3	5	9	5640	MCS+
58	2	3	4	9	662	MCS+
63	3	3	3	9	40	MCS+
70	1	3	6	10	514	MCS+
74	2	3	5	10	2254	MCS+
79	3	3	4	10	256	MCS+
84	4	3	3	10	90	MCS+
102	2	4	6	12	502	PTCS
105	3	4	5	12	1584	PTCS
109	4	4	4	12	452	PTCS
112	3	4	6	13	1954	PTCS
115	4	4	5	13	1109	PTCS
120	4	5	6	15	1163	rPTCS

Appendix Table 3: GCS combinations and total scores with imputed verbal scores for intubated patients. GCS combinations are numbered based on the same, but non-imputed, combinations in

Appendix

Supplementary Table 1. Imputation based on Rutledge[3] Equation 1 (Imputed GCS Total Score = (-.3756+(Motor Score*.5713)+(Eye Score*.4233)) allows only one verbal score to be imputed for each combination of eye and motor scores. The imputed verbal score is rounded and summed with the eye and motor scores to produce an imputed total score. This results in an artificial decrease in the potential variability of disorder of consciousness (DoC) diagnoses within each GCS total score. Based on the imputation, an MCS- diagnosis and total scores of 11 and 14 are not mathematically possible, highlighting a further limitation of the imputation approach.

Appendix