

DOTTORATO DI RICERCA IN *NEUROSCIENZE*

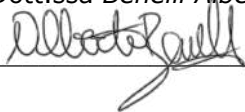
CICLO XXXVII

**Cognitive Fatigue and Non-Invasive Brain Stimulation: New Insights from Healthy
Individuals and Potential Applications in Multiple Sclerosis and Astronauts**

Settore Scientifico Disciplinare
MED /26

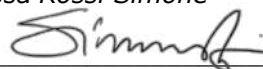
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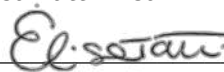
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Abbreviations List

NiBS:Non Invasibve Brain Stimulation

tRNS: transcranial random noise stimulation

tDCS: transcranial direct current stimulation

tACS: transcranial alternating current stimulation

ECT: Electroconvulsive therapy

TMS: Transcranial Magnetic Stimulation

MS: multiple sclerosis

CF:Central Fatigue

MS_F:Multiple scleroris patients with fatigue

MS_NF:Multiple scleroris patients without fatigue

VR: virtual reality

HRV: Heart Rate Variability

RMSSD:root mean square of successive differences

EEG: Electroencephalography

EMG: Electromyography

MRI: magnetic resonance imaging

rsfMRI:resting-state functional magnetic resonance imaging

fMRI:functional Magnetic Resonance Imaging

FC: Functional connectivity
rsFC:resting state functional connectivity
DTI:Diffusion Tensor Imaging
FA:Fractional Anisotropy
MD: Mean Diffusivity
MEP:motor-evoked potentials
ICF: Intracortical Facilitation
SICI: short intracortical inhibition
RMT:resting motor threshold
CS:conditioning stimulus
TS: test stimulus
CSP:cortical silent period
ISI:inter-stimulus interval
LTP:long-term potentiation
LTD:long-term depression
tES:transcranial electric current stimulation
rTMS:repetitive TMS
FoE:Figure-of-Eight
DBU:Downward-Bending U-shape
UBU: upward-bending U-shaped
GABA:gamma-aminobutyric acid
NMDA:N-Methyl-D-aspartic acid
M1: primary motor cortex
S1: Primary sensory cortex

SMN:Sensory Motor Network
DMN:Default Mode Network
SMA: supplementary motor area
L-VGCC:L-type voltage-gated calcium channel
HD:high-definition
PET:positron emission tomography
MEG: magnetoencephalography
HD-tDCS:tDCS high-definition electrodes
net-tDCS:network-targeted tDCS
STDP:spike-timing-dependent plasticity
lf-tRNS:low frequency tRNS
hf-tRNS:high frequency tRNS
BOLD:blood oxygen level dependence
EPSP:excitatory postsynaptic potentials
IPSP: inhibitory postsynaptic potentials
MRS:magnetic resonance spectroscopy
SAEs: serious adverse events
MAEs: mild adverse events
AEs: moderate adverse events
CNS: central nervous system
RT:reaction time
CLT: Cognitive load theory
BG:basal ganglia
dACC: dorsal anterior cingulate cortex

vmPFC: ventromedial prefrontal cortex

NAcc: nucleus accumbens

ACC: anterior cingulate cortex

TH:thalamus

PCC: posterior cingulate cortex

MFG: middle frontal gyrus

OFC: orbitofrontal cortex

PMA:Premotor area

IC: Insular cortex

SN:salience network

RN:reward network

CCN: Cognitive control network

TOT:time-on-task

MDD: Major Depression Disorder

FDA: Food and Drug Administration

HRP: Human Research Program

FSS: Fatigue Severity Scale

mFIS: Modified Fatigue Impact Scale

VAS:Visual Analog Scale

IL-6: interleukin-6

TNF α : tumor necrosis factor-alpha

IFN γ : interferon-gamma

IL-10: interleukin-10

IL-1 β : interleukin-1beta

HCs: healthy controls
HPA: hypothalamic-pituitary-adrenal
PPC: posterior parietal cortex
RRMS: Relapsing-remitting multiple sclerosis
SDMT: Digit-Symbol Modality Task
EP: evoked potential
ERP: Event-Related Potential
ERD: event-related desynchronization
ERS: event-related synchronization
TMS-EEG: TMS combined with simultaneous EEG
CBT: Cognitive behavioral therapies
MNI: Montreal Neurological Institute
VAS-F: Virtual Analog Scale for Fatigue
EDSS: Expanded Disability Status Scale
EC: Eyes closed
EO: Eyes open
9-HPT: 9 hole peg test
TWT: timed 25-foot walk test
SRT: Selective Reminding Test
SPART: Spatial Recall Test
PASAT: Paced Auditory Serial Addition Test
WLG: Word List Generation
MADRS: Montgomery-Åsberg Depression Scale
NfL: neurofilament light chain

GFAP:glial fibrillary acidic protein

IP-10: CXCL10

MCP-1: CCL2

IL-8: CXCL8

FDI: First Dorsal Interosseous

TR: repetition time

TE: echo time

EOG: Electro-oculography

T1w: T1-weighted image

T2w: T2-weighted image

ROI: Region of Interest

LPM: Lesion Probability Map

MSQOL-54: Multiple Sclerosis Quality of Life-54

BMI: Body Mass Index

TPR: True Positive Rates

FN: False Negative Rates

FNR: False Negative Rates

SCC: Superior Calcarine Cortex

SFG: superior frontal gyrus

FP: Frontal Polar

CST: Cortico spinal tract

SLF: Superior Longitudinal Fasciculus

Karolinska Sleepiness Scale: KSS

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Abstract

Fatigue has been a highly debated topic in the literature for decades. It remains an unclear area, despite being an obstacle to daily demands. When we talk about fatigue, we refer not only to the feeling of tiredness related to challenging tasks, but also to a symptom that affects many diseases, such as multiple sclerosis (MS).

My Ph.D. project is the result of collaboration between the Kayser Aerospace Company and the Brain Stimulation and Neurophysiology Laboratory to test a noninvasive brain stimulation treatment protocol aimed at reducing cognitive fatigue in astronauts, who face numerous difficulties during long space flights and need to perform at their best without limitations. The aim of this thesis is to verify the effectiveness of Non-Invasive Brain Stimulation (NiBS) techniques in reducing cognitive fatigue, with the future goal of applying these methods to both astronauts and the clinical population with MS. I conducted two studies to collect preliminary results on the efficacy of NiBS as a countermeasure for cognitive fatigue, applying it to healthy individuals and also testing MS patients using various neuroimaging and neurophysiological techniques to gain new insights into central fatigue symptom (CF). The first study focused on evaluating the effectiveness of a NiBS protocol using transcranial random noise stimulation (tRNS) during a virtual reality task with healthy subjects. Participants, comparing the real stimulation protocol with a placebo, reported a statistically significant reduction in fatigue measured by a Visual Analog Scale, as well as a statistically significant improvement in task performance, without significantly altering other physiological parameters such as heart rate variability. Therefore, these results, which confirm the safety and

efficacy of the tool, will pave the way for new studies involving astronauts. Finally, to apply an innovative stimulation protocol to MS, it was necessary to explore the symptom of CF in detail, which was, indeed, the objective of the second study. Thanks to collaboration with the Department of Neurology of Le Scotte Hospital in Siena, I tested MS patients using different neurophysiological and neuroimaging techniques, such as Transcranial Magnetic Stimulation (TMS) and Electroencephalography (EEG), along with cognitive tests and blood sample data. We observed that intracortical facilitation, a TMS parameter related to the glutamatergic system, was the highest weight among all significant differences in explaining the symptom. Based on these results, the next step will be to extend the use of tRNS to the clinical population of MS, adapting the protocol based on the findings of the second study to treat the CF symptom.

1 Non-Invasive Brain Stimulation

1.1 NiBS background

The neuroimaging development in the last century allowed us to investigate the structural and functional correlations between brain and behavior, as well as the underlying cognitive processes. Nevertheless, the understanding that correlative linkages by themselves cannot prove that a measurable neuronal activity is required or sufficient for a behavior or mental process to occur took hold. These concerns led to the need for further investigation using methods that would enable us to directly examine how experimentally generated alterations in brain processes impact behavior and the underlying mental functions. The elaboration of these techniques dates back more than 30 years to the discovery by Merton and Morton that delivering short electrical currents to a person's scalp may activate the cortex underneath it. This experiment was groundbreaking because it showed that the human brain may be stimulated electrically without having to open the skull, allowing a non-invasive and safe modulation of neuronal functions in the brain [1]. The neuroplasticity process, which is continuous, self-organizing, and adaptable, is common in the cortical regions and allows the brain to learn and adapt to novel environmental circumstances [2]. Previous studies have demonstrated that non-invasive brain stimulation (NiBS) protocols are capable of producing various effects in

the human brain that resemble those of neuroplasticity, modulating cortical excitability. Depending on the mechanism used, the effects can last for minutes to hours after stimulation stops [3]. NiBS refers to a set of technologies and techniques capable of modulating brain excitability through transcranial stimulation. Two major modalities of NiBS are TMS and transcranial electric current stimulation (tES) [4]. NiBS has become increasingly popular due to its potential to influence neuroplasticity, cortical excitability, and behavior [5, 6]. The most common techniques are TMS and tES [5, 7]. In this thesis, I will show both the results of NiBS techniques to evaluate inhibitory and excitatory mechanisms in the motor cortex in MS patients who reported fatigue, and the results of a study we conducted on the modulation of cognitive fatigue in healthy subjects.

1.2 Transcranial Magnetic Stimulation

1.2.1 Theoretical background

Transcranial magnetic stimulation (TMS) was introduced as a neurophysiological technique in 1985 when Anthony Barker, who described for the first time a novel method of directly stimulating the human motor cortex by a non-contact and non-invasive technique using a pulsed magnetic field [8]. Actually, its origins can be traced back to when, in 1832, the chemist Michael Faraday discovered electromagnetic induction: since then, progress has been unstoppable. Faraday demonstrated that an electric current passing through one coil could induce a current in a nearby coil. The current in the first coil produces a magnetic field, which in turn causes the current to pass through the second coil. In TMS, the second coil is replaced by brain tissue and the induced electric

field stimulates neuronal activity. TMS stimulator delivers a large current in a short period of time; The current in the TMS coil then produces a magnetic field that, if it changes rapidly enough, will induce an electric field sufficient to stimulate neurons [9]. A TMS device consists of a series of capacitors connected to a coil of wire that has inductance and resistance. When a rapidly varying electric current passes through a coil of wire placed on the scalp, a variable magnetic field is generated that penetrates the skull. This generates eddy currents that lead to action potentials in brain neurons [10]. The technology of TMS stimulators has improved over the years, with the use of different high-power solid-state devices and coil configurations. The electrical signal generator for TMS coils is usually composed of three basic components: a capacitor (C) for energy storage, a switch to temporally connect the coil to the capacitor (usually a silicon-controlled rectifier, SCR) and a high-voltage source to load the capacitor when it is not connected to the coil. The practical TMS coil can be represented by an inductance (L) in series with a small resistance (R). The three components of the RLC harmonically oscillate to produce a sine-shaped decaying electrical current that produces a magnetic field when passing through the coil [11]. In contrast to tES, TMS does not directly apply electrical current via electrodes attached to the scalp but through inductive electromagnetic stimulation. The TMS stimulator passes a short-lasting current through the coil, generating a strong time-varying electromagnetic field perpendicular to the transducing coil, which is placed tangentially on the head. The magnetic field is not attenuated by the tissue surrounding the brain (e.g., skin and bone) and induces a phasic electric field in the targeted tissue. This field can depolarize excitable structures within the brain, such as neurons. If the electric field induced by TMS is sufficiently strong to depolarize the membrane potential of a given

neuron above a certain threshold, an action potential will be triggered. However, even TMS-induced subthreshold depolarization can have neuronal effects and affect ongoing endogenous activity [12].

1.2.2 Coils and stimulation protocols

Among the existing TMS coils, we can find the circular coil, the Figure-of-Eight (FoE) coil, one of the most used, the Butterfly coil (V-shaped), the Cone coil (double circular), the Downward Bend U-shaped coil (DBU), the Upward Bend U-shaped coil (UBU), the Halo coil and the Heshed coil (H-coil). FoE coil configuration is composed of two circular coils of about 5 to 15 cm in diameter, either very close to each other or overlapped at their intersection. The electrical current moves in the opposite direction at each circular coil, producing a convergent induced electric field at the intersection. If the direction of the current is the same in both coils, the induced electric field at the center would theoretically be zero, which allows for the production of a simulated TMS with the same FoE coil by changing the direction of the electric current in one of the circular coils. This coil is useful when TMS is required in small superficial cortical regions (about 5 mm), for instance, to produce motor evoked potentials (MEP) and for the treatment of some psychiatric disorders. In 2018, the U-shaped downward bend coil was designed to follow the shape of the head to improve focalization and intensity in the treated zone. However, based on the results, although the bending improved the intensity of the induced electric field, it reduced the focality for certain angles compared with the FoE coil. In 2020, the same group improved the focality by bending the UBU coil, to reduce the effect of the winding. In 1992, Roth and colleagues proposed the so-called H-coil to produce a focalization zone into the head. The coil design was made

numerically to produce a summation of the electric field at a certain region of the brain. This coil can be customized to produce focalized stimulation in deep regions of the brain with little effect in the cortical zones [11] (Fig.1). Refinement of the coils is ongoing, with the goal of finding the right tool in terms of focality and controllability, avoiding confounders such as skin heat and noise emitted during the stimulus. In addition, new cooling systems have made it possible to use coils as exploratory tools with single-pulse protocols and treatments with repetitive protocols.

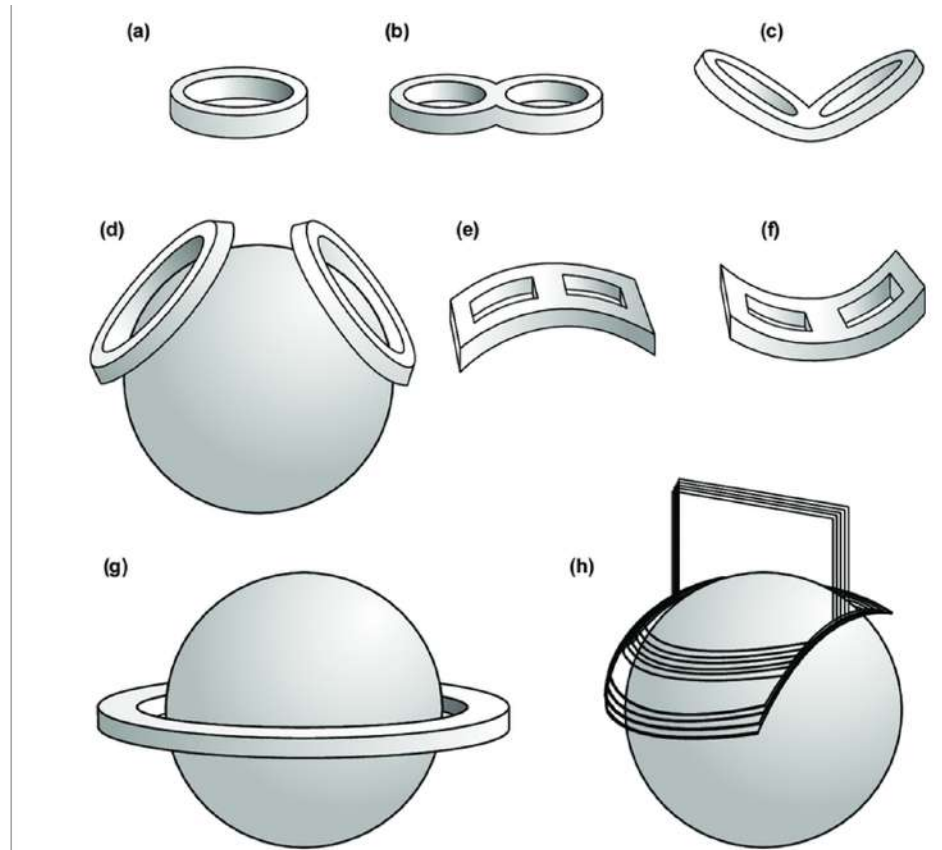


Figure 1. TMS coils. (a) Circular coil, (b) Figure-of-Eight (FoE) coil, (c) Butterfly (V-shape) coil, (d) Cone (double circular) coil, (e) Downward-Bending U-shape (DBU) coil, (f) Upward-Bending, U-shape (UBU) coil, (g) Halo coil, and (h) Hesed coil (H-coil). [11] .

1.2.3 Action mechanism: what is being stimulated?

The TMS stimulator passes a short-lasting current through the coil, generating a strong time-varying electromagnetic field perpendicular to the transducing coil, which is placed tangentially on the head. The magnetic field is not attenuated by the tissue surrounding the brain (e.g., skin and bone) and induces a phasic electric field in the targeted tissue. This field can depolarize excitable structures within the brain, e.g. neurons. If the electric field induced by TMS is sufficiently strong to depolarize the membrane potential of a given neuron above a certain threshold, an action potential will be triggered. However, even TMS-induced subthreshold depolarization can have neuronal effects and affect ongoing endogenous activity. Despite its widespread use, relatively little is known about how TMS engages the cortical target region and how target engagement propagates within the brain. To simulate the neural response to TMS, axonal bends, branch points, and terminations are the most likely sites of action potential initiation by TMS [13]: the TMS-induced electric field directly interacts with axons in the targeted cortex. The direct neural response to the TMS-induced electric field is complex, involving a mixture of neuronal populations [14]. Despite increasing use in both research and clinical practice, there is still no consensus concerning which structures are the target of TMS. All that we know at present has been well encapsulated within the review by Siebner and colleagues (2022)[12], who pointed out that the primary target of TMS is axonal terminals in the crown top and lip regions of cortical gyri. The induced electric field can also excite loops of myelinated axons in the juxtacortical white matter below the crown of the gyri. Neuronal excitation spreads ortho- and antidromically along stimulated axons and causes secondary excitation of connected neuronal populations within local intracortical microcircuits in the target area. Axonal and trans-synaptic spread

of excitation also occurs along cortico-cortical and cortico-subcortical connections, impacting neuronal activity in the target network. Local and remote neural excitation depends critically on the functional state of the stimulated target area and network. While it seems quite clear which preferential target is the axon, it is still unclear which neurons are stimulated by TMS. Much evidence recognizes that TMS effectively excites inhibitory interneurons in the stimulated brain area. Cortical inhibitory interneurons release the neurotransmitter γ -aminobutyric acid (GABA) on both excitatory and inhibitory neurons. It is commonly believed that the inhibitory effects of TMS are mediated by inhibitory interneurons through a GABA-ergic suppression of the excitatory effects of TMS, although "shunting inhibition" caused by an activity-driven increase in transmembrane conductance has been suggested as an alternative mechanism. In paired-pulse TMS paradigms using short intervals between stimuli, neural excitation of the first conditioning pulse can acutely increase membrane conductance in the dendritic tree of cortical pyramidal cells. This "leaky" membrane conductance may reduce the trans-synaptic current flow from the dendritic tree to the soma induced by the second TMS pulse [15]. TMS may also directly activate the axons of inhibitory interneurons, which show considerable spatial dispersion and arborization in grey matter [16]. These studies, all carried out by stimulating Primary Motor Cortex (M1), have highlighted that an important factor determining the susceptibility of intracortical interneurons to TMS is the relative degree of axonal myelination: therefore, the myelinated axons of inhibitory interneurons are also targets for TMS. An alternative hypothesis postulates that TMS primarily induces neuronal excitation at the level of the myelinated axon of pyramidal neurons [17]. Thus, TMS-induced excitation of axon terminals projecting from pyramidal cells onto an inhibitory interneuron would elicit synchronously synaptic

inputs and raise the likelihood of action potential initiation at low stimulus intensities, as suggested by human TMS studies [18, 19] (Fig.2).

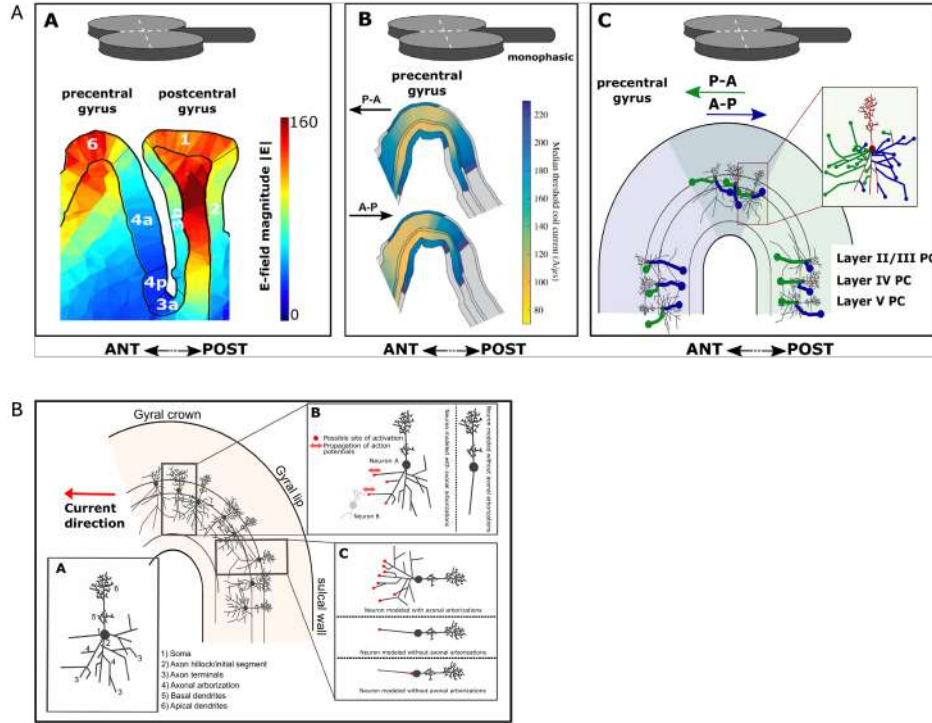


Figure 2. Cognitive fatigue neurophysiological parameters. Panel A: Inset A shows the electric field distribution; Inset B shows the activation thresholds by layer; Inset C shows the axon depolarization. Panel B: Inset A shows a drawing of a single pyramidal cell; Inset B shows Activation of pyramidal cell in the crown or lip region; Inset C shows Activation of a pyramidal cell in the lip region of the gyrus or in the sulcal wall [12] .

1.2.4 Principal physiological effects

As shown in the studies reported in the previous section, TMS is most often applied over the M1 as it can induce muscle twitches called MEPs, measured by electromyography (EMG) [20]. The MEP amplitude depends on both the stimulation intensity and the corticospinal integrity; it is commonly measured from the negative trough to the positive peak and referred to as "peak-to-peak amplitude." In terms of stimulation

mechanisms, studies have shown that the induced action potential causes descending volleys in the corticospinal tract, which can include D-waves (direct waves) followed by I-waves (indirect waves) [21]. TMS does typically not directly activate descending axons, which causes an early D-wave. The mechanism is rather based on an indirect activation of excitatory synapses of the pyramidal neurons in layer 5 of the motor cortex, producing I-waves. Depending on the directionality of the induced current, different MEP amplitudes can be produced. In general, the largest MEP is produced by a posterior-anterior direction producing an I1-wave as the first wave. A lateral-medial direction can produce a D-wave, and the anterior-posterior direction typically produces an I3-wave. The amplitude of the MEP is also dependent on whether the muscle is at rest or contracting. Higher MEPs are measured in contracting muscles due to the higher level of activity in the motor neurons [7]. TMS has multiple stimulation paradigms but is most applied in single, paired, or repetitive pulses [6]. These paradigms can be used to measure different aspects of cortical excitability, corticospinal tract integrity, or modulate cortical plasticity [7]. TMS has been used to quantify different parameters of cortical excitability over the M1, such as resting motor threshold (RMT), recruitment curves, MEP latency, short intracortical inhibition (SICI), and intracortical facilitation (ICF) [22]. RMT is the lowest threshold for producing an MEP during rest, which reflects cortical excitability [7]. The RMT can be determined with the relative frequency method by finding the minimum amount of intensity to induce an MEP of $\geq 50 \mu V$ in 5 out of 10 trials [22, 23]. RMT is also a measure of axonal excitability: N-methyl-D-aspartate (NMDA) antagonists have been shown to reduce RMT, while GABA-ergic drugs do not affect RMT [19]. The variance in RMT can be attributed to multiple factors such as the strength of the corticospinal projections, the

size of the cortical representation, and the difficulty of accessing the muscle with TMS; RMT is lower for the hand areas compared to lower limbs [24]. The recruitment curve is defined by the different MEP amplitudes depending on the increase of the stimulation intensity [7]. Recruitment curves can assess the excitability of neurons that are less excitable or more distant from the center of the TMS pulse [24]. The MEP latency is a measure of corticomotor conduction time and reflects the time between the TMS pulse and the peripheral MEP response. The latency is affected by distance; muscles closer to the stimulation site (facial muscles) have a shorter latency than muscles in the lower extremities (leg muscles) [7, 22]. MEP latencies are reflective of the integrity of white matter fibers depending on the fiber diameters or the myelin sheet thickness [22]. Paired-pulse stimulation paradigms have been used to measure cortical excitability and physiology. With the use of a mono-focal double pulse applied over the M1, it is possible to quantify neuroplastic changes in the inhibitory and excitatory motor networks and reflect interneuronal influences [19] by paired-pulse protocols such as SICI and ICF [25]. More specifically, a subthreshold conditioning stimulus (CS) is given, which is big enough to activate cortical neurons but too small to induce an MEP. This is followed by a suprathreshold test stimulus (TS) after a short interval. The CS influences the amplitude of the TS depending on the inter-stimulus interval (ISI) between the two pulses. A short interval between 1-6 ms has an inhibitory effect on MEP amplitude-SICI- while longer intervals between 8-30 ms facilitate the MEP amplitude-ICF- [7, 19]. SICI has been proposed to be mediated by GABAergic (activation of inhibitory circuits mediated by GABAa interneurons), and ICF by glutamatergic mechanisms [24]. The modulation of SICI has been relevantly related to motor learning as a measure of motor cortical plasticity [26]. Finally, using a single-pulse protocol, it is possible to identify the

cortical silent period (CSP) following the MEP, which corresponds to the interruption of voluntary muscle activity in a muscle of interest, through EMG [27], as it takes place after the excitatory response (i.e., the MEP). CSP has both cortical and spinal origins: the first half of its duration appears to be determined by various spinal mechanisms and the second half by the activation of GABA_B intracortical interneurons, which gives rise to a long-lasting inhibition [28]. The duration of the CSP gradually increases with the intensity of TMS. In addition to stimulation protocols involving a single or double pulse, the most commonly used protocols in clinical treatments involve repetitive stimulation: repetitive TMS (rTMS). These protocols, which like the others can be presented at different intensities, generate different effects depending on the discharge frequency of the pulses (e.g., inhibitory frequency $<1\text{Hz}$ or excitatory frequency $>5\text{Hz}$). These protocols generate long-lasting processes called long-term potentiation/depression of excitatory synaptic transmission (LTP/LTD): LTP is the potentiation of a synaptic connection when the presynaptic action potential comes before the post-synaptic potential; in contrast, LTD is the weakening of the synapse if the presynaptic action potential follows the post-synaptic one [29]. This evidence suggests that, in addition to changes in neural excitability, several other mechanisms may contribute to the lasting effects (long-term effects) of NiBS and probably reflect short-term activity-dependent forms of modulation of synaptic efficacy. When we talk about synaptic plasticity, we refer to that phenomenon of continuous remodeling of brain function in response to external stimuli, through short-term and long-term changes in internal connections, and which represents one of the main adaptive properties of the CNS [30] (Fig.3).

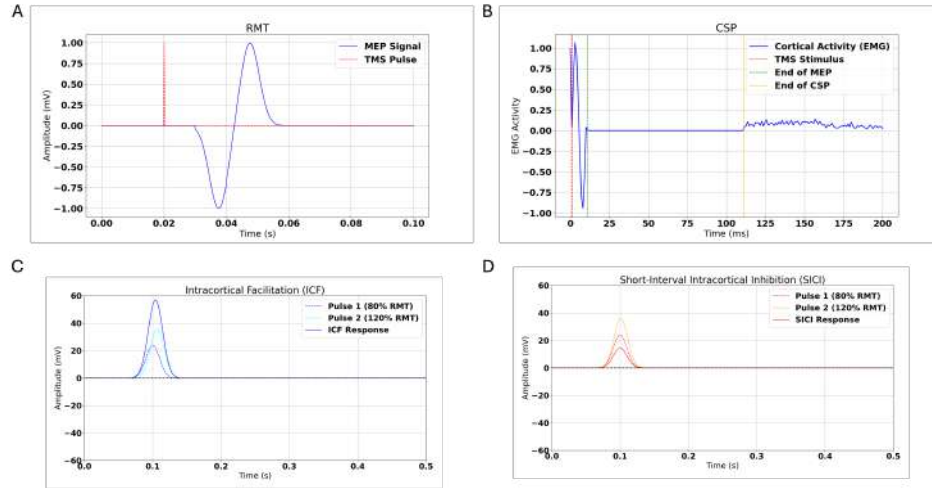


Figure 3. Summary of TMS techniques. Panel A: Resting motor threshold MEP; Panel B: Cortical silent period; Panel C: Intracortical facilitation; Panel D: Brief intracortical inhibition.

1.2.5 safety

The safety guidelines for the use of TMS in research and clinical protocols were encapsulated in the work of Rossi et al. [31]. The most significant risk of rTMS is that of inducing a seizure during the stimulation session, but this risk is quite low. Another concern is potential hearing loss due to the loud clicks of TMS, but this risk is mitigated with ear plugs. The most common side effect of TMS is headache and mild discomfort up to transient pain at the site of administration, which affects up to 40% of the patients, but is temporary and responds to analgesic medication. In addition, the safety profile can vary for populations. For example, although the results are encouraging for the pediatric population, more studies are needed [4].

1.3 Transcranial Electrical Stimulation (tES)

The first use of electrical stimulation as a technique to modulate cortical activity and excitability dates back to the discovery of electricity itself. However, in more modern years, Electroconvulsive Therapy (ECT) emerged in the 1930s [32]. It was used as a treatment for a patient in 1939 [33], but was unfortunately shown to induce seizures using strong electrical currents. With new electrical stimulation techniques, this side effect has been largely resolved by the application of a weak current. The three main tES techniques are: transcranial direct current stimulation (tDCS), transcranial alternating current stimulation (tACS) and transcranial random noise stimulation (tRNS) [34] (Fig.4).

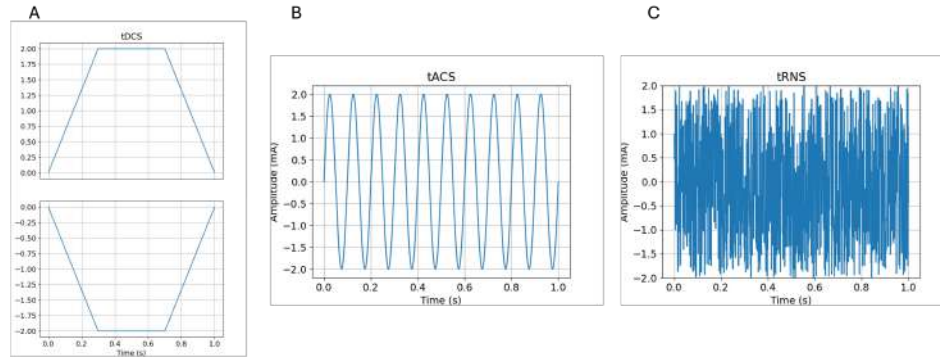


Figure 4. Summary of tES techniques. Panel A. top: anodal tDCS; bottom: cathodal tDCS. Panel B. tACS at 10Hz. Panel C. tRNS with frequency range between 0Hz and 640Hz

1.3.1 Transcranial Direct Current Stimulation (tDCS)

As early as 1804, physicist Giovanni Aldini applied galvanic (direct) currents to treat individuals suffering from melancholia. With the discovery of ECT in the 1930s, the use of galvanic current brain stimulation was abandoned. However, throughout the 1960s, it

reemerged with investigations focused on treating schizophrenia and depression, as well as altering mood and alertness. This reemergence was short-lived, as pharmacological advances in psychiatry placed other research in its shadow. Research on direct-current brain stimulation has been reduced again. In 1998, Priori and colleagues were among the first to apply controlled polarized direct current to the scalp, producing small changes in cortical excitability [35]. Two years after Priori's experiments, Michael Nitsche and Walter Paulus published their seminal paper that established much of the modern approach to tDCS [36].

Action mechanism: what is being stimulated?

tDCS applies a weak current (0-2 mA) through regions of the brain via two electrodes, an anode (+) and a cathode (-), which may shift regional cortical excitability to a state of excitation or inhibition. A subthreshold current passes from the anode to the cathode site, generating weak electric fields in surface neuronal populations, which in turn change neuronal excitability and spontaneous firing patterns [37]. These changes in neuronal properties are dependent on both polarity (direction of current) and intensity (amount of current) [38, 39]. Traditionally, anodal stimulation involves placing the anode on the scalp over the target location and the cathode at a distant inert location, such as the contralateral supraorbital area or shoulder. In contrast, cathodal stimulation applies the cathode to a target location, and the anode to an inert location. As stimulation begins, changes occur primarily in the membrane potential of pyramidal neurons in layers V/VI, leading to depolarization or hyperpolarization of neurons in the target region [40]. Anodal tDCS depolarizes target neurons, whereas cathodal tDCS hyperpolarizes those same neurons. The underlying principle for these effects comes

from neuronal-electric field interactions, suggesting that electric fields polarized from the dendrites toward the axon will depolarize the axon and soma, but hyperpolarize the dendritic tree; the axon and soma will be hyperpolarized if electric fields point from the axon to the dendrites [34]. tDCS-induced neuroplastic changes may be associated with modulation of neuronal ionic channels, specifically the voltage-gated calcium channel type L (L-VGCC) and the NMDA receptors [15].

Types of electrodes and montages

One experimental variable that strongly affects the behavioral or physiological impact of tDCS is the arrangement (fitting) and size of the electrodes used [34]. The application of new montages, which combine the old concept of bifocal stimulation with 5-7 cm sponge electrodes, has increased the efficacy of protocols and reduced side effects (e.g. burning, pain, discomfort, and itching). Recent work has expanded the range of mounting options, allowing for high-definition (HD) montages in addition to traditional anode-cathode pair setups; this enables more focused stimulation using smaller arrays (e.g. 1 cm) of high-definition tDCS electrodes (HD-tDCS) [41, 42]. An array of HD-tDCS electrodes, known as the 4 1 ring, is a more targeted stimulation method than conventional tDCS [42, 43]. HD-tDCS is a reliable method for targeting specific areas of the cortical region, it can produce plasticity changes that may outlast those induced by conventional tDCS [44], and has been shown to reduce perception of pain in patients with fibromyalgia [45, 46] and in experimental pain [47]. Although tES interventions typically focus on a single cortical target, it is widely recognized that many behavioral manifestations of neurological and psychiatric diseases are not only the result of abnormalities in an isolated brain region, but represent alterations in

brain networks [48]. In this context, when specifications for the location and type of stimulation effects are available, brain networks become the target of neuromodulatory interventions. Advances in neuroimaging such as positron emission tomography (PET), EEG, magnetoencephalography (MEG) and resting-state functional connectivity MRI (rs-fcMRI) allow us to visualize brain networks in humans with unprecedented clarity and noninvasively. In parallel, technologies allow the use of more than two electrodes for stimulation, facilitating multifocal stimulation of brain networks [49]. The efficacy of this approach has been demonstrated in the work of Mencarelli et al. (2020) [50], which tested this novel approach for multichannel network-targeted tDCS (net-tDCS). This method was optimized to increase the excitability of the sensorimotor network (SMN) while inducing cathodal inhibitory modulation in prefrontal and parietal brain regions negatively correlated with SMN, using a multichannel tES device compatible with magnetic resonance imaging. The results showed that net-tDCS induced a greater increase in functional connectivity (FC) over SMN compared to bifocal tDCS (control condition), during and after stimulation.

1.3.2 Transcranial Alternating Current Stimulation (tACS)

Theoretical background

Low-intensity AC methods utilize different electrode montages and current characteristics. The use of alternating currents with a similar montage as that used in tDCS is known as tACS [51]. tACS involves the direct delivery of alternating electric currents to the scalp; the current travels through the skull to affect primarily cortical neurons. This alternating current has a sinusoidal waveform, where the voltage gradually changes from positive to negative every half-cycle. Consequently, the current flows from an an-

odal electrode to a cathodal electrode in one half cycle and reverses direction in the second half-cycle [49]. The underlying concept of an alternating current is to simulate the natural rhythmic patterns of electrophysiological activity in the brain, which can be detected by EEG and MEG [52].

Action mechanism and physiological outcomes

Such rhythmic patterns, oscillating at a certain frequency, are referred to as brain oscillations. Various specific brain oscillations have been coupled with several brain functions and states [53]. Moreover, connectivity and communication between distant cortical regions have been shown to be associated with the synchronization of brain oscillations within these regions [54, 55]. In this context, tACS is also employed to couple or decouple two connected neuronal circuits by synchronizing or desynchronizing their oscillations, respectively. Accordingly, tACS may represent a potential therapeutic tool by modifying altered brain oscillations and connectivity patterns that have been identified in various psychiatric and neurological disorders [56]. The parameters of the alternating current can be customized in terms of frequency, amplitude, phase shape, phase timing, and the duration and number of stimulation sessions. The stimulation frequency is typically set to EEG frequencies to modulate the associated brain processes [56]. tACS has two main effects: online effects (those that coincide with the stimulation length) and offline or after-effects (those that extend beyond the stimulation period), even if we have no certain evidence regarding the latter; both involve entrainment of brain oscillations at the stimulation frequency. This method falls under modulation rather than stimulation techniques, meaning that, similar to tDCS, it causes the cell bodies and dendrites of cortical neurons to oscillate their membrane potentials toward

depolarization or hyperpolarization; it is not strong enough to alter the frequency of action potentials but controls their timing in a frequency- and location-specific manner [57]. This alternating variation in membrane potential is thought to be sufficient to change the probability that a neuron will generate action potentials [58]. The influence of tACS depends not only on amplitude and frequency but also on the three-dimensional orientation of both neurons and the penetrating current [40]. Its effects arise from manipulating the membrane potential of neurons that are aligned with the introduced electric field, primarily pyramidal cells in layer V. These cells are extremely sensitive to current changes due to their elongated soma-dendritic axis [40]. Moreover, they possess characteristic properties, including intrinsic resonance, neuroplastic activity, and cortico-cortical projections [59, 60, 61]. Consequently, once stimulated by tACS, these cells exhibit specific frequency resonance, long-term after-effects, and long-range oscillatory cortical connectivity (more details on resonance and after-effects are discussed below). In 2018, Liu and colleagues proposed five different neuronal mechanisms that could translate the previously mentioned cellular effects of tACS into whole network activity on a larger scale [62]. First, stochastic resonance: a wide range of tACS-affected neurons, differing in their momentary probability to generate action potentials, will respond stochastically to be either polarized or hyperpolarized, leading to the absence of a theoretical ‘minimum effective threshold’. Second, rhythm resonance occurs when the tACS frequency matches that of the endogenous oscillations, resulting in the stimulatory current wave striking the endogenous one at a similar phase every cycle. Third, temporal biasing of spikes: the spike timing of neurons is regulated by the interaction between the stimulation and internal currents, potentially working synergistically to excite the same group of neurons during each cycle of stimulation. Fourth, network

entrainment: the entrainment of an endogenous irregular activity necessitates an external current with sufficiently stronger amplitude. Finally, imposed pattern: to overcome endogenous regular oscillations and introduce a new oscillation, the strongest stimulation is required [62]. The authors attributed the online and offline tACS effects to two synergistic phenomena: entrainment and neuroplasticity, respectively. Entrainment, by definition, occurs when an external rhythmic system affects another naturally occurring one, forcing it to follow its oscillating frequency. During tACS, the external driving current compels the endogenous brain oscillations to synchronize in terms of frequency and phase [63]. For neuroplasticity to elicit offline effects, long-term plasticity should occur in one of two primary forms: LTP and LTD, both results of spike-timing-dependent plasticity (STDP). Offline effects of tACS have been shown to last for 70 minutes after one stimulation session lasting 20 minutes [64].

1.3.3 Transcranial Random Noise Stimulation (tRNS)

Theoretical background

tRNS is a non-invasive electrical stimulation of the brain in which a weak alternating current oscillating at random frequencies is delivered through the scalp using a pair of electrodes. The frequency band of tRNS can encompass a full range (typically 0.1 to 640 Hz) or can be delivered at low or high frequency (lf-tRNS or hf-tRNS; by convention, respectively, ranging from 0.1 to 100 Hz and 101 to 640 Hz). It is a relatively recent brain stimulation technique, but in recent years its popularity has increased dramatically. The modulatory effects of tRNS, which involve mainly the high frequency band, have been investigated with different motor, sensory, and cognitive tasks [2, 65].

Action mechanism: what is being stimulated?

tRNS involves the application of alternating currents at a mix of frequencies, between 0.1 and 100 Hz for "low" frequency stimulation, and between 101 Hz and 640 Hz for "high" frequency stimulation. The arbitrary division of "low" and "high" frequencies is based on one of the early tRNS papers [2], with 640 Hz being at the high end of physiologically measured human brain oscillations [66]. tRNS generates a random level of current for each sample of the signal. These random current levels are normally distributed and result in zero-mean Gaussian white noise. Therefore, tRNS is a biphasic stimulus and is not considered directional. The absence of a DC field means that tRNS might not induce a homeostatic effect since the electrical field is constantly changing [67]. However, it is possible to add directionality by incorporating a DC field (i.e., combining tRNS with tDCS), which allows the neuronal membrane response to adapt to the field over time and induce a homeostatic effect. One study found preliminary evidence that 10 minutes of offline tRNS involving a DC offset (anodal stimulation) is more likely to increase cortical excitability [68] compared with tRNS alone, although the apparent difference was not statistically significant. It is speculated that tRNS with a DC offset combines characteristics of direct current stimulation (polarizing the resting membrane potential) and random noise stimulation (potentially introducing noise into the system) to induce changes in cortical excitability [68]. A key characteristic of tRNS is that it is polarity independent, i.e., it is neither anodal nor cathodal [69]. In one study that demonstrated this polarity independence, the authors asked three groups of participants—those receiving tRNS, those receiving tRNS with reversed electrodes, and those receiving sham stimulation—to undertake a visual perceptual learning task during which stimulation was applied (online; intensity: 1.5 mA, 100–640 Hz) over the

occipital cortex, with the second electrode placed on the right upper arm [70]. Reversing the cable connections between the groups showed that tRNS improved learning performance irrespective of its polarity. The polarity independence and absence of a uniform electrical field direction mean that it is possible to use both electrodes to simultaneously stimulate different cortical areas. Polarity independence can be beneficial when targeting multiple nodes in specific brain networks. However, electrode placement should be considered carefully to avoid stimulation of unwanted sites. Whether tRNS enhances excitability under both electrodes equally is yet to be evaluated. For example, excitability changes under both electrodes could be tested by applying TMS over the M1 in each hemisphere to elicit MEPs, before and after stimulation with tRNS. Several independent modeling studies have demonstrated that, due to the resistive properties of the skull and tissue, tES applied at 2 mA peak-to-peak generates $<0.5\text{V/m}$ electric fields in the human brain. This is sufficient to generate 0.1–0.2mV changes in the membrane potential of stimulated cells [62]. This is too small to induce action potentials, but large enough to bias activity via alterations in the resting membrane potential. Importantly for tRNS, the human brain, skull, and soft tissue have ohmic properties, i.e., induced fields are independent of stimulation frequency [57]. This means that all applied frequencies can, in principle, reach the cortex. However, neuronal membranes might act as a low-pass filter [71]. Thus, even though all frequencies reach the brain, the induced change in membrane potential might be smaller than estimated. Moreover, the cut-off frequency is dependent on the type of neuron. For example, layer-4 stellate neurons have a higher cut-off frequency than layer-5 pyramidal neurons [72]. Therefore, neuronal elements could be affected differently depending on the stimulation frequency. In this context, computational modeling could be beneficial to generate

testable hypotheses concerning which neuronal populations respond to tRNS. One such model applied to tDCS found that including pyramidal cells and interneurons resulted in a more accurate simulation of the results of an in-vivo tDCS experiment [73]. tRNS is also less affected by cortical folding, thus reducing variability in outcomes due to anatomical differences between participants. Moreover, tRNS allows for the stimulation of a larger population of neurons (both excitatory and inhibitory). For example, with tDCS, tangential DC currents applied to a symmetrical dendritic arbor will cancel each other out at the axon hill, whereas with tRNS, the cell would be depolarized regardless of the orientation of the current flow [2].

Principal neurophysiological basis of both online and offline tRNS effects

When comparing the benefits of tRNS with other tES methods, it induces less discomfort, which is helpful for blinding [74], and could increase comfort when stimulating over long periods. Several studies have shown that tRNS has a greater neuromodulatory influence compared to other tES methods, reflected in greater neurophysiological and behavioral after-effects [75, 76, 77], making it a promising method to improve human behavior. Recently, it has been shown that tRNS (2 mA peak-to-baseline amplitude) can cause an acute online increase in cortical responsiveness, assessed with TMS, during its application over the M1 [78]. In that study, tRNS was applied in short bursts of 3 s, and cortical excitability was evaluated with single-pulse TMS between 1.3 and 1.7 s during the stimulation. There was a small but reliable decrease in RMT acutely. The observed effects were not likely due to neuroplastic changes since tRNS conditions were always interleaved with other active control conditions, thereby minimizing the influence of long-term excitability changes. Moreover, there was no systematic change

in excitability over time for trials without tRNS, making it unlikely that these results were affected by long-term neuroplasticity. The underlying neurophysiological mechanism of online excitability modulation with tES is unclear, but Liu and colleagues have proposed several hypotheses [62], including but not limited to, entrainment of network patterns and stochastic resonance; entrainment is unlikely to result from adding noise but might be achieved using other tES protocols like tACS. Stochastic resonance has been proposed by several authors as a potential working mechanism of tRNS [79]. Stochastic resonance is a phenomenon whereby small amounts of noise can be of benefit to the output of a system [80], i.e., where noisy electrical fluctuations boost neural signals and enhance detectability of weak events. Stochastic resonance occurs when an optimal level of noise is added to a weak signal. At a neuronal level stochastic resonance can occur when physiological (excitatory postsynaptic potentials (EPSP) and inhibitory postsynaptic potentials (IPSP)) and exogenous (tES) polarizing mechanisms are added. An EPSP is a depolarization of a postsynaptic cell following the influx of positively charged ions (such as Na^+), whereas an IPSP is a hyperpolarization due to an influx of negative ions (e.g. Cl^-) or efflux of positive ions (e.g. K^+). tRNS might cause an acute repetitive opening of sodium channels [81, 82, 83]. A small amount of noise can then bias the probability or timing of neuronal firing when a cell is close to threshold. At a behavioral level, the stochastic resonance hypothesis has been tested by presenting weak stimuli (visual, auditory, or tactile) to bring neurons close to firing threshold and then applying tRNS as a way of adding neural noise. An optimal amount of noise results in improved behavioral performance, whereas too little or too much noise results in no performance enhancement or even a reduction of performance. Of note is that much of the SR work has been conducted when noise is applied to the peripheral

nervous system (e.g., visual or auditory white noise). How peripheral and central noise (e.g., induced with tRNS) are treated equally at neural level remains unknown. Some tRNS studies have demonstrated an inverted U-relationship between tRNS intensity and behavioral performance [79], but some have not shown this relationship [84]. A potential idea is to test whether, and how, peripheral noise and central noise interact. Related to stochastic resonance is the hypothesis of temporal summation of neural activity. tRNS might lead to the membrane potential of stimulated neurons approaching their response threshold due to induction of direct temporal summation [2]. That is, since tRNS might repeatedly open sodium channels and cause a second sodium influx, resulting in further depolarization of the neuron; due to the polarity independence of tRNS this should occur at both electrodes. Such effects might be expected when the time it takes to change the membrane potential of a neuron is sufficiently long to permit the summation of two tRNS stimuli delivered in close succession. This temporal summation would interact with neurons that are activated by a task, which could result in the strengthening of activity-dependent processes and thus potentially behavioral improvements [70]. Comparative studies of the tRNS effects in humans and non-human animal models could shed light on the underlying mechanisms to optimize stimulation protocols. For example, using intracranial recordings it should be possible to develop a better understanding of the physiological impact of tRNS in vivo [85, 86]. Moreover, tDCS has been shown to induce after-effects on the glial and vascular systems; whether tRNS can influence these as well is unknown and requires further investigation [85, 86]. Prolonged tRNS stimulation can induce neurophysiological after-effects. tRNS offline effects refer to experiments in which participants are not engaged in a specific task during stimulation. The impact of tRNS on cortical excitability was first demonstrated by

Terney and colleagues [2]. They stimulated the left motor cortex with high-frequency tRNS (HF-tRNS, 10 min, 1 mA peak-to-peak) and tested cortical excitability using TMS delivered over the M1 to induce MEPs. They found increased MEP amplitudes (increased excitability) after tRNS when subjects were passively seated during stimulation, and this effect lasted 60 minutes post-stimulation [2]. In a separate study, Herpich and colleagues [87] found that HF-tRNS (20 min, 1 mA, offline) produced increased cortical excitability in the visual cortex, and this effect lasted 60 minutes post-stimulation, demonstrated by a lower TMS intensity needed to elicit phosphenes. Various factors can influence the tRNS effect on cortical excitability, including intensity and current density, stimulation duration, and frequency range. For example, a single study showed that 10 minutes of stimulation (0.1–640 Hz, offline) targeting the primary motor cortex with low-intensity tRNS (0.4 mA) reduced cortical excitability, while higher-intensity tRNS (1 mA) increased excitability. Stimulation intensities between 0.6 mA and 0.8 mA did not influence excitability [76]. The inhibitory effect did not emerge until 20 minutes after stimulation, whereas the increase in excitation with 1 mA occurred immediately after stimulation. The authors suggest that their results could stem from different sensitivities of excitatory and inhibitory synapses to different stimulation intensities, and that inhibitory mechanisms may have a delayed onset compared with excitatory protocols. It is therefore important to assess cortical changes at various time points to prevent missing any possible tRNS effects. These results show that stimulation intensity is critical in determining the tRNS effect on excitability. Similar intensity effects have been observed for other tES methods, such as tDCS which can have either facilitatory or inhibitory effects depending on stimulation intensity [38, 88]. It has been suggested that increasing stimulation intensity while keeping its duration constant might activate

counter-regulatory mechanisms to prevent excessive brain excitation [89]. Individual differences in brain anatomy could explain some of the variation in the tRNS effects intensity on excitability and behavior because the electrical field induced at a target location can vary greatly as a function of skull thickness and cerebrospinal fluid depth [90]. Current modeling could be used to determine the electrical field strength at the target location, for example, by combining an individual's MRI with toolboxes such as SimNIBS [91]. Direct physiological validation of these models is currently limited but some studies have shown that individual current modeling can be useful [79]. One caveat is that most software packages model electric fields for direct current stimulation, which produces different current strengths relative to random noise stimulation because the latter is weaker in its power. This is because tRNS applies a distribution of different intensities (applied with different frequencies) where the peak intensity occurs least often, in contrast to tDCS, which always applies a single intensity value. As it may not always be possible to create individual head models, it is important to report tRNS intensity and electrode size to determine current density. Electrical fields can influence subcutaneous nerves that signal to the brain and thus can indirectly affect brain circuits even when subjects are not aware of the stimulation [62]. Stimulation duration is another parameter that modulates after effects on cortical excitability. A minimum stimulation duration of 5 minutes (HF-tRNS, 1 mA) is considered necessary to induce after effects on corticospinal excitability in the motor system [92], while the minimum duration for after effects in sensory or other systems has yet to be examined. In relation to stimulation duration, several tDCS studies have demonstrated the influence of break duration on neuroplastic changes between repeated rounds of stimulation. For example, 5 minutes of anodal tDCS increases neuronal excitability for 3 minutes; however, if two

5-minute periods of tDCS are applied, separated by a 3-minute break, then the second session has an inhibitory effect [93]. Several other tDCS and TMS studies have also demonstrated that the effect of stimulation depends on stimulation history, formalized in a model of homeostatic plasticity [94, 95]. Understanding how repeated applications of tRNS are influenced by homeostatic plasticity is important to inform the timing of stimulation sessions when these are used to enhance performance spanning multiple training sessions. The range of frequencies applied is important in determining the after effects of stimulation. tRNS applied at 100–640 Hz (1.5 mA for 10 minutes) has been found to increase MEP amplitudes at 10 and 20 minutes after stimulation, though this change was not evident immediately after stimulation [65]. Other frequency bands (100–400 Hz; 400–640 Hz; sham) did not influence cortical excitability. These results suggest that a broad frequency spectrum is necessary to induce after effects. Despite these neurophysiological after effects, the exact physiological mechanism of tRNS offline effects is unknown. Pharmacological evidence (in vivo) has suggested that sodium (Na^+) channel blockers reduce tRNS-induced changes in cortical excitability [96]. This sodium dependence is consistent with evidence showing that adding noise to cell slices (in vitro) causes an acute repetitive opening of sodium channels [81, 82, 83] and can enhance action potential generation in mouse sensory neurons [97]. Continuous opening of sodium channels would lead to membrane depolarization, which could result in LTP-like changes. tDCS research suggests that LTP-like mechanisms occur after more than 3 minutes of stimulation [2, 96]. tRNS-induced plasticity is also sensitive to GABA_A receptors [96], and a recent study has suggested that GABA levels can be reduced after prolonged stimulation with tRNS in juvenile mice [98]. It is unknown if similar effects occur in the human brain, but this could plausibly be tested by measuring GABA levels

following tRNS with magnetic resonance spectroscopy (MRS) [99]. The effects of tRNS lasted for approximately 60 minutes after 10 minutes of stimulation; the physiological mechanisms underlying these effects are not well known, but it is suspected that they may be due to the repeated opening of sodium channels [100] or to the increased sensitivity of neuronal networks to modulation [101]. Studies with functional magnetic resonance imaging (fMRI) have shown a reduction in blood oxygen level dependence (BOLD) after the use of tRNS, relating the change in blood flow to the energy used by brain cells [92]. Finally, to underline the strong after effects of this technique, Snowball et al. [102] reported an improvement in calculation- and memory-recall-based arithmetic learning with tRNS that was associated with hemodynamic responses, suggesting efficient neurovascular coupling in the Dorsolateral Prefrontal Cortex (DLPFC). These changes were maintained up to 6 months after stimulation, implying that long-term neuromodulatory effects are observed [34].

1.3.4 tES safety

Low-intensity tES in humans, which includes tDCS, tACS, and tRNS or their combinations, appears to be safe. Until now, no serious adverse events (SAE) have been reported in more than 18,000 sessions administered to healthy subjects and neurological and psychiatric patients [58]. Moderate adverse events (AE), defined by the need for intervention, are rare and include skin burns with tDCS due to suboptimal electrode-skin contact. Very rarely have mania or hypomania been induced in patients with depression (11 documented cases), but a causal relationship is difficult to prove due to the low incidence rate and limited number of subjects in controlled studies. Mild AEs (MAEs) include headaches after stimulation, as well as tingling and burning

sensations occurring during tDCS at peak-to-basal intensities of 1-2 mA and during tACS at peak-to-peak intensities greater than 2 mA. AEs are frequently reported by individuals receiving placebo stimulation. The profile of AEs in terms of frequency, magnitude and type is comparable in healthy and clinical populations, and this is also the case for more vulnerable populations, such as children, elderly people, or pregnant women. Combined interventions (e.g., coapplication of drugs, electrophysiological measurements, neuroimaging) were not associated with further safety issues. Safety is established for low-intensity ‘conventional’ tES defined as <4 mA, up to 60 min of duration per day. Animal studies and modeling evidence indicate that brain injury could occur at predicted current densities in the brain of 6.3–13 A/m² that are over an order of magnitude higher than those produced by tDCS in humans. Using AC stimulation, fewer AEs were reported compared to DC [58].

2 Cognitive fatigue

2.1 Theoretical background

"Fatigue is the best pillow." This is how Benjamin Franklin defined fatigue in 1726, giving a positive connotation to what has been considered a highly debated condition across many professional fields and disciplines. Today, despite the lack of a clear understanding of what fatigue is, and especially without precise knowledge of its causes, we can accept the definition provided by the scientific community which describes it as "a decrease in the ability and efficiency of mental and/or physical activities caused by excessive mental and/or physical exertion" [103]. Why do people experience fatigue? It is possible to summarize the meaning of fatigue as a normal and important response to either physical or mental activity, emotional stress, boredom, or lack of sleep. At the end of the 19th century, an influential book entitled "Fatigue", written by the Italian physiologist Angelo Mosso [104], Professor of Physiology at the University of Turin, was one of the first concerning the biological basis of fatigue during exercise. Although his interest was focused on exercise-induced fatigue, the author introduced two concepts that can be generalized to other fatigue manifestations: 1) fatigue can be defined as a condition which has both a central and peripheral origin, the last considered as a chemical force transformed into mechanical work 2) to quote his own words: "At first

sight it may seem to be an imperfection of our body, but it is in fact one of its most marvelous perfections. Fatigue, which increases faster than the amount of work done, saves us from the damage that less sensitivity would cause to the organism.” Based on this explanation, fatigue is a necessary condition for human beings to be able to rest after a specific amount of effort. According to this principle, should we define fatigue as a negative or positive element in daily life? Since that the focus of modern society is on performance, regardless of the problems that may result, fatigue is considered as both an enemy and a limitation to the goal achievement. This condition is nothing more than the result of an accurate reading of the ”energy reserves” available; therefore, it is a protective mechanism for the person himself [105]. Reducing fatigue to maintain high levels of performance is the scientific goal of many researchers. The question then arises: what are the consequences of reducing fatigue to maintain a high performance level? Unfortunately, no longitudinal studies have been carried out to observe this, so it is difficult to find an answer. The matter becomes more complicated when we are faced with clinical conditions, in which fatigue is reported as a symptom (e.g. multiple sclerosis (MS)), having a negative effect on the person’s quality life. The sensation of fatigue is often characterized by pronounced discomfort, need for rest, and loss of motivation [103, 106]. It is primarily a subjective experience, although it is widely recognized as a symptom of disease or a side effect of therapies [107]. Being able to define fatigue is a challenge itself because of the multiple, often conflicting, definitions that can be found in the literature; moreover, even more difficult is the definition of cognitive fatigue, as most of the literature has focused on describing muscle fatigue. Fatigue can be divided into physical, psychosocial and cognitive types [108]. While psychosocial fatigue is purely subjective in its measurement, physical and cognitive fatigue can be assessed

both subjectively (using questionnaires and rating scales) and objectively (regarding the latter, sometimes its definition overlaps with fatigability definition, which we will see later). Subjective cognitive fatigue refers specifically to a persistent perceived feeling of exhaustion, and includes trait and state components. Trait fatigue represents the overall state of a patient that gradually changes over time, whereas state fatigue represents the fluctuation in subjectively perceived fatigue levels over time [109]. Trait fatigue can be assessed using self-report questionnaires, whereas state fatigue can be assessed using visual analog scales (VAS) or numerical rating scales. Physical fatigue manifests as a decrease in muscle strength during exertion or a decrease in a person's ability to generate force. The underlying mechanisms vary and may include the accumulation of metabolites in muscle fibers or errors in motor signal processing by the motor cortex [110], which may result in reduced EMG signals [111]. Cognitive fatigue, on the other hand, is characterized by a sense of tiredness or exhaustion accompanied by disengagement from the current task, resulting in impaired cognitive and behavioral performance, not related to sleepiness [112]. Ongoing research continues to explore the underlying causes of cognitive fatigue. It is important to distinguish fatigue as a research focus from "fatigability," which refers to the measurable decline in performance during physical or cognitive tasks relative to a baseline [113]. Fatigability is state-dependent and can be assessed objectively using behavioral or electrophysiological parameters [114]. In summary, while subjective fatigue is a qualitative experience, objective fatigability allows for quantitative assessment, highlighting the multidimensional nature of this phenomenon [106]. Instead, what is defined as peripheral fatigue concerns reduced neuromuscular junction effectiveness and processes beyond neuromuscular function such as metabolic and biochemical changes within the muscle [115]. Finally, the etiological

component of fatigue related to the central nervous system (CNS), which defines central fatigue (CF) [116], has been described as a decrease in voluntary muscle activation, which is directly related to decreased frequency and synchronization of motor neurons and reduced impulses from the motor cortex[117, 118] (Fig.5). Following this digression on the fatigue classification, what I'm going to present in the thesis will be related to cognitive fatigue in healthy subjects, and CF in MS disease.

	Description	Reference
Physical fatigue	<i>"decrease in muscle strength during exertion or a decrease in a person's ability to generate force. The underlying mechanisms vary and may include the accumulation of metabolites in muscle fibers or errors in motor signal processing by the motor cortex"</i>	Hoda M. Abd-Elfattah, et al., 2015
Cognitive fatigue	<i>"sense of tiredness or exhaustion accompanied by disengagement from the current task, resulting in impaired cognitive and behavioral performance, not related to sleepiness"</i>	Peng Qi et al., 2019
Central fatigue	<i>"progressive exercise-induced reduction in voluntary activation of a muscle"</i>	Gandevia, 2001
Peripheral fatigue	<i>"reduced neuromuscular junction effectiveness and processes beyond neuromuscular function such as metabolic and biochemical changes within the muscle"</i>	Korzeniewski B., 2019
Fatigability	<i>"measurable decline in performance during physical or cognitive tasks relative to a baseline"</i>	Kluger B.M. et al., 2013

Figure 5. Fatigue Classification. The following figure shows the most important types of fatigue

2.2 Cognitive fatigue assessment scales

To evaluate the presence and state of fatigue, the scales and techniques currently in use are divided into subjective and objective methods. Among subjective scales, a further subdivision distinguishes between trait fatigue (a characteristic of the individual) and state fatigue (determined by the situation). The objective assessment of fatigue (fatigability) is conducted either through task performance (behavioral measures) or electrophysiological methods, such as evaluating the amplitude and latency of the P300 [119]. As highlighted by the studies of Karim et al. (2024) [120] and Jesús Díaz-García et al. (2021) [121], the scales most commonly used for subjective fatigue evaluation are divided into those addressing fatigue as a trait (a characteristic of the individual) or as a state (determined by the situation). Subjective fatigue questionnaires assess the level of fatigue using Likert scales with score ranges from 1 to 7 or 1 to 10. The VAS-F it's a scale which consists of 18 items relating to the subjective experience of fatigue, in terms of energy levels. Each item asks respondents to place an "X," representing how they currently feel, along a visual analogue line that extends between two extremes (e.g., from "not at all tired" to "extremely tired") [122]). The aforementioned questionnaires pertain to the evaluation of fatigue state and are utilized for both healthy individuals and patients who clinically exhibit fatigue as a symptom. Moreover, it is crucial to investigate sleepiness levels using specific scales, such as the Karolinska Sleepiness Scales [123], as well as the degree of experienced mental workload, for example, with the NASA Task Load Index [124]. This investigates not only perception of workload but also motivation to perform the task, boredom and self-awareness. In this section I'm not going to delve into the details of scales for assessing fatigue as a trait of the

individual (e.g., Fatigue Severity Scale (FSS), Modified Fatigue Impact Scale (mFIS)) [119], as these are more commonly associated with clinical conditions, such as MS, rather than with healthy individuals.

2.3 Neurophysiological and neuroimaging parameters of cognitive fatigue

When we refer to cognitive fatigue, we are referring to a condition that is closely linked not only to a reduction in cognitive capacity such as processing speed, reaction time (RT), sustained attention following an excessive cognitive workload [125, 126], but mostly to work and driving accidents. To understand the cognitive fatigue condition, it is important to outline another concept: mental workload; this refers to an essential component in tasks that require dynamic decision-making as well as other real-time, high-risk, situations. Mental workload can be defined as the amount of mental activity required to complete any task by working memory. The latter is essential to process information for short periods of time; for example, it is required for tasks such as arithmetic operations, reading, and learning [127]. The workload generated while performing any task is determined by the rate at which the information processing required to complete such a task occurs. From literature, we know that there are different kinds of mental workload, and this is best explained in The Cognitive Load Theory (CLT), which was developed in the 1980s and expanded significantly in the 1990s. It is a significant theory that offers a framework for research into cognitive processes [128]. As we know, the learning process of new information or skills depends on numerous variables, but the degree to which pertinent elements interact is crucial. This is called

element interactivity and varies along a continuum from low to high: low-element interactive content can be understood and learned independently without considering any other element. While the components of high-element interactivity can be taught separately, understanding them requires simultaneous processing of all the components and their interconnections. As a result, complex interactive material is challenging to comprehend [128]. One type of cognitive load, intrinsic cognitive load, is caused by element interactivity because the demands on working memory are inherent to the subject matter being learned. Along with element interactivity, how information is given to learners and the learning tasks they must complete can also place a cognitive strain on them. When the cognitive load is considered unnecessary and obstructive, it is called extraneous or ineffective cognitive load [128]. These two kinds of cognitive load are cumulative, so extraneous cognitive load is most significant when intrinsic cognitive load is high. In the opposite case, the total cognitive load may not exceed working memory capacity, so levels of extraneous cognitive load may be less relevant. Because of this, experimental designs that aim to lower cognitive load work best when element interactivity is high [128]. Returning to the evaluation of mental workload, it is relevant to outline the importance that the workload level remains optimal, because an imbalance can cause two different but equally wrong responses: - High workload: can lead to unplanned and unbalanced perils. - Low workload: can lead to disengagement from the assignment. An imbalance of mental workload can lead to a state of cognitive fatigue, so the two concepts are strictly related, as the state of fatigue depends on the entity of mental workload, which instead depends on the duration and the complexity of the performing task [127]. Indeed, when intellectually demanding activities are performed for an extended period, mental workload rises, and fatigue is likely to result [129]. It

is also assumed that long-time work will lead to a growth in cognitive fatigue, which will influence the efficiency of the worker involved and the well-being of the production system. As a result, accurate mental workload assessments will not only help employees work more efficiently but will also promote safety in the workplace, lowering accidents and increasing productivity [130].

2.3.1 Neuroimaging results

Thus far neuroimaging studies shed new light on cognitive fatigue and its brain underpinnings. Most of the functional imaging studies regarding cognitive fatigue, underlying fatigue network, suggested that the main regions involved in the development of cognitive fatigue are the basal ganglia (BG) and frontal lobes [131]. So, it is likely that the mechanism underlying fatigue may involve abnormal function of the motor system, as well as areas of the dopaminergic system in the BG, which are associated with motivation and reward [131]. Nakagawa and colleagues [131] studied the association between fatigue and brain structures in healthy individuals, particularly young subjects, finding coherent results with the theory that fatigue can result from a failure to integrate limbic inputs and motor functions in the BG, which consequently affects the striatal–thalamic–frontal cortical system. One important work by Wylie and colleagues (2020) defined the "fatigue brain network". Considered the Striatum, the DLPFC, the dorsal Anterior Cingulate Cortex (dACC), the ventromedial Prefrontal Cortex (vmPFC) and the anterior Insula, as seed regions, showed a FC reduction between DLPFC, caudate nucleus, insula/putamen, and the supplementary motor area (SMA), and an increased FC between dACC and more posterior regions, such as the inferior parietal lobule (bilaterally) and the angular gyrus, along with increased cogni-

tive fatigue [132]. The DLPFC is a critical area of the brain for executive functions, such as working memory, action planning, information integration, decision-making, and regulation of inhibitory control, which is necessary for the self-control of behavior [133, 134]. The vmPFC projects to the ventral striatum and it seems to be engaged in processing affective information and subjective goal value [134]. The striatum represents the main BG component, and it is divided into a ventral and a dorsal component: the ventral striatum, represented by the nucleus accumbens (NAcc) and the olfactory tubercle, mediates rewarding experiences and motivation. During decision-making, it has consistently been shown that the ventral striatum determines the potential value of rewards in relation to costs [135]. The NAcc is praised the most for its involvement in incentive-based learning, evaluation, and reward processing [136]. The dorsal striatum, represented by the NAcc and the putamen, plays a crucial role both in habitual behavior regulation and in the selection and initial aspects of decision-making [136]. A main role in the cognitive fatigue network seems to be taken by the anterior cingulate cortex (ACC), a component of the limbic system [137]. Indeed, research has disclosed how the ACC has a fundamental role in connecting actions to their consequences. Therefore, the ACC might not merely convey whether an activity is likely to result in an error and what effect is anticipated from it. It might also be a key component of a system for encoding whether a particular activity is beneficial, in light of the value of the envisioned outcome and the expense of carrying out the action [138]. Thereby, proving its importance in processes like error and conflict monitoring, outcome prediction, and effort management. Also, the ACC seems to work in conjunction with the ventral striatum to mediate cost-benefit decisions and persistence in accomplishing a goal [138], consistent with various studies which have brought to light how cognitive fatigue may

be related to imbalance between effort and reward. Moreover, cognitive fatigue seems to correlate with the activation of different areas depending on the kind of task. For instance, it's been seen how cognitive fatigue in a face encoding task increases activity in the bilateral thalamus (TH) and caudate, whereas in a word encoding task, it increases activity in the right TH, posterior cingulate cortex (PCC), and middle frontal gyrus (MFG) [139]. To better understand the pathophysiology underlying cognitive fatigue, we must discuss about the dual regulation system. Several studies have revealed that a main role in the regulation of motor output from the M1 is played by the balance between the two components, of the dual regulation system, the "facilitation system" and the "inhibition system". The "facilitation system" is activated by mental workload and motivation, and maintains cognitive task performance in the presence of cognitive fatigue. It is composed by a neural circuit that interconnects the limbic system, BG, TH, orbitofrontal cortex (OFC), DLPFC, (ACC), premotor area (PMA), SMA, and M1. It seems that cognitive fatigue may lead to mental function decline, right through the impairments of these brain regions, particularly enhancing the response inhibition components of executive function [140]. According to Tanaka and Watanabe (2012), [141] a motivational input, mainly through an amplified dopaminergic drive, increases motor output to the peripheral system by raising activity in the SMA and then M1 through the circuit explained above; doing so, compensates for the effects of cognitive fatigue. On the other hand, over-activation of the facilitation system, such as activation of the system repeatedly or for an extended period, can lead to system dysfunction and might result in chronic or cumulative cognitive fatigue [140]. The inhibition system, formed by the Insular Cortex (IC) and PCC, hinders performance on cognitive tasks. Although both high- and low-demand mental task trials lead to cognitive fa-

tigue, it has consistently been found that the high-demand task trials lead to a lower decline in cognitive performance than the low-demand task trials. The reason behind this can be found in the activation of the mental inhibition system during low-demand mental tasks, in addition to the activation of the mental facilitation system during high-demand, fatigue-producing mental tasks [140]. Likely, the neurological mechanisms of cognitive fatigue are intimately related to the system of mental inhibition, due to the IC and PCC involvement in the fatigue network [140]. However, instead of directly suppressing task performance, the IC and/or PCC may inhibit task performance through other brain regions such as the frontal area: the DLPFC known for its role in executive functions, receives input from both the facilitation and inhibition systems [16]. Furthermore, has been shown that cognitive fatigue not only affects cognitive performance but can also suppress physical task performance during exercise trials, due to higher perceived exertion [110, 142, 143]. These findings suggest that the mental inhibition system and the physical inhibition system share neural substrates [140] (Fig.6).

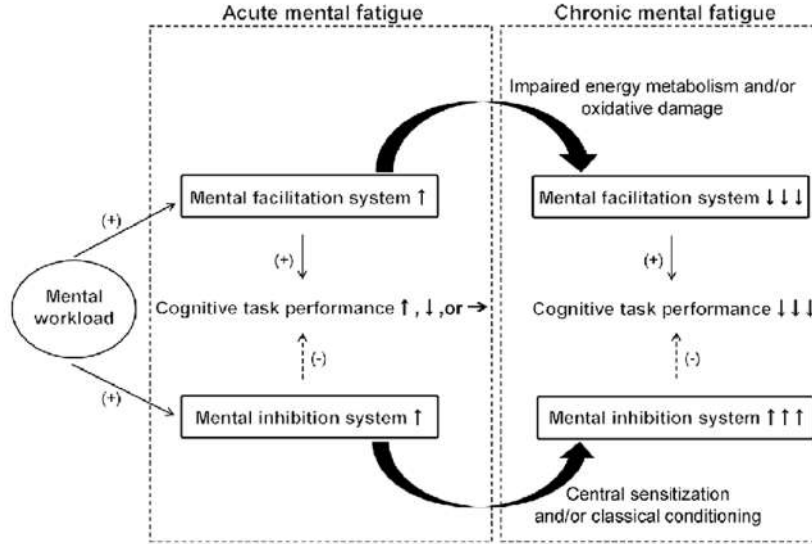


Figure 6. Dual regulation system. Acute mental workload activates the mental facilitation and inhibition systems to cause acute mental fatigue. The activated mental facilitation system maintains or improves cognitive task performance, whereas the activated mental inhibition system impairs cognitive task performance. Cognitive task performance is regulated by these two systems of the dual regulation system. Repeated and prolonged mental workload causes dysfunction of the facilitation system due to impaired energy metabolism or oxidative damage and overactivation of the inhibition system through central sensitization or classic conditioning. These alterations in the dual regulation system result in severely reduced cognitive task performance, i.e., chronic mental fatigue. [140] .

Why these changes occur is not yet entirely clear, however, it is interesting to see how the major nodes of the fatigue network are themselves part of other networks essential for behavioral control, such as: - the salience network (SN), which includes the anterior insula; - the reward network (RN), which includes the striatum and vmPFC; - the cognitive control network (CCN), which includes the DLPFC and anterior insula [132]. As described above, both motivation and reward are core aspects of human life. The brain regions that control the physiological and cognitive processing of reward are collectively referred to as the mesolimbic system or the reward system. The synchronized release of many neurotransmitters is necessary for reward. However, dopamine

is the most relevant neurotransmitter being involved in motivational processes control and reward-seeking behavior [136]. Reward processing is complex and made up of two temporally separate phases: reward-anticipation and reward-outcome. The first one is related to a motivational process, reward-outcome, instead, is characterized by the pleasure of achieving rewards. Both stages have been associated with a fronto-striatal reward circuit involving the BG and cortical target regions (e.g., the OFC, the medial PFC, and ACC). Specifically, the impairment of the "non-motor functions of basal ganglia" seems to be very relevant in cognitive fatigue, leading to effort-reward imbalance [144]. While traditionally associated with controlling and regulating motor functions, the basal ganglia are now widely recognized for their significant role in processes such as learning, motivation, addiction, and reward-guided behavior, as supported by extensive current research [144]. So, the involment in high-order activities, has been explained by the presence of widespread BG's projections to functionally distinct areas of the PFC and TH. For instance, the putamen and dorsal CN communicate with the DLPFC in addition to receiving projections from the motor and PMC. The ACC instead, sends projections to the striatum [144]. The fatigue onset is largely dependent on the loss of motivational effect from striatal-thalamic impulses to the frontal lobe. The evaluation of potential advantages and energy expenditures may be key to cognitive fatigue [131]. When tasks must be completed for an extended period, motivation wanes. For instance, it is known that after a long drive we feel as we have exhausted many of our cognitive resources, starting to feel the sensation of fatigue and find it challenging to carry on. This feeling is often associated with a perception that the effort required to continue driving exceeds our capabilities. So, when we reach this fatigued state, it is common to slow down and to be more cautious than before [145]. Since that the brain

is also able to detect changes in the payoff matrix, it generates fatigue to signal itself that the effort necessary to complete the task no longer justifies the rewards. Instead, if a reward is outlined for the task at hand that is inducing fatigue, research has shown how the individual's behavior and performance changes, continuing the task despite the feeling of fatigue [140]. Boksem and colleagues (2006) designed a study where subjects had to sustain an attention task for several hours, resulting in an increase in RT as an expression of fatigue, suggesting that participants became fatigued as the task went on. At the end of the first task, subjects were asked to continue performing for the possibility that they might be rewarded financially if they outperformed the other participants, thus introducing a motivation and a reward. Once they were given a new goal, participants kept performing willingly despite the fatigue. These findings imply that the cortico-striatal network, which has been demonstrated to be activated by effort and reward components of activity, can influence cognitive fatigue [146]. All that is consistent with the concept mentioned above of an overlap between fatigue and reward networks. Studies showed a critical role of the right fronto-parietal network in mediating time-on-task effects (TOT), and that these effects endure after a period of heavy cognitive workload [147]. Studies divide this network into two different areas: "bottom-up": where the input is provided by the inferior parietal cortex, temporal-parietal junction and the TH. In this area the attention is aimed to detect signals that are intrinsically alerting. "top-down": includes DLPFC and ACC that perform functions under conscious control, like focusing attention towards specific signal features, inhibiting unwanted distractions, or modulating attention via motivation [147].

2.3.2 Electrophysiological results

In addition to neuroimaging findings related to cognitive fatigue, electrophysiological markers, such as those obtained from EEG, contribute to more accurate profiling and detection of fatigue. The efficacy of the EEG-based approach for identifying fatigue has been validated by research that suggests the EEG signal may be a very predictive and accurate measure of alertness [148]. An individual's alertness levels are likely to decline if an activity is performed continuously and repeatedly, for instance in a long-distance drive, because generates an increase in cognitive load. This increase can be shown by patterns in the EEG parameters that appear coherent with a progressive rise in subject fatigue levels. In particular, it has been discovered that the alpha band is the most crucial element, in the EEG spectrum, for determining attention level; since the onset of fatigue, it has been observed that the distribution, amplitude, and frequency of alpha waves vary. A decline in alertness, is reflected by the EEG pattern as a gradual increase in alpha energy, more specifically lower alpha bands (8 -10 Hz). Instead, a drop in the alpha band can be associated with a rise in alertness, often correlated to a conscious effort [129]. In literature, the presence of particular EEG graph elements, which are alpha spindles, and defined as a short burst in the alpha band, seems to indicate a sensation of drowsiness and fatigue, detected for instance during a monotonous driving task. Spindles are also coherent with driving error events. Alpha bursts have a frequency between the higher portion of the theta band and the lower portion of alpha bands, so around 7 – 9 Hz. The topographical distribution, seems to be predominant in the central and parietal areas [149]. Other parameters that have been noted to be correlated with a decrease in vigilance are an increase of theta band, mainly in frontal and central areas, linked with declines in cognitive task

performance, a decrease of the beta band (14-30 Hz), especially in posterior regions [129]. Along with all these changes in EEG power, there is a decline in performance efficiency and an increase in response time [129, 150, 151]. Finally, increased mental effort has been observed related to a theta synchronization in the frontal regions and lower alpha desynchronization in the parietal and occipital regions [152]. Thus, EEG is shown to be a technique that can assess mental effort and a state of fatigue in healthy subjects undergoing a control task. An increase in theta power in frontal areas after a demanding task is one of the clearest features related to mental fatigue after cognitive exertion. The work of Wascher and colleagues (2014) shows precisely how, following the performance of a 4-hour task, the power extracted for theta (6-7 Hz) and alpha (9-10 Hz) activity trended differently as time elapsed increased. In fact, alpha peaked after 1 hour from the start of the task, while the increase in theta was constant throughout the entire 4 hours. This shows how much more sensitive theta is to cognitive effort [153]. Both theta and alpha change not only following long demanding tasks, but also following a simultaneous paradigm involving 3/4 tasks. In fact, according to the work of Puma and colleagues (2018), an increase in the power of the EEG alpha (8-12Hz) and theta (4-8Hz) bands reflects an increase in the involvement of cognitive resources for the completion of one to three subtasks in a multitasking environment [154]. These values peaked when performance declined. Consistent differences in alpha and theta band power levels were associated with task performance levels: higher performance was correlated with lower band power. These findings are confirmed by the review of Tran and colleagues (2020) who show that an overall increase in EEG activity following mental fatigue; in particular, large increases in theta bands, in the frontal, middle and posterior sites, and alpha bands, in the middle and posterior sites [155]. As reported

above, not only did theta and alpha show a relationship with cognitive fatigue, but beta was also seen to be correlated with cognitive effort and cognitive fatigue, especially in posterior areas. Indeed, the work of Xavier and colleagues (2020) shows, in a group of clinicians who worked for 33 hours straight, that there was a Correlation with Stroop Test results indicating that those who have higher alpha (8-13Hz), beta (13-20Hz), and lower relative theta (4-8Hz) powers at the frontal region at post-call rest have higher percentage of correct congruent trials [156]. As reported above, not only did theta and alpha show a relationship with cognitive fatigue, but beta was also seen to be correlated with cognitive effort and cognitive fatigue, especially in posterior areas. The beta waves (18-30Hz) have the greatest amplitudes in the frontal and central zones [157] and are considerably associated with decision-making ability or completing mathematical tasks[158]. When humans have their eyes open and are listening and thinking during analytical problem-solving, judgment, decision-making, and processing information about their environment, these waves are created[159]. Beta waves increase the body's energy level, and brain work, allowing for greater information processing and integration. As shown above, the concepts of reward, cognitive effort and motivation are closely linked to each other. This revealed dissociable neural signatures of reward and effort, with BG beta(12-20Hz) oscillations tracking effort on a single-trial basis and PFC theta (4-7Hz) signaling previous trial reward, with no effects of net subjective value [160].

2.3.3 TMS results

A final aspect not too much debated in the literature is to observe whether cognitive fatigue in healthy subjects has any effect on cortical excitability as assessed by

TMS. The effect of strenuous and fatiguing physical activity on cortical excitability has been assessed by TMS, showing a shift in the balance of excitation and inhibition, with a long-lasting reduction in corticomotor excitability and a concomitant increase in SICI targeting M1 following fatigue exercise [161]; TMS studies have also demonstrated a reduction in the amplitude of MEP in homologous muscle after unimanual fatigue exercise [162]. However, although the negative effect of cognitive fatigue on physical performance is known, it has not yet been tested whether fatigue induced by demanding cognitive tasks correlates with changes in cortical excitability indices. The only available study on this topic is by Amanda J. Morris and Anita D. Christie (2020), where they investigated the effect of cognitive fatigue tasks in young and elderly women and saw that the maximum force output decreased significantly after the cognitive fatigue task ($p = 0.02$) and this was not different between the age groups ($p = 0.32$). The length of the CSP increased significantly in the young and old groups ($p = 0.06$), suggesting that cognitive fatigue may cause an increase in cortical inhibition; this, being associated with increased inhibition could explain the difficulty of maintaining high levels of attention focus and energy recruitment [163]. The effect of cognitive fatigue on cortical excitability will undoubtedly be the subject of future studies, to enhance the efficacy of treatments, including proposing the combination of multiple methods. Most importantly, it will help us to delineate even better why and how cognitive fatigue and exercise interact. Cognitive fatigue is also associated with an increase in parasympathetic nervous system activation, indexed by an elevation in the root mean square of the subsequent RR interval differences (RMSSD) [164, 165], a time-domain parameter of Heart Rate Variability (HRV) [166], following the disengagement of cognitive tasks. Controlled by the autonomic nervous system, HRV reflects the body's ability to

self-regulate, particularly during demanding cognitive tasks. Increases in HRV during these tasks have been associated with increased self-regulation effort, while studies have found strong correlations between cognitive fatigue and increased heart rate. However, as we will see in the discussion part of Chapter 4, although the HRV parameter has been observed to be related to cognitive fatigue, there is no clear connection between them [151] (Fig.7).

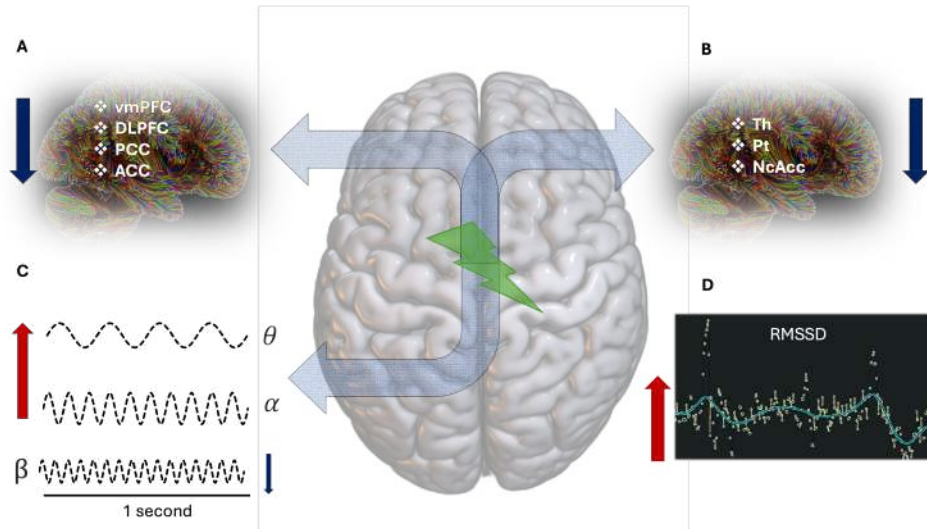


Figure 7. Cognitive fatigue neurophysiological parameters. Panels A and B report the reduction in FC reported in a state of cognitive fatigue. Panel C reports EEG changes associated with a state of fatigue. Panel D reports cardiac changes associated with a state of fatigue.

2.4 Astronauts fatigue induced by spaceflight

Among the great challenges of recent decades, space exploration is one of the most fascinating. Investing in this field has enabled many changes not only from an engineering point of view, concerning the equipment and operation of space shuttles but also in terms of safety for the astronauts themselves. When we talk about space exploration,

however, we also need to point out the negative effects of missions that not only negatively affect performance within the spacecraft but also continue once they return to Earth. Solving astronauts quality of life issues during and after a long journey (6 or more months) is the challenge that many professionals are facing. All difficulties faced by astronauts can be related to sleep disorders [167, 168, 169], motion sickness [170], and feeling of fatigue accompanied by another condition termed brain fog [171]. These states are closely related to several factors such as the absence of gravity, environmental isolation, the radiation they are exposed to [172], and finally the many activities they constantly have to perform increasing levels of alertness and stress. What follows from all these conditions is predominantly an increase in perceived fatigue, which finds many similarities with many clinical conditions such as post-COVID-19, chronic fatigue syndrome, and MS. As previously described, environmental stressors most often result in cognitive deficits related to short-term memory, learning, spatial orientation, motor function, emotion recognition, risk decision-making, vigilance, RT, processing speed, circadian regulation, and cognitive fatigue [173]. Fatigue represents a significant risk factor that has the potential to impact the health and performance of astronauts during spaceflight missions. This risk may be influenced by several factors, including exposure to artificial and transmitted light, individual vulnerability to sleep loss and circadian dynamics, and work/sleep schedules [174]. Perceived fatigue exhibits numerous parallels with various clinical conditions observed on Earth. This may result from repeated exposure to cosmic rays and thus be similar to the side effects of radiation therapy for patients with cancer, underscoring the deleterious effects of ionizing radiation on the CNS [175]. Another element running parallels to the fatigue condition is the presence of depression, due to both environmental isolation within the spacecraft

and sleep disturbances [176]. Some veteran astronauts [177] have suggested that periods of high intensity workloads may potentially lead to feelings of mental and physical fatigue; medical and aviation industries indicate that increased and intense demanding tasks, particularly in conjunction with disturbed sleep and fatigue, could potentially lead to significant health issues and performance errors. This could, in turn, result in an increased incidence of injuries, accidents, or even death [18]. The research on the side effects of space travel has been observed not only on the behavioral outcomes but also on the brain changes associated with them. Techniques utilized to quantify brain alterations encompass both in-situ methodologies, such as EEG, which can be deployed within the spacecraft by having the astronaut wear a headset to record electrical brain activity, and non-portable equipment (e.g. MRI), which precludes their use during travel [178]. Among the causes that generate an increased feeling of fatigue, in this case, "ocular" fatigue, we find Spaceflight-Associated Neuro-Ocular Syndrome, which also underlies cognitive impairments or effects on performance such as neuro vestibular issues [179]. This could also be due to reduced FA in the temporo-parietal junction zone. (Fig.8).

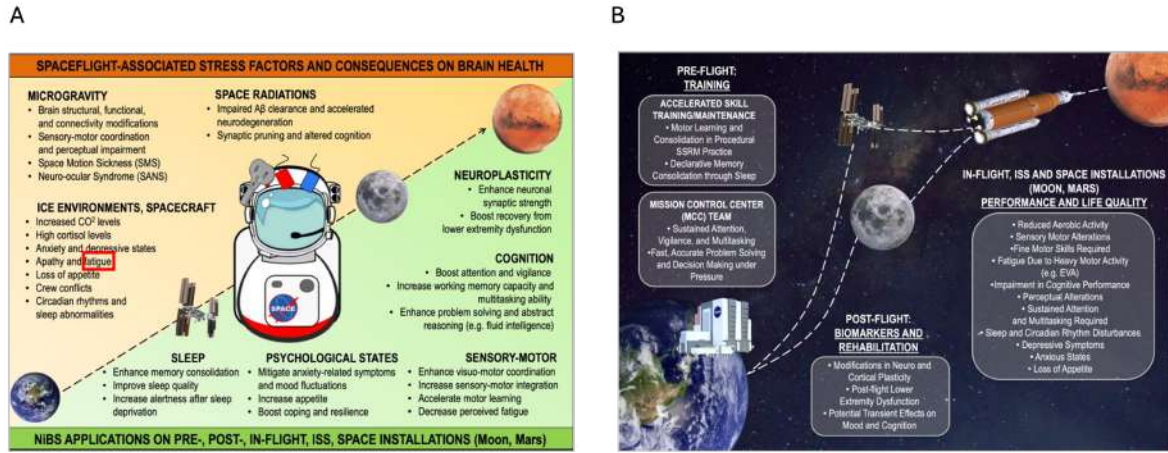


Figure 8. NiBS application in Space. Panel A summarizes both the side effects associated with space travel and the possible application of NiBS techniques to counteract them. Panel B shows the areas pivotal for space exploration that could potentially benefit from NiBS applications [180].

2.4.1 NiBS as fatigue countermeasure application

NiBS techniques are widely used to explore and treat many clinical disorders (e.g. Major Depression Disorder (MDD)); the Food and Drug Administration (FDA) approved the repetitive TMS for MDD patients classified as "treatment-resistant". Among NiBS, TMS and tES may represent a valid set of potential countermeasures to target a wide range of spaceflight risks identified by the Human Research Program (HRP) roadmap (NASA IG-18-021) [180]. The use and effectiveness of these techniques for astronauts were showcased by the review of Romanella and colleagues [180], in which the major areas of use of NiBS methods were reported both with respect to cognitive performance such as memory, processing speed, and attentional skills, but also on physical performance in which not only athletes' performance was improved but also physical fatigue was reduced. Regarding the latter topic, the greatest efficacy is attributed to tDCS. Many groups of researchers have shown that the application of tDCS can modulate the

perception of fatigue and exertion [133, 181, 182, 183]. In these studies, the stimulation target was the M1, where the anodal electrode was placed. Increased endurance and reduced muscle fatigue were observed in athletes, having training more similar to the astronauts. The effectiveness of tDCS was not only observed if the anodal electrode was placed on M1 but also on the DLPFC [183]. This result seems to stem from the fact that DLPFC is a crucial region of inhibitory control that plays an important role in endurance performance. The authors thus demonstrated that tDCS with the anode electrode on the L-DLPFC can improve both inhibitory control and endurance cycling performance in healthy individuals. This introduces another very important concept when talking about fatigue, namely its psychological component, mainly related to the individual's perception of fatigue. In fact, various authors suggested that the improvement in workload was achieved by a significant reduction in the perception of effort [184, 185]. Increased perception of fatigue also occurs, as mentioned earlier, in other conditions that astronauts may experience, such as a depressed or anxious state [186] or sleep-related disorders [167]. What has not been addressed enough in the literature is the reduction of cognitive fatigue, resulting from cognitive work overload in which people are forced to maintain high levels of reactivity, the ability to shift attentional focus quickly but also maintain focus on different stimuli, and maintain high performance at working memory tasks. All of these cognitive demands are also common to astronauts, who have to deal with situations in which they may be faced with multiple tasks at once in even potentially dangerous situations. This element finds great correspondence with many daily life activities, especially driving. Indeed, as with astronauts, for truck drivers, where the journey can be very tiring, maintaining good responsiveness and alertness, without becoming overwhelmed by fatigue, is crucial to one's safety. A re-

cent study I recently completed with my group in Siena [187] showed the effectiveness of a NiBS technique in reducing cognitive fatigue while maintaining good task performance. In this study, we applied the tRNS during a virtual reality (VR) driving truck simulation. The tRNS target in this case, based on the available literature, was the facilitatory system previously observed by Ishii and colleagues [140] (all details regarding the study by Benelli and colleagues, 2024, will be explained in the dedicated chapter). This certainly represents a new approach to verify the effectiveness of NiBS techniques as a fatigue countermeasure. Based on all the results described here, it is increasingly likely that they will be used by astronauts both before and after flight, but also during flight, mainly because the devices for these stimulations are light and easy to handle, and therefore potentially implementable in the instruments on board the spacecraft.

3 Multiple sclerosis

3.1 Central fatigue symptom in MS disease

3.1.1 Subjective description

CF in healthy individuals refers to a “progressive exercise-induced reduction in voluntary activation of a muscle” [117], showing the CNS component in the genesis of fatigue. CF, however, is also a chronic symptom in several clinical conditions such as Parkinson’s, COVID-19, side effects of chemotherapy, but especially MS. In neurological disorders, this term is used to refer to “declines in performance on a cognitive task, changes in motivation, effects on CNS function” [188] or as a “reversible motor and cognitive impairment, with a reduced motivation and an increased desire to rest” [189]; from the patients’ perspective, fatigue is usually reported as “excessive tiredness,” “malaise,” or “weakness” [190]. The neurological disorder most affected by CF is definitely MS, where reported by at least 80% of the patients, and it is described by 55% of them as the most disabling symptom significantly affecting their quality of life [191]. Fatigue is often present in the early stages of the disease [142], and sometimes, it is present even before the onset of the disease itself. It is more frequently present in primary and secondary progressive phenotypes than in relapsing-remitting ones [192].

There is no universally accepted definition of MS-related fatigue, but its main features are an exaggerated perception of effort and limited resistance to sustained physical and cognitive activity. Within clinical guidelines, fatigue is described as a “significant lack of physical and/or mental energy perceived by the individual or caregiver to interfere with usual or desired activity” [113]. MS chronic fatigue has been defined as “fatigue that is present for any amount of time on 50% of days for more than 6 weeks, that limits functional activities or quality of life” [189]. Chaudhuri and Behan [193] offer a definition which at a minimum is testable, defining CF as: “the failure to initiate and/or sustain attentional tasks (‘cognitive fatigue’) and physical activities (‘physical fatigue’) requiring self-motivation (as opposed to external stimulation)”. It is necessary to distinguish between primary and secondary fatigue: primary fatigue is due to the disease process; it is a result of damage to the nerves, and it is MS specific, while secondary fatigue results from drugs or concomitant manifestations such as sleep disorders and mood disorders [194]. Being a subjective symptom, MS-related fatigue can only be measured via self-report: there are many questionnaires in clinical practice, but the FSS and mFIS are considered the gold standard for MS-related fatigue evaluation [191].

3.1.2 Pathophysiological features

The pathophysiology of primary fatigue is complex, multifactorial, and not completely clear; both peripheral and central factors contribute to its onset, but it is certainly the latter that play the main role [195]. One of the most interesting theories regarding how fatigue occurs in MS is certainly that proposed by Annapoorna Kuppuswamy [196], which draws attention to the fact that chronic fatigue experienced in many neurological conditions is the result of a difficulty in attributing the correct effort for a motor action

or recognizing one's own physical capabilities. This, which manifests itself even at rest, has a major impact on a person's quality of life, also because neuronal firing in a resting condition consumes 20% of the body's energy, while task-related energy consumption accounts for less than 5% [197]. This mismatch between one's own capabilities and the external environment generates a condition of continuous metabolic suffering. MS fatigue has been considered a disorder of movement preparation, as evidenced by altered pre-movement motor cortex excitability, and movement preparation involves prediction of sensory outcomes—a key feature of sensory attenuation [196]. The following section will describe the main biological, neuroimaging and neurophysiological features of CF in MS.

The role of cytokines and inflammation

Among the factors contributing to the occurrence of this symptom, it is generally recognized that inflammation plays a key role in the development and persistence of fatigue: a correlation has been found between high levels of some inflammatory cytokines (including interleukin-6 (IL-6) and tumor necrosis factor-alpha ($\text{TNF}\alpha$)) and the presence of this disabling symptom [198]. Among the authors, Hessen and colleagues [199] found a significant correlation between serum levels of the proinflammatory cytokines interferon-gamma ($\text{IFN}\gamma$) and $\text{TNF}\alpha$ and levels of fatigue (measured with m-FIS), while no significant results were found for interleukin-10 (IL-10) [199]. These results agree with those obtained from other similar studies which conclude for a relevant role of cytokines (especially $\text{TNF}\alpha$) in the pathogenesis of this complex symptom [198]. Subsequent research also found a significant relationship between IL-6 and fatigue levels [200]. However, in order to define the role of cytokines in the pathophysiology of fatigue, more

homogeneous studies would be needed. According to Hanken and colleagues [201], the subjective feeling of MS-related fatigue could be a form of the so-called "inflammation-induced sickness behavior": according to this hypothesis, fatigue would derive from cytokine-mediated activity changes within those brain areas involved in interoception and homeostasis, such as the IC, ACC, and hypothalamus [201]. As demonstrated by many authors, the presence of high levels of pro-inflammatory cytokines causes subjective tiredness even in healthy individuals [202]. The term "illness behavior" refers to behavior that occurs when a healthy individual gets sick and it can be considered an adaptive response to acute infection or injury [203]. It is characterized by symptoms such as fatigue, depression, anhedonia, anorexia, and increased pain sensitivity, and appears to be caused and regulated by pro-inflammatory cytokines: the main ones are interleukin-1alpha ($IL-1\alpha$), interleukin-1beta ($IL-1\beta$), $TNF\alpha$, and IL-6, which are produced at the site of infection by activated immune cells and then act centrally [202]. The role that immune factors play in MS-related fatigue is confirmed by the fact that patients often experience a higher level of fatigue during relapses, when there is increased immune activation with increased release of pro-inflammatory cytokines such as $TNF\alpha$, IL-1, IL-6, and lymphotoxin α [204]. The elevated levels of pro-inflammatory cytokines at the peripheral level cause the activation of the afferent interoceptive fibers, including the afferents fibers from the vagus nerve; these are responsible for the innervation of specific brain areas such as the IC (particularly the anterior insula), ACC, and hypothalamus, which are areas involved in interoception and the maintenance of homeostasis [201]. If subjective fatigue in MS patients reflects increased levels of pro-inflammatory cytokines, it can be hypothesized that anti-inflammatory treatment has beneficial effects on this symptom. Indeed, glatiramer acetate, which has anti-inflammatory properties,

reduces fatigue in MS patients [205]. Similarly, Natalizumab, a drug that has been shown to reduce circulating plasma levels of $\text{TNF}\alpha$, IL-6, and IL-10, and cerebrospinal fluid levels of IL-1 β , IL-6, and interleukin-8 (IL-8), seems to have a positive effect on fatigue [206]. The beneficial effect that regular aerobic exercise has on MS-related fatigue may also be related to its anti-inflammatory effects: in fact, it inhibits the production of pro-inflammatory cytokines while stimulating the release of anti-inflammatory ones ($\text{TNF}\alpha$ and $\text{IFN}\gamma$) [207]. It seems that activation of microglia also plays a key role in the genesis of fatigue: correlating microglial substantia nigra activation to fatigue scores in MS patients, it was found that MS patients with fatigue have a higher microglial activation of the substantia nigra compared to healthy controls (HCs)[207]. Subsequently, it was also found a correlation of fatigue scores with microglia activation in diffuse cortical and subcortical gray matter regions, including the right precuneus, parahippocampal gyrus, putamen, TH, and juxtacortical white matter [208]. According to the model proposed by Hanken and colleagues [201], fatigue in MS patients has different characteristics depending on the progression of the disease. At the onset of the disease, inflammatory processes probably predominate and cause subjective fatigue, while in the latter stages of the disease, atrophy of specific brain regions progresses, which could predominantly contribute to objective fatigue. Therefore, according to this hypothesis, anti-inflammatory treatment could show beneficial effects on MS-related fatigue only at the onset of the disease, losing effectiveness in the subsequent phases.

The role of Hypothalamic–pituitary–adrenal axis

The hypothalamic-pituitary-adrenal axis (HPA axis) is an important neuroendocrine pathway consisting of three components: the hypothalamus, the pituitary gland, and

the adrenal glands. Through a complex system of direct influences and feedback interactions, it manages to modulate various physiological functions, including the body's response to stress. A dysfunction of the neuroendocrine system, in particular, the HPA axis, could contribute to the onset of fatigue. Gottschalk and colleagues found significantly elevated serum levels of adrenocorticotrophic hormone (ACTH) in fatigued MS patients compared to those of nonfatigue [209]. However, this study contrasts with the findings in the literature showing that endocrine dysregulation in chronic fatigue is related to hyporeactivity of HPA [210]. Therefore, this correlation should be investigated with further studies. Furthermore, researchers have hypothesized a link between this endocrine dysfunction and elevated levels of pro-inflammatory cytokines, highlighting the profound interconnection between the immune and endocrine systems. The involvement of the hypothalamus in the pathophysiology of MS fatigue is also supported by the MRI findings: According to the latter, the hypothalamus showed a positive association with higher processing speed [211] in MS patients than in HC [212]. A possible role of neurotransmitter and circadian rhythm disruptions in the precipitation of MS-related fatigue has been recently hypothesized [191]. At the neurotransmitter level, it seems that the presence of dysregulation in excitatory and inhibitory cortical pathways contributes to the presence of primary fatigue in this autoimmune disease: Indeed, GABA and glutamate levels in the prefrontal cortex have shown a correlation with fatigue levels: in particular, a negative correlation for GABA and a positive one for glutamate [213]. Beyond all that has been mentioned, it is recognized that at the base of this disabling symptom there are certainly some functional and structural abnormalities of the brain that we will discuss below.

Neuroimaging features related to central fatigue in MS

Fatigue derives from a complex interplay of structural alterations and changes in FC in cortical/subcortical areas and pathways within the frontal, parietal, temporal, and BG structures. Overall, MS fatigue appears to be related more to functional rather than structural neural alterations. At the level of macrostructural alterations, fatigue seems to be related to atrophy in the cortical and subcortical gray matter, white matter, and corpus callosum. Thanks to voxel-based morphometry studies, it was possible to find a link between MS fatigue and both regional atrophy (at the level of deep structures of the right hemisphere such as Nacc, forceps major, TH, and striatum) and diffuse atrophy at the level of cortical areas involved in important cognitive functions such as attention and reward (right inferior temporal gyrus, left superior frontal and precentral gyrus, frontal and parietal lobes, left central sulcus, and posterior parietal cortex (PPC)) [214]. Several studies have reported an association between fatigue and the presence of thalamic atrophy along with BG and frontoparietal cortex volume loss [215]. The thalamic involvement in the mechanisms underlying fatigue has been demonstrated both by anatomopathological techniques (which highlight demyelination, neuroinflammation, and neurodegeneration in this area) and by neuroimaging techniques (which demonstrate thalamic atrophy already in the early stage of the disease). Additionally, advanced neuroimaging techniques have shown indirect signs of demyelination and axonal loss in the TH of MS patients with fatigue [216]. Analyzing the microstructural changes, through Diffusion Tensor Imaging (DTI), fatigue has been associated with white matter integrity in fronto-striatal and parieto-striatal networks, the left frontal and the right temporal white matter, the cingulum bundle, and the left thalamo-frontal pathways [217]. These studies have also shown a lower fractional anisotropy (FA) index

in non-fatigued MS patients, suggestive of reduced structural integrity of the amygdala; therefore, the integrity of the amygdala appears to be related to the perception of fatigue [201]. Tartaglia and collaborators, using noninvasive proton magnetic resonance spectroscopy, measured N-acetyl aspartate levels and correlated them with levels of fatigue (as measured by the FSS scale). Study results show that diffuse periventricular axonal injury is related to increased fatigue in MS patients [218]. Given that the amygdala plays an important role in mediating the communication of inflammatory processes from the peripheral immune system to the CNS [219], this result supports the theory linking fatigue to inflammation and impaired "immune to brain" communication at the early stage of MS disease. In addition, this structure plays a crucial role in processes such as motivation, emotional processing, decision-making, and behavioral processes: consequently, reduced amygdala activity in MS no-fatigued patients could also be associated with changes in emotion, motivation, and behavior. Despite some evidence regarding structural damage underlying this symptom, there is more and more evidence of functional causes, therefore linked to alterations not only in the activity of individual areas, but also in their communication. A first example of how structural damage cannot play a major role in the onset of the symptom comes from the study of the recent findings by [220] who showed that fatigue in a cohort of 440 Relapsing Remitting Multiple Sclerosis (RRMS) patients was not related either to neuroimaging structural parameters or neurodegenerative features (e.g., brain atrophy). Cortical functional alterations are related to a dysfunction in a cortico-subcortical pathway centered on the TH, which is a determining factor in the onset of fatigue. However, there are conflicting opinions on the meaning of this dysfunction. In fact, while some studies report a reduction in the activity and connectivity of the thalamic network in fatigued

subjects, other studies describe instead an increase in these measures: these discrepancies could reflect different stages of the disease. In the early stages, there may be hyperactivation of the cortical-subcortical network due to compensatory mechanisms for MS-related structural damage, and therefore fatigue is not present. Over time, the reduction of functional reserve, together with the effort resulting from hyperactivation, could lead to the onset of fatigue. When these plasticity phenomena are no longer able to compensate for the reduced structural connectivity, FC decreases, and fatigue becomes chronically present [216]. Dysfunction of the thalamo-striatal-cortical circuits could be the pathophysiological basis of fatigue, not only related to MS but also to other neurological disorders such as Parkinson's disease. Inflammatory processes in different brain areas can certainly contribute to the pathogenesis. In fact, primary fatigue in MS is probably caused by a combination of disrupted neural circuits and a pathological immune response [221]. Few studies have analyzed brain activity and MS-related fatigue during cognitive tasks: DeLuca and colleagues [222] found increased fatigue-related activation in the frontal and parietal regions, as well as BG and TH, during a modified version of the Symbol Digit Modality Task (SDMT). Subsequently, Engström and colleagues [223] sought to explore the thalamo-striatum-cortical theory of cognitive fatigue in MS patients with self-reported fatigue: for this purpose, they used a complex working memory task during fMRI. Their results effectively confirmed the involvement of the thalamo-striatum-cortical network in the MS fatigue pathophysiology. Perceived fatigue during a cognitive task seems to be associated with impaired activation of the PPC and reduced FC between the posterior and frontal parietal cortices and subcortical structures such as the striatum and TH [223]. Other studies focused on the local neural activity of the SMN, where the M1 has been shown to be more excitable at rest

and hyperactive during motor execution in MS patients with fatigue, and its inhibition is reduced after exercise [224, 225]. As MS-related fatigue intensifies, the SMA shows increased activation [226]. Specogna and colleagues [227], using an fMRI study, measured cortical activation during a finger tapping test (with the right hand) in 24 RRMS patients with and without fatigue and 15 HCs. Comparison between HCs and non-fatigued patients demonstrated greater activation in the latter of the primary sensorimotor cortex bilaterally, right supplementary motor cortex, left premotor cortex, left cerebellum, and the superior parietal lobule bilaterally. In patients with fatigue, compared to those without fatigue, activation of the putamen, the right premotor area, and DLPFC was instead highlighted (brain areas involved in motor planning and conscious motor adaptation) [227]. From these results, it can be deduced that patients with fatigue show greater activation of the motor attentional network during the execution of a motor task. Therefore, in summary, motor areas show BOLD hyperactivity related to increasing fatigue, while parietal areas show BOLD hypoactivity and decreased FC related to increasing fatigue. Patients with fatigue, in addition to abnormal recruitment of the SMN (M1 and SMA), show impaired BG functions, including motivation and reward processing, thus supporting the hypothesis that fatigue underlies motor and non-motor network dysfunction. At the molecular level, the release of pro-inflammatory cytokines probably interferes with BG-cortical loops, influencing motivated action and resulting in fatigue [228]. Indeed, an imbalance in the interhemispheric FC between homologous sensorimotor areas appears to be associated with increasing fatigue; this imbalance was highlighted both at rest and during motor execution in relation to increased fatigue [229]. As fatigue levels increased, impaired FC at rest was also found between the temporo-parietal hemispheric homologous regions [230]; a reduced com-

munication between S1 and M1, or a poor responsiveness of M1 to S1 projections, may play a role in MS fatigue [219]. Results emerging from the study of rsFC of both the default mode network (DMN) and SMN in fatigued and non-fatigued MS patients are interesting: comparing them with HCs, fatigued patients showed increased rsFC in the PCC and reduced rsFC in the ACC of the DMN. Based on these results, it can be stated that fatigue in RRMS is mainly related to a functional rearrangement of non-motor resting state networks [224]. fMRI was also used in a pilot study to examine how cognitive fatigue affected brain activation during conditions of variable cognitive loads of rapid information processing (with visual stimulations). With increasing levels of cognitive fatigue, in the HCs group there was evidence of greater activation of anterior brain regions (i.e., superior and MFG, IC, and superior temporal gyrus), associated with greater speed, to meet the increased activity demands in the high cognitive load condition. The MS group, on the other hand, did not show greater activation in these anterior areas but rather, as the difficulty of the task and therefore the fatigue increased, the MS group showed a continuous activation of multiple posterior regions (i.e., precuneus, lingual gyrus, and middle occipital gyrus) without any speed improvement [231]. Furthermore, abnormal FC of striatal nuclei with cortical regions was found in MS patients with fatigue. There is evidence of reduced FC of the caudal nucleus (particularly the ventral superior striatum) with SMN and frontal, parietal, and temporal cortex regions in fatigued MS patients compared to non-fatigued MS patients and HCs. Finally, the DLPFC showed reduced connectivity with the parietal cortex, precuneus, and PCC, and hyperconnectivity with the rostral inferior parietal lobe in MS patients with fatigue [232]. The connections of the superior ventral striatum overlap many brain areas related to reward regulation, attention, and motor functions [233]. Reduced FC

of the ventral cortical striatum could represent a correlate of the imbalance between effort and reward (perception of high perceived costs and low benefits), which has been recognized as a central feature of fatigue and associated with cortico-striatal circuit dysfunction [232]. All previous results are grouped in Figure 9, showing not fatigue brain areas but also those areas involved in Depression and finally those overlapping among depression and fatigue.

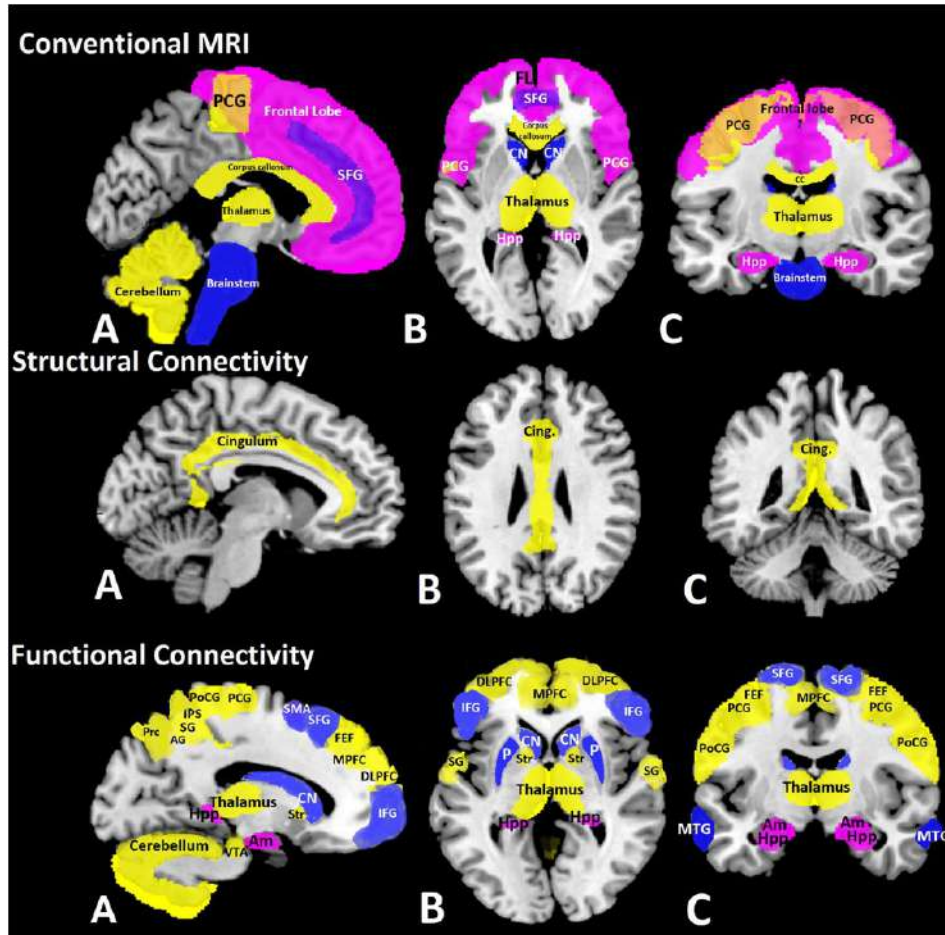


Figure 9. Brain regions related to fatigue and MDD conditions Sagittal (A), axial (B), and coronal (C) view of brain regions suggested to be involved in depression (magenta), fatigue (blue) or both* (yellow) in > 1 study, using conventional MRI, structural and functional connectivity. Brain regions were extracted from brain atlases available in FSL and superimposed on a template T1w image, available in MRICron. Results from included publications were compiled and summarised in this figure by the authors of this study, using MRICron. AG=angular gyrus (as a region of default mode network), Am=amygdala, CC=corpus callosum, Cing=cingulum, CN=caudate nucleus, DLPFC=dorsolateral prefrontal cortex, FEF=frontal eye field (as a region of dorsal attention network), FL=frontal lobe, Hpp=hippocampus, IFG=inferior frontal area, IPS=intraparietal sulcus (as a region of dorsal attention network), MPFC=medial prefrontal cortex (as a region of default mode network), MTG=middle temporal gyrus, P=putamen, PCG=precentral gyrus, PoCG=postcentral gyrus, Prc=precuneus (as a region of default mode network), SFG=superior frontal gyrus, SG=supramarginal gyrus, SMA=supplementary motor area, STG=superior temporal gyrus, Str=superior ventral striatum, VTA=ventral tegmental area. Overlapping brain regions between symptoms, in at least 1 study for each symptom [220].

Neurophysiological and biological hallmarks

TMS results TMS is a valuable tool used in MS patients to investigate the neurophysiological basis of some symptoms such as fatigue, which are not yet fully explained by conventional structural damage. The available studies regarding electrophysiological correlates of MS-related fatigue, particularly those involving intracortical excitability and corticospinal excitability measures obtained by TMS, have often yielded inconsistent results. This area thus requires further research and elucidation. First, demyelination and neurodegeneration in MS might lead to abnormalities of cortical excitability and MEP; moreover, an increase in MEP latency is a significant predictor of fatigue severity [234]. TMS studies investigating the correlation between CSP and fatigue have produced inconsistent results. For example, the study by Chaves and colleagues showed that CSP (or rather, an extended CSP) can predict fatigue [235]. Based on this concept, these researchers hypothesized that improving the fitness of these patients could ameliorate this symptom by decreasing GABAergic-mediated intracortical inhibition and thereby shortening the CSP. However, in another study, this correlation between prolonged CSP and fatigue severity was not found [236]. These inconsistencies are likely due to clinical and methodological differences between the two studies: they analyze different disease phenotypes (mainly relapsing-remitting disease versus progressive disease, respectively) and use different fatigue assessment scales (VAS vs. mFIS, respectively). Santarnecchi and colleagues found shorter CSP in MS patients with fatigue versus HCs. After prolonged index-to-thumb tapping, CSP prolongation involved both "fatigued" and "unfatigued" intrinsic hand muscles, suggesting a cortical spread of CF mechanisms. However, treatment with amantadine annulled differences in CSP duration between controls and patients, in parallel with improvement of fa-

tigue scores [237]. Regarding double-pulse parameters, different studies have found similarly conflicting results. Liepert and colleagues Liepert and colleagues discovered a correlation between SICI and fatigue levels.: specifically, SICI in fatigued patients was significantly reduced before a fatiguing exercise compared to HCs and non-fatigued MS patients; this difference remained after exercise [225]. The SICI reduction found in fatigued MS patients indicates possible disinhibition in the motor cortex mediated by downregulation of GABA activity. Thus, this TMS study demonstrated reduced intracortical inhibition, before and after exercise in fatigued MS patients, suggesting that fatigue in MS may reflect downregulation of GABA activity. This increased cortical excitability could represent a compensatory mechanism for conduction deficits in demyelinated pyramidal tract fibers intended to overcome premature fatigue. Furthermore, it has been shown that in MS patients with fatigue, a longer post-exercise period is required to normalize motor thresholds [238], and knowing that motor threshold is modulated by voltage-gated Na⁺ channels [239], Na⁺ channel dysfunction could contribute to the presence of fatigue [240]. Moreover, a correspondence was found between the severity of fatigue and the time required for normalization of motor threshold after cessation of exercise [225]. These results contrast with those obtained in another study, which found significantly higher SICI in fatigued compared to non-fatigued patients [236]. The increased SICI in fatigued MS patients could reflect increased GABAergic activity and thus an alteration of the GABAergic/glutamatergic balance. Therefore, insufficient facilitation mechanisms might be responsible for an inability to overcome the functional deficit, leading to exhaustion of compensatory mechanisms and increased fatigue. Finally, a third study found a similar pattern in fatigued MS patients compared to non-fatigued patients and HCs [241]. Other studies have also explored the neuro-

physiological correlates of MS-related fatigue during motor task performance, aiming to investigate possible correlations of this symptom with movement preparation or execution phases. Interesting results emerged comparing fatigued MS patients, non-fatigued MS patients, and HCs: after the motor task, significantly reduced premovement-ICF was found in fatigued patients compared to the other two groups [241]. This result was associated with frontal lesion load, worsening motor performance, and increased fatigue levels. From these data, it is deduced that alterations involving brain networks dedicated to movement preparation may be implicated in the mechanisms underlying MS-related fatigue. A further difference between the three subject groups is that in fatigued MS patients, unlike non-fatigued MS patients and HCs, no increase in post-exercise MEP amplitude was observed during handgrip facilitation. Therefore, fatigued patients were less able to increase motor output through motor facilitation; this loss of ICF could be explained by a dysfunction in the corpus callosum, which causes a lack of reduction of inhibition of the contralateral cortex during handgrip facilitation. As previously demonstrated, levels of fatigue appear to correlate with the time required for resting motor threshold to return to pre-exercise levels [225].

Electrophysiological results This symptom appears related to EEG power spectral density of specific frequencies as well as to features such as evoked potential (EP) or microstate analysis. The term microstates refers to spontaneous broadband EEG activity at rest that can be described by a limited number of topographies (maps) of scalp potentials that remain stable for a certain period (60-120 ms) before rapidly switching to a different topography. This suggests that the scalp potential field reflects the momentary state of global neuronal activity and that changes in the topography of this field indicate changes in the global coordination of neuronal activity over time

[242]. It has been demonstrated that microstate analysis decomposition within separate, narrow frequency bands could provide more fine-grained information for capturing the spatiotemporal complexity of multichannel EEG. In the study by Baldini and colleagues, they observed that in fatigued MS patients, a significant decrease of microstate F, associated with the salience network, was present in the broadband and beta band. Moreover, microstate B, associated with the visual network, showed a significant increase in fatigued patients compared to healthy subjects in broadband and beta bands. Multiple linear regression showed that high cognitive fatigue was predicted by both an increase and decrease in delta band microstate B and beta band microstate F [243]. In the work of Vecchio and colleagues, it was observed that FC in the resting state with eyes open changes along with fatigue symptoms in the beta1 band (13-20 Hz) in the left dominant sensory network hemisphere, revealed through cortical EEG source analysis of network graph theory. They observed that fatigue increases with the progressive loss of small-world organization in sensory areas. These network alterations spread to the primary sensory areas, particularly the PPC, which receives visual information from the primary visual areas, somatosensory information from the primary somatosensory area, and motor information from the primary motor, supplementary motor, and premotor areas [244]. The increasing prevalence of altered connectivity in the parietal region, parallel to increased fatigue, reinforces the idea that perception plays a central role in the generation of fatigue and that prolonged perception of fatigue reduces the motivational drive, thereby increasing the perception of fatigue [245]. The recent review by Hamilton and colleagues grouped studies investigating cognitive fatigue with EEG [246]. These studies showed that fatigue symptoms increased with higher resting FC in the temporoparietal region in the beta band, and a positive correlation was found

between fatigue levels and frontofrontal connectivity in the beta and theta bands. Additionally, considering event-related potential (ERP) studies during EEG using cognitive performance tasks, although the ERP response after the cue stimulus in MS patients with fatigue did not differ significantly from HCs, significant differences related to distractor stimulus and ERP amplitude were noted among participants between the two groups [246]. The pioneering study by Leocani and colleagues [247] showed an increase in event-related desynchronization (ERD) at 18-22 Hz and a decrease in event-related synchronization (ERS) post-movement at 18-22 Hz in frontal regions in a group of MS patients who complained of fatigue compared with normal subjects and non-fatigued MS patients [247]. Since the ERD of the 10- and 18-22 Hz rhythms is considered a correlate of cortical activation, whereas the ERS of the same rhythms is a correlate of cortical inactivity, corresponding in time to corticospinal inhibition at transcranial magnetic stimulation [24, 248], these results are consistent with a central origin of fatigue in MS and indicate cortical dysfunction even during a simple motor task, resulting in hyperactivity during movement execution and failure of the inhibitory mechanisms that intervene after the movement is over. Finally, modern methods of neurophysiological investigation, including TMS combined with simultaneous EEG (TMS-EEG), provide outcome measures derived from EEG responses to TMS (i.e., evoked potentials or brain oscillations) that can be used as neurophysiological markers of excitability or connectivity for any brain area, including regions where TMS does not generate a proxy for cortical/corticospinal excitability, such as MEPs or phosphenes [249]. Based on this new approach, Leodori and colleagues observed that although there were no differences in MEPs or CSPs between MS and HCs, patients showed post-fatigue increases in TMS-evoked potential propagation from M1 to the rest of the cortex and

in source-reconstructed activity within the sensorimotor network, contrasting with the reduction observed in HCs [250].

Multiple sclerosis fatigue	MS fatigue definition	Reference
	"Significant lack of physical and/or mental energy perceived by the individual or caregiver to interfere with usual or desired activity"	<i>Kluger et al., 2013</i>
	"Fatigue that is present for any amount of time on 50\% of days for more than 6 weeks, that limits functional activities or quality of life"	<i>Mills et al., 2007</i>
	MS Fatigue Key Role of Inflammation	
	Correlation exists between high inflammatory cytokines (IL-1 α , IL-1 β , TNF α , IL-6) and fatigue symptoms	<i>Hessen et al. 2006</i>
	Significant correlations between fatigue levels and pro-inflammatory cytokines (IFN γ , TNF α) but not IL-10	<i>Hanken et al. 2018</i>
	MS Fatigue HPA Axis Dysfunction	
	Elevated ACTH levels in fatigued MS patients compared to non-fatigued ones	<i>Gottschalk et al. 2005</i>
	MS fatigue related to a HPA hyporeactivity	
	MS fatigue MRI and fMRI features	
	MS fatigue correlates with atrophy in cortical and subcortical gray matter, white matter, and corpus callosum	<i>Ribbons et al., 2017</i>
	MS fatigue correlates with thalamic atrophy and volume loss in basal ganglia/frontoparietal cortex	<i>Bernitsas et al., 2017; Capone et al., 2020</i>
	Reduced FA in the amygdala associated with MS fatigue	<i>Hanken et al., 2018</i>
	Increased MS fatigue-related activation in frontal, parietal, basal ganglia, and thalamic regions during cognitive tasks	<i>De Luca et al., 2008</i>
	Impaired activation in the posterior parietal cortex and reduced connectivity during cognitive tasks in MS-fatigued patients	<i>Engstrom et al., 2013</i>
	Hyperexcitable motor areas (M1, SMA) with greater activation in MS-fatigued patients during motor tasks	<i>Bisecco et al., 2018</i>
	Decreased functional connectivity in striatal nuclei and cortical regions among MS_fatigued patients	<i>Jaeger et al., 2019</i>
	MS fatigue TMS features	
	Prolonged CSP predicts MS fatigue symptom	<i>Chaves et al., 2019</i>
	No correlation between prolonged CSP and MS fatigue severity	<i>Chalah et al., 2021</i>
	Shorter CSP in MS-fatigued patients compared to HCs	<i>Santarnecchi et al., 2015</i>
	Reduced SICI in MS-fatigued patients	<i>Liepert et al., 2005</i>
	Higher SICI in MS-fatigued patients	<i>Chalah et al., 2021</i>
	Similar SICI patterns across MS-fatigued, MS non-fatigued patients, and HCs	<i>Morgante et al., 2011</i>
	Reduced ICF was observed in MS-fatigued patients compared to MS non-fatigued patients and HCs after a motor task	
	MS fatigue EEG features	
	Significant decrease in microstate F (salience network) and an increase in microstate B (visual network) in MS-fatigued patients compared to HCs, particularly in broadband and beta bands	<i>Baldini et al., 2024</i>
	Changes in resting state functional connectivity in the beta1 band (13-20 Hz) in the left sensory network, linked to increased MS fatigue and loss of small-world organization	<i>Vecchio et al., 2017</i>
	MS fatigue symptoms were associated with higher resting functional connectivity in the temporoparietal region (beta band) and frontofrontal connectivity in beta and theta bands	<i>Hamilton et al., 2024</i>
	Increased event-related desynchronization (ERD) and decreased event-related synchronization (ERS) at 18-22 Hz in MS-fatigued patients	<i>Leocani et al., 2001</i>
	MS-fatigued patients exhibited increased TMS-evoked potential propagation and source-reconstructed activity in the sensorimotor network post-fatigue, contrasting with HCs	<i>Leodori et al., 2023</i>

Figure 10. MS fatigue features The figure shows the main scientific contributions that best describe the characteristics of the symptom fatigue in MS.

3.1.3 Available treatments for central fatigue in MS

MS-related fatigue is a complex and multidimensional symptom composed by physical, psychosocial, and cognitive domains, and therefore its treatment requires a personalized plan that can address all its dimensions. I will focus on the nonpharmacological treatments, specifically on NiBS techniques. While pharmacological treatments such as Amantadine Hydrochloride, Modafinil, Pemoline, Carnitine, and potassium channel blockers have shown some positive but still debated and controversial results, several non-pharmacological alternatives seem to provide encouraging outcomes, including aerobic exercise, whole body cryostimulation, cognitive behavioral therapies (CBT), and NiBS [251]. NiBS techniques, such as tDCS and TMS, have been explored as potential treatments for fatigue in MS. While initial findings are promising, more robust clinical trials are necessary to establish their efficacy and determine the most effective stimulation parameters and long-term effectiveness. A recent study by Chalah and colleagues examined the effects of bifrontal tDCS on fatigue in MS patients. The study included eleven MS patients experiencing fatigue, who were randomly assigned to receive two blocks of tDCS—one active and one sham—over five consecutive daily sessions. The bifrontal tDCS involved placing the anode and cathode over the left and right prefrontal cortices, respectively. The treatment order was crossed over, with a 3-week interval between the two blocks. Results indicated that only the active tDCS, not the sham tDCS, led to a significant improvement in fatigue by the fifth day of treatment ($p < 0.05$). Furthermore, the positive effect of active tDCS on fatigue appeared to last for at least one week following stimulation ($p = 0.05$) [236]. Similarly, Tecchio and colleagues found that anodal tDCS applied over bilateral somatosensory areas was effective in reducing fatigue in mildly disabled MS patients [252]. Another study showed improvement in fa-

tigue scores in approximately half of the patients who underwent both active and sham anodal tDCS over the left DLPFC. However, no significant differences were observed between the effects of the two conditions [253]. While outcomes from bilateral stimulation of the sensorimotor and motor cortex have shown promise [252, 254, 255], the effects of left DLPFC stimulation remain controversial. Two studies reported negative results [251, 256], while others documented positive outcomes [236, 253]. Nevertheless, existing studies are still quite limited, and the effectiveness of these techniques in alleviating fatigue in MS patients is currently considered poor and mainly short-lived. A recent study by Salemi and colleagues (2019) investigated the application of tRNS as a countermeasure to reduce fatigue in MS. They recruited 17 RRMS patients, dividing them into two groups for either real or sham stimulation over 10 days, targeting the M1 of the dominant side or contralateral to the most impaired limb. In the “real group,” beneficial effects were observed only on the mFIS; the absence of effects on cognitive and psychosocial domains suggests that tRNS on M1 may primarily target the physical aspect of fatigue [257]. As previously discussed, adopting an individualized treatment approach is the most effective method for symptom reduction. This was demonstrated by Tecchio and colleagues (2022) [258], who applied a tDCS protocol with the anodal electrode on S1 (bilaterally) (Fig.11) and the cathodal electrode on Oz. Their novel treatment, based on evidence that fatigue in MS is characterized by hyperexcitability of M1 and hypoexcitability in S1, along with reduced connectivity between S1 and M1, involved a contoured anodic electrode placed on S1, designed for each subject on his own S1 shape; this personalized approach prevented the stimulation from adversely affecting M1. The proposed home treatment lasted for 5 consecutive days, resulting in a reduction of fatigue, as assessed by the mFIS, in 10 of the 15 patients recruited. This

study highlights not only the efficacy, safety, and feasibility of a customized treatment but also the potential for patients to administer treatment at home after professional training. The inconsistent results from various studies likely reflect a lack of treatment personalization based on neurophysiological, neuroimaging, and biomolecular indices. The study we conducted about, which will be describe chapter 4, aims to introduce the concept of personalized treatment by exploring possible biomarkers to achieve this goal.

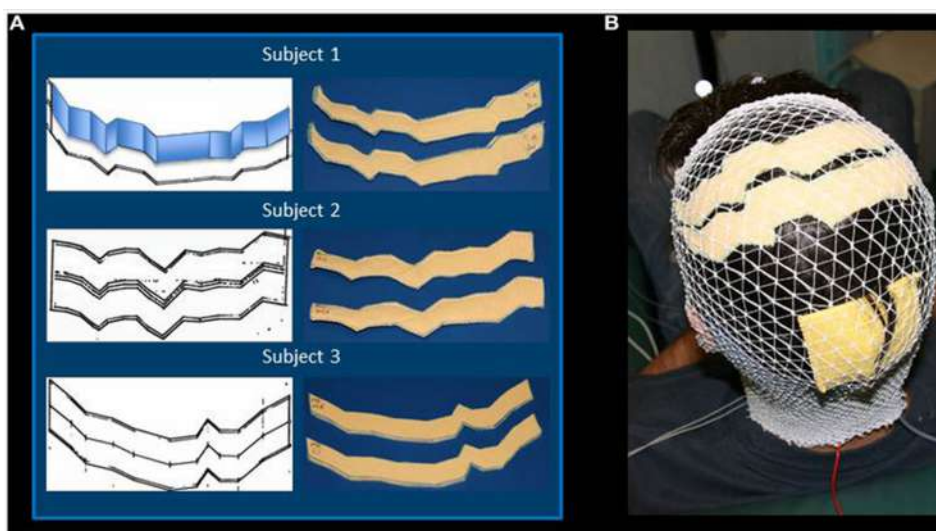


Figure 11. Regional Personalized Electrode Shaping. The sponge electrodes are shaped for each subject in two steps: first, the left and right central sulci are drawn on a piece of paper using SoftTactic software from a volumetric MRI; second, the central sulcus is fitted with 2 cm-wide parallelograms (an example is shown for Subject 1). For each S1 and M1 electrode, the shape is drawn on and cut out of two sponge sheets; these two sheets are sewn together to allow the insertion of conductive material (A). The stimulating electrodes are positioned using a neuronavigation procedure (the reference landmark is visible frontally). Electrodes are secured using an elastic cotton cap (B) [258].

4 Reduction of Cognitive Fatigue and Improved Performance at a VR-based Driving Simulator using tRNS

4.1 Abstract

Cognitive fatigue increases accident risk reducing performance, especially during complex tasks such as driving. We evaluated whether tRNS could mitigate cognitive fatigue and improve driving performance. In a double-blind study, thirty participants performed a VR driving truck task during real (n=15) or sham (n=15) tRNS applied bilaterally on the “anti-fatigue network”. They completed two 30-minute driving sessions while their driving performances were constantly monitored; heart rate was also monitored to evaluate arousal (RMSSD). tRNS was applied only during the first driving session to evaluate both online and offline stimulation effects. The primary outcome was cognitive fatigue reduction and performance improvement in the second (non-stimulated) driving session. Real tRNS significantly improved driving performances in the second driving session reducing cognitive fatigue. These results might also lead to the use of tRNS in those neurological disorders characterized by fatigue.

4.2 Study protocol

Subjects were tested under the same experimental protocol within a randomized double-blinded study design. Blindness was deployed using the Matlab programming language, which randomly assigned a number for each condition with no knowledge of the experimenter. Using the same script again, the conditions were randomized equally among participants. The experiment was conducted in a quiet environment to minimize the influence of external stimulations. The protocol included 3 sequential drive trials (practice trial, first and second drive) with a truck driving experience (Euro Truck Simulator 2) by connecting the Steam gaming platform to head-mounted Oculus Quest 2. Driving routes were the same for all participants. The practice trial consisted of a 15-minute training session without any brain stimulation to practice driving in VR. The first 30-minute drive involved the administration of stimulation either tRNS (0Hz-500Hz) or Sham, applied in a double-blind and randomized controlled manner among participants; tRNS Stimulation represented the experimental condition and the Sham stimulation represented the control condition. The following 30-minute drive was performed without any stimulation, to verify the possible stimulation after effect on the driving performance, the main goal of the study. HRV recordings were made during the first and second drives, as well as between the practice trial and the first drive, and between the first and second drives. A third HRV recording was taken at the end of the second drive. We decided to perform all 3 trials on the same day to induce the maximal cognitive workload inducing more cognitive fatigue. Participants engaged in the VR sessions were sitting on a chair, blind to the type of stimulation applied. Each participant also underwent two cognitive assessments (before the practice trial and after

the second drive) by performing several tasks in a fixed order. To evaluate cognitive abilities changes before and after the two drives we used different cognitive tasks: visual search task (in this task multiple objects are presented and a target is identified among them; subjects were asked to identify the target stimulus as fast as possible. Not only the number of correct responses is taken into account but also the speed of stimulus processing), and useful visual field task (in this task a stimulus was shown briefly and then made to disappear by asking the subject to return as quickly as possible to where she/he saw it; not only is the number of correct responses taken into account but also the speed of execution), Go-NoGo task (with this test, the ability to inhibit automatic responses is assessed as target stimuli are presented interspersed with confounding stimuli rapidly; the subject is asked to press as fast as possible when the target stimulus appears and not press when the confounding stimulus appears. Not only is the number of correct responses taken into account but also the speed of execution) and simple RT task (with this test, the speed processing is assessed; the subject is asked to press as fast as possible when the target stimulus appears. Only the execution speed is taken into account). Each task required approximately 3–5 min; they were executed using a desktop PC controlled by Presentation software (Versions 13 and 14, NeuroBehavioral Systems, Albany, CA, USA). Fig. 11A shows the experimental setting and design.

4.3 Materials and Methods

Participants In this exploratory, double-blind, parallel arm study, 32 healthy young adults (all right-handed; 19 males and 13 females; mean age: 25.3 ± 3.7 years; education: 14.6 ± 2.4 years) were recruited from students at the University Hospital of Siena, Italy. Two of them withdrew for personal reasons; therefore, analyses were carried out

on 30 subjects (17 males and 13 females; mean age: 24.9 ± 3.5 years; education: 14.5 ± 2.3 years). Demographic details for all participants are provided in the Supplementary Materials in Table S3. All subjects were licensed to drive and were inexperienced in operating a VR driving simulator. Each subject declared their willingness to participate in the study and signed a written informed consent; the Local Ethics Committee approved the research (Code: Brainsight 21-24). Participants were assigned to the active group (9 males and 6 females; mean age: 24.5 ± 10.7) or sham group (8 males and 7 females; mean age: 25.2 ± 11.3) in a blind, and randomized manner. Taking into account the exploratory nature of the study, the sample size was defined using G*Power 3.1 [259] on the following basis: since we were interested in one specific contrast (tRNS vs. Sham) alpha was set at $0.05/2=0.025$. Power was set at 0.60, indicating that 30 subjects are essential to have a 60% probability of recognizing a difference between two conditions as statistically significant (at two-sided alpha 0.025). Subjects with a history of epilepsy, sleep disturbances, migraine, psychiatric drug therapy, and history of other neurological or psychiatric disorders were excluded. Simulator Sickness Questionnaire (SSQ) was used to assess the degree of cybersickness resulting from the use of Virtual Reality [260].

4.3.1 Euro Truck Simulator 2

We chose Euro Truck Simulator 2 because it offers the opportunity to use real maps while driving a truck. The game has been customized to increase the smoothness of the steering wheel, and pedal pressure to recreate a real driving as much as possible. During the simulation, all participants ran 3 routes of different durations (as shown above). In the practical test section, of mild difficulty, the course was set with a reduced percentage

of traffic, a sunny sky, and no abrupt behavior from other drivers. The second and third guides had a higher difficulty degree: rainy sky, high traffic, abrupt behavior by other drivers, and increased police presence to carry out checks. This distinctive setting was aimed at recreating a highly challenging environment. Participants were required to maintain a high level of attention while performing multiple tasks simultaneously: watch the road, maintain a safe speed within the limits, and pay attention to other drivers while following the route correctly as a navigator. Variables of interest extracted from the simulator were the driving performances obtained based on the damages caused to the truck and the cumulative monetary losses.

4.3.2 Transcranial Random Noise Stimulation

tRNS was administered through a 32-channel hybrid EEG/tCS neurostimulation system (Starstim; Neuroelectronics, Barcelona, Spain). The device was connected by cable to the computer. Hybrid electrodes (NG Pistim) were used, consisting of an upper part containing the sintered Ag/AgCl core with a diameter of 12 mm, screwed onto a lower base such that a circular area of about 3.14 cm² was covered. The electrodes were placed on a 32-channel neoprene EEG headset with holes corresponding to the positions of the International 10-20 EEG System. The scalp portion below the electrode was prepared by inserting 15 ml of sterile sodium chloride solution (0.9%) to avoid uncomfortable sensations on the skin and reduce impedances, which were always kept below 20 khom. Gel (Signa, Parker Laboratories, Inc.) was applied to optimize signal conductivity and lower impedance. Electrode impedance was checked before starting each tRNS session to ensure safety and maximal efficacy of stimulation, as well as to ensure familiarization of participants with the tRNS-induced scalp sensations (e.g., slight tingling). tRNS was

applied for 30 minutes with a maximum intensity of 2 mA on each electrode and of 4 mA total across all electrodes, preceded by a 30-s ramp-up period and followed by a 30-s ramp-down period, while research and clinical personnel carefully monitored for eventual side effects for the entire duration of each session. For Sham stimulation, only a ramp up and ramp down of 30-s were set, without any stimulation in between.

4.3.3 Biophysical Modeling

To determine the appropriate electrode montage for our target, an open-source simulation software (SimNIBS v3.2) was employed. Utilizing computational modeling through the Finite Element Method (FEM), SimNIBS incorporates MRI scan segmentation, mesh generation, and E-field computations. This allows for the projection of current distribution and accurate calculation of the electric field generated by various NiBS techniques [261]. The software presents a realistic volume conductor head model, defaulting to the FEM model generated from T1 - and T2 - weighted images and segmentation from the SimNIBS example dataset [91]. The data sample, acquired ethically from a healthy subject under the approval of the Ethics Committee of the Medical Faculty of the University of Tübingen [262], comprises white matter, gray matter, cerebrospinal fluid, bone, and scalp tissue volumes for a comprehensive simulation. Simulations were based on isotropic conductivities reported in Thielscher, Antunes, and Saturnino (2015)[261], with values corresponding to gray matter (0.276 S/m), cerebrospinal fluid (1.790 S/m), bone (0.010 S/m), and scalp (0.250 S/m). The final mesh, encompassing gray and white matter, scalp, bone, and cerebrospinal fluid, consisted of approximately 200,000 nodes and 3.6 million tetrahedral elements (see Windhoff, Opitz, and Thielscher 2013)[262] for further modeling details). Drawing on literature data, we proposed an

innovative stimulation montage targeting both cortical and subcortical areas directly and indirectly, respectively. Taking a cue from the study regarding the facilitation system by Ishii and colleagues (2014)[140] and other studies that only considered DLPFC [119, 263, 264] as the target stimulation area, we decided to focus on the SMA (MNI coordinates left: -28; -2; 52 right: 28; 1; 52), bilaterally, MFG (MNI coordinates left: 45; 53; -7 right: 44; 34; 20) and M1 (MNI coordinates left: -36; -19; 48; right= 38; 18; 45). Using the DSI Studio tractography software tool, subcortical projections of the cortical target region revealed fiber connections to subcortical structures such as the striatum, TH, and the caudate nucleus. Importing subcortical nuclei as ROIs from the ATAG_basal_ganglia Atlas in DSI Studio, we observed fibers passing through these ROIs using automatic fiber tracking. A connectivity matrix was then created by loading Brodmann's anatomical atlas to observe cortical areas reached by the fibers, including DLPFC, SMA, and M1. Based on simulation results and the model, we identified a montage capable of targeting regions. Specifically, we placed eight electrodes over C1, C2, C3, C4, FC3, FC4, FC5, and FC6. On C1 level, an intensity of 0.85 mA with a phase angle of 0° was used; on C2 level, an intensity of 0.85 mA with a phase angle of 180° was used; on C3 level, an intensity of 1.3 mA with a phase angle of 0° was used; on C4 level, an intensity of 1.3 mA with a phase angle of 180° was used; on FC3 level, an intensity of 0.75 mA with a phase angle of 0° was used; for a total of 2.5 mA; on FC4 level, an intensity of 0.75 mA with a phase angle of 180° was used; on FC5 level, an intensity of 1.1 mA with a phase angle of 0° was used; and on FC6 level, an intensity of 1.1 mA with a phase angle of 180° was used; for a total of 4 mA (Fig.11B).

4.3.4 HRV recording

In all subjects, heart rate variability was measured using a Polar H10 heart rate monitor device placed on the chest. Specifically, we were interested in the detection of the RMSSD parameter, related to the activity of the vagal parasympathetic system. HRV was recorded before (6 minutes), during the first VR drive experience (30 minutes), after the first drive trial (6 minutes), during the second VR drive experience (30 minutes), and after the second drive trial (6 minutes). Measures of RMSSD variations were expressed as millisecond percentage. For the resting recording, subjects were asked to rest with their eyes open for the first 3 minutes and then to close their eyes for the following 3 minutes. They were asked not to think about anything in particular and to remain as relaxed as possible. Kubios HRV software, Standard Version [265], was used to perform HRV analysis on individual cardiac traces and to identify outliers and artifacts (using the automatic correction feature). An automatic beat correction algorithm was utilized to correct for erroneous interbeat intervals [266]. This algorithm accurately identifies missed and extra beat detections with an accuracy rate of 100% and ectopic beats with an accuracy rate of 97%. The detected artifacts are rectified using cubic spline interpolation.

4.3.5 Outcome measures and data analysis

Drive performance, RMSSD, VAS-F, cyber sickness, and side effects. The primary aim was to verify fatigue scores after each trial in the different stimulation conditions. Through the [123]VAS-F, participants were asked to report their fatigue status by choosing a score from 0-10 (0=absence of fatigue; 10=worst possible fatigue). In addition

to the level of fatigue, driving performance (damages to the truck and monetary losses incurred because of traffic violations) was compared. Damage refers to the mechanical damage to the vehicle on 5 key components of the player's truck: engine, transmission, chassis, cab, and wheels. This parameter is quantified as the average percentage value of damage among these vehicle elements. Loss refers to monetary loss expressed in dollars and simulates both the penalties that the driver incurs due to traffic violations while driving and the money that is deducted to pay for repairs to the vehicle in the event of mechanical damage. RMSSD data refer to the variation between RMSSD recorded before, during, and after the VR drive for each condition. A standardized side-effects questionnaire addressing general discomfort, headache, itching, and tingling during tRNS [267] was also administered for each experimental condition. The practical trial values obtained and corrected all the results shown from the first and second drives, both behavioral and physiological. Cognitive test scores were made considering the differences between the first and second administrations.

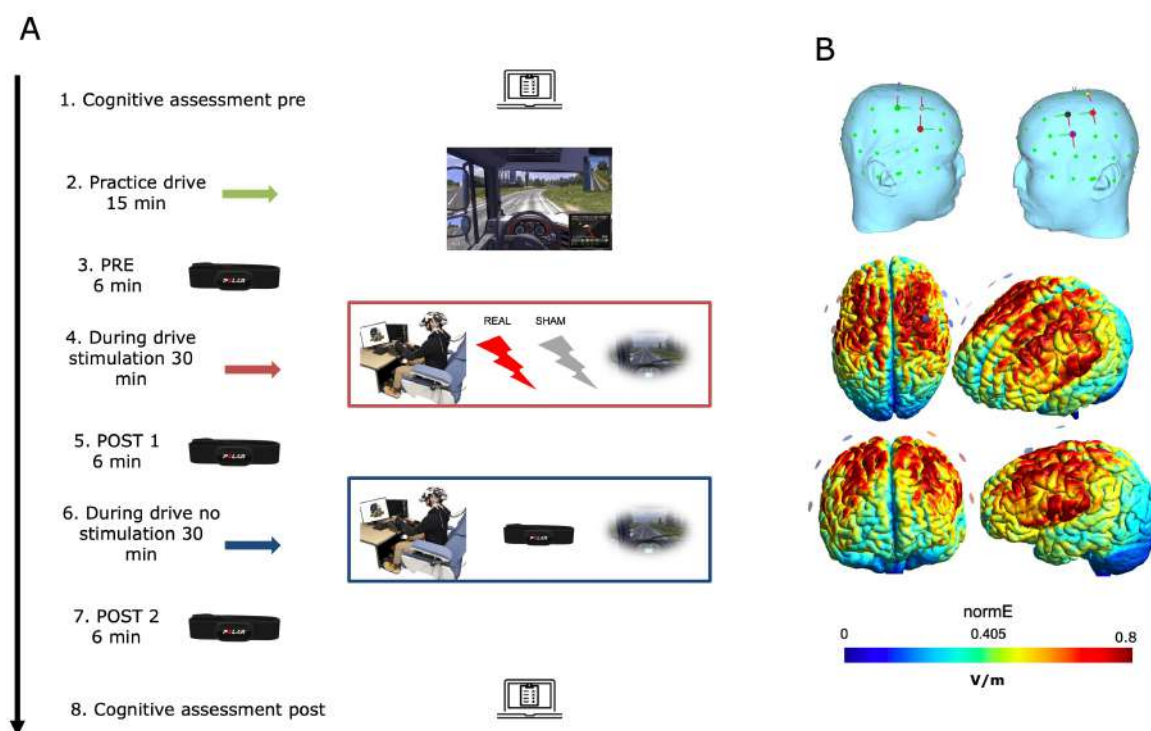


Figure 12. Study design. (A) Timeline of the study. Initially, tests used for cognitive assessment were administered (applied before and after driving tests); subjects were driving while wearing a VR headset (either during the first drive with real or placebo stimulation or during the second drive). (B): tRNS montage with 8 electrodes chosen to stimulate bilaterally SMA (MNI coordinates left: -28; -2; 52 right: 28; 1; 52), bilaterally MFG (MNI coordinates left: 45; 53; -7 right: 44; 34; 20) and M1 (MNI coordinates left: -36; -19; 48; right= 38; 18; 45): C1 (.85mA), C2 (-.85mA), C3 (1.3mA), C4 (-1.3mA), FC3 (.75mA), FC4 (-.75mA), FC5 (1.1mA) and FC6 (-1.1mA). The figure graphically shows the arising E-field (represented in NormE) resulting from the montage in V/m on a healthy example subject.

4.4 Statistical Analysis

JAMOVI® v.2.4 software (Jamovi project, 2023) was employed to analyze both behavioral differences in Drive Performance and VAS-F scores, the RMSSD cardiac parameter, SSQ (Simulator Sickness

Questionnaire), and stimulation side effects questionnaire. Within the scope of behavioral data analysis, the dependent variables consisted of self-reported Drive Performances (3 levels: practice trial, first drive performance, and second drive performance) and VAS-F (3 levels: VAS-F PRE, VAS-F POST 1 and VAS-F POST 2), while the selected independent variable was the stimulation condition (2 levels: tRNS and Sham). In analyzing physiological data, RMSSD (RMSSD PRE, RMSSD during the first drive, and RMSSD during the second drive) represented the dependent variables, and stimulation condition was the independent variable. Through the same procedure, we tested the levels of Cyber Sickness induced by VR and the stimulation Side effects. The Shapiro-Wilk test was used to check the normality of data distribution. The study employed the nonparametric Kruskal-Wallis Test to examine differences between groups. The grouping variable was the condition (real or sham), and the dependent variables were RMSSD, VAS-F, damages, and loss. The effect size (ϵ^2) is reported. Two separate analyses were conducted, each considering the four variables of the first and second drives. The Bonferroni correction method was applied to each drive analysis, correcting α to $0.05/4 = 0.0125$.

4.5 Results

Normal distribution was violated for all behavioral and physiological variables. Scores above or below two standard deviations from the mean were identified and eliminated using JAMOV[®] v.2.4 software. Comparing both behavioral results and physiological values, no differences were reported on truck damages and monetary loss for violations of the driving code (i.e., the two main outcome measures addressing performance) between the two subjects' groups during the practical trial, pointing out an equal baseline level among them (Tab.1); there were also no differences in each single cognitive test (Tab.4). Instead, significant differences between groups receiving real or sham stimulation during the first drive emerged only during the second drive trial (without stimulation) and concerned both truck damages and monetary loss (Tab.1) as well as Visual Analog Scale of fatigue (VAS-F) (Tab.3).

4.5.1 Driving Performance

Table 1 shows the different outcomes between the sham and the real tRNS condition regarding truck damages and monetary loss, comparing both the practice tests to reveal any basic difference

during the first and second drives. Significant differences between the two conditions are shown in Fig.13. The results show that there is no difference either during the test or compared with the first guide. Instead, compared to the second drive there is a significant increase in performance as shown by a reduction in fines and truck damage and a decrease in perceived fatigue

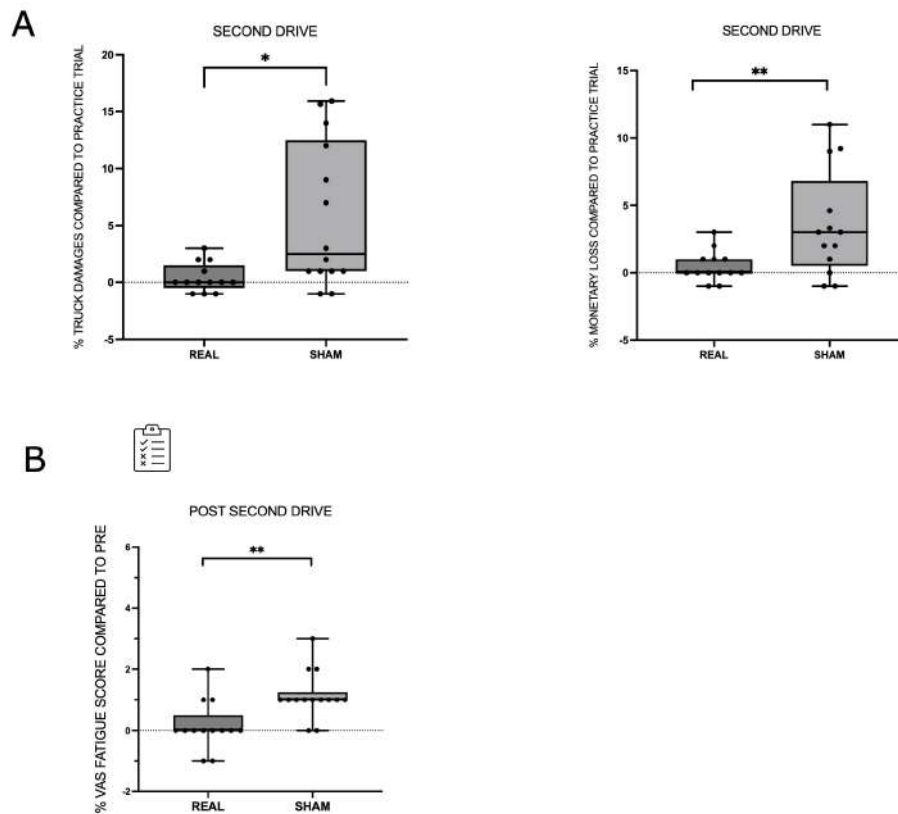


Figure 13. tRNS effects on driving performance and fatigue perception related to second driving session. (A): The graph shows the significant score differences between the sham and real condition regarding both damages (left) and monetary loss (right). The black line inside the graphs represents the median. (B): Shows significant differences in fatigue perception comparing sham and real conditions. * $p < .05$; ** $p < .01$

Table 1. Damages and monetary loss (US dollars)

	Sham	Real	P-value	ϵ^2
practice drive damages	2%	6%	0.236	0.005
practice drive loss	\$806	\$1227.5	0.548	0.013
first drive damages	4.6%	1.8%	0.765	0.004
first drive loss	6.9%	-1.5%	0.137	0.088
second drive damages	5.75%	0.38%	0.011	0.246
second drive loss	3.54%	0.26%	0.009	0.272

4.5.2 RMSSD

Table 2 shows the disparities in outcomes between the sham and real conditions in terms of cardiac activity during trials between Real and Sham. No significant differences were observed comparing RMSSD between conditions, despite the fact that a reduction in RMSSD following the Real condition can be observed with respect to both the first drive and the second drive, especially regarding the second one.

Table 2. Damages and monetary loss (US dollars)

	Sham	Real	P-value	ϵ^2
RMSSD pre	37.2	47.6	.430	0.022
RMSSD during first drive	-6.4%	-19.3%	0.068	0.145
RMSSD during second drive	-0.9%	-20.6%	0.053	0.139

Note: RMMSD= Root Mean Square of Successive RR interval differences

4.5.3 VAS-F

Table 3 illustrates variations in results between the sham condition and the real condition concerning perceived fatigue using the VAS-F. Results show a reduction in perceived fatigue, as assessed by the VAS-F, only relative to the second drive after Real stimulation.

Table 3. Perceived fatigue

	Sham	Real	P-value	ϵ^2
VAS-F pre	3.2	4	.074	0.110
VAS-F post first drive	0.9%	0.6%	0.127	0.090
VAS-F post second drive	1.14%	0.15%	0.003	0.346

Note: VAS-F = Visual Analog Scale Fatigue

4.5.4 Cognitive Test

Table 4 shows the results between the sham and real conditions of cognitive tests administered. No changes were observed in the cognitive assessment after the driving sessions compared with the initial assessment.

Table 4. Cognitive Test

4.5.5 SSQ

Table 5 shows no significant differences in SSQ scores between the two groups.

4.5.6 Side effects

Figure 14 shows the statistically significant differences between the sham condition and the real condition on side effects questionnaire scores. Stimulation-related side effects have, as expected, shown significantly higher scores after true stimulation than after Sham stimulation

	Sham	Real	P-value	ϵ^2
SRT reaction time	6.20 ms	-0.02 ms	0.322	0.036
GNG accuracy	0%	0%	0.678	0.006
GNG reaction time	-8.5 ms	-16.4 ms	0.141	0.080
UFOV reaction time	-244.5 ms	-304.3 ms	0.876	8.68e-4
UFOV accuracy	0%	0%	0.604	0.009
Visual search reaction time	-183.4 ms	-191.6 ms	0.144	0.082
Visual search accuracy	0%	0%	0.438	0.023

Note: SRT = Simple Reaction Time; GNG= Go No Go; UFOV= Useful Field of View

Table 5. Perceived Cyber Sickness

	Sham	Real	P-value	ϵ^2
SSQ post first drive	5.6	9.5	.341	.457
SSQ post second drive	6.6	9.8	.440	.155
SSQ total	6.3	9.6	.420	.247

Note: SSQ = Simulator Sickness Questionnaire

4.6 Discussion

Despite being a growing issue in the demanding modern society, cognitive fatigue is still understudied and there is a need for better characterization of the problem to develop possible countermeasures. There are no drugs to counteract cognitive fatigue in healthy individuals other than natural remedies with mixed effects [268]. Drugs counteracting fatigue are those used to reduce fatigue during diseases or therapies such as cancer therapy: they might have toxic effects [269] and hence their use is not justified in healthy people. The primary aim of the current study was to assess the feasibility and effectiveness of tRNS in mitigating cognitive fatigue and enhancing performance in healthy individuals within a real-world ecological context, specifically during a VR truck driving experience. The choice of tRNS



Figure 14. tRNS side effects This figure presents the disparities in side effects between the two conditions, revealing elevated scores for all items in the real condition. **** $p < .0001$

over other NiBS techniques was mainly dictated by two reasons: tRNS' effects on cortical excitability have been shown to be stronger than those of tDCS [75], and longer-lasting after effects than tDCS have been shown as well [2, 78, 98]. Last, the use of tRNS has been associated with improvements in performance in complex cognitive tasks relevant to the task used in the present study [270]. Repetitive transcranial magnetic stimulation was not considered given the limitations of using such a solution outside the laboratory settings. To achieve our goal, the application of tRNS was biophysically modeled to target what is referred to as the cortico-subcortical "anti-fatigue network," which includes regions such as the bilateral SMA, the MFG, and the M1 [132]. Previous research in the field of neuroenhancement highlighted a significant advantage of NiBS: its functional effects persist for up to 60 minutes after stimulation [271]. The experimental design was thought to allow better evaluation of subjects both during and after stimulation. Results confirm that the neurophysiological effects described for tRNS on cortical excitability can be translated at a behavioral level. Indeed, during the initial VR drive, no significant differences were observed between the sham group and those who received real tRNS. More importantly, significant performance improvements, in terms of reduced truck damages and less monetary expenses for violations of the driving code, emerged during the second drive, only in individuals who had previously undergone the driving trial during real tRNS. This suggests no immediate effects of tRNS (i.e., during the first drive), but rather a prolonged neuromodulatory after-effect, that is particularly relevant from a translational point of view. Such lasting behavioral benefits, that overall reflect improved allocation of spatiotemporal and attentional resources and more efficient reactivity,

are not associated with a significant reduction of the HRV-RMSSD parameter. However, we observed a gradual reduction from the first to the second drive that might reach statistical significance by expanding the sample size, given that there are distinct patterns in the parasympathetic system activity following real and sham stimulation. However, physiological relationships between cognitive fatigue and neurovegetative activity [272] are complex and often discordant: when measured by RMSSD, some argue that a decrease in parasympathetic tone is associated with high cognitive fatigue [273, 274], while others conclude that a decrease in parasympathetic tone corresponds to an increased state of cognitive fatigue [165, 275]. Our result, however, would indicate that after real stimulation, subjects report a decrease in parasympathetic tone indicative of an increase in alertness [276] useful in real-life situations without experiencing fatigue, as demonstrated by scores on the VAS-F scale. This reduction could also be due to the nature of the task itself, as it is very stimulating, which (as explained in the introduction) could lead to a decrease in RMSSD. Although the subjects did not report negative experiences related to the stimulation after the first drive, significant differences emerged from the side effects questionnaire between the sham and real stimulation, and this latter result could explain the consequent decrease in parasympathetic tone [277] during the second drive. The fact that side effects were greater (albeit still small) during real stimulation than during sham reinforces the importance of the beneficial effects of tRNS during drive performances. Even though the results indicate differences between the two groups, these did not show significant changes in the level of general cognitive performance at the final assessments. This suggests that the effects of tRNS stimulation while driving are independent of changes related to the overall subjects' cognitive abilities, at least those investigated by the employed tests. This could potentially lead to the standardization of tRNS administrations based solely on the required duration of the effect, irrespective of the individual cognitive ability level, thus opening the door to large-scale practical applications in various settings with imbalances in mental workload.

Cognitive fatigue is not merely a disruption in cognitive functioning, but rather a multifaceted symptom arising from the concurrent activation and deactivation of overlapping neural networks [132]. It is noteworthy that the regions identified as part of the cortico-subcortical anti-fatigue network also play a role in the reward network, reinforcing the notion that cognitive fatigue is strongly linked to an imbalance between effort and reward [144]. Furthermore, the results underscore the pivotal role

of motivation and reward in balancing the mental facilitation and inhibition systems. Motivational input, primarily through enhanced dopaminergic and norepinephrine tone [278], activates the facilitation system, effectively compensating for the effects of cognitive fatigue [140].

A last point to consider is the cybersickness symptom, one of the major VR side effects. The use of NiBS techniques has already yielded positive results in reducing this symptom as shown by the study [279] where the transcranial alternating current stimulation at 10 Hz applied on the vestibular cortex significantly reduced cybersickness in healthy subjects undergoing a VR-based rollercoaster ride. In the current study, however, considering that both the type of stimulation and target areas were different, participants' cybersickness remained unchanged throughout driving trials. The trend of slightly increased cybersickness in the real group compared with the sham group did not affect driving performance.

In conclusion, this study presents a novel approach involving non-invasive brain stimulation to sustain high levels of focus, particularly in occupations characterized by substantial workloads, such as professional drivers. The implications of these findings extend beyond this specific context and hold significant potential for enhancing performance and safety in other demanding professions. Considering also the strong link between cognitive fatigue and physical fatigue [142, 280], and the existing utilization of noninvasive brain stimulation techniques like tDCS in the context of physical fatigue [133, 182], the findings of this study open the door to the prospect of introducing a novel treatment approach and launching further research endeavors aimed at improving athletic performance in endurance activities like marathons or triathlons. The proposed approach is also worth testing in neurological disorders where fatigue is a central symptom, such as multiple sclerosis or Parkinson's disease, to accelerate their rehabilitation program. The latest field of potential translational application may be the gaming and e-sport world; demanding games such as first-person shooter, racing, and real-time strategy games. The last area of potential translational application may be the world of gaming and e-sports; challenging games such as first-person shooters, racing, and real-time strategy games. By decreasing fatigue perception, this stimulation technique would maintain both a high focus on the right target as well as good visuomotor coordination.

4.7 Limitations of the study

The findings suggest that tRNS when precisely targeted to the cortical areas of the brain “anti-fatigue” network, could represent a novel strategy for alleviating cognitive fatigue and enhancing performance compared to placebo stimulation. This study, however, has several limitations, primarily related to the relatively limited sample size, which could be expanded in future research endeavors. Due to experimental time constraints, EEG analysis could not be conducted, but assessing EEG activity before, during, and after stimulation could offer further validation of the positive effects of tRNS on cognitive fatigue by visualizing cortical activity dynamics. The stimulation method may have an impact on changing cardiac activity [281], so in a future phase of the study, it will be appropriate to make a recording of the RMSSD during stimulation alone (i.e., without concomitant tasks) to obtain an individual baseline during stimulation to adjust the parameter obtained during the task with its stimulation-related baseline. Additionally, future research could benefit from longer-term follow-ups to investigate the eventual sustained effects of tRNS. Since these findings emanated from a single-day experiment and a single session of stimulation, future studies should investigate whether subjects undergoing sequential stimulations over an extended period could yield even more pronounced performance improvements. It is also important to acknowledge the subjective nature of reporting cognitive fatigue as a limitation, which is inherent in cognitive fatigue evaluations; however, the observed changes in parasympathetic activity mitigate the intrinsic weakness of the subjective report. Subsequent investigations could explore the effects of tRNS without practical driving tests during stimulation to determine if the behavioral and physiological responses observed in this study remain consistent or undergo changes.

5 Neurophysiological and imaging hallmarks of central fatigue in Multiple Sclerosis

5.1 Abstract

CF affects 80% of MS patients, with 55% deeming it the most disabling symptom. Current research often lacks a multidimensional approach by exploring neurophysiological and structural factors separately. This study aimed to define the structural and functional bases of fatigue in MS using a multidimensional framework. Here, we recruited 41 RRMS patients and 21 HCs. Based on FSS, patients were classified as fatigued (MS_F: 19, $FSS \geq 4$) or non-fatigued (MS_NF: 22, $FSS < 4$). Participants underwent a three-day investigation consisting of TMS, resting state EEG, structural (FLAIR, T1, DTI), rsfMRI, clinical assessment, blood test for the detection of Neurofilament light chain and Serum Glial Fibrillary Acidic Protein as well as cytokines levels detection. Our results showed a higher theta band power in the MS_F group compared to the MS_NF group in frontocentral regions during eyes-open condition, and both lower theta band power, and alpha band power over on central and frontal regions; a reduction in power was observed, considering higher frequencies, in both beta and gamma bands diffusely over front, middle and rear areas during eyes-closed condition. Lower TMS-based ICF compared to MS_NF has been observed as well. Finally, increased FC between the Supracalcarine Cortex right and both Frontal Pole left, and Superior Frontal Gyrus left and among MS_F patients, a greater myelin sheath disruption was evident mostly in the corticospinal tracts and corpus callosum measured by FA. No differences were found in fMRI measurements, lesion number/volume, brain volumes, clinical measures, and biomarkers between groups. Finally, using the Matlab Decision Tree application as classification learner, a reduction of ICF was identified as the main predictor of fatigue, with an 87.2%

accuracy. We concluded that MS CF is marked by resting fatigue, as showed by EEG, underpinned by altered axonal integrity; reduced glutamatergic activity as indexed by reduced ICF, regulates this state. These findings might be used as new clinical tests and may constitute the neurophysiological background of new neuromodulatory approaches for treating fatigue.

5.2 Materials and Methods

In this cross-sectional study, we recruited 41 RRMS patients and 21 HCs (F: 14; mean [$\pm$ SD] age: 41.8 ± 10.8 years). All patients were fully right-handed according to Oldfield's questionnaire [282], and MS diagnosis was made according to the McDonald criteria [283]. Based on the FSS [284], patients were classified as fatigued **MS_F**: 19, FSS > 4 ; 14 F; mean [$\pm$ SD] age: 41.8 ± 10.8 years; Expanded Disability Status Scale (EDSS): 2.0 [0–3.5]; disease duration: 107.7 ± 90.7 months. **MS_NF**: 22, FSS < 4 ; 11 F; mean [$\pm$ SD] age: 42.4 ± 9.6 years; median [range] EDSS: 1.0 [0–2.5]; disease duration: 107.5 ± 91.2 months. The study protocol was approved by the local ethics committee, and all subjects gave their informed consent to participate in the experiments. Participants underwent a three-day investigation consisting of TMS, resting-state EEG, structural (FLAIR, T1, DTI), rsfMRI, clinical assessment, blood test for the detection of Neurofilament light chain and Serum Glial Fibrillary Acidic Protein, as well as cytokine levels detection. Exclusion criteria were contraindications for TMS (epilepsy, heart pacemaker, metallic implants in the brain, pregnancy) [31], relapses in the previous 3 months, paresis of the upper limbs, ingestion of drugs known to influence motor cortex excitability (e.g., antidepressants, benzodiazepines, neuroleptics, anticonvulsants), and the occurrence of new neurological symptoms within four weeks before the investigations.

5.2.1 Study protocol

Figure 15 shows the experimental setting and design. The study consists of 4 phases: the first phase includes MRI (50 min long); the second phase includes a neuropsychological assessment (60 min long), the third phase a TMS session (30 min long) and, finally, a rest EEG recording (3 min Eyes-closed (EC)/3min Eyes-open (EO)). All 4 phases of the study were performed on different days except the TMS and EEG recording, which were performed on the same day 2 hours apart: stimulation first

and then EEG. Patients' blood samples were taken during their own clinic visits or checkups.

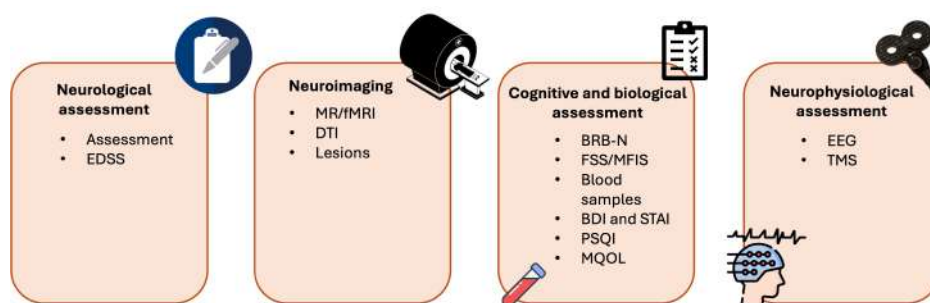


Figure 15. Summary of multidimensional investigations. From left to right, the four phases of the process are evident: the initial phase encompasses recruitment, neurological and clinical assessment; the second phase is associated with neuroimaging investigation; the third phase is related to the collection of biological samples and cognitive and psychological assessment; and the fourth phase is concerned with the collection of neurophysiological parameters.

5.2.2 Neuropsychological, Psychological, and Clinical Assessment

All participants underwent a neuropsychological evaluation with the Rao Brief Repeatable Battery [285]. To assess dexterity and movement, the 9-hole peg test (9-HPT) and the timed 25-foot walk test (TWT) were administered. The Brief Repeatable Battery evaluates the most frequently impaired cognitive domains in MS, incorporating tests of verbal learning and memory (Selective Reminding Test [SRT]), including Long-term Storage, Consistent Long-term Retrieval, and delayed recall; visual or spatial learning and memory (10/36 Spatial Recall Test [SPART]) and its delayed recall; complex attention and information processing speed (Paced Auditory Serial Addition Test [PASAT] and Symbol Digit Modalities Test [SDMT]); and verbal fluency on a semantic stimulus (Word List Generation [WLG]). Depression was evaluated using the Montgomery-Åsberg Depression Scale (MADRS) (score range: 0–60, with the highest score indicating more severe depression) [286].

5.2.3 Blood samples

Serum samples collection

Serum samples were collected between 8 and 10 a.m. following an overnight fast. The blood was immediately centrifuged to separate the serum, which was then collected into sterile polypropylene tubes and stored at -80 degrees Celsius.

NfL and GFAP Assay

The concentrations of neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) were determined in patients' serum samples using commercially available immunoassay kits for NfL and GFAP – Simoa™ assay Neurology 2-Plex B (GFAP, NfL) Assay Kit (Catalog #103520; Quanterix, Billerica, MA, USA). The assays were conducted on the semi-automated ultrasensitive SR-X Biomarker Detection System (Quanterix). Samples were diluted at a ratio of 1:4 and randomly distributed on 96-well plates. Quality control (QC) samples, provided with the kit, exhibited concentrations within the predefined range, and the coefficient of variance across the plates was maintained below 10%. All samples were analyzed blindly under alpha-numeric codes, and diagnostic codes were disclosed only after QC-verified NfL and GFAP concentrations were reported to the database manager. Concentrations were measured in pg/ml and documented in the database.

Cytokines Assay

Concentrations of Cytokines in patient serum sample were determined using a multiplex bead-based flow cytometry assay (LEGENDplex HU Essential Immune Response Panel, BioLegend, San Diego, CA, USA) which include detection of IL-4, IL-2, CXCL10 (IP-10), IL-1 β , TNF- α , CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN- ϵ , IL-12p70, CXCL8 (IL-8), TGF- β 1 (Free Active Form). The FACSCanto II flow cytometer and LEGENDplex™ version 8.0 software (BioLegend San Diego, California, USA) were utilized for sample analysis. Samples were diluted at a ratio of 1:2 and randomly distributed on the plates. Concentrations were measured in pg/ml and recorded in the database. Before analysis, the cytometer underwent calibration using set-up beads according to the manufacturer's protocol.

5.2.4 TMS recordings and analyses

TMS was performed with a butterfly focal 70-mm coil connected to an ATEs magnetic stimulator (EBNeuro, Italy). With the participant seated, the TMS coil was maintained tangentially to the scalp with the handle pointing backward and laterally at an angle of 45° from the midline, perpendicular to the central sulcus. This coil position delivers posterior-anterior directed pulses that are known to produce large MEPs resulting from the gradual recruitment of indirect waves [287, 288]. The cortical representation of the First Dorsal Interosseous (FDI) was targeted within the left M1. The use of a focal coil guaranteed stable simultaneous MEPs from the considered hand muscle. To measure EMG activity and collect the MEPs, foam surface electrodes were placed on the belly of the FDI muscle, with the ground and reference electrodes placed on the styloid process and the interphalangeal joint of the index finger, respectively. Once the best-fitting scalp position for the coil targeting M1 was identified, it was maintained throughout the experiment using a neuronavigational system (BrainNET, EBNeuro Ltd, Florence, Italy) with infrared cameras (Polaris Vicra, NDI, Waterloo, Canada). An MRI template for each participant was uploaded in the neuronavigational software, and a co-registration procedure was performed using scalp landmarks (nasion, vertex, and the two preauricular points) and additional landmarks positioned on a plastic glasses frame worn by the subjects. The coil was calibrated using an in-house algorithm based on five landmarks specific to the device. During TMS, the software provided online visual feedback, allowing the investigators to maintain a consistent coil orientation, rotation, and distance (with a tolerance of 2 mm for each spatial axis: x, y, and z) throughout the session. Corticospinal responses (or MEPs) were recorded using surface electrodes placed with a belly-tendon montage. The analysis time base was 50–100 ms, and bandpass acquisition filters were set at 20–20,000 Hz. The gain of amplifiers was adjusted to fully display the negative-positive evoked peaks of MEPs without saturation. Resting Motor Thresholds (RMT) were determined as the minimum TMS intensity, expressed as a percentage of the maximal stimulator output (0–100%), necessary to elicit 5 out of 10 MEPs with a peak-to-peak amplitude of 50 μ V during muscle relaxation [23]. To measure CSP, 15 pulses at 120% of RMT were delivered [23] while the subject pressed between the index finger and thumb, exerting 20% of maximal contraction. The background EMG activity was monitored on a computer, and subjects were asked to maintain an active contraction (index finger against thumb) at approximately 20% of their maximum force until instructed to relax [289]. The

EMG signal was continuously displayed to both the subject and experimenter as feedback to maintain consistent contraction levels during CSP measurement. CSP was measured as the length of time from the MEP onset ($+2SD$ from baseline EMG mean) to the return of voluntary tonic EMG activity. CSP duration was obtained by averaging 10–15 traces per subject [23]. MEP latency was calculated from the valid MEPs recorded during RMT assessment as the time in milliseconds from the TMS stimulus to MEP onset, defined as the point where the MEP exceeded $\pm 2SD$ from the EMG background activity [23]. Paired TMS was applied to test SICI and ICF [225]. The first subthreshold stimulus was delivered at 80% of RMT, followed by a test stimulus set at 120% of RMT, producing a MEP of approximately 1 mV. A 3-ms ISI was used for SICI, and a 10-ms ISI for ICF. For each ISI, 15 stimuli were administered randomly 5–8 seconds apart. This paired-pulse paradigm is known to test the excitability of intracortical neuronal circuits [19, 21, 290]. Recordings were made during complete muscle relaxation. All subjects' recordings were taken from the right FDI muscle with surface electrodes employing biphasic stimuli for RMT, CSP, SICI, and ICF. Results were stored on an EMG machine (Next, EB Neuro) and analyzed offline.

5.2.5 EEG preprocessing and analyses

HD EEG data were acquired using the g.tec amplifier (g.[®]HIamp, g.[®]USBamp) and a 64-channel cap to record 3 minutes with eyes closed and 3 minutes with eyes open at rest. Sixty-four electrodes were placed on the scalp of each participant following the international 10-20 EEG system. Two additional electrodes were placed vertically above and beneath the left eye (electrooculography (EOG)), and electrodes for the left and right mastoid placements were incorporated into the EEG cap. The recording electrodes were digitized at a sampling rate of 256 Hz, and the impedance for all electrodes was maintained below 10 k Ω . The ground electrode was positioned at the Nz location. Preprocessing was conducted using the open-source EEGLAB toolbox [291] for MATLAB (Mathworks, Inc.). The continuous signal was first filtered using a Finite Impulse Response filter between 1 and 90 Hz and Notch filtered at 50 Hz (46–54 Hz). Independent Component Analysis was performed to remove stereotypical artifacts such as eye blinks, muscular activity, and heartbeat. Components were retained after a visual inspection of the power spectral density, topographical maps, and time course of each estimated component. Channels previously removed due to poor signal quality were reconstructed using spherical spline interpolation. External channels were excluded without subsequent interpolation to optimize

the analysis on the 61 averaged and referenced channels. Spectral power analysis was carried out using the MATLAB-based Fieldtrip Toolbox [292]. A grand average was computed for each group among all subjects to perform the time-frequency analysis. The "multitaper" method was used to estimate the power spectrum, employing multiple tapers (window functions applied to the signal to minimize spectral leakage) to reduce variance. The Hanning window was chosen to refine the analysis further. The frequencies of interest for the analysis ranged from 1 Hz to 90 Hz, with increments of 0.5 Hz. After computing the time-frequency of the grand average, a cluster-based permutation test was conducted. The method for neighborhood definition was set to 'triangulation'. Statistical analysis was performed using the 'montecarlo' method, and the test statistic was an independent samples t-test with a cluster-based correction. The cluster statistic method was set to 'maxsum', and a minimum of three neighboring channels was required to form a cluster. The distribution tails were set to zero. The significance level for the test was set at 0.05, and the number of randomizations for the permutation test was set to 10,000. Using this approach, comparisons were made between the three groups. Analyses were conducted for the theta, alpha, and beta frequency bands, based on the literature. Both the eyes-open and eyes-closed conditions were analyzed separately.

5.3 MRI acquisition and processing

MRI data were acquired with an eight-channel head coil on a three-Tesla MR scanner (Philips Medical Systems, Best, The Netherlands) located at Meyer University Hospital, Florence. A sagittal survey image was used to identify anterior and posterior commissures. Sequences were acquired in the axial plane parallel to the bicommissural line. A dual-echo, turbo spin-echo sequence (repetition time (TR)/echo time (TE)₁/TE₂ = 4000/10/100 ms, voxel size = 1×1×3 mm) yielded proton density (PD) and T2-weighted (T2W) images. DTI data were an echo-planar imaging sequence (TR = 7036 ms; TE = 196 ms; voxel size = 2.5 mm³) with 32 diffusion directions and b-value = 900 s/mm². DTI acquired had these parameters: TR = 7036 ms; TE = 96 ms, flip angle = 90°, voxel size = 2.5 mm³, 50 slices, diffusion sampled on the half sphere with 32 diffusion directions and b-value = 900 s/mm²; fMRI acquired had these parameters: 200 volumes, TR = 3000 ms, TE = 35 ms, flip angle = 90°, voxel size = 1.87×1.87×4 mm, 30 slices. A high-resolution T1-weighted image (T1W, TR = 10 ms, TE = 4 ms,

voxel size = 1 mm³) was also acquired for image registration, anatomical mapping, and analysis of GM concentration. All images were visually checked and analyzed centrally. Brain tissues and structures masks were created using an in-house developed pipeline, using FSL tools (<https://fsl.fmrib.ox.ac.uk>). To investigate voxel-wise differences in GM and WM volumes, we first created a study-specific GM and WM mask template. This was performed by registering each subject-specific GM and WM mask to a standard-specific space. Then, each patient-specific GM and WM mask was registered on the GM and WM template previously created.

5.3.1 fMRI Preprocessing and analyses

Functional and anatomical MRI data processing and statistical analyses were performed using the CONN toolbox (RRID: SCR_009550) version 22.a [293] and SPM version 12 release 7,771 (Wellcome Department of Imaging Neuroscience, UCL, London, United Kingdom) in MATLAB R2022a (The MathWorks Inc., Natick, MA, United Kingdom) using CONN's default minimal preprocessing pipeline. This pipeline includes the functional realignment and unwarp for intermodality coregistration of all scans to the first scan, slice-timing correction ([294]) compensating for acquisition time differences among different slices, outlier detection [295] identifying individual scans with suprathreshold frame-wise displacement and/or global signal change values, direct functional normalization [296] projecting functional images into standard MNI reference space resampled to 2mm isotropic voxels, and spatial smoothing with a 8 mm full width at half maximum Gaussian kernel. Anatomical data preprocessing comprised direct segmentation and normalization [297] which iteratively performed tissue segmentation into six tissue classes, including gray matter, white matter, and CSF using SPM12 posterior tissue probability maps, and normalization to IXI-549 MNI space, resampling the output anatomical images to 2 mm isotropic voxels. Group-level analyses were performed using a General Linear Model [298]. For each connection, a separate GLM was estimated, with first-level connectivity measures at this connection as dependent variables (one independent sample per subject and one measurement per task or experimental condition, if applicable), and groups or other subject-level identifiers as independent variables. Connection-level hypotheses were evaluated using multivariate parametric statistics with random effects across subjects and sample covariance estimation across multiple measurements. Inferences were performed at the level of individual clusters (groups of similar connections). Cluster-level inferences were based on parametric statistics within and between each pair of ROIs [299], with ROIs

identified using a complete-linkage hierarchical clustering procedure based on ROI-to-ROI anatomical proximity and functional similarity metrics. Results were thresholded using a combination of a $p < 0.05$ connection-level threshold and a familywise corrected $p\text{-FDR} < 0.05$ cluster-level threshold [300]. Analyses were performed on structural atlas Atlas, to observe any differences between groups.

5.3.2 Brain Volumetric assessment

Brain volumes were segmented on the T1-weighted (T1-W) image, the sequence that provides the primary contrast between grey matter and white matter tissues. Before segmentation, T1-W images were lesion-filled to avoid bias in grey matter volume measurement [301]. Brain segmentation was performed using SIENAX [302], a part of the FSL (FMRIB Software Library) suite [303]. SIENAX estimates normalized brain, grey, and white matter volumes using its pipeline. SIENAX starts by extracting brain and skull images from the whole-head input data [302]. The brain image is then affine registered to a standard space [304, 305] using the skull image to determine the registration scaling, primarily to obtain the volumetric scaling factor for normalizing head size. Next, the brain image is segmented into cerebrospinal fluid, grey matter, and white matter [306]. Grey and white matter brain volumes are then determined by multiplying the volumes of these segmented masks by their respective scaling factors. The normalized whole brain volume is calculated as the sum of the grey and white matter volumes. Subcortical structures (TH, Caudate, Putamen, Pallidus, Hippocampus, Amygdala, Nacc) were segmented using FIRST, as provided by the FSL library, and their volumes were normalized using the scaling factor obtained from SIENAX [307].

5.3.3 WM lesions detection and analyses

To assess lesion distribution, white matter lesions were semi-automatically segmented on FLAIR/T2-hyperintense images using an in-house developed deep learning tool, and lesion masks were created. The accuracy of lesion segmentation was double-checked for each subject by an expert user (R.C.) and manually edited when necessary, using FSL View. Lesion volumes were subsequently obtained. Cortical lesions were assessed on double inversion recovery (DIR) images. Binarized lesion masks for each patient were registered onto the MNI152 standard brain through FSL tools using linear registration (FLIRT, FMRIB Linear Image Registration Tool) [304], followed by nonlinear registration (FNIRT, FMRIB Non-linear Image Registration Tool) [308]. A lesion probability map (LPM) was generated

by merging and averaging all lesion masks that were registered onto the standard brain. Means, medians, and proportions of demographic and clinical features were calculated for patients (and their subgroups) and HCs. Differences were evaluated using ANOVA. Voxel-wise analyses were performed using permutation testing, as implemented in the FSL "randomize" program [309]. Differences in grey matter volumes and lesion load between each patient group and the control group, as well as between each pair of disease groups, were assessed using design matrices within general linear models, with disease as the variable of interest and age and sex as covariates. Voxel-wise analyses were carried out using GM and lesion masks as input images for the "randomize" tool (i.e., the dependent variable). Within these masks, the tool detects regions with increased GM atrophy or increased lesion load. For significant results ($p < 0.05$, corrected for multiple comparisons), the number of voxels (nV) is reported for GM atrophy, and both the number of voxels and the coordinates of the most significantly different voxels are reported for lesion load (LPM).

5.3.4 WM preprocessing and DTI analyses

DTI data were preprocessed using the FSL program Eddy, which corrects for eddy current-induced distortions, subject movement, and signal dropout [310]. The FMRIB Diffusion Toolbox (FDT) was used to obtain both FA and Mean Diffusivity (MD) images by fitting a diffusion tensor model at each voxel. FA and MD images for all subjects were first registered to a standard-space image (FMRIB58_FA) using FNIRT [240]. These images were then smoothed using a 3-mm sigma Gaussian kernel (equivalent to 7-mm FWHM). For each MS patient, lesion mask information was incorporated during the FNIRT step to avoid over-distortion of lesion areas. Voxelwise statistical analysis of both FA and MD data was conducted using TBSS (Tract-Based Spatial Statistics) [240], part of the FSL suite [303]. First, FA and MD images were created by fitting a tensor model to the raw diffusion data using FDT, followed by brain extraction with BET [302]. All subjects' FA and MD data were then aligned to a common space using the nonlinear registration tool FNIRT, which applies a b-spline representation of the registration warp field [311]. Next, the mean FA and MD images were generated and thinned to create mean whole-brain FA and MD skeletons, representing the centers of all tracts common to the group. Each subject's aligned FA and MD data was then projected onto this skeleton, and the resulting data were used for voxelwise cross-subject statistics.

5.4 Statistical Analysis

Statistical analyses on either behavioral, biological, structural MRI or physiological variables derived from TMS were performed with JASP software (JASP Team (2024) and SPSS software while fMRI and EEG analyses were performed with Matlab CONN and Fieldtrip toolboxes, respectively; normality of variable distributions were tested with the Shapiro-Wilk test. Kruskal-Wallis analyses on age, gender, education and Body Mass Index (BMI), EDSS, MADRS, and disease duration were applied respectively. As the TMS values, blood, lesion, DTI scores followed non-normal distribution, they were analyzed by the method Kruskal-Wallis using the MADRS scores as covariates. Dunn's test was used as a post-hoc test for Kruskal-Wallis; all reported results are $p < .05$, Bonferroni corrected. To verify that these variables correlated with the FSS variable, we used a Spearman correlation analysis. For analysis on the parametrically distributed data (such as brain volumes and fMRI scores), we used ANCOVA analysis, MADRS scores have been used as covariates to test differences between groups. Finally, to explore significant variable weights able to classify MS_F group, we used a Decision Tree in MATLAB with the Classification Learner application, the 'Statistics and Machine Learning Toolbox (version 11.4), and as input we chose all significant outputs. For this last analysis, we considered MS patients only because the variable to be estimated was the group variable (1=MS_F; 2=MS_NF), without thus including HCs.

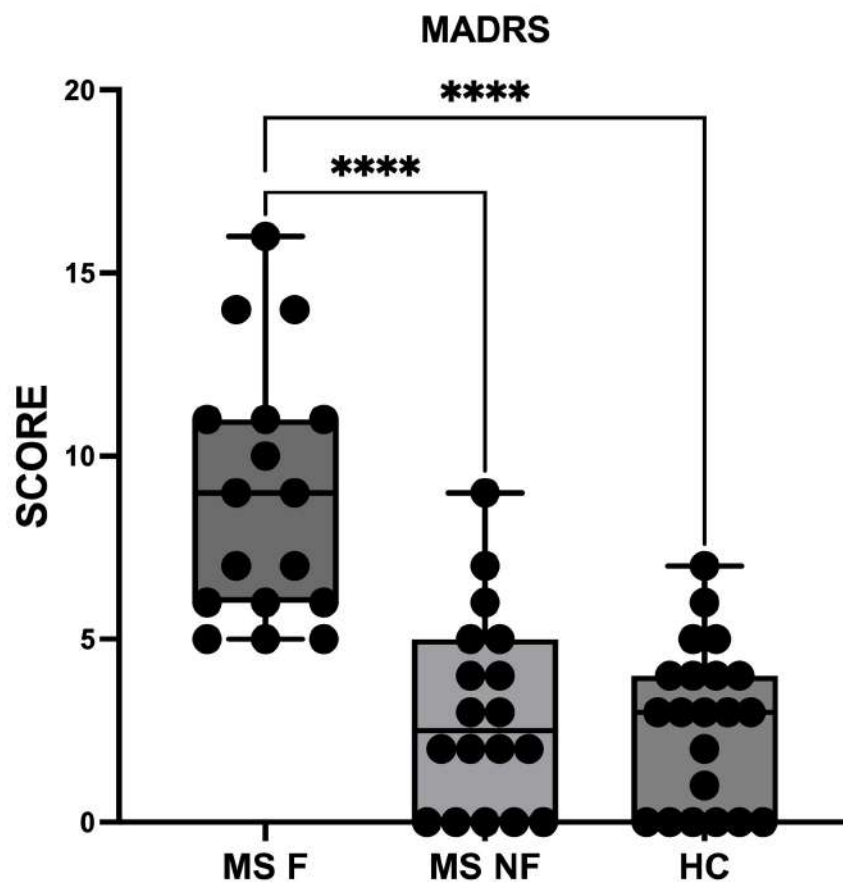
5.5 Results

No differences existed among the 3 groups according to both genders ($p = 0.193$) and age ($p = 0.969$); on the other hand, regarding the education level, the healthy group reported a higher level than the group of patients with fatigue ($p = 0.013$). No differences were found between the groups concerning Body Mass Index (BMI) ($p = 0.091$), and disease duration ($p = 0.995$) but a significantly higher EDSS for patients with fatigue compared to patients without fatigue ($p = 0.005$). Additionally, patients with fatigue reported a significantly higher score at MADRS than both the HCs group ($p = < .001$) and patients without fatigue ($p = 0.012$) (Fig. 15) and lower scores at Multiple Sclerosis Quality of Life-54 (MSQOL-54) ($p = 0.018$). Descriptive results have been reported in Table 6.

Table 6. Descriptive results

	Sample	Age	Gender	BMI	Ed	Treat	Dur	FSS	EDSS
MS F	19	41.8 $\pm$ 10.8	0=13;1=6	24.8 $\pm$ 5.8	13.3 $\pm$ 3.5	0=7; 1=5	107.7 $\pm$ 90.7	5.2 $\pm$ 0.8	1.7 $\pm$ 1.0
MS NF	22	42.4 $\pm$ 9.6	0=11;1=11	25.4 $\pm$ 5.3	14.1 $\pm$ 3.1	0=12; 1=5	107.5 $\pm$ 90.9	2.4 $\pm$ 0.8	1.1 $\pm$ 0.3
HCS	21	41.7 $\pm$ 10.7	0=14;1=7	22.1 $\pm$ 2.8	16.4 $\pm$ 2.8	-	-	-	-

Note: MS_F=Multiple Sclerosis with Fatigue; MS_NF=Multiple Sclerosis without Fatigue; HC=Healthy Controls; EDSS=Expanded Disability Status Scale; FSS=Fatigue Severity Scale; BMI=Body Mass Index; Ed=Education; Treat=Pharmacological Treatment (0=1st line; 1=2nd line; Dur=DISEASE DURATION (months); Gender=(0=F;1=M)

**Figure 16.** MADRS comparisons between groups. ****p<.0001

5.5.1 TMS

Using the MADRS variable as a covariate ICF (Statistic = 28.73; $p < 0.001$) reported different results among groups. Specifically, as shown in Fig. 17 patients with fatigue reported reduced ICF ($p < .001$) compared with the group of patients without fatigue; the reduction of ICF in MS_F patients was definitely lower also versus HCs, but this result did not reach significance. Furthermore, a significant difference was also observed between the MS_NF group and HCs ($p = .005$). When using the EDSS and MSQOL_54 variables as covariates, comparing only the two groups of patients, no differences were found.

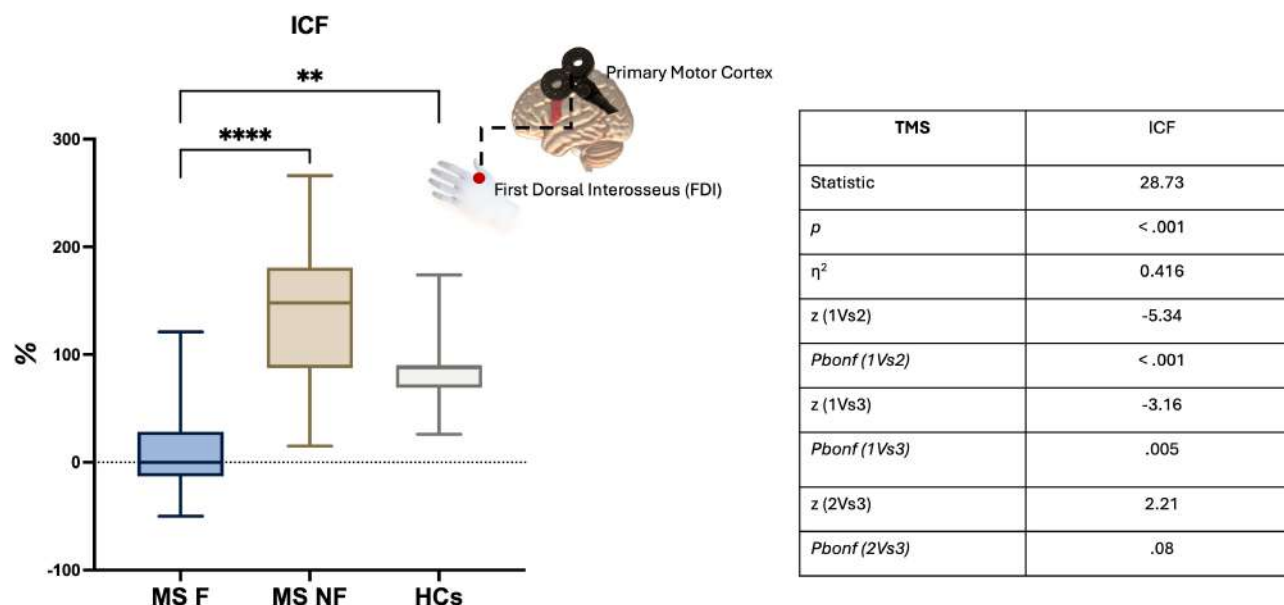


Figure 17. The graph represents the ICF comparisons between the 3 groups. ** $p < .005$, **** $p < .001$

5.5.2 Blood samples

No differences were observed concerning the blood sample variables between the two groups of patients. The blood samples were used to evaluate the concentrations of NfL and GFAP, as well as the levels of IL-4, IL-2, CXCL10 (IP-10), IL-1 β , TNF- α , CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN- γ , IL-12p70, CXCL8 (IL-8), and TGF- β 1.

5.5.3 WM lesions

No differences were observed concerning the number of lesions and lesion volume between the two groups of patients.

5.5.4 DTI

Figure 18 presents the white matter bundles in which patients with fatigue exhibited lower FA than those without fatigue, with the MADRS score as a covariate. All significant different bundles between MS_F and MS_NF are the Genu of Corpus Callosum, the Left Cortico spinal tract (CST), the Left Posterior Limb of internal capsule, the Right Posterior thalamic radiation, the Right External Capsule, the Right Cingulum, Right Superior, the Longitudinal Fasciculus (SLF), and the Right Inferior fronto occipital Fasciculus.

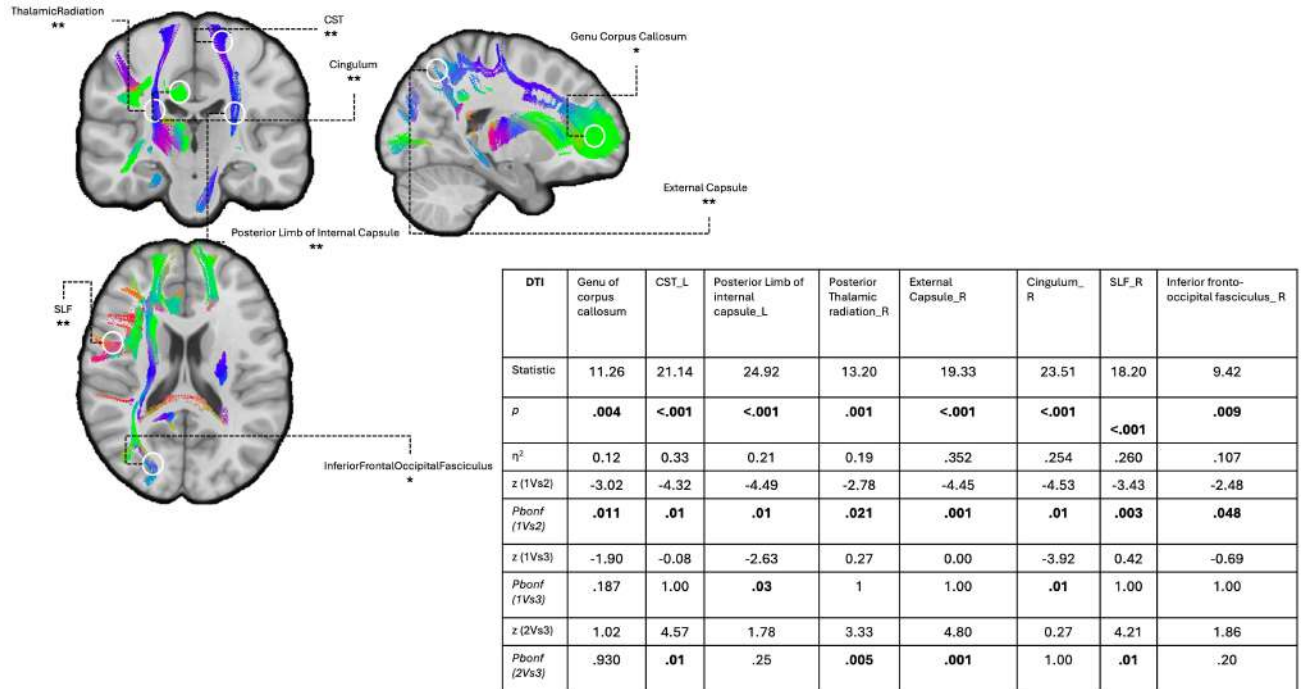


Figure 18. shows the WM bundles in which FA is reduced in the MS_F group compared with the MS_NF group. * $p < .05$, ** $p < .01$

5.5.5 EEG

Figure 19 shows topographic maps of the differences between the 3 groups in theta, alpha, and beta frequency bands with open and closed eyes. The figure reports significant results for $p < .05$ for the eyes-open condition, while for the eyes-closed condition with a $p < .025$. Resting EEG analysis showed higher theta band power in the MS_F group compared to the MS_NF group in frontocentral regions during eyes-open condition, and both lower theta band power, and alpha band power over central and frontal regions; a reduction in power was observed, considering higher frequencies, in both beta and gamma bands diffusely over front, middle and rear areas during eyes-closed condition.

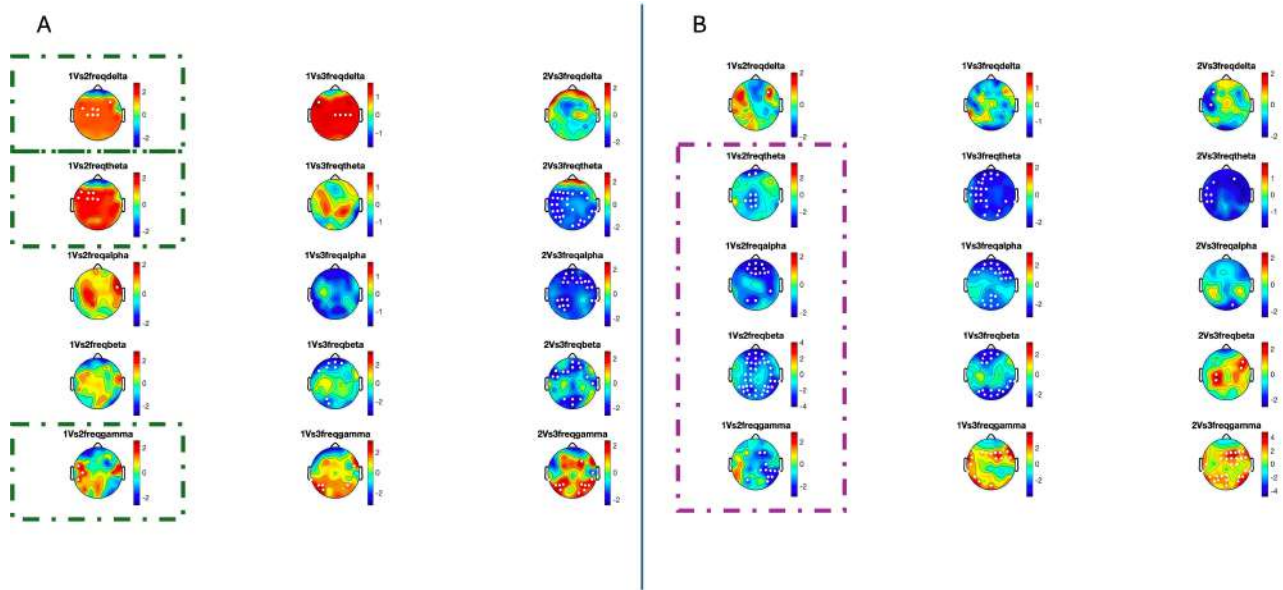


Figure 19. Panel A reports the significant differences between 1 (MS_F), 2 (MS_NF) and 3 (HCs) in the all frequency bands during the eyes-open condition. Panel B reports the significant differences between 1 (MS_F), 2 (MS_NF) and 3 (HCs) in the all frequency bands during the eyes-close condition.

5.5.6 fMRI

Figure 20 shows the statistical significance difference results related to the comparison between MS_F and MS_NF groups showing increased functional connectivity between the Supracalcarine Cortex right (SCC r) and both Frontal Pole left (FP l), and Superior Frontal Gyrus left (SFG l) within MS_F group. Group comparisons have been performed using an ANCOVA statistic because data

followed a normal distribution verified via the Shapiro Wilk normality test. Comparing SCCr FPI functional connectivity, the ANCOVA revealed a significant effect of GROUP ($F(2, 58) = 10.263$, $p < .001$, $\eta^2 = 0.261$) and MADRS ($F(1, 58) = 5.297$, $p = .025$, $\eta^2 = 0.067$). These results suggest that both GROUP and MADRS contributed meaningfully to explaining variability in SCCr FPI. Post-hoc pairwise comparisons revealed that group 2 had significantly higher SCCr FPI scores compared to Group 1 (Mean Difference = 0.208, SE = 0.047, $t = 4.441$, $p < .001$, Bonferroni-adjusted); group 1 had significantly higher SCCr FPI scores compared to Group 3 (Mean Difference = 0.175, SE = 0.051, $t = 3.421$, $p = .003$, Bonferroni-adjusted) and finally no significant difference was observed between Group 2 and Group 3 (Mean Difference = 0.033, SE = 0.043, $t = 0.771$, $p = 1.000$, Bonferroni-adjusted). Comparing SCCr SFGI functional connectivity, the ANCOVA revealed a significant effect of GROUP ($F(2, 58) = 6.309$, $p < .003$, $\eta^2 = 0.174$) and MADRS ($F(1, 58) = 0.305$, $p = .583$, $\eta^2 = 0.004$). These results suggest that GROUP only contributed meaningfully to explaining variability in SCCr FPI functional connectivity. Post-hoc pairwise comparisons revealed that group 2 had significantly higher SCCr FPI scores compared to Group 1 (Mean Difference = 0.137, SE = 0.043, $t = 3.217$, $p = .006$, Bonferroni-adjusted); group 1 had not significantly different SCCr FPI scores compared to Group 3 (Mean Difference = 0.036, SE = 0.047, $t = 0.770$, $p = 1.000$, Bonferroni-adjusted) and finally significant difference was observed between Group 2 and Group 3 (Mean Difference = 0.102, SE = 0.040, $t = 2.569$, $p = .038$, Bonferroni-adjusted).

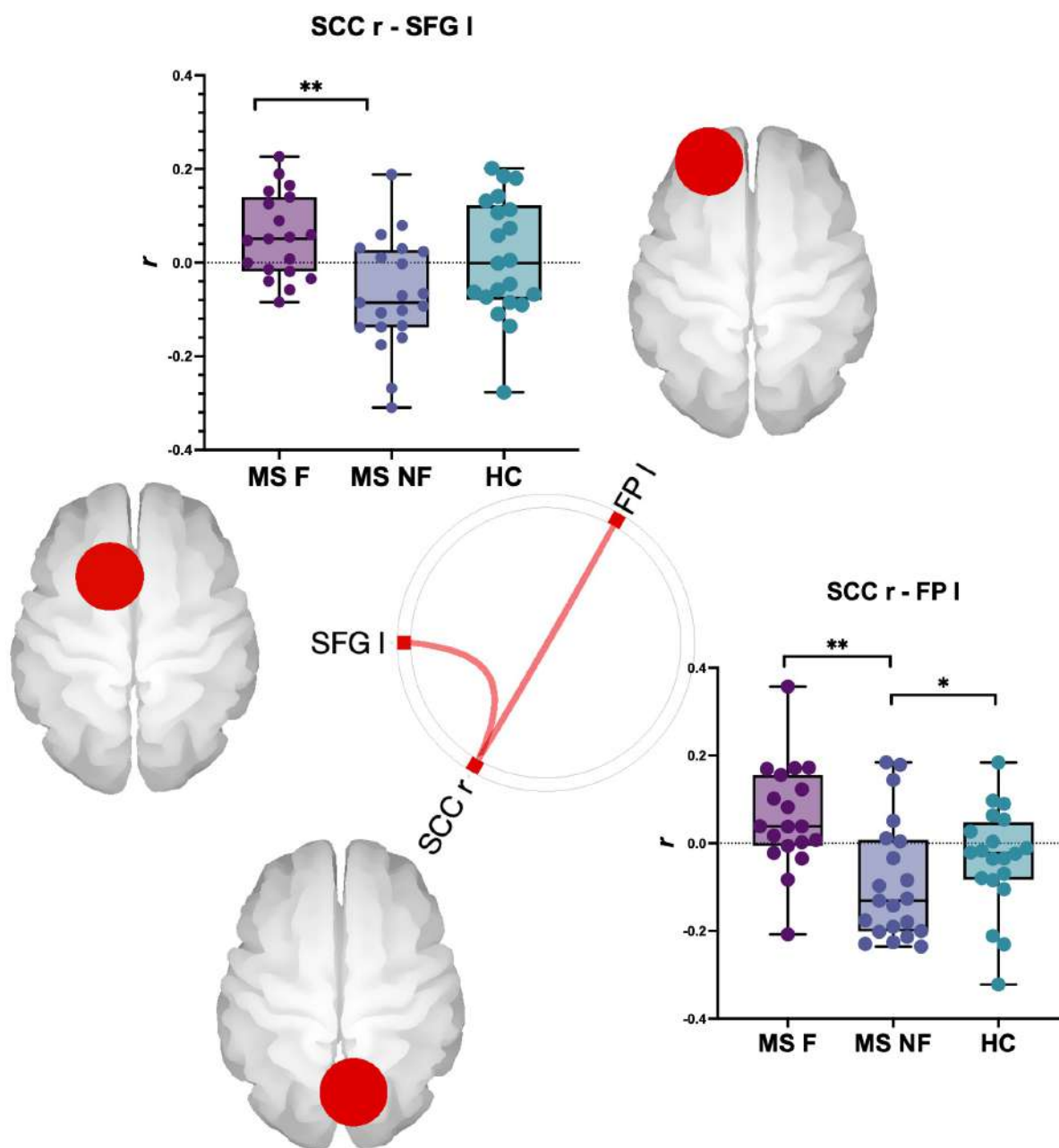


Figure 20. The ROIs displayed represent the right Frontal Pole Left (FP I), the Superior Frontal Gyrus Left (SFG I), and the SupraCalcarine Right (SCC r). Comparisons between multiple sclerosis patients with fatigue (MS.F), multiple sclerosis patients without fatigue (MS.NF), and healthy control subjects (HCs) * $p < .05$, ** $p < .01$

5.5.7 Brain Volumetric Assessment

Comparing cortical and subcortical volumes between the groups, the only observed differences concern the thalamus (TH) and the caudate nucleus. For the caudate nucleus, the analyses revealed a significant effect of the group variable ($F = 5.607$, $df = 2$, $p = .006$, $\eta^2 = 0.162$), indicating a moderate effect size. The covariate, MADRS, did not significantly contribute to the model ($F = 2.866$, $p = 0.096$, $\eta^2 = 0.041$). Post hoc tests with Bonferroni corrections showed a significantly lower volume in the MS F group compared to the HC group (Mean Difference= -1.118 , $p = .004$). For thalamus volume data, the results indicated a highly significant main effect of the group ($F = 12.116$, $p < 0.001$, $\eta^2 = 0.293$), reflecting a large effect size. Similar to the caudate nucleus analysis, the MADRS covariate was not significant ($F = 2.539$, $p = .117$, $\eta^2 = 0.031$). Post hoc comparisons revealed significant differences between the MS F and HC groups (Mean Difference= -2.490 , $p < .001$) as well as between the MS NF and HC groups (Mean Difference= -1.815 , $p < .001$). These findings suggest that HC is markedly different from both MS F and MS NF groups.

5.5.8 Neuropsychological, Psychological, and Clinical Assessment

Analyses performed on cognitive test scores from the Rao Brief Repeatable Battery reported no significant differences between the groups.

5.5.9 Correlation results

Figure 21 shows the results of correlations between the FSS variable and all other statistically significant variables in distinguishing patients with and without fatigue.

FSS	Spearman (ρ)	p value
MADRS	0.8	<0.0001
ICF	-0.7	<0.0001
Genu_CC	-0.7	<0.0001
CST_L	-0.5	0.0018
Post_LimC_L	-0.8	<0.0001
PTR_R	-0.6	<0.0001
Ext_Caps_R	-0.4	0.0132
Cing_R	-0.6	<0.0001
SLF_R	-0.7	<0.0001
FoF_R	-0.8	<0.0001
fMRI_SCC r - SFG l	0.7	<0.0001
fMRI_SCC r - FP l	0.6	0.0002
EO_theta_F1	0.4	0.0139
EO_theta_FC1	0.3	0.0408
EC_beta_FP1	-0.5	0.0018
EC_beta_FPZ	-0.5	0.0018
EC_beta_FP2	-0.4	0.0038
EC_beta_PO7	-0.4	0.024
EC_beta_PO3	-0.3	0.0307
EC_beta_O1	-0.4	0.0098
EC_beta_TP10	-0.6	0.0001

Figure 21. The figure shows all correlation results between FSS (Fatigue Severity Scale) and all the other statistically significant variables

5.5.10 Classification results

I implemented a decision tree model as a classification method to distinguish the MS_F and MS_NF groups. With the available data set, the model achieved a prediction accuracy of 82.1% , allowing reliable distinction between these two groups. The variables included in the classification model are the ICF values, the significant EEG bands power values, the significant rsfMRI FC, and the statistically significant FA values. In addition, I isolated individual parameters to observe their “weight” in deter-

mining accuracy in belonging to one group or another. Then, creating new models, I calculated for all True Positive Rates (TPR), i.e., the proportion of observations correctly classified by true class, False Negative Rates (FN) and Error Rates. False Negative Rates (FNR), the proportion of observations misclassified by true class, and finally the accuracy rate. Accuracy is the ratio of (true positives (TP) + true negatives (TN))/(TP+TN+false positives (FP)+false negatives (FN)). Figure 22 illustrates the prevalence of the individual variable in the context of symptom fatigue.

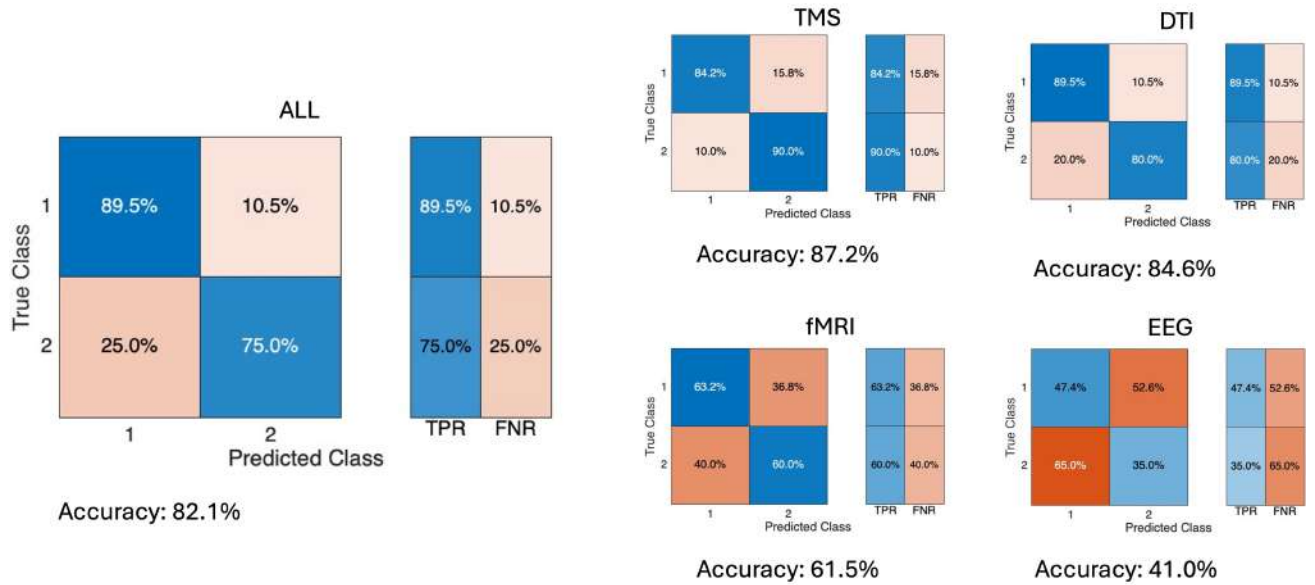


Figure 22. The figure shows all the decision tree models tested, displaying for each model their own TPR (True Positive Rates), FNR (False Negative Rates), and accuracy.

5.6 Discussion

5.6.1 Study aim

In this study, we aimed to address fatigue as a genuinely complex and multifactorial symptom—often overlooked in existing literature. Our experimental design took a multidisciplinary approach to studying this symptom and identifying the key components of CF in MS. The study was based on classifying the discriminative parameters of CF in MS. We sought to understand why some

patients in the same clinical population experience fatigue while others do not report it as a clinical symptom.

5.6.2 Study results

blood samples results

Among our results, despite existing studies linking fatigue with inflammation [201], no differences were found between the two patient groups. This lack of difference may be attributed to the anti-inflammatory therapy that all patients were undergoing at the time of evaluation, as they had not suspended their current therapy and were not in the acute stages of relapse, which is associated with elevated inflammation levels. Additionally, patients did not report clinically significant cognitive deficits, and no significant differences were observed between groups, even though literature links fatigue to a certain degree of cognitive decline [312].

Cognitive and psychological results

Although no differences were found in the examined cognitive domains, the *MS_F* group reported higher scores on the MADRS scale, indicating a higher level of depression. Despite this significant difference, the mean score for the *MS_F* group still falls within the mild depression range (scoring from 7 to 19) [313]. Since fatigue is associated with depression [314], interpreting the results is challenging. We considered the MADRS scale scores as a covariate to observe the combined effect of the FSS and MADRS, rather than focusing solely on the FSS. Determining whether depression and fatigue existed before the MS diagnosis or developed because of the diagnosis remains complex. It is difficult to establish a clear cause-and-effect relationship between fatigue and depression.

MRI and fMRI results

Although the literature suggests significant correlations between lesion load (both amount and size) [315] and brain structure volumes such as the thalamus [216, 316], no differences were found when comparing *MS_F* to *MS_NF*, suggesting that the symptom may be more functional than anatomical. This seems to be in line with recent findings by [220] who showed that fatigue in a cohort of 440

RRMS patients was not related to neuroimaging structural parameters related to neuroinflammation (e.g., WML) and neurodegenerative features (e.g., brain atrophy). However, reduced FA was found in the *MS_F* group compared to the *MS_NF* group, indicating a decrease in both fiber density and axonal diameter [317]. This reduction was observed mostly in the right associative bundles (such as the Cingulum, Superior Longitudinal Fasciculus, Inferior fronto-occipital fasciculus, and external capsule), as well as the Genu of the corpus callosum, left corticospinal bundles (specifically the Posterior limb of the internal capsule), and Posterior thalamic radiation of the right. These findings, which are consistent with those of previous studies [224], suggest the possibility of a slowdown in the passage of inputs not only underlying the pathology itself but also, more importantly, an increased energy requirement, which could result in a condition of chronic fatigue. Regarding the fMRI results, SCC, probably related to mental effort in domains such as visual processing, may cause fatigue in different systems. The higher FC between SFG, FP and SCC could underline the compensatory effort made by the frontal areas to cope with the mental effort related to visuospatial processing, through an increased cognitive control, decision-making and reward.

Neurophysiological results

EEG results

Neurophysiological measurements revealed some significant differences that partially align with existing literature. Resting EEG analysis showed higher theta band power in the *MS_F* group compared to the *MS_NF* group in frontocentral regions during eyes-open condition, and both lower theta band power, and alpha band power over central and frontal regions; a reduction in power was observed, considering higher frequencies, in both beta and gamma bands diffusely over front, middle and rear areas during eyes-closed condition. What is certainly striking is not only the significance of beta reduction, but also the stark difference between a closed-eye condition and an open-eye condition. In the open-eye condition, the presence of environmental stimuli requires significant cognitive effort for processing, regardless of the activity. This may lead to a changed perception of one's abilities and the stimuli themselves; in the closed-eye condition, with the disappearance of stimuli we no longer find

an electrophysiological correlate of a cognitive effort, in fact we find a reduction in alpha and theta, but c the difficulty to “rest,” as evinced by an overall lowering of power in beta. Although there is limited literature on spectral power density analysis for CF in MS, evidence suggests increased FC at rest in beta and theta bands during fatigue [318]. A notable reduction in microstate F, linked to the VAN, within the beta band, was consistent with our fMRI and EEG findings, showing reduced beta band power during eyes-closed conditions in the *MS_F* group. Considering the influence of GABAergic interneurons on beta oscillation generation [319] and their top-down inhibitory effects [320], the reduction in beta power suggests inhibitory difficulties in the fatigued subjects, leading to increased energy expenditure. In healthy individuals, theta band activity has been associated with cognitive fatigue and cognitive effort [153, 155], which supports our findings. Instead, the total difference between the two conditions may be the common point between the various theories expressed so far. Furthermore, as reported in chapter 2, in a eyes-open condition an increased beta wave indicates that an individual is cognitively active, acquiring and processing information to solve difficulties. The fact that a reduction in beta power is observed in *MS_F* patients could indicate a lack of sufficient energy to allow adequate analysis and response.

TMS results

Although previous studies have linked CF with TMS neurophysiological output measures such as SICI and CSP, related to GABAergic interneurons [225, 236, 241], our study found only a significant difference in ICF. The *MS_F* group showed reduced ICF, which is associated with glutamatergic intracortical circuits [321], indicating a dysfunction in glutamate recruitment [213]. Lower glutamate levels, consistent with findings in major depressive disorder [322], contradict the literature linking reduced glutamate levels to increased inflammatory response [323], suggesting that anti-inflammatory drug therapy might influence these results. Decreased glutamatergic levels could not only be explained by the anti-inflammatory effect of the ongoing therapy but could also explain the reduced energy of the patients [324] and the constant perception of fatigue. This result finds partial agreement with the work of [213], although fatigue in MS may primarily stem from reduced glutamatergic activation rather than GABAergic/glutamatergic dysregulation. The persistent fatigue perception among patients could

indeed result from an imbalance between the glutamatergic and gabaergic systems and a functional reorganization of brain activity, as these would not allow an adequate analysis of the environmental context and thus a consequent response to stimuli.

Decision Tree model

Finally, classification analysis revealed that ICF was the most discriminating factor between *MS_F* and *MS_NF* groups. This finding suggests that reduced activation of the glutamatergic system underlies the symptom, challenging some previous studies linking the glutamatergic system to increased inflammation and fatigue.

5.7 Conclusion

This study suggests that a multidisciplinary approach to symptom investigation may be beneficial and outlines a profile, or "fingerprint," of CF in MS. Fatigue is characterized by persistent cognitive effort, even at rest, and difficulty returning to a restful state, as shown by EEG findings. fMRI studies reveal excessive energy expenditure to respond to the mental effort related to visuospatial processing. Structural connectivity and TMS results suggest that a slowdown in the passage of inputs, resulting in increased energy demand, coupled with reduced glutamate levels—which is linked to a lack of energy—might explain chronic fatigue. Considering our findings, we believe that a rapid examination, such as that promoted by TMS and specifically the application of the ICF detection protocol, could be a valuable addition to the evaluation of fatigue and the assessment of new treatments. Furthermore, it is possible that new treatments could be developed to counteract this disabling symptom, for example through the application of NiBS protocols. NiBS protocols, such as tDCS, have demonstrated promising results in reducing symptoms [56, 258]. Additionally, findings in reducing fatigue in healthy subjects [187] could potentially contribute to new clinical evidence.

6 Future tRNS application on MS central fatigue and astronauts fatigue

6.1 Thesis aims

The dual goal of this thesis was achieved by showing a potential countermeasure of cognitive fatigue in healthy subjects, using NiBS application, and a drafting of neurophysiological parameters of MS fatigue using an innovative, comprehensive, multidisciplinary approach. In the first study, the aim was to demonstrate the efficacy of tRNS as an efficient method to reduce cognitive fatigue in healthy subjects involved in a driving truck VR simulation. The primary challenge in this work was identifying the optimal stimulation targets. In the existing literature on NiBS, concerning cognitive fatigue, the most targeted areas have been the DLPFC and M1: we explored the application of NiBS techniques beyond the stimulation of single areas, focusing on activating an entire brain network, namely what we have called "anti fatigue network". The areas included in the anti-fatigue network were selected based on two primary sources: the work of Wylie and colleagues, 2020 [132], which demonstrated the FC between brain regions during cognitive fatigue, and the work of Ishii and colleagues, 2014 [140], which highlighted the role of facilitation and inhibition systems. In contrast, the second study adopted a multidimensional approach to investigate the neurophysiological parameters of MS fatigue; this involved moving beyond the use of single methods to consider a broader range of techniques. This is not intended to be a discussion of the results per se, but rather to demonstrate that the findings

of the first study can be applied to MS and, more importantly, that they have the potential to be effective. As described in Chapter 1, NiBS techniques have previously been used to reduce fatigue in MS patients, thus demonstrating their effectiveness and safety. The use of tRNS on that network termed “anti-fatigue” [187] represents a new potential target for reducing fatigue in MS patients as well. The theoretical concept behind it is represented by the fact that using our the montage we used, tRNS was not able to activate stimulated areas only, but also the deepest structures related to the limbic network. As described in the second chapter, fatigue represents a physiological mechanism in response to a given energy expenditure. Based on this description, we understand that fatigue is necessary for the human being and that reduced fatigue might interfere with its internal homeostasis leading to consequences, as yet unexplored.

6.2 New perspectives

For this reason, our hypothesis in using this kind of stimulation protocol concerns a reinforcing action of that energy reserve described at the beginning, not acting only preparing the cortical areas to better respond to environmental stimuli by making the cortex more excitable, but also on the motivational drive. Lastly, with this kind of stimulation, we are also proposing a new intervention with the aim of facilitating neuronal firing by increasing cortical excitability. Thus, the use of stimulation should not be oriented to act directly on the fatigue perception, although studies such as that of Kuppuswamy (2017) [196] show that an increase in fatigue is related to a dysfunctional perception of external stimuli and one’s own abilities, but to prepare the person to respond adequately to environmental stimuli; in this way, the reduction of fatigue perception will be the most powerful effect, but it will still be an effect dictated by the increase in the person’s energy reserve and motivational drive. In fact, in our study we acted on those areas whose activation and connectivity were found to be reduced in a state of fatigue, and that had higher fatigue perception as consequence.

6.2.1 New perspectives: Astronauts

Based on the results obtained, the next steps will involve the use of tRNS on astronauts, starting from the category of workers defined as “astronaut-like”, such as submariners. In fact, they perform

very cognitively demanding activities, in environments similar to those of astronauts during their missions. Before proceeding with the use of this method on astronauts, it will be appropriate to calibrate the stimulation, again through the use of the biophysical model, also based on the changes (such as those related to the Cerebral Spinal Fluid) that displace the astronaut's brain, changing for example the cortical excitability threshold [325, 326], potentially modifying the tissue conductivity (Fig.23).

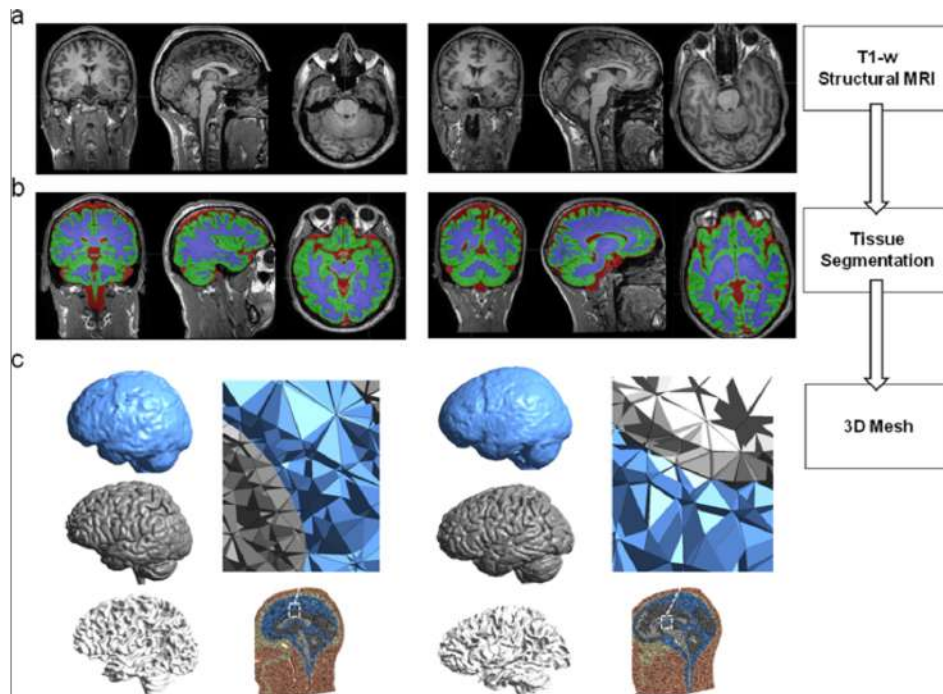


Figure 23. Image processing and mesh creation diagram. The pipeline of image processing to create a 3D mesh for running simulations is shown for two participants in the study: one cosmonaut (left) and one control participant (right). a) T1-weighted (T1-w) anatomical scans were collected for each participant at three time points (pre-flight, post-flight, and follow-up). The figure shows MRIs collected pre-flight for both participants. b) The SimNIBS pipeline was used to segment the T1 anatomical image into tissue classes (skin, bone, CSF, eyes, gray matter, and white matter). For visualization, CSF is shown in red, gray matter in green, and white matter in blue. c) The head volume 3D meshes, composed of tetrahedral elements (CSF: blue; gray matter: gray; white matter: white), were then computed. This process allows MNI coordinates to be transformed into personalized subject space, ensuring accurate targeting [326].

Once the protocol has been adapted for astronauts, the next logistical step will be to implement the device inside the spacecraft. A final aspect will be to help the astronauts not only during the

flight, but also upon their return to Earth by accelerating their recovery.

6.2.2 New perspectives: MS

If we instead consider fatigue in MS, this, as we have seen in literature, appears to be cognitively linked to an altered perception, highlighted by alterations of M1 and S1 BOLD activities, and in the connectivity between these ones. Based on the outcomes of our study, we found also structural alterations (reduced FA) that disrupt the transmission of electrical signals and neuronal firing. Furthermore, as highlighted in the previous chapter, fatigue seems increasingly connected with reduced activation of the dopaminergic system (which also impacts motivational drive, in addition to initiating an action). The neurons responsible for dopamine release, as we described above, are part of the fronto-striatal circuits, which include deep structures such as the basal ganglia. Based on this evidence, the cascading reaction that is created could originate from the immune system, which characterizes the pathology itself, potentially linked to a deficit in dopamine synthesis. According to the gating hypothesis, dopamine might modulate cognition due to its interaction with other neurotransmitters in the PFC. When there is too much dopamine, the "gate" for excitatory inputs from glutamate neurons to post-synaptic PFC cells shuts down, while too little dopamine allows interference between different inputs [327]. Following this logic, the dopamine imbalance hypothesis explains the symptom of fatigue as a result of either too much or too little dopamine [328]. Looking at our results, it would seem that the connection between what the literature names as a reduction in dopaminergic activity, is more closely linked to a reduction in glutamatergic activation, as shown by ICF. Despite the studies focusing on dopamine have been more interested in the PFC without considering M1, considering that the PFC is closely related to the aforementioned subcortical structures, as is M1, this certainly lead the groundwork for investigating whether the dopaminergic gating hypothesis for fatigue could be extended including the role of M1. Finally, to complete the picture of the brain network involved in MS fatigue, it is appropriate to mention the TH, plays a regulatory role both directly, as shown in the previous chapter, and indirectly on fatigue through its interactions with the dopaminergic system, based on its cortico-striatal connections. Based on this evidence, the role of tRNS will be to facilitate neuronal firing by stimulating the activation of the glutamatergic system and dopaminergic release, as the stimulation setup, through stimulation via the cortical projections of the basal ganglia, is potentially able to stimulate deeper areas. Furthermore, although further simulations are needed,

the stimulation intensity will be designed to compensate for the disruption of myelin sheaths in the affected tracts, preventing the slowing down of the transmitted electrical signal. In order to increase the effect of tRNS on both patients and healthy subjects, as has already been observed for other NiBS methods such as tDCS, it will be important to combine it with cognitive (e.g., working memory and attentional tasks) and motor (e.g., treadmill and bicycle) stimulation techniques. In fact, as shown in previous sections, the combined effect of stimulation and both mental and cognitive training techniques can enhance the effect of NiBS protocols.

7 Publications

Romanella SM & **Benelli A**, Palmisano A, Tatti E, Neri F, Cinti A, Rossi S, Santarnecchi E. “Induce sleepiness with optimized Transcranial Alternating Current Stimulation” (in prep).

Neri F, Cappello C, Viberti F, Donniacuo A, Burzi L, Cinti A, **Benelli A**, Smeralda CL, Romanella SM, Santarnecchi E, Mandalà M, Rossi S. “rTMS of the auditory association cortex improves speech intelligibility in patients with sensorineural hearing loss”. *Clinical Neurophysiology*, 160:38-46. (2024). <https://doi.org/10.1016/j.clinph.2024.02.007>

Neri F, Cappello C, Pitinca ML, Taddei S, Viberti F, Cinti A, **Benelli A**, Santarnecchi E, Mandalà M, Rossi S. ”Speech perception improvement through rTMS in hearing loss”. *Brain Stimulation*. 16(1):156. <https://doi.org/10.1016/j.brs.2023.01.128>

Rossi S, Cinti A, Viberti F, **Benelli A**, Neri F, De Monte D, Giannotta A, Romanella SM, Smeralda C, Danniaucuo A, Prattichizzo D, Pasqualetti P, Santarnecchi E, Mandalà M. “Frequency-dependent tuning of the human “sixth sense” by transcranial oscillatory currents”. *Clinical Neurophysiology*. 153, 123-132. (2023). <https://doi.org/10.1016/j.clinph.2023.06.013>

Benelli A, Neri F, Cinti A, Pasqualetti P, Romanella SM, Giannotta A, De Monte D, Mandalà M, Smeralda CL, Prattichizzo D, Santarnecchi E, Rossi S,. “Frequency dependent reduction of cyber-sickness in Virtual Reality by transcranial oscillatory stimulation of the vestibular cortex”. *Neurotherapeutics*. 20, 6. 1796-1807. (2023). <https://doi.org/10.1007/s13311-023-01437-6>

Cinti A, Romanella SM, **Benelli A**, Neri F, Rossi S, Santarnecchi E. “Optimized Neuromodulation for First-person Gaming”(in prep).

Benelli A, Memoli C, Neri F, Romanella SM, Cinti A, Giannotta A, et al. Reduction of Cognitive Fatigue and Improved Performance at a VR-based Driving Simulator using tRNS. *iScience*. 2024 Jul;110536. <https://doi.org/10.1016/j.isci.2024.110536>

Neri F, Cappello C, Viberti F, Donniacuo A, Burzi L, Cinti A, **Benelli A**, Smeralda CL, Romanella SM, Santarnecchi E, Mandalà M, Rossi S. “rTMS improves speech intelligibility in patients with sensorineural hearing loss“. *Clinical Neurophysiology*, 160, 38-46. (2024). <https://doi.org/10.1016/j.clinph.2024.02.007>

Neri F, Cappello C, Viberti F, Donniacuo A, Burzi L, Cinti A, **Benelli A**, Smeralda CL, Romanella SM, Santarnecchi E, Mandalà M, Rossi S. “rTMS increases speech perception ability in deaf patients“. *Brain Stimulation*, 16(1), 253-254. (2023). <https://doi.org/10.1016/j.brs.2023.01.410>

Agnorelli C, Cinti A, Barillà G, Lomi F, Scoccia A, **Benelli A**, Neri F, Smeralda LC, Cuomo A, Santarnecchi E, Tatti E, Fagiolini A, Rossi S. “Neurophysiological correlates of ketamine-induced dissociative state in bipolar disorder“. (submitted)

Benelli A & Reilly J., Marquez J., Romanella SM., Santarnecchi E. “Functional Networks related to Cancer-Related Cognitive Impairment in Bbreast Ccancer Ppatients“. (in prep)

Tatti E, **Benelli A**, Cortese R, Cinti A, Serbina A, Saed S, Luchetti L, Battaglini M, Cudeiro J, De Mauro A, Plantone D, Righi D, Lisini T, d’Aurizio N, Massucco E, Neri F, Stromillo ML, Giannotta A, Lomi F, Scoccia A, Lai G, Ghilardi MF, De Stefano N, Olivelli M, Rossi S. “Insights into Central Fatigue in Multiple Sclerosis: Movement-Related Beta Oscillatory Activity and its Association with Brain Neurophysiology and Structure“. (in prep)

Benelli A, Cortese R, Tatti E, Massucco E, Luchetti L, Battaglini M, Cudeiro J, de Mauro A, Zhang J, Plantone D, Pasqualetti P, Righi D, Neri F, Stromillo ML, Cinti A, Giannotta A, Lomi F, Scoccia A, Lai G, De Stefano N, Olivelli M, Rossi S. “Neurophysiological hallmarks of central fatigue in Multiple Sclerosis“. (in prep)

Neri F, Breccia D, **Benelli A**, Ferrone S, Romanella S, Mencarelli L, Rossi S, Olivelli M, Mandalà M. “Functional head impulse testing for detection of vestibular dysfunction in Multiple Sclerosis” (submitted)

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