SPECT imaging of cerebral blood flow changes induced by acute trigeminal nerve stimulation in drug-resistant epilepsy. A pilot study

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Abstract

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Title

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Author

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Declaration of Competing Interest

None of the authors has potential conflicts of interest to be disclosed.

Abstract

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CONCLUSION: Short-term TNS is followed a global increase in cortical perfusion, namely in the temporal and limbic lobes.

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Highlights:

- Patients with drug-resistant epilepsy showed global hypoperfusion at brain SPECT.
- After trigeminal nerve stimulation perfusion increased in the temporal and limbic lobes.
- TNS effects may reflect activity changes in cortical areas associated to epilepsy.

Keywords: Cerebral blood flow; Cerebral perfusion; Drug-resistant epilepsy; Brain SPECT; Trigeminal nerve stimulation.

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1. Introduction

It is estimated that up to one third of patients with epilepsy are drug-resistant (DRE) (Kwan, 2004, 2010; Löscher and Schmidt, 2011). Most of them are not suitable candidates for surgery due to difficult location of the epileptogenic focus, identification of the seizure focus within eloquent areas, presence of multiple foci or refusal for surgical treatment by patients (Schulze-Bonhage, 2019).

For this subset of DRE patients, in 1997 vagal nerve stimulation (VNS) was approved by the U.S. Food and Drug Administration as an alternative and/or adjunctive treatment option (Schulze-Bonhage, 2019).

Although VNS is generally safe and well tolerated, this therapy is not risk-free owing to the implantation-related invasiveness and costs (Ben-Menachem et al. 2015) and, mainly, to the vegetative side effects associated to VNS (Howland, 2014). These drawbacks, along with a 49.4% rate of non-responders (Englot et al., 2011; Gschwind and Seeck, 2016), have led researchers to propose the transcutaneous stimulation of the trigeminal nerve (TNS) as a valid alternative to VNS for the treatment of DRE (DeGiorgio et al., 2003, 2006, 2009; Fanselow et al., 2000). Subsequent studies have shown that TNS is a safe and promising treatment for DRE in the long term (DeGiorgio et al., 2013; Pop et al., 2011; Soss et al. 2015; Olivié et al., 2019; Gil-López et al., 2020), with additional positive effects on sleep quality, mood and quality of life (Slaght and Nashef, 2017; Olivié et al., 2019).

The sites of the antiepileptic effects of TNS in the central nervous system (CNS) and its mechanisms of action are yet to be elucidated. Studies carried out in animal models evidenced that trigeminal afferents activated by short-term TNS protocols target the nucleus of the solitary tract, the locus coeruleus and the dorsal raphe nucleus (Mercante et al., 2017). These nuclei are crucial to establish ascending connections with other brainstem and forebrain structures (i.e. parabrachial nucleus, cerebellum, reticular formation, hypothalamus, thalamus, amygdala, hippocampus, entorhinal cortex, endopyriform gyrus), that are thought to be implicated in the inhibitory control of

seizures (Bari and Pouratian, 2012; George et al., 2000; Magdaleno-Madrigal et al., 2002; Mercante et al., 2017; Moseley and DeGiorgio, 2014; Rutecki, 1990; Wang et al., 2016). In line with anatomical evidences (Mercante et al., 2017), physiological studies carried out in humans indicate the brainstem as the structure mainly targeted by TNS with a long-term depression-like mechanism (Mercante et al. 2015; Pilurzi et al. 2016). In addition, short-term TNS has been shown to influence EEG activity both in healthy and DRE subjects, suggesting that the cerebral cortex is eventually involved by TNS (Ginatempo et al., 2018, 2019).

Despite the increasing therapeutic use of TNS in DRE and in a number of other neuropsychiatric conditions (Shiozawa et al., 2014), no neuroimaging procedures have been undertaken to investigate the anatomo-functional correlates of TNS.

Therefore, the main purpose of this study was to explore which cortical areas are targeted by acute TNS in DRE patients using functional imaging modalities commonly used in the evaluation and diagnosis of epilepsy. To this aim, we used brain perfusion single photon emission computed tomography (SPECT), which represents the most used functional procedure in ictal and interictal epilepsy evaluation (Buch et al., 2008; Mielke et al., 1994).

2. Material and Methods

2.1 Patients

Ten patients (44 ± 9.0 years old; 7 males) referring to the Center for Diagnosis and Treatment of Epilepsy, Unit of Neurology, University of Sassari, and diagnosed with DRE in accordance with the criteria of the International League Against Epilepsy (Kwan et al. 2010), participated in this study. Exclusion criteria were: history of migraine, diabetes, trigeminal neuralgia, psychiatric disorders, cardiovascular diseases, history or signs of neurological disorders other than DRE, pregnancy and seizure attack in the 24 hours preceding the SPECT scan. All patients underwent EEG examination and magnetic resonance imaging (MRI).

The study was approved by the local Ethics Committee (Prot 982/2/L ASL n. 1 Sassari, 17/11/2011) and written informed consent was obtained from all participants.

2.2 TNS

TNS was delivered bilaterally to the infraorbital nerve (ION) through an electrical neurostimulator (Winner, Fisioline Biomedical Instrumentation, Verduno, CN, IT) connected to 26-mm disposables, hypoallergenic, silver-gel self-adhesive stimulating electrodes (Globus, Domino s.r.l., Codognè, TV, IT) with the cathode placed over each infraorbital foramen and the anode 2 cm lateral, to stimulate V2 branches of trigeminal nerve. In each subject, perceptual and pain thresholds were bilaterally calculated and stimulation intensity was set, for each ION, to the maximal intensity that the subject could sustain comfortably, and always below the pain threshold. The stimulus consisted of trains of a symmetric biphasic square wave pulse (duration 0.25 ms, frequency 120 Hz), delivered in a cyclic modality (30 s ON and 30 s OFF) for 20 min, according to protocols described in previous works (Ginatempo et al., 2019).

2.3 Brain SPECT

Brain SPECT was performed according to International guidelines (Kapucu et al., 2009) at baseline (T0) and after a 20-minute TNS session (T1), which were carried out two weeks apart.

Patients were asked to avoid eating, smoking and the intake of CNS stimulants (such as caffeine, cola, energy drinks), alcohol, and any drugs known to affect cardiovascular function in the 12 hours preceding the SPECT scan. They were positioned in a quiet, dimly-lit room in a supine comfortable position with an eye-mask and were instructed not to speak, read, or move from at least 5 min before to 5 min after the injection of the radiopharmaceutical tracer (99mTc HM-PAO) through an intravenous cannula inserted before TNS administration. The 99mTcHM-PAO complexes are lipophilic and of small size, thus they circulate with the blood to the brain and pass the blood-brain-barrier. Intracellularly, the tracer is rapidly metabolized and retained unmodified for a relatively

long time (4-6 hours) since a steric hydrophilic transformation of the 99mTcHM-PAO occurs by the intracellular glutathione reductase (Asenbaum et al.,1998). The tracer retention in the brain is very stable when the early back-diffusion has ceased, and only a small loss of tracer (approximately 0.4%/hour) is observed in most human cases during the following 24 hours (Andersen, 1989). Hence, the steady-state 99mTc-HMPAO of the distribution images, obtained from 10 min to 2-3 hours after the injection of the tracer, agrees more closely with regional cortical blood flow (rCBF) and can be used to evaluate the functional status of the brain (Zerarka et al., 2001). The 99mTc HM-PAO was prepared using pertechnetate coming from a generator eluted in the previous 24 hours, which eluted was not older than 2 hours. Radiochemical purity (>80%) was determined on each vial prior to injection. A dose of 740 Mega-Becquerel of tracer was injected intravenously within 30 min since radioligand reconstitution and one minute before the end of the TNS procedure. Images were acquired within 30-60 minutes from injection using a dedicate dual-head gamma camera equipped with fan-beam collimators (rotation: 180°, matrix size: 128x128, zoom factor: 1, frame time: 30 seconds, angular step: 3 degrees).

2.3.1. Image processing

Unprocessed projection data and sinogram forms were evaluated in a cinematic display prior to a filtering process to assess the presence of motion or other potential artefacts, then the filtered back-projection reconstruction was applied.

Transaxial slices were reformatted into at least three orthogonal planes. Transverse sections were generated parallel to a given anatomic orientation (e.g. anterior commissure-posterior commissure line) ensuring a high degree of standardization in the plane orientation. Subsequent coronal and sagittal sections were created on the basis of transaxial slices. Finally, the set of transaxial, sagittal and coronal slices were produced in an automated standard color scale with a section thickness of 2.1 mm. Finally, the cortical uptake of 99mTc-HMPAO was normalized and expressed as a percentage of the maximum uptake observed in the cerebellum.

166

167 **2.3.2 SPECT Data analysis**

168 A qualitative evaluation of images was performed separately by two experienced nuclear medicine
169 physicians (SN and AS) by visual interpretation of tracer uptake in cortical regions based on the
170 French Color Standard International Scale. Each patient's dataset was normalized individually to the
171 mean cerebellar pixel values.

172 Brain SPECT perfusion deficits were scored by visual qualitative analysis with respect to
173 localization (frontal, parietal, temporal and occipital cortex), lateralization (left or right hemisphere)
174 and severity of hypoperfusion. A consensus reading was reached in case of discordant evaluations.
175 The scores were subdivided into four grades, as follows: grade 1 = normal (80-100%); grade 2 =
176 slight (60%-80%); grade 3 = moderate (60%-50%); grade 4 = severe (<50%).

177 Quantitative assessment was performed using the NeuroGam® software (GE Medical System,
178 Segami Corp., Columbia, MD, USA) to evaluate any statistically significant TNS-induced changes
179 in the five cerebral lobes of both hemispheres. To accurately locate abnormal rCBF, the areas of
180 cerebral cortex were analyzed in the Brodmann areas (BAs) (Fu et al. 2015, 2018).

181 The mean rCBF for each region was obtained through a predefined algorithm which converts the
182 mean pixel value in each corresponding region of interest into a percentage of the maximum pixel
183 value in the entire volume of brain. This software uses an affine anatomical coregistration by blocks
184 of data defined in the Talairach space. Patient images were normalized to determine the z-scores
185 which were calculated automatically by the system for each patient, as the number of standard
186 deviations from normative data stored in the NeuroGam® software package, obtained from age-
187 and gender-matched healthy control subjects.

188 A surface mapping was done according to color scale depending on the number of standard
189 deviations from normal values.

190

191 **2.4. Statistical analysis**

All statistical analyses were conducted using the SPSS statistical software (version 20.0, IBM, New York, USA). Odds ratio (OR) calculations were performed with RevMan 5.4 software (The Cochrane Collaboration). Analyses of individual data were carried out employing the NeuroGam® software to create a z-score map, which calculates z-scores using the following equation: *z-score= (control mean−individual value) / control SD.*

This is equivalent to presenting the individual data in terms of standard deviations (SDs) from the NeuroGam® normative database obtained from matched healthy controls, which distribution is set as follows: *z=-1.5 to z=+1.5.*

Values outside that distribution in either direction were considered abnormal. In particular, z-scores <-1.5 were interpreted as "decreased uptake" whilst z scores >+1.5 were interpreted as "increased uptake" in comparison to normative data.

To test for any differences in tracer uptake between patients' z-scores in basal conditions and following TNS, a three-fold approach was followed: qualitative, quantitative/dichotomous (i.e. changed Vs. unchanged), and quantitative/scalar (i.e. by comparing z-scores before and after TNS). Scores deriving from the French Color Standard International Scale were analyzed by the non-parametric Wilcoxon Signed-Rank Test. Quantitative analyses were carried out on dichotomous data. Data were analyzed by lobes and BAs, and corrections (Bonferroni) for multiple comparisons applied. OR and their 95% confidence interval (95% CI) were calculated by comparing the number of events showing increased tracer uptake (responders) *versus* those showing no change (non-responders) following TNS.

To test for within-subjects' differences in perfusion following TNS delivery, the standardized mean differences (SMD) of the pre-to-post change were calculated. According to Cohen (1988), these effect sizes were categorized as follows: trivial (<0.2), small (<0.5), moderate ($0.5 \leq d < 0.8$) and large (≥ 0.8).

To study and weigh the influence of the history of epilepsy in years on the findings, bivariate correlations with baseline (pre), post-TNS and pre-to-post data were carried out by calculating the Pearson's interclass correlation coefficients.

For all the comparisons and analyses, significance was set at the 0.05 level.

3. Results

3.1 Patients characteristics

The demographic and clinical characteristics of the participants are detailed in Table 1. Patients had an average seizure frequency of 3.0 seizures per month. None of our patients exhibited a picture of epileptogenic encephalopathy but focal slow wave activity, within a normal background. Only in P09 and P10, who had undergone pre-surgery evaluation, the EEG recording of seizure activity allowed to localize a temporal epileptogenic zone and define a multifocality in the former and a stable unilateral epileptogenic focus in the latter. In particular, in P09 seizure started at different temporal EEG electrodes including both temporal lobes. The remaining patients (P01-P08) had interictal unspecific or epileptiform EEG abnormalities with constant-side regional localization and a tendency to spread contralaterally, with the exception of P03, who showed independent and asynchronous activity on both sides. Consequently, in patients P01-P08 the epileptogenic zone could only be hypothesized from the site of the structural lesion, when present, from clinical ictal semiology of localizing value, and/or from interictal EEG data. When linking symptomatology to the EEG findings, epileptic foci were localized in the temporal lobe (60% of cases), frontal lobe (20%), fronto-temporal lobe (10%) and temporo-occipital lobe (10%). As evidenced by the MRI, the most common etiology was cryptogenic (70% of cases), with only 30% of patients (P01, P03 and P05) exhibiting an anatomical lesion with probable epileptogenic meaning (but often without a clear functional correlate, e.g. P01).

241 The intensity of ION stimulation ranged from 6.0–12.0 mA. During the SPECT and TNS
242 procedures, no epileptic seizures were clinically detected neither perceived by the patients.
243
244 **3.2. Qualitative and quantitative analyses of SPECT data**
245 A representation of SPECT images used for qualitative and quantitative analysis is provided in
246 Figure 1 for a representative subject (P04) of whom an EEG trace is also shown (Figure 2). Table 2
247 summarizes the results of the qualitative analysis. All patients presented a certain degree of cerebral
248 hypoperfusion at baseline, which involved focal areas only in 30% of them and both hemispheres in
249 70% of cases. The cortical areas that were more frequently found hypoperfused were the temporo-
250 mesial (70%), temporo-parietal (60%) and temporal (20%) areas. After TNS, 70% of patients
251 exhibited a decrease in the French Color Standard International Scale hypoperfusion score, and a
252 global increase in the cortical tracer uptake. In particular, the Wilcoxon Signed-Rank test revealed a
253 significant reduction in the median hypoperfusion score after TNS (baseline, 3; 95% CI 2.64 to
254 3.36; after TNS, 1.5; 95% CI 0.91 to 2.09; p=0.015).
255 The calculation of the OR, which was run by comparing over 5 lobes on both hemispheres the
256 number of events showing increased tracer uptake Vs. events showing no change following TNS,
257 evidenced an overall significant general effect favoring a TNS-induced increase in rCBF (OR =
258 4.96; 95% CI 2.02 to 12.18; p=0.0005), showing that the increase in rCBF was 4.96 times more
259 likely to occur than no change. When data were analyzed by lobe, Bonferroni-corrected multiple
260 comparisons revealed significant differences only in the temporal (OR = 16.00; p=0.003) and limbic
261 (OR = 16.00; p=0.003) and lobes (Figure 3a), the latter including the area surrounding the corpus
262 callosum, the cingulate cortex and the parahippocampal gyri.
263 When correcting by lobe and by side (Figure 3b), the calculation of the OR evidenced that,
264 following TNS, patients showing a bilaterally increased tracer uptake in the limbic (left and right:
265 p=0.02) and temporal lobe (left and right: p=0.02) were significantly more than those who
266 displayed no change (Figure 3b).

Temporal and limbic lobes were, therefore, further inspected by calculating the OR for the specific BAs of each lobe. Within the temporal lobe, following TNS, patients with increased uptake were significantly more than those displaying no change in left BA 21 (OR= 45.00; p=0.02), right BA 22 (OR= 71.40; p=0.007), and left BA 38 (OR= 13.50; p=0.04). After correcting for multiple comparisons over 6 areas, a significant difference emerged only for right BA 22 (p=0.04).

Regarding the limbic lobe, after correcting for multiple comparisons over 5 areas, a significant difference was detected in right BA 23 (OR= 36.00; p=0.04).

The comparison of the z-scores of the change in perfusion following TNS revealed a significant increase in tracer uptake by 0.59 SD (95% CI 0.27 to 0.90; baseline: -2.41±0.59 z-scores; post-TNS: -1.98 z-scores; p=0.001) in the temporal lobe, and by 0.43 SD (95% CI 0.06 to 0.81; baseline: -2.28±0.94 z-scores; post-TNS: -1.69 z-scores; p=0.03) in the limbic lobe.

Bivariate correlations revealed no significant relationship between the history of epilepsy in years and the perfusion at baseline (r=0.46; p=0.18), after TNS (r=0.34; p=0.33) and the pre-to-post change in perfusion (r=0.41; p=0.22), as assessed quantitatively by z-scores. Likewise, no relationship emerged when correlating the history of epilepsy with the qualitative estimates of perfusion (as assessed with the hypoperfusion score through subjective visual inspection via the French Color Standard International Scale) at baseline (r=0.50; p=0.14), at the post (r=0.11; p=0.77), and pre-to-post change (r=0.30; p=0.40).

4. Discussion

The present study investigated the responses of cortical brain regions to acute TNS administration in DRE patients. To this purpose, the variations in rCBF were studied using 99mTc HM-PAO brain perfusion SPECT. Data provided first evidence that, in these selected patients, short-term TNS was

associated to a consistent increase of rCBF in the temporal and limbic lobes, within the bounds of a global increase in cortical perfusion.

The length since epilepsy diagnosis in our cohort was found not to influence baseline cerebral perfusion, post-TNS perfusion as well as the pre-to-post data, assessed either qualitatively or quantitatively. The influence exerted by the early onset and duration of epilepsy on SPECT imaging remains controversial, since these features were positively correlated to the extent of hypoperfusion in the intercritical SPECT by some studies (Duncan, 1992; Duncan et al., 1992; Avery et al., 2001) but not by others (Andersen et al., 1996). Although the spread in illness duration exhibited by our sample was found uncorrelated with SPECT data, the role played by the clinical history in the observed changes of perfusion following TNS delivery needs cautious interpretation.

All patients exhibited a global hypoperfusion at baseline, which is compatible with their clinical condition characterized by a long-term intake of pharmacological polytherapy (Spanaki et al. 1999; Joo et al. 2008). Qualitative analysis evidenced that, compared to baseline, 70% of patients exhibited a global increase of cortical tracer uptake after 20 minutes of TNS. In particular, the specific cortical areas that appeared hypoperfused at baseline (mainly temporo-mesial, temporo-parietal and fronto-temporal and temporo-occipital areas), showed a significant decrease of the hypoperfusion score, as assessed with the French Color Standard International Scale. It is noteworthy that both the localization of the hypoperfusion and its reduction after short-term TNS converge with the epileptic focus (temporal in 80% of cases), which was clearly determined in P09 and P10 and supposed on the basis of electro-clinic signs in the remaining patients. Although this concordance corroborates the idea that SPECT reflects those changes in cortical perfusion that are associated to functional changes in neuronal activity, namely the epileptic focus, it has to be interpreted with due caution since interictal SPECT has been associated to limited diagnostic accuracy and variable correlation with MRI data (Lee et al., 2000; Siegel et al., 2002; Thadani et al., 2004; Tepmongkol et al., 2015). In addition, our sample was small and heterogeneous, with

epileptogenic lesions of diverse nature and interictal specific or aspecific focality (P01-P08), contrasting EEG and clinical data (e.g. P07). Furthermore, EEG lateralization was uncertain (e.g. P03) in all but two patients (P09 and P10), which do not allow mapping an electric network and thus making any correlation of the EEG information with the SPECT signal. Moreover, the lack of EEG co-registration in our study does not allow proving that the hypoperfusion observed in the interictal SPECT might be the result of subclinical electric seizures (Lee et al., 2000).

The quantitative analysis confirmed the observations made following the visual inspection of SPECT scans. In particular, a significant increase in rCBF was found in the temporal and limbic lobes of both hemispheres. More specifically, the calculation of the OR evidenced that, after TNS, patients showing increased tracer uptake in the limbic (80%) and temporal (80%) lobes were significantly more than those displaying no change. Furthermore, the quantitative analyses of z-scores to assess the magnitude of the changes in perfusion from baseline to post-TNS, confirmed significant TNS-induced increases in perfusion only in the above said lobes.

Taken together, these data indicate that, in DRE patients, short-term TNS is able to produce a global increase in cortical perfusion and, within this frame, the temporal and limbic lobes of both sides were particularly responsive to TNS. Since a close relationship exists between perfusion distribution and the metabolism of ^{99}mTc HM-PAO (Silverman, 2004), changes in the bio-molecular structure of neurons as well as in the neurophysiopathological substrate are believed to subtend the information obtained with ^{99}mTc HM-PAO brain SPECT (Palumbo et al., 2014; Nuvoli et al., 2016). In this light, we offer that TNS-induced changes in cortical blood perfusion may result from a change in the functional activity of cortical neurons, which may have occurred after TNS, in line with two recent studies showing EEG changes both in healthy individuals and in DRE patients following short-term TNS (Ginatempo et al., 2018, 2019).

Neurophysiological studies performed in healthy subjects suggested that TNS action is exerted mainly at the brainstem than cortical level (Mercante et al., 2015), with a long-term depression-like

mechanism (Pilurzi et al., 2016). The connections of the trigeminal nerve within the CNS are considered neuroanatomically strategic in influencing several cortical structures, among which those involved in cognitive, affective and hyper-arousal symptoms (Bathla and Hegde, 2013; Cook et al., 2016). In fact, trigeminal afferents projecting to the spinal trigeminal nucleus reach not only the somatosensory cortex, via the lemniscal pathway, but also the nucleus of the solitary tract, the locus coeruleus and the dorsal raphe nucleus of the ipsilateral side (Mercante et al., 2017). The nucleus of the solitary tract is considered the relay station mediating the TNS-induced changes in several areas of the brain, such as the amigdala, the entorhinal cortex, the structures of the limbic system and the hippocampus, (Fanselow, 2012; Magdaleno-Madrigal et al., 2010; Mercante et al., 2017). The latter also receives a dense bilateral projection from the locus coeruleus (Loy et al., 1980), which regulates hippocampal and cortical functions through its noradrenergic projections (Loy et al., 1980; Hansen and Manahan-Vaughan, 2015). In addition, the nucleus of the solitary tract and the locus coeruleus are potential relay stations mediating the neuromodulatory influence exerted by trigeminal afferents on limbic structures (Sasa and Takaori, 1973). These experimental data support an indirect action of TNS on cortical structures through a main action exerted at the brainstem level from where trigeminal afferents are widely distributed to the above cerebral structures, which have been associated to temporal lobe epilepsy (Bone et al., 2012), are considered as highly epileptogenic (Bertram et al., 1998; O'Shea et al., 2000; Sato et al., 1998; Vismer et al., 2015) and major contributors to the spread of epileptic activity to other brain areas (Majak et al., 2002; Stevens et al., 1988). In light of these experimental data, the mechanisms by which TNS exerts its antiepileptic effects have been mainly attributed to its regulatory action on the abovementioned brainstem and forebrain structures, which play a pivotal role in the physiopathology of the epilepsy (Wang et al., 2016; Mercante et al., 2017). In this regard, TNS may act within the frame of an epileptic network, a system composed of cortical and subcortical regions that are anatomically and functionally connected, where changes in activity in any part of it can influence the others (Spencer, 2002; Kramer et al., 2012). In this context, a TNS action exerted

mainly at brainstem level may spread to diverse cortical areas, such as temporal and limbic ones. The convergence between SPECT signal (which evidenced significant effects of TNS in the temporal and limbic regions) and the anatomo-electro-clinical signs indicating a picture of temporal lobe epilepsy in 80% of cases, might allow considering also a possible direct action of TNS on these regions.

Even though the present data do not allow claiming that the cortical rCBF changes are causally related with the antiepileptic action of TNS, it stands to reason that such modifications of cerebral perfusion could indirectly influence the neuronal activity or rather represent the result of a TNS-induced change in neuronal activity. In this regard, a correlation between rCBF redistribution and epileptogenicity has been previously reported, with a rCBF increase in the temporal lobe leading to a reduction of epileptic seizures in a linear way (Weinand et al., 1997).

4.1 Study limitations

Some limitations to this pilot study need to be acknowledged. One relates to the heterogeneous and small sample size. However, DRE is a rare condition and samples of this magnitude are common in studies conducted in this category of epileptic patients. Moreover, given that PET and SPECT imaging studies using TNS are not available, those using VNS enrolled samples from 3 to 12 patients (Ko et al., 1996; Ring et al., 2000; Vonck et al., 2000; Sucholeiki et al., 2002; Barnes et al., 2003; Henry et al., 2004).

Another limitation of the study is the lack of a control group, which may raise concerns on the specificity of the TNS effects for epilepsy. Unfortunately, due to the invasiveness of the SPECT procedure, the institutional ethics committee did not approve the study in healthy controls, which prevented the enrolment of a control group. Possibly for the same ethical concerns, none of the PET and SPECT studies using VNS as peripheral neuromodulation method in epilepsy had a healthy

control group (Ko et al., 1996; Vonck et al., 2000; Ring et al., 2000; Sucholeiki et al., 2002; Barnes et al., 2003; Henry et al., 2004).

Finally, the lack of a sham TNS session is another limitation which leaves it open whether any change in imaging parameters is due to the intervention or reflects unspecific effects related to repeated measuring or placebo effects. A sham TNS had been planned in patients but, again, was listed among the ethical concerns due to the need to perform multiple administrations of radioactive tracer. To partially compensate for this limitation, we chose to renounce a sham TNS SPECT and rather perform a baseline and a real TNS SPECT to be able to compare the baseline with our normative dataset and the real TNS SPECT with baseline, so that each patient could serve as her/his own control.

Although these limitations may reduce the impact of this pilot study, its preliminary results offer useful cues for the interpretation of clinical works describing the antiepileptic effect of TNS but lacking mechanistic explanations, and should be viewed as a starting point for further research over a larger sample, integrated by a control condition and a control group.

4.2. Conclusions

This study provides first evidence that short-term TNS induces a consistent increase in cortical perfusion of the temporal and limbic lobes. However, the main issue about the network involved by TNS within different brain regions, remains unresolved and further large controlled studies are needed to confirm the efficacy of TNS in epilepsy, as it is the case of attention-deficit/hyperactivity disorder, for which TNS has received the approval of the Food and Drug Administration (FDA News Release, 2019).

Epilepsy is a network disease process involving multiple regions of the brain in the genesis of seizures or in the maintenance of epileptogenicity. Therefore, further studies are needed to improve

413 the anatomical knowledge of the cortical areas involved by trigeminal nerve stimulation. In this
414 regard, the clinical benefits of TNS vs sham have been documented in DRE only with chronic
415 protocols of stimulation (De Giorgio et al. 2013) and, more recently, in temporal DRE in
416 comparison with medical therapy (Gil-López et al., 2020). Therefore future research should
417 investigate the effects of chronic TNS on neurophysiological and neuroimaging outcomes and
418 correlate the findings with the clinical effects of TNS in epileptic patients.

419

420

421 **References**

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630

631 **Figure legends**

632

633 **Figure 1. Brain perfusion SPECT of a representative patient (P04) with drug-resistant**
634 **epilepsy.**

635 A) The qualitative transaxial SPECT slices at baseline, compared with B) those obtained after 20
636 minutes of transcutaneous trigeminal nerve stimulation (TNS) evidenced a global increase in the
637 cortical tracer uptake. C) The quantitative analysis performed with the NeuroGam® software
638 showed, at baseline, areas of decreased cerebral blood flow in the left limbic lobe (z-score = -1.8),
639 in the left temporal lobe (z-score =-2.1) and in parietal lobe bilaterally (z-score = left lobe: -3.4:
640 right lobe: -2.7). D) Following TNS, an overall increase in cerebral perfusion was associated with a
641 reduction of the hypoperfusion in the left limbic lobe (z-score =-1.4), left temporal lobe (z-score = -
642 0.7) and in the parietal lobe bilaterally (z-score = left lobe: -1.2: right lobe: -0.6).

643

644 **Fig, 2. EEG trace recorded from the representative subject (P04) whose brain perfusion**
645 **SPECT is shown in Figure 1.**

646 Awake scalp EEG showing a posteriorly dominant symmetrical and reactive alpha rhythm with
647 brief trains of interictal and repetitive spike and waves complexes in the right anterior temporal
648 region, spikes with phase reversal at F8 and synchronous sharp waves and spike waves in the right
649 posterior temporal region.

650

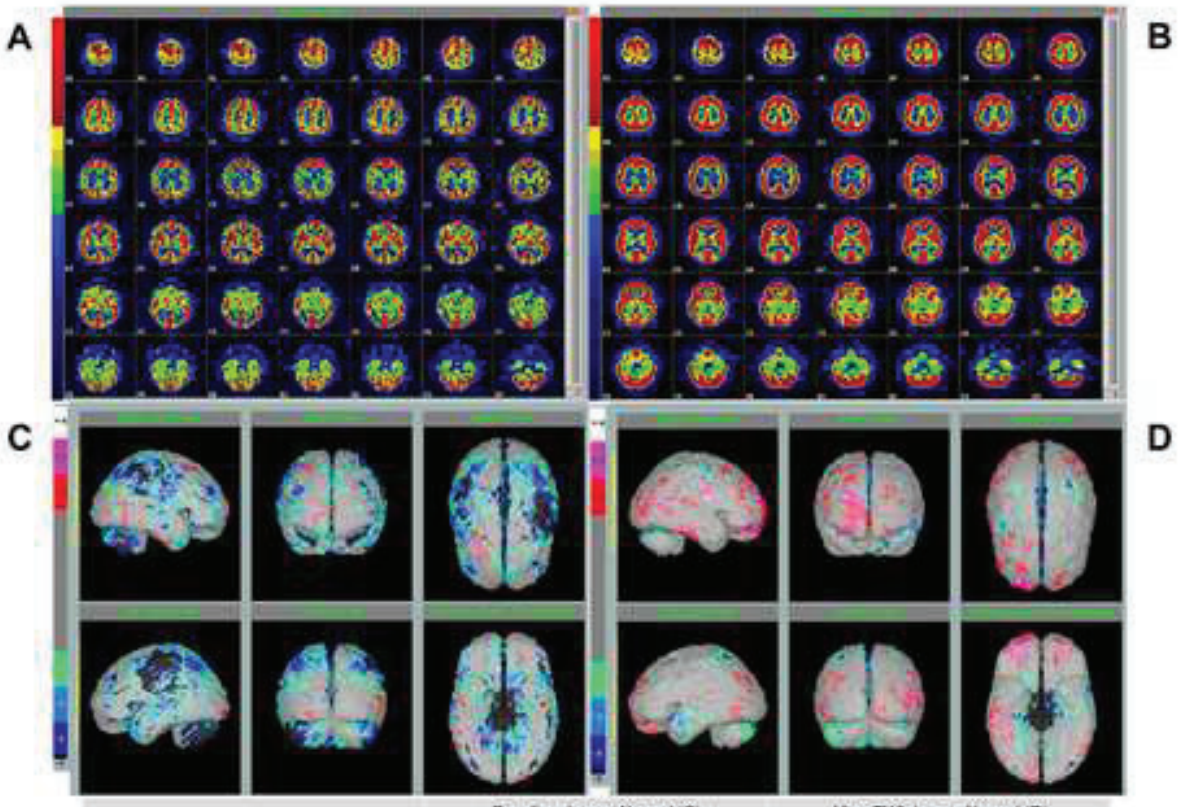
651 **Fig. 3. Calculation of the Odds Ratio over five lobes of both cerebral hemispheres**

652 A) Comparing the number of events showing increase in tracer uptake/events showing no change
653 between the trigeminal nerve stimulation (TNS) and baseline conditions in 5 lobes on both

hemispheres, the calculation of the odds ratio evidenced an overall significant effect favoring a TNS-induced increase in rCBF (70% of cases; p=0.0005). B) The analysis of the odds ratio by lobe and by side evidenced a significant bilateral increase of tracer uptake in the limbic (left and right: p=0.02) and temporal (left and right: p=0.02) lobes following TNS.

Figure 1

Click here to access/download;Figure;Figure 1.tif



	Baseline data vs Normal (C) Mean standard deviations	After TNS data vs Normal (D) Mean standard deviations
Frontal lobe Left Roi	-1.4	-0.1
Frontal lobe Right Roi	-0.8	0.9
Limbic lobe Left Roi	-1.8	-1.4
Limbic lobe Right Roi	-1.5	-1.0
Occipital lobe Left Roi	-1.2	-0.4
Occipital lobe Right Roi	-1.1	0.2
Parietal lobe Left Roi	-3.4	-1.2
Parietal lobe Right Roi	-2.7	-0.6
Temporal lobe Left Roi	-2.1	-0.7
Temporal lobe Right Roi	-1.4	-0.3

Figure 2

Click here to access/download;Figure;Figure 2.tif

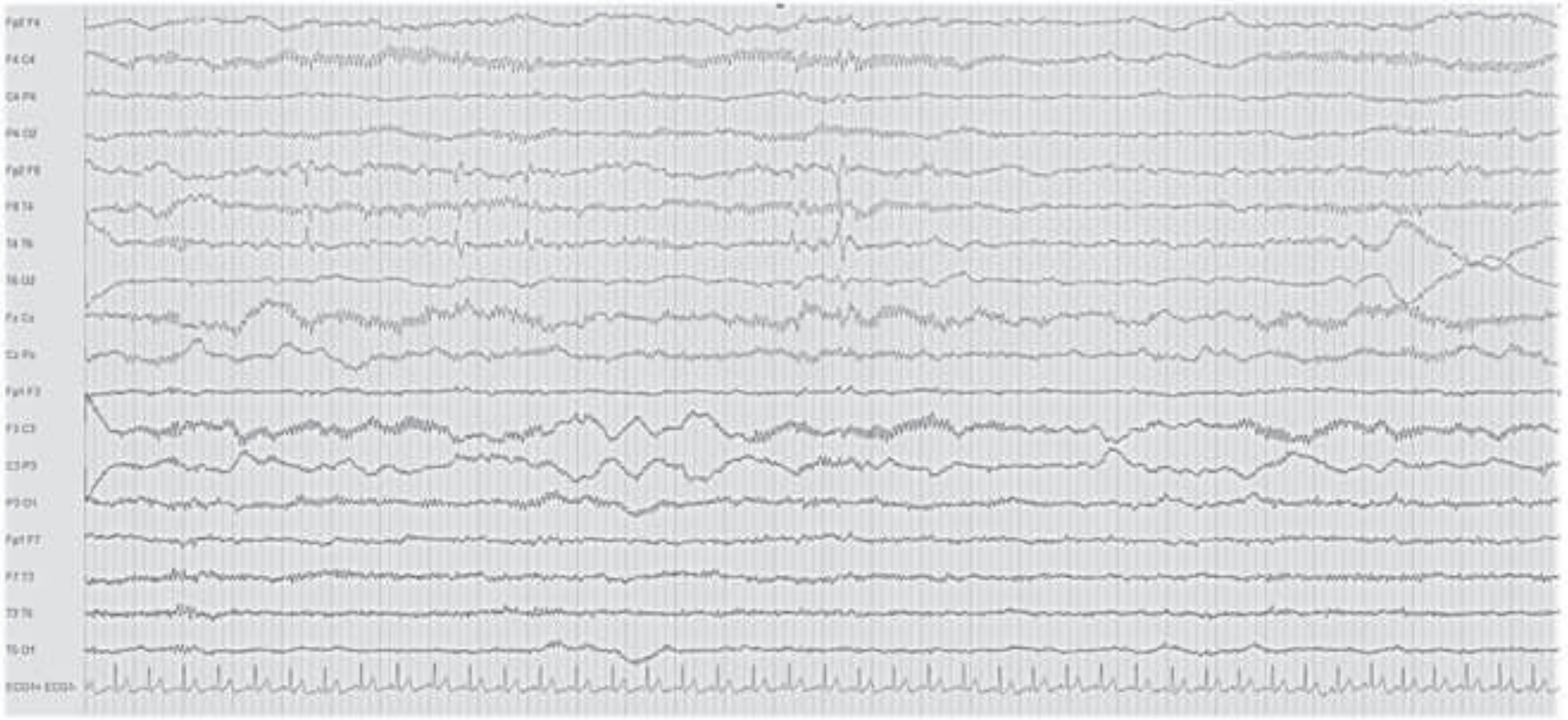
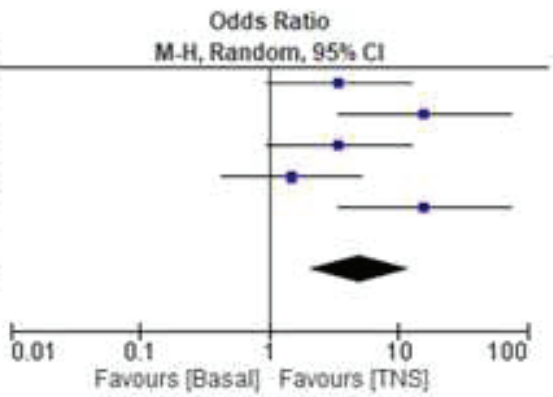


Figure 3

A

Study or Subgroup	rCBF increase Events	Total	No change Events	Total	Odds Ratio M-H, Random, 95% CI
Frontal	13	20	7	20	3.45 [0.94, 12.65]
Limbic	16	20	4	20	16.00 [3.40, 75.34]
Occipital	13	20	7	20	3.45 [0.94, 12.65]
Parietal	11	20	9	20	1.49 [0.43, 5.19]
Temporal	16	20	4	20	16.00 [3.40, 75.34]
Total (95% CI)		**100**		**100**	**4.96 [2.02, 12.18]**
Total events	69		31		

Test for overall effect: Z = 3.50 (P = 0.0005)



B

Study or Subgroup	rCBF increase Events	Total	No change Events	Total	Odds Ratio M-H, Random, 95% CI
Frontal left	7	10	3	10	5.44 [0.80, 36.87]
Frontal right	6	10	4	10	2.25 [0.38, 13.47]
Limbic left	8	10	2	10	16.00 [1.79, 143.15]
Limbic right	8	10	2	10	16.00 [1.79, 143.15]
Occipital left	7	10	3	10	5.44 [0.80, 36.87]
Occipital right	6	10	4	10	2.25 [0.38, 13.47]
Parietal left	6	10	4	10	2.25 [0.38, 13.47]
Parietal right	5	10	5	10	1.00 [0.17, 5.77]
Temporal left	8	10	2	10	16.00 [1.79, 143.15]
Temporal right	8	10	2	10	16.00 [1.79, 143.15]
Total (95% CI)		**100**		**100**	**4.60 [2.42, 8.74]**
Total events	69		31		

Test for overall effect: Z = 4.66 (P < 0.00001)

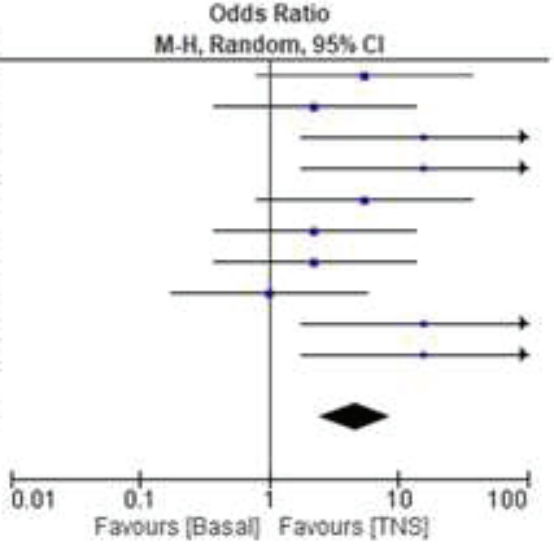


Table 1. Clinical and demographic features of patients with drug resistant epilepsy

PATIENT ID	SEX	AGE (years)	SIMPTOMATOLOGY	EPILPEPTIC FOCUS	MRI	AEDs	NUMBER OF SEIZURES (/month)	DURATION OF EPILEPSY (years)
P01	M	46	Alteration in consciousness and loss of motor control, contralateral eyes deviation, rare generalized tonic-clonic seizures	Left Temporal-Occipital	Left Occipital Gliosis	LTG+LEV+PER	3	18
P02	M	44	Loss of contact, behavioral automatism, generalized tonic-clonic seizures	Left Fronto-Temporal	Cryptogenic	LEV+LAC	2	42
P03	F	38	confusion and loss of contact (psychomotor state), rare generalized tonic-clonic seizures	Bilateral Temporal	Bilateral Peritrigonal Nodular Heterotopia	LTG+ LEV	3	18
P04	M	29	Psychomotor state, generalized tonic-clonic seizures	Right Temporal	Cryptogenic	LEV+LAC	4	2
P05	M	51	Left clonic seizures and secondarily generalized tonic-clonic seizures	Right Frontal	Right Frontal Focal Cortical Dysplasia	LEV+CBZ	2	3
P06	F	32	Right facial clonus without alteration of consciousness, rare generalized tonic-clonic seizures	Left Frontal	Cryptogenic	VPT+LEV	2	6
P07	M	54	Auditory aura, psychical hallucinations (dreamlike experience and elation) Alteration of consciousness, rare generalized tonic-clonic seizures	Left Temporal (EEG: Bilateral Frontal)	Cryptogenic	PRM+TPM	4	33
P08	F	38	Auditory and labyrinthine auras and loss of contact , rare generalized tonic-clonic seizures	Left Temporal	Cryptogenic	LTG+LEV+PER	4	15
P09	M	58	Automotor seizures with distal automatism and limb dystonia, loss of contact and postictal drowsiness	Bilateral Temporal	Cryptogenic	VPT+LEV	2	9
P10	M	49	Cephalic aura, unresponsiveness, masticatory movements, automatism involving right hand, loss of postural tone and fall, post-ictal aphasia	Right Temporal	Cryptogenic	CBZ+PRM+TPM	3	30

F, female; M, male; EEG, Electroencephalogram; MRI, Magnetic Resonance Imaging; AEDs, Antiepileptic drugs; LTG, lamotrigine; LEV, levetiracetam; LAC, lacosamide; CBZ, carbamazepin; VPT, valproate; PRM, primidone; PER, perampanel; TPM, topiramate; Epileptogenic focus defined by pre-surgery video EEG in P09 and P10; presumed on the basis of anatomo-electro-clinical features in P05, P06 and P08; Expressed on the basis of the symptomatogenic zone and localizatory symptom (in contrast with intercritic EEG) in P07; hypothetical in P01, P02, P03, P04, due to aspecific semiology and intercritic EEG with no localizatory and lateralization value.

Table 2. Data obtained from the qualitative analysis of SPECT imaging of patients with drug-resistant epilepsy at baseline and after 20 minutes of transcutaneous bilateral stimulation of the infraorbital branch of the trigeminal nerve (baseline and TNS sessions separated by two weeks).

Patient ID	Extension of hypoperfusion	Localization	Lateralization	baseline Hyp_score	TNS Hyp_score	Cortical tracer uptake after TNS
P01	FA	TM and FT	Left	3	1	-
P02	WA	T	Bilateral	3	1	Global increase
P03	WA	TM, P T	Bilateral Left	2	2	Global increase
P04	FA	TP	Bilateral	2	1	Global increase
P05	WA	FP, TP, TM	Bilateral	3	3	-
P06	FA	TP TM	Bilateral Left	2	1	Global increase
P07	WA	T, TM F	Bilateral Left	3	3	-
P08	WA	TP, TO	Bilateral	3	1	Global increase
P09	WA	TP, TM, O	Bilateral	3	2	Global increase
P10	WA	TP, TM	Bilateral	4	2	Global increase
Group median data P1 -P10				3	1.5*	

TNS, trigeminal nerve stimulation; Hyp, hypoperfusion; **FA**, focal area; **WA**, wide area; **T**, temporal; **M**, mesial; **TM**, Temporo-mesial; **F**, frontal; **P**, parietal; **O**, occipital. *Significant for $p<0.05$ (calculated by Wilcoxon Signed Rank Test).