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NEUROIMAGING AND FLUID DIAGNOSTIC AND PROGNOSTIC BIOMARKERS IN ADULT PATIENTS WITH STATUS EPILEPTICUS

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2. Abstract

2.1. Abstract (English version)

Status Epilepticus (SE) is a common neurological emergency characterized by high short term morbidity and mortality. Identifying diagnostic and prognostic biomarkers could help in the evaluation and management of SE patients. Beside clinical-electroencephalographic evaluation, a multimodal approach based, on the measurement of neuro-glial injury biomarkers and on the identification of acute neuroimaging alterations related to SE could help both rapidly identifying those patients who will eventually develop short and long term consequences of SE and assisting the diagnosis in more doubtful cases. This thesis aims to: 1. determine the cerebral CT perfusion (PCT) patterns correlated to SE and define their role in supporting the diagnosis of NCSE; 2. define the profile changes of serum and CSF biomarkers of neuro-glial degeneration and their potential prognostic role; 3. define the usefulness of such a multimodal evaluation to improve SE management in clinical practice. This is a prospective monocentric collection of adult patients with SE. Enrolled patients underwent to the acquisition of serum samplings during SE within 72 hours from its diagnosis. Neuro-glial injury biomarkers (neurofilament light chain [NfL] and S100B) were measured. The same fluids' biomarkers were measured in control groups: age and sex-matched healthy controls (HC) and epileptic patients (EP). Outcome measures were: 30 days mortality, disability and treatment refractoriness. In NCSE, an analysis of the characteristics of PCT and EEG patterns and their relationship was then made. Twenty-one focal NCSE patients were studied with PCT and EEG in the acute phase. Eighteen patients (86%) had focal hyper-perfusion patterns and 3 (14%) a normo-perfusion patterns. In patients with hyper-perfusion patterns there was a perfect (100%) concordance in spatial localization of focal multilobar cortical hyper-perfusion and focal ictal activity. Among the hyper-perfused patients, all the 12 patients with continuous pattern (CP)

showed cortical hyper-perfusion while only 3 out of 6 (50%) with waxing and waning pattern (WWP) had cortical hyper-perfusion (χ^2, p = 0.03).

In 87 SE patients serum levels of NfL and S100B were measured. In SE group, serum levels of NfL and S100B were significantly higher compared to EP and HC groups (p < 0.001). NfL above 70.25 pg/ml were found to be an independent predictor of 30 days mortality after correction for age (OR 5.71 95% CI 1.09-29.98 p = 0.04). NfL above 33.4 pg/ml showed to be an independent predictor of 30 days disability after correction for age (OR 3.9 95% CI 1.41-10.64 p = 0.009); NfL above 19.75 pg/ml appeared as an independent predictor of refractoriness development (OR 8.2 95% CI 2.08-32.58 p = 0.003). Serum S100B levels appeared significantly higher in patients who died and in those who worsened within 30 days even though they did not predict outcome. Moreover, no significant differences were found in serum levels of S100B between responsive and refractory SE. Comparing patients who developed epilepsy and those in whom SE remained an isolated event, no differences in serum NfL levels were found while serum levels of S100B were significantly lower in patients who developed epilepsy. Nevertheless, neither NfL nor S100B were found to be predictors of epilepsy development after SE.

In conclusion, hyper-perfusion PCT patterns represent an imaging biomarker to assist and support the diagnosis of NCSE in the emergency setting, especially in doubtful cases. Serum NfL levels seem to be helpful for the prognostication in terms of short term death, functional outcome and refractoriness development.

Key words: biomarkers, diagnosis, prognosis, biological fluids, status epilepticus

2.2. Abstract (Italian version)

Lo Stato Epilettico (SE) è una frequente emergenza neurologica caratterizzata da elevata morbidità e mortalità nel breve termine. Biomarcatori prognostici e diagnostici possono aiutare nella valutazione e nella gestione di questi pazienti. Un approccio multimodale, che aggiunga, a fianco della valutazione clinico-elettroencefalografica, la misurazione di biomarcatori neuroradiologici e di danno neuro-gliale può permettere di migliorare l'identificazione di coloro che svilupperanno sequele a breve e lungo termine ed assistere la diagnosi, specialmente nei casi più dubbi. Scopi della tesi sono: 1. Determinare i patterns perfusionali di CT (PCT) dello SE e definirne il ruolo diagnostico nei casi di SE non convulsivo (NCSE); 2. Definire i livelli sierici e liquorali di biomarcatori di danno neuro-gliale e valutarne il ruolo prognostico; 3. Definire l'utilità di un approccio multimodale nella diagnosi e prognosticazione dello SE.

I pazienti arruolati sono stati sottoposti a prelievo sierico durante lo SE, entro 72 ore dalla sua diagnosi per la misurazione di Neurofilamenti a catena leggera (NfL) e di proteina S100B. Gli stessi biomarcatori sono stati misurati nei gruppi di controllo: controlli sani (HC) e pazienti con diagnosi di epilessia (EP) omogenei per età e genere. Le misure di outcome misurate sono: mortalità e livello di disabilità a 30 giorni dallo SE e sviluppo di refrattarietà al trattamento. Nei pazienti con NCSE, è stata effettuata un'analisi delle caratteristiche dei patterns perfusionali ed EEG.

Ventuno pazienti con NCSE sono stati studiati con PCT ed EEG. In 18 pazienti (86%) è stato evidenziato un pattern di iper-perfusione ed è stata osservata una concordanza spaziale perfetta (100%) tra la sede multilobare dell'iper-perfusione e la sede dell'attività critica focale all'EEG. Tutti i pazienti con patterns epilettici continui all'EEG (12 pazienti) presentavano un pattern di iper-perfusione mentre soltanto 3 su 6 (50%) con patterns EEG discontinui (WWP) avevano iper-perfusione (χ^2, $p = 0.03$).

Per 87 pazienti è stato possibile ottenere i livelli sierici di NfL e S100B. Nei pazienti con SE, i livelli di entrambi i biomarcatori sono risultati significativamente maggiori rispetto a quelli rilevati nei gruppi di controllo ($p < 0.001$). NfL sierici > 70.25 pg/ml sono risultati un predittore indipendente di mortalità a 30 giorni dopo correzione per età e refrattarietà (OR 6 95% CI 1-36.12 $p = 0.05$); NfL > 33.4 pg/ml sono risultati predittore indipendente (dopo correzione per età) di sviluppo di disabilità a 30 giorni (OR 3.9 95% CI 1.41-10.64 $p = 0.009$); NfL > 19.75 pg/ml si sono mostrati un predittore di sviluppo di refrattarietà al trattamento (OR 8.2 95% CI 2.08-32.58 $p = 0.003$). I livelli sierici di S100B invece sono risultati essere significativamente maggiori nei pazienti deceduti o peggiorati clinicamente ma non sono risultati essere predittori per questi ouctomes. SE responsivi e refrattari non hanno presentato differenze significative nei livelli di S100B. Comparando coloro che hanno sviluppato un'epilessia a coloro per i quali lo SE ha rappresentato un evento isolato, non si sono osservate differenze significative nei livelli di NfL mentre i livelli di S100B sono risultati significativamente ridotti in coloro che hanno sviluppato epilessia successivamente. Tuttavia nessuno dei due biomarcatori rappresenta un predittore di sviluppo di epilessia dopo uno SE.

In conclusione, i patterns CT di iper-perfusione rappresentano un biomarcatore neuroradiologico per supportare la diagnosi di NCSE utile soprattutto nei casi più dubbi. Inoltre i livelli sierici di NfL sono utili per la prognosticazione in termini di mortalità e morbidità a breve termine e sviluppo di refrattarietà al trattamento.

Parole chiave: biomarcatori, diagnosi, prognosi, liquidi biologici, Stato Epilettico

3. General introduction

3.1. Definition of Status Epilepticus

The Status Epilepticus (SE) is a neurologic emergency. Through the years, SE has been defined in different ways and its definition changed many times.

The first formal definition of SE was formulated by Gastaut in 1970 in the first ILAE Classification of Seizures in which the SE was defined as "a seizure that persists for a sufficient length of time or is repeated frequently enough to produce a fixed and enduring epileptic condition" (Gastaut, 1970). In the subsequent ILAE revision (1981), the SE was defined as "a seizure that persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur" ("Proposal for Revised Clinical and Electroencephalographic Classification of Epileptic Seizures.," 1981).

In 2001, the Glossary of Descriptive terms (J. Engel, 2001) defined SE as "A seizure that shows no clinical signs of arresting after a duration encompassing the great majority of seizures of that type in most patients or recurrent seizures without interictal resumption of baseline central nervous system function".

All these definitions highlight the central feature of the Status Epilepticus: either a unique prolonged seizure or the repetition of single seizures; both without spontaneous resolution that imply the need for a pharmacological intervention to end it. Nevertheless, in all these definitions, the timeframe to classify it as a SE episode it is not precisely defined.

Given the lack of a unique and precise definition of SE, operational definitions with different length of time have been used in textbooks, papers and clinical studies. In the majority of cases, SE was defined as a seizure that lasts for at least 30 minutes ("Guidelines for Epidemiologic Studies on Epilepsy. Commission on Epidemiology and Prognosis, International League Against Epilepsy," 1993). This definition is certainly useful for epidemiological studies but from a pragmatic point of

view it seems important to start a treatment as early as possible, in particular for generalized convulsive SE (GCSE), to avoid serious consequences. For this reason, Lowenstein et al. (Lowenstein et al., 1999) in 1999 lowered the time threshold at 5 minutes for GCSE as "…≥5 minutes of continuous seizure or two or more discrete seizures between which there is incomplete recovery of consciousness".

In 2015, the ILAE Task Force on Classification of Status Epilepticus (Trinka et al., 2015) proposed a new and unique definition of SE as "a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms which lead to abnormally prolonged seizures (after time point t1). It is a condition that can have long-term consequences (after time point t2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures".

This definition focuses, for the first time, on the "time dimension" defining both the time after which a seizure becomes a SE (t1) so when a treatment must be started; and the time after which the prolonged seizure activity starts to cause neuronal damage (t2) defining how aggressively a treatment should be.

At present, these time points, mostly based on evidences derived from animal studies, are clearly defined for GCSE while for the other forms of SE there is lack of evidences thus they are only indicative. The major time points for the different seizures types are summarized in **Table 1.**

Table 1. Operational dimensions with t_1 indicating the time that emergency treatment of SE should be started and t_2 indicating the time at which long-term consequences may be expected

Type of SE	Operational dimension 1 Time (t_1), when a seizure is likely to be prolonged leading to continuous seizure activity	Operational dimension 2 Time (t_2), when a seizure may cause long term consequences (including neuronal injury, neuronal death, alteration of neuronal networks and functional deficits)
Tonic–clonic SE	5 min	30 min
Focal SE with impaired consciousness	10 min	>60 min
Absence status epilepticus	10–15 min[a]	Unknown

[a]Evidence for the time frame is currently limited and future data may lead to modifications.

Table 1: Time points for the definition of SE. (Trinka et al. Epilepsia 2015)

3.2. Pathophysiology of Status Epilepticus and basis for treatment refractoriness

In the early 19th century, Trousseau wrote about SE that "in the status epilepticus, something happens [in the brain] which requires an explanation". This is clearly showed by the observation that in animal models of SE, seizures rapidly become self-sustaining and continue long after the withdrawal of the epileptogenic stimulus, whether chemical or electrical. Ongoing seizures produces many different changes in the brain thus there is not only a single mechanism that is responsible for the evolution from a single seizure to a SE but different mechanisms that can cause an "excitation-inhibition imbalance". Different changes appears at different times. In the very first milliseconds to seconds after SE beginning, proteins phosphorylation appears. This induces ionic channels to open or close, neurotransmitters and modulators release and receptors desensitization. Between seconds and minutes, receptors trafficking starts. The existing receptors can move from the synaptic membrane into endosomes, or they can be mobilized from storage sites to the synaptic membrane, and this process drastically changes excitability by altering the number of inhibitory and excitatory receptors available in the synaptic cleft. This is particularly important for the synaptic γ-aminobutyric acid type A (GABA-A) receptors: with the persistence of seizures, GABA-A receptors are more and more internalized, while, at the same time, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) and N-methyl-D-aspartate receptor (NMDA-R) move to the synaptic membranes (Naylor, 2005). This important change, on one hand increases excitability and reduces inhibition, while, on the other hand explain the phenomenon of the increasing benzodiazepine resistance and of the contemporary increasing of anti-NMDA-R antagonists effectiveness in ongoing SE. Moreover, interestingly, extra-synaptic GABA-A receptors are not endocytosed, raising the possibility that stimulation of those extra-synaptic receptors might be useful in the treatment of long lasting status epilepticus (Goodkin et al., 2007). In the minutes to hours' time range, changes in neuropeptide modulators happen. These changes are often

maladaptive, with increased expression of pro-convulsive neuropeptides (tachykinins, substance P and neurokinin B) and depletion of inhibitory neuropeptides (dynorphin, galanin, somatostatin and neuropeptide Y) contributing to a state of raised excitability. Finally, in the hours, days, and weeks after the seizures beginning, long-term changes in gene expression happen. Many changes in gene expression are the result of seizure induced neuronal death, and of the neuronal re-organization (Betjemann & Lowenstein, 2015; Chen & Wasterlain, 2006). All these changes can produce different long term irreversible brain damages among which the most commonly recognized is mesial temporal sclerosis.

Besides these mechanisms that determine and sustain the SE, motor forms of SE and among them in particular GCSE itself, determine the development of systemic effects that need immediate recognition and correction to avoid further consequences.

The very beginning of GCSE is dominated by the body's attempt to maintain homeostasis: blood pressure, central venous pressure, blood glucose increase, and the patient become tachycardic. Thus, cerebral blood flow, brain glucose and oxygen utilization increase in the initial phases of a seizure to maintain cerebral homeostasis. Passing time, the homeostatic mechanisms start to fail. It is generally theorized that after 30 minutes (but in some situations this could also begin before), homeostatic failure begins and the patient may need systemic support. Cerebral blood flow, brain glucose, and parenchymal oxygenation decrease and potentially play a role in the cell damage associated with GCSE. Respiratory and metabolic acidosis, electrolyte imbalance (for example, hyperkaliemia), hyperthermia, and rhabdomyolysis may all occur (Chen & Wasterlain, 2006). All these systemic alterations can potentially worsen the brain damage. In this view, although the outcome of SE is mainly due to the etiology of SE itself, the rapid management of SE and the prevention of its systemic complications can potentially avoid or at least reduce the morbidity connected to SE.

This has been well seen in many different animal models of SE (provoked by anti-GABA drugs, glutamatergic drugs, cholinergic drugs and electrical stimulation) and there are clear human evidences (Scott et al., 1998).

3.3. Epidemiology of Status Epilepticus

SE is a relatively common condition with estimated year incidence of 50 000 – 60 000 in the USA (Betjemann & Lowenstein, 2015). In epidemiological studies conducted before the new ILAE definition of SE was implemented, incidence rates ranged from 3.5 to 41 per 100 000 per year in North America (Betjemann et al., 2015; DeLorenzo et al., 1996; Dham et al., 2014; Hesdorffer et al., 1998a; Logroscino et al., 2001; Wu et al., 2002), 9.9 to 27.2 in Europe (Coeytaux et al., n.d.; Govoni et al., 2008; Jallon et al., n.d.; Knake et al., 2001; Vignatelli et al., 2003, 2005), 1.3 to 5.2 in Asia (Ong et al., 2015; Tiamkao et al., 2014, 2015) and 10.8 in Africa (Bhalla et al., 2014). This significant variability in SE incidence is related to both, differences in SE definitions (in particular the duration criteria) among the studies and different distribution of risk factors due to the variation of the population composition (thus, making comparisons about the incidences of SE among studies particularly difficult). The first population-based epidemiological study utilizing the new definition of SE, found a 10% increase in incidence of SE compared to preceding publications (Leitinger et al., 2019). This study showed cumulative incidence of the first non-hypoxic SE of 36.1 per 100.000 adults per year. This was higher than most of the previously reported figure. The high incidence was moderately associated with reduced diagnostic time in a new definition of SE (5 minutes for convulsive and 10 minutes for focal motor or absence SE) and a high proportion of elderly population with increased risk of developing SE (in the elderly aged over 60 years, incidence increases up to 79.9 per 100 000 per year). According to the age of onset, the incidence of SE shows a bi-modal, U-shaped age distribution: the first peak occurred in the first year of life and the second

peak occurred in the elderly population, representing the largest group (Logroscino et al., 2001) of patients at risk for developing SE (Coeytaux et al., n.d.; DeLorenzo et al., 1996; Govoni et al., 2008; Jallon et al., n.d.; Vignatelli et al., 2005).

In the study by Leitinger et al (Leitinger et al., 2019), the high incidence of SE was also the result of an high proportion of Non-Convulsive Status Epilepticus (NCSE, 34.4%) in that study population, as it was more frequently diagnosed due to the new Salzburg consensus electroencephalographic (EEG) criteria for NCSE (Leitinger, Beniczky, et al., 2015).

SE is a condition characterized by a relatively high short term mortality rate. In the majority of the studies, mortality is referred as 30-days case fatality and in some others it is detected at hospital discharge. It varies from 5% to 46% (Coeytaux et al., n.d.; DeLorenzo et al., 1996; Giovannini et al., 2015; Govoni et al., 2008; Jallon et al., n.d.; Knake et al., 2001; Logroscino et al., 1997, 2005; Vignatelli et al., 2003, 2005) with differences among studies related to different inclusion criteria (e.g. inclusion or exclusion of post-anoxic SE, inclusion or exclusion of paediatric population, age distribution etc) and partly related to different aetiologies composition of the different studied populations, since mortality is generally high in acute symptomatic SE due to hypoxia/anoxia or cerebrovascular disease and low in SE due to low levels/withdrawal of anti-seizure medications (ASMs) and withdrawal of alcohol (DeLorenzo et al., 1996; Govoni et al., 2008; Logroscino et al., 2005; Vignatelli et al., 2003) and in patients with remote symptomatic aetiologies.

Refractory SE (RSE) develops in 23% to 43% of patients with SE (Mayer et al., 2002; Novy et al., 2010), with a mortality rate of 17% to 65% (Drislane et al., 2009; Giovannini et al., 2015; Holtkamp, 2005; Mayer et al., 2002; Novy et al., 2010; Rossetti et al., 2005; Sutter, Marsch, et al., 2013). RSE and Super-Refractory Status Epilepticus (SRSE) are more likely to develop in patients with acute brain insult, such as stroke, trauma or infection rather than in those with chronic epilepsy.

3.4. Classification of Status Epilepticus

As it was for the definition of SE, its classification has changed many times too. In the first classifications (Gastaut, 1970; "Proposal for Revised Clinical and Electroencephalographic Classification of Epileptic Seizures.," 1981) the only distinction was between partial and generalized SE.

In 2001 ILAE classification (J. Engel, 2001) based on the seizures' classification, more details were added: Generalized Status Epilepticus (GSE) was divided in Generalized tonic-clonic SE (GCSE), Clonic SE (CSE), Absence SE (ASE), Tonic SE (TSE), Myoclonic SE (MSE); while Focal Status Epilepticus (FSE) recognized Epilepsia partialis continua (EPC), Aura continua, Limbic SE and Hemiconvulsive SE with hemiparesis (HHS).

The 2006 ILAE classification (J. Engel, 2006) outlined nine different possible types of SE: Epilepsia partialis continua of Kojevnikov, Supplementary motor area (SMA) status epilepticus, Aura continua, Dyscognitive focal (psychomotor, complex partial) status epilepticus, Tonic–clonic status epilepticus, Absence status epilepticus, Myoclonic status epilepticus, Tonic status epilepticus, Subtle status epilepticus.

The 2015 last classification of SE (Trinka et al., 2015) introduces four axis of classification according to which every episode of SE is defined:

- Semiology
- Etiology
- EEG correlates
- Age

Semeiology

SE is firstly divided according to the presence or absence of prominent motor phenomena. If there are prominent motor phenomena, these are subsequently classified according to the type of motor activity seen. If there are subtle motor phenomena or no motor phenomena at all, the SE is defined as Non Convulsive Status Epilepticus (NCSE) and it is subdivided according to the level of consciousness (with or without coma). In **Table 2** the classification in relation to the clinical presentation is shown.

Table 2. Axis 1: Classification of status epilepticus (SE)
(A) With prominent motor symptoms
A.1 Convulsive SE (CSE, synonym: tonic–clonic SE)
A.1.a. Generalized convulsive
A.1.b. Focal onset evolving into bilateral convulsive SE
A.1.c. Unknown whether focal or generalized
A.2 Myoclonic SE (prominent epileptic myoclonic jerks)
A.2.a. With coma
A.2.b. Without coma
A.3 Focal motor
A.3.a. Repeated focal motor seizures (Jacksonian)
A.3.b. Epilepsia partialis continua (EPC)
A.3.c. Adversive status
A.3.d. Oculoclonic status
A.3.e. Ictal paresis (i.e., focal inhibitory SE)
A.4 Tonic status
A.5 Hyperkinetic SE
(B) Without prominent motor symptoms (i.e., nonconvulsive SE, NCSE)
B.1 NCSE with coma (including so-called "subtle" SE)
B.2 NCSE without coma
B.2.a. Generalized
B.2.a.a Typical absence status
B.2.a.b Atypical absence status
B.2.a.c Myoclonic absence status
B.2.b. Focal
B.2.b.a Without impairment of consciousness (aura continua, with autonomic, sensory, visual, olfactory, gustatory, emotional/ psychic/experiential, or auditory symptoms)
B.2.b.b Aphasic status
B.2.b.c With impaired consciousness
B.2.c Unknown whether focal or generalized
B.2.c.a Autonomic SE

Table 2: Semeiological axis. (Trinka et al. Epilepsia 2015)

Etiology

The cause of SE is defined "Known or symptomatic" when a certain cause of SE is found. Relating to the temporal relationship between the beginning of SE and the beginning of the cause, it is subdivided in "Acute", "Remote" and "Progressive". When the cause is not certain the term "Unknown or Cryptogenic" should be used.

In this classification disappeared the term "Idiopathic" previously used to indicate a SE in the context of Idiopathic Epilepsy Syndrome because it is theorized that in these syndromes when an episode

of SE appeared it is always triggered by something, so that it could be classified as Acute Symptomatic. In **Table 3** the etiological axis is represented.

Table 3. Etiology of Status Epilepticus
Known (i.e., symptomatic)
Acute (e.g., stroke, intoxication, malaria, encephalitis, etc.)
Remote (e.g., posttraumatic, postencephalitic, poststroke, etc.)
Progressive (e.g., brain tumor, Lafora's disease and other PMEs, dementias)
SE in defined electroclinical syndromes
Unknown (i.e., cryptogenic)

Table 3: Etiological axis. (Modified by: Trinka et al. Epilepsia 2015)

EEG correlates

Since there are no specific patterns for any types of SE and there are no evidence-based EEG criteria for SE; for each episode of SE the EEG should be classified for:

- Location: generalized (including bilateral synchronous patterns), lateralized, bilateral independent, multifocal;
- Name of the pattern: Periodic discharges, rhythmic delta activity or spike-and wave/ sharp-and-wave plus subtypes;
- Morphology: sharpness, number of phases (e.g., triphasic morphology), absoluteand relative amplitude, polarity;
- Time-related features: prevalence, frequency, duration, daily pattern duration and index, onset (sudden vs. gradual), and dynamics (evolving, fluctuating, or static);
- Modulation: stimulus-induced vs. spontaneous;
- Effect of intervention (medication) on EEG.

Age

Five different groups are identified:

- Neonatal (0 to 30 days);

- Infancy (1 month to 2 years);
- Childhood (> 2 to 12 years);
- Adolescence and adulthood (> 12 to 59 years);
- Elderly (≥ 60 years)

3.5. Treatment of Status Epilepticus

Based on the response to treatment, SE is generally divided in four stages/phases:

- Early SE or Stage I
- Established SE or Stage II
- Refractory SE or Stage III
- Super-refractory SE

Early SE or Stage I

Early SE or Stage I is the very first phase of SE. Benzodiazepines are worldwide considered by all guidelines the first-line treatment for SE (Meierkord et al., 2010; Minicucci et al., 2020; Riviello et al., 2006). This class of drugs increases neurons hyper-polarization by binding to GABA A receptor with subsequent increased chloride flux into the neurons, so enhancing inhibitory neurotransmission. The most common used benzodiazepines are: Lorazepam, Diazepam, Midazolam (intravenus administration is considered off label for SE treatment in Italy) and Clonazepam (intravenus route still not available in Italy). Each benzodiazepine could have different routes of administration (Intravenous [IV], Intramuscular [IM], Rectal, Buccal and Intranasal [IN]). Intravenous route is the most used and preferred in-hospital route of administration while the others are quite often preferred in out-of-hospital settings (and in particular intranasal, buccal Midazolam or rectal Diazepam in children treatment (Trinka et al., 2016) whenever an IV line is not

ready available. A Cochrane review (Prasad et al., 2014) on anticonvulsant therapy for SE, found that in a pre-hospital setting, when an IV line is not ready for use, IM Midazolam appears to be non-inferior to IV Lorazepam for seizures and SE interruption and as safe as IV Lorazepam. On the other hand, when an IV line is available, IV Lorazepam carries a lower risk than Diazepam of seizures and SE continuation. From a pharmacokinetic point of view, Lorazepam is less lipid-soluble than Diazepam so that, on one hand, its action starts later than Diazepam´s action but, on the other hand, it does not undergo to the rapid redistribution into peripheral tissues as Diazepam goes and its action persist in time preventing seizures relapse. Thus, IV Lorazepam has to be the preferred drug in early in-hospital SE treatment (Trinka et al., 2016).

Established SE or Stage II

If SE is not controlled by benzodiazepines administration, the SE enters the Stage II. In this stage a therapy with an Anti-seizure medication is the choice. The most important international guidelines (Meierkord et al., 2010; Minicucci et al., 2020; Riviello et al., 2006) suggest the use, as a first line therapy for this stage, of IV Phenytoin/Fosphenytoin.

The efficacy of *Phenytoin (PHT)* is worldwide accepted even if it carries some important risk of systemic side effects (such as cardiac arrhythmias and hypotension) as well as local side effects related to extravasation (local irritation, thrombophlebitis, compartment syndrome, 'purple glove syndrome' and tissue necrosis) (Trinka et al., 2016).

Thus, whenever there is any contraindication to use Phenytoin, IV Valproic Acid or Phenobarbital should be chosen.

Valproic Acid (VPA) enhances the GABA inhibitory action. It has a broad spectrum of efficacy and it has a very rapid onset of action. It has few risks of adverse events (mostly nausea, dizziness, thrombocytopenia) and it carries virtually no cardiovascular and respiratory risks or sedating

properties (Trinka et al., 2014). The major concern is about the possibility of developing hyper-ammonaemia and the related hepatic encephalopathy (Embacher et al., 2006; König et al., 1999). A review of 2014 (Trinka et al., 2014), shows that Valproic Acid is as effective as phenytoin in the treatment of established SE with a lower profile of adverse events compared to phenytoin. Given the presence of few, small randomized control trials (RCTs), international guidelines consider Valproate as an alternative first line therapy to Phenytoin in the treatment of established SE.

IV *Phenobarbital (PB)* is found to be at least as effective as a combination of Diazepam and Phenytoin (Shaner et al., 1988) and it was showed not inferior to Lorazepam in the initial treatment of SE (Treiman et al., 1998). Phenobarbital carries a high risk of Centran Nervous System (CNS) depression (sedation, hypotension and respiratory failure) especially if administered after benzodiazepines used and for this reason its use is nowadays mostly avoided outside the intensive care environment.

There are emerging evidences favouring the use of new, third generation, antiseizure medications (e.g. Levetiracetam, Lacosamide, Perampanel, Brivaracetam) in SE treatment even though randomized, controlled trials are not still available.

Levetiracetam (LEV) acts with a not completely understood antiseizure mechanism, mostly based on the interaction with Synaptic vesicle glycoprotein 2A (SV2A) and, to a less extent, on Ca2+ calcium channels and on glutamatergic transmission. It is a broad spectrum drug with action against all types of seizures with few drug to drug interactions, low adverse events risk profile and without hepatic metabolism. For all these reasons it is now broadly used in everyday clinical practice.

Recently, the results of a randomized, single blind, control study, the Established Status Epilepticus Treatment Trial (ESETT trial) which compared the effectiveness and side effects of IV Levetiracetam, Fosphenytoin and Valproic Acid in the treatment of established Convulsive SE have been published. The compared ASMs were equally effective stopping clinical seizures and improving level of

consciousness by 60 minutes after the start of drug infusion without the need of any additional ASM (47%, 45% and 46% of cases respectively) and with a similar rate of adverse effects (Chamberlain et al., 2020; Cock & on behalf of the ESETT Group, 2011).

Lacosamide (LCS) acts as enhancer of slow inactivation of Na+ channels and reduces the activity of Collapsin response mediator protein 2 (CRMP2). It has few drug to drug interactions, hepatic metabolism and it carries few side effects (the most worrying of which is III degree atrio-ventricular conduction block, mostly reported in patients with known cardiovascular risk factors (Garcés et al., 2014; Krause et al., 2011). To date, there are not prospective randomized control trials on its used in SE, thus Lacosamide is still placed as a second line agents for the treatment of established SE after the failure of phenytoin, valproate or levetiracetam or when these drugs are contraindicated (Trinka et al., 2016).

Perampanel (PER) is a selective, non-competitive, antagonist of glutamate AMPA receptors. At present, the experience related to the use of Perampanel in clinical practice is limited to non-randomized monocetric studies in which Perampanel was mostly reserved to the treatment of Refractory and Super-Refractory SE. Nevertheless, PER appears to have good efficacy (Alsherbini et al., 2020; Beretta et al., 2018; Brigo et al., 2018; Ho et al., 2019; Leo et al., 2018; Strzelczyk et al., 2019).

Brivaracetam (BRV) as Levetiracetam, interacts with the SV2A protein with a greater affinity compared to that of Levetiracetam. Moreover, it is more lipid-soluble thus it can pass through the blood-brain barrier (BBB) rapidly (Gillard et al., 2011; Nicolas et al., 2016). Up to now, there are studies of real world evidence on the use of Brivaracetam for the SE treatment that shows efficacy in 27-57% of cases especially when it is used soon (within 6 hours from the beginning of SE) (Orlandi et al., 2021; Santamarina et al., 2019; Strzelczyk et al., 2017).

Refractory Status Epilepticus (RSE) or Stage III

If seizures continue despite the treatment with at least two trials of intravenous antiseizure medications the SE is defined as refractory. In literature there are different definitions of this condition some of which are based on the time elapsed since the beginning of SE, some others are based on the numbers of failed trial of antiseizure medications (2 or 3 drugs) and on the need of an anaesthetic therapy. This happens in 23-43% of patients (Holtkamp, 2005; Mayer et al., 2002; Novy et al., 2010). In this scenario, especially in patients with motor forms of SE, frequently the patient has to be admitted to the Intensive Care Unit (ICU) and anaesthetic therapy is needed. Irrespective of the potential serious complications associated with the use of anaesthetics, early seizure termination is of vital importance in order to avoid neuronal injury in refractory convulsive SE.

The dosages of anaesthetics has to be tailored on the single patient based on the EEG monitoring. In general the therapy is titrated to reach either the complete disappearance of seizures (seizures suppression) or a deeper suppression of electrical activity intermingled with bursts of slow activity of variable length (a burst suppression pattern, BS). The level of anaesthesia has to be monitored using either repetitive EEG, at least once every 24 hours, or, if there is the possibility, starting a continuous EEG monitoring (c EEG) (Ferlisi & Shorvon, 2012). There are no randomized control trials for this stage and guidelines are based upon retrospective case series. The anaesthetics most frequently used as the first line choice in this stage are *Midazolam*, *Propofol* and *Thiopental* (or *Pentobarbital*). There are no evidences of differences related to efficacy and mortality rate among them and the choice is usually done on the basis of prior experience, usual practices and the profile of the adverse events for each of them (Claassen et al., 2002; Ferlisi & Shorvon, 2012).

Propofol has a mechanism of action not completely understood but it could act as an antagonist of glutamate NMDA receptor (Trinka et al., 2016) and modulating GABAergic function (Shorvon & Ferlisi, 2011). Its action starts rapidly and ends rapidly after the cessation of its infusion with a rapid

regain of vigilance even after prolonged infusion (Ferlisi & Shorvon, 2012). Propofol infusion could create hypotension and cardio-respiratory depression but at a lesser extent when compared to other anaesthetics. The most serious and frightful complication is the Propofol Infusion Syndrome (PRIS), characterized by metabolic acidosis, rhabdomyolysis, renal insufficiency and cardiac failure (Mirrakhimov et al., 2015).

Midazolam is a benzodiazepine drug with great anticonvulsant properties but with short action. Thus at this stage, Midazolam is used as a continuous infusion for prolonged periods. As the other benzodiazepine it could exert cardio-respiratory depression (Trinka et al., 2016). Moreover, one of the most important problem related to its use is that the prolonged administration generates frequently a reduction in its response (a phenomenon known as tachyphylaxis) thus dosage's adjustments are frequently needed.

Barbiturates such as *Thiopental* or *Pentobarbital* acts mostly on the GABA A receptor increasing the GABAergic transmission. Their action is way longer than that of Propofol thus when they are stopped the sedation effect could continue for long time. Moreover, they create hypotension, cardio-respiratory depression, gut paralysis, immunodepression and increased infection risk and they have many drugs interactions (Ferlisi & Shorvon, 2012; Trinka et al., 2016).

In recent years, *Ketamine* gained attention. It exerts its action as an antagonist of NMDA receptor of Glutamate thus it could also carry a neuroprotective effect beside the anticonvulsive one (Fujikawa, 1995). Moreover, compared to the other anaesthetics it does not depress cardio-respiratory function. At present, guidelines recommend its use as a second line therapy after the failure of the aforementioned anaesthetics or whenever their use has contraindications (Alsherbini et al., 2020; Shorvon, 2011).

Finally, there are Inhalation halogenated anaesthetics (in particular Isoflurane). Since they are still quite rarely used there is currently Oxford level 4, grade D evidence to use Isoflurane in adults and

children with RSE (Trinka et al., 2016). Up to now they could not be recommended as a first line therapy in RSE but their administration could be reserved to the subsequent stages (Shorvon & Ferlisi, 2011).

During this phase it is also recommended to continue ASMs therapy, administered the support therapy, pay attention to and treat the possible general complications and try to find and treat the underline cause (because the resolution of the underline cause could represent sometimes, per se, the SE treatment).

Super-Refractory Status Epilepticus (SRSE)

Super-refractory status epilepticus can be defined as SE that continues despite 24 hours of general anaesthesia or recurs on the weaning of anaesthetic therapy (Shorvon & Ferlisi, 2011). It generally develops in patients with severe acute brain injuries or as a de novo condition (e.g. New Onset Refractory Status Epilepticus/Febrile Infection related Epilepsy Syndrome, NORSE/FIRES) in patients with no history of previous epilepsy or other pre-existing relevant neurological disorders and without a clear acute or active structural, toxic, or metabolic cause (Gaspard et al., 2018).

This stage represents a “terra incognita” in which any clinical evidence is completely lacking and treatment is totally based on results from case reports and small case series in which patients at this stage, are frequently treated with multiple drugs at the same time and it is extremely difficult to find out evidences of efficacy for each of them. The backbone of the therapy in this stage is still anaesthetic therapy. It is common clinical practice to continue it for an initial period of 24 hours and then reverse it in a very slow way to avoid withdrawal seizures. If the condition persists anaesthetic therapy is re-established. Thereafter, anaesthetic therapy’s institution/withdrawal patterns should be every 24-48 hours initially and then at 5-7 days-cycles (Shorvon & Ferlisi, 2011). Besides anaesthesia, ASMs should always be administered throughout the course of anaesthesia, so that

when the anaesthetic agent is withdrawn there should be an adequate antiseizures coverage. Even if for each of them the evidences are scarce or anecdotical, an incredible number of different therapies could be tried at this stage: Ketamine, Lidocaine, Magnesium sulphate, Pyridoxine, Steroids and Immunomodulant/Immunosuppressive therapy, Ketogenic diet, Hypothermia and in extreme cases resective neurosurgery. Moreover, it is increasingly recognized that different brain stimulation' techniques could play a role even at this stage of refractoriness. Among them we recognize: Transcranial magnetic stimulation (TMS), Vagal nerve stimulation (VNS), Deep brain stimulation (DBS), Electroconvulsive therapy (ECT) (Ferlisi & Shorvon, 2012; Shorvon, 2011; Shorvon & Ferlisi, 2011; Trinka et al., 2016).

In any case, too rapid changes in the therapeutic regimen should be avoid since they are recognized to be linked to an increased risk of seizures recurrence (Ferlisi & Shorvon, 2012; Shorvon, 2011; Shorvon & Ferlisi, 2011).

Finally, even at this stage it is important to search and to treat the underlying cause and to handle with all the possible complications (Ferlisi & Shorvon, 2012; Shorvon & Ferlisi, 2011).

Figure 1 reports the four steps treatment approach for SE.

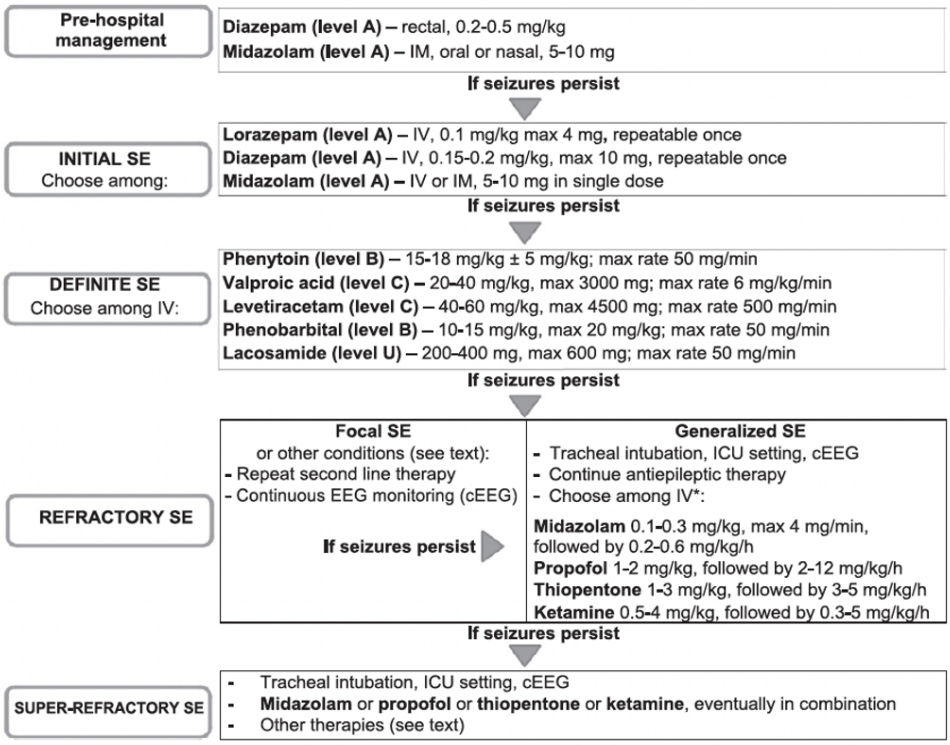

Figure 1: Workflow for pharmacological intervention. * Titration should aim to suppress seizures or burst suppression. (Minicucci et al, Epilepsy&Behavior 2020)

4. Neuroimaging and fluid biomarkers in Status Epilepticus

4.1. Diagnostic issues in Status Epilepticus

The diagnosis of SE relies upon a clinic-electroencephalographic evaluation. In the forms of SE associated with overt motor manifestations, the diagnosis is mainly clinical while in the non-convulsive forms of SE a correct diagnosis can be challenging and the gold standard for the diagnosis is the EEG. In the past decades many different EEG criteria for the diagnosis of NCSE have been formulated (Beniczky et al., 2013; Hirsch et al., 2005; Kaplan, 2007; Young et al., 1996). At present, the diagnosis of NCSE is based on clinical suspicion (acute appearance of quantitative or qualitative disturbances of consciousness without major motor phenomena) and relies upon the evaluation of EEG and the application of the EEG Salzburg criteria (SCC) (Leitinger, Beniczky, et al., 2015; Leitinger et al., 2016). Nevertheless, it is noteworthy that EEG can also present patterns of undetermined ictal significance, that may fall in the so called ictal-interictal continuum (IIC) or Possible NCSE (P-NCSE) such as lateralized periodic discharges (LPDs). However, a rapid diagnosis is crucial, to initiate appropriate antiseizure treatment and in these "gray" situations the decision to treat is not always straightforward.

Hence, the need to search for and to add elements to develop a multimodal evaluation based on *a)* neuroimaging information (identification of the hemodynamic patterns or the peri-ictal abnormalities that support the diagnosis) and *b)* fluid diagnostic biomarkers for the rapid diagnosis of non-convulsive forms of SE.

4.2. Neuroimaging biomarkers of Status Epilepticus

In the past two decades we observed an extensive use of different neuroimaging techniques to evaluate patients with SE in the peri-ictal and postictal phases. This recent development is underscored by the increasing numbers of reports on this topic even though the majority of the

existing literature on neuroimaging in SE consists of case reports, small case series and most of the studies are of a retrospective nature. Different neuroimaging techniques could evidence the different brain alterations that accompany the persistence of seizures.

MRI in particular, may show a broad spectrum of abnormalities that are either the causes or the consequences of sustained epileptic activity named peri-ictal Magnetic Resonance Imaging (MRI) abnormalities (PMA).

In particular, when there is continuous seizure activity an increase in metabolic demand (meaning an increase O_2 and glucose consumption) happens. At the beginning the increased metabolic demand is coupled with an increase in cerebral blood flow (CBF) thus cellular energy status is generally maintained. At this stage, many different studies have shown that perfusion techniques such as CT perfusion (PCT) (Hauf et al., 2009; Romoli et al., 2022), MRI arterial spin labelling (ASL) (Matsuura et al., 2015) or Single-Photon Emission Tomography (SPECT) (Alavi & Stecker, n.d.; Kutluay et al., 2005) could show an hyper-perfusion pattern located both in the area of the maximal ictal activity and involving the propagating areas too. Besides, metabolic neuroimaging and in particular Fluoro-deoxy-glucose Positron Emission Tomography (FDG-PET) could evidence the increase metabolism generally involving both the seizures onset zone and the areas involved by seizures propagation (Franck et al., 1986).

These techniques are particularly useful in NCSE, where they can demonstrate signs of focal cerebral hyper-perfusion and increased metabolism, which could be theoretically useful either to diagnose status epilepticus or to localize the brain regions involved by ictal activity. The use of CT perfusion has been proved feasible and with good sensitivity to detect focal hyper-perfusion in NCSE in two retrospective studies (Hauf et al., 2009; Payabvash et al., 2015). Moreover, other two studies have specifically evaluated the sensibility of ASL in small groups of patients with NCSE (Matsuura et al., 2015; Shimogawa et al., 2017).

Neuroinflammation processes that accompany, since the beginning, SE determine a key element in the pathophysiology of SE represented by the opening of the BBB that determines extravasation and vasogenic edema to appear. This stage could be evaluated by MRI Transverse Relaxation Time 2/Fluid Attenuation Inversione Recovery (T2/FLAIR) and Diffusion Weighted Imaging/Apparent Diffusion Coefficient (DWI/ADC) sequences that show an increment of signal intensity that could affect different brain structures involved in complex cortico-subcortical networks activating during SE.

If ictal activity persists, as occurs in SE, an increase in glucose utilization and oxygen extraction is needed, which cannot be adequately matched by the enhanced blood flow. As a result, the blood flow–metabolism ratio become uncoupled, leading to a reduction of high-energy adenosine phosphates and tissue hypoxia, thereby stimulating anaerobic glycolysis. This determines many different changes at biochemical and molecular levels (e.g. lactate accumulation, failure of Na+/K+ adenosine triphosphate (ATPase) pump, water and Na^+ influx) ultimately leading to cytotoxic edema to appear. At this stage MRI DWI/ADC sequences can show the typical increased signal in DWI and decreased ADC coefficient characteristic of cytotoxic edema.

DWI sequence, can be more sensitive in detecting epileptogenic areas as compared to post-ictal EEG (EEG sensitivity drops dramatically with the time after cessation of a seizure or a SE) (Mendes & Sampaio, 2016).

These alterations have been documented in animal models of SE and correlated to histopathologic findings of vasogenic edema, cytotoxic edema and neuronal loss (Engelhorn et al., 2007; Nakasu et al., 1995).

Peri-ictal abnormalities have been observed in different cortical areas (Cianfoni et al., 2013; Juhász et al., 1998; Kim et al., 2001; Lansberg et al., 1999), as well as involving subcortical

structures such as the mesolimbic structures, (Cartagena et al., 2014; Cianfoni et al., 2013; Giovannini et al., 2018; Kim et al., 2001; Milligan et al., 2009) the pulvinar nucleus of thalamus (Giovannini et al., 2018; Huang et al., 2009; Katramados et al., 2009; Milligan et al., 2009), the splenium of corpus callosum (Giovannini et al., 2018; Milligan et al., 2009; Xiang et al., 2014), the contralateral cerebellum (a sign known as crossed cerebellar diaschisis) (Huang et al., 2009; Milligan et al., 2009; Sabatini et al., 2016; Zaidi et al., 2013), the insular cortex and basal ganglia (Cianfoni et al., 2013; Huang et al., 2009), and the claustrum (Cartagena et al., 2014; Meletti et al., 2015).

These alterations are frequently transitory in nature, and thus they completely or partially disappear during the follow-up. However, in some cases, especially in prolonged SE, these alterations could be present for a long time and they can generate permanent structural damages such as cortical laminar necrosis, mesial temporal sclerosis, and focal brain atrophy, with a consequent permanent functional damage and risk of chronic epilepsy development (Canas et al., 2010; Cartagena et al., 2014; Chatzikonstantinou et al., 2011; Choy et al., 2010; Cole, 2004; Cornwall et al., 2022; Giovannini et al., 2018; Mendes & Sampaio, 2016; Parmar et al., 2006; Pohlmann-Eden, 2004).

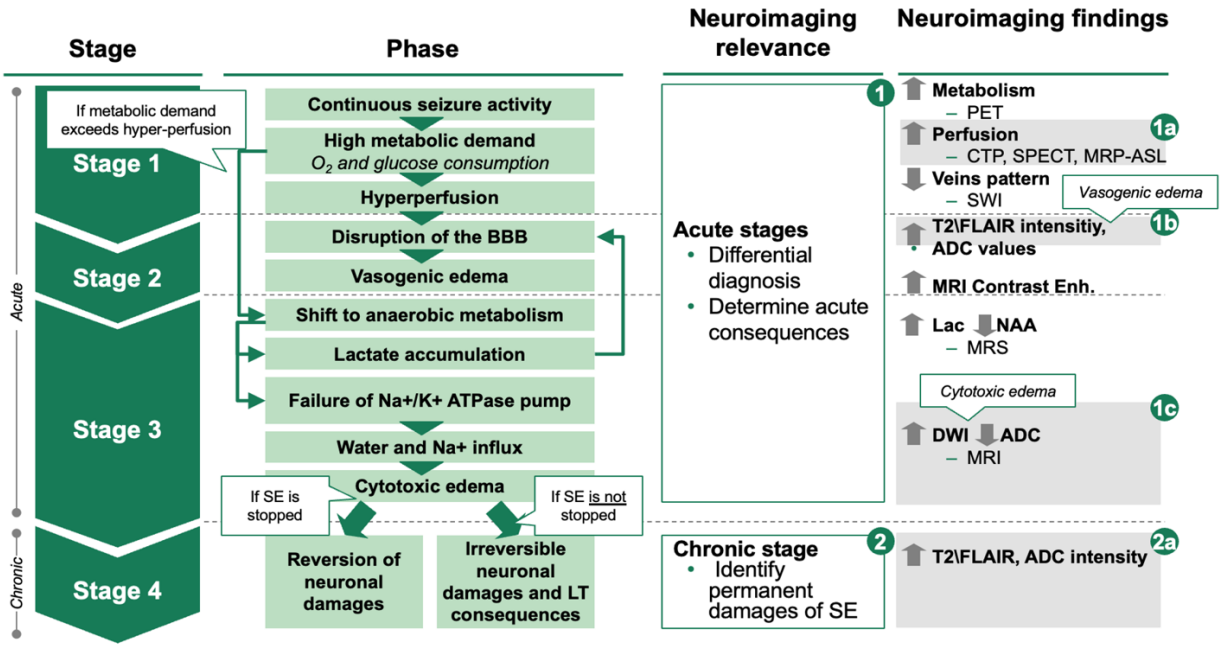

Figure 2: Neuroimaging key in all four stages of the Status Epilepticus' acute and chronic alterations pathogenesis. (Personal oral communication, IEC 2017).

4.3. Neurodegeneration, neuroinflammation and epileptogenesis after Status Epilepticus

4.3.1. Neuroinflammation

Neuroinflammation plays a key role in the process leading to neurodegeneration and epileptogenesis after SE. CNS inflammation can activate microglia (the innate immune system of the CNS) and astrocytes, resulting in damage to the BBB. Increased permeability of the BBB plays an important role letting leukocytes, albumin, and other detrimental factors to massively enter the CNS resulting in an intensification of the inflammation and hyperactivity of neurons (decreasing the seizure threshold by increasing the extracellular levels of potassium and glutamate). Furthermore, excessive bioelectric discharges, seizures and injured neurons might indirectly contribute to inflammation through cell-to-cell signalling with microglia and facilitating BBB leakage, which eventually promote inflammation in a vicious circle (Serrano et al., 2011; Simani et al., 2020).

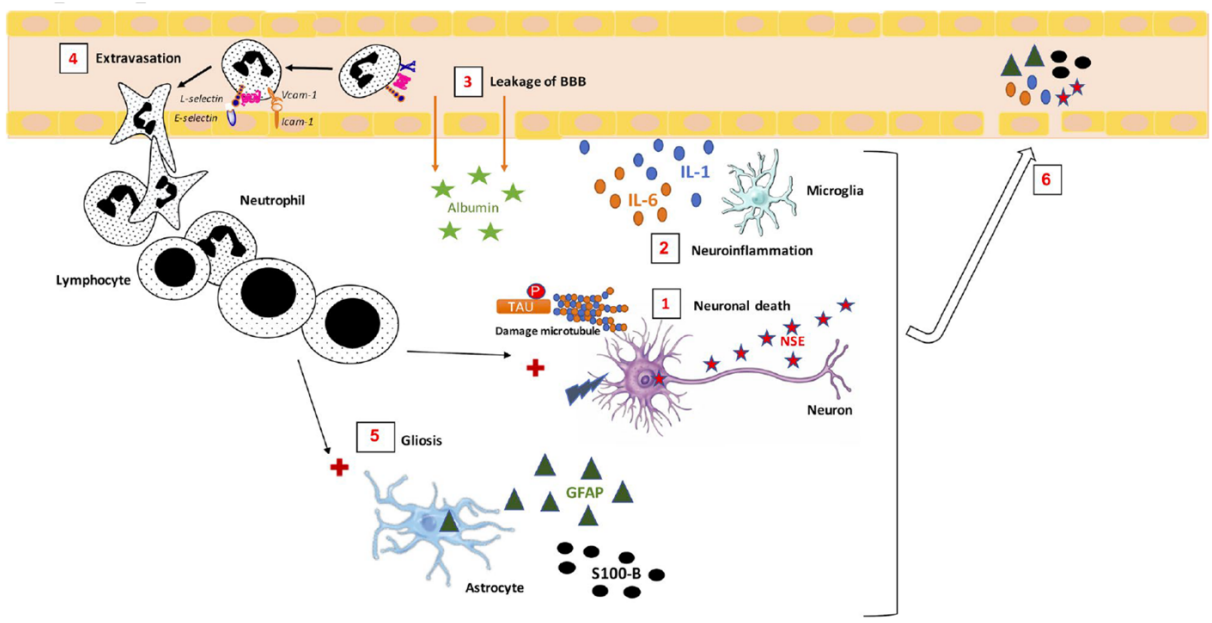

Figure 3: Neuroinflammation and Neuro-glial degeneration in SE. (Hanin et al Epilepsia 2019)

Different animal models in which SE has been induced either by chemoconvulsant drugs or electrical stimulation of various brain areas showed that after SE a rapid and intense inflammatory cascade appears. The pathophysiological interactions among the various inflammatory molecules, and the exact sequence of events are very complex and not yet completely understood. A major role in the inflammatory pathway is played by Cyclooxygenase 2 (COX-2) protein which levels are already raised 30-60 minutes after the beginning of SE. When neurons are damaged they could release a family of molecules known as alarmins among which prostaglandine E2 that takes part of a COX2 dependent process leading to microglia activation thus increasing different inflammatory interleukins' transcript levels such as Interleukin 1β (IL-1β), Tumor Necrosis Factor α (TNF-α), Interleukin 6 (IL-6) that ultimately contribute to the determination of hippocampal injuries. In mice knockout for COX2 in those neurons in which it is generally induced by SE, there was less severe cytokines burst, leakage of BBB and ultimately hippocampal gliosis (Serrano et al., 2011) without diminishing the effect on seizures suggesting that neuroprotection does not seem to derive from less severe seizures per se (Dingledine et al., 2014).

Other major inflammatory pathways that are enhanced after SE involved the activation of IL-1 receptor type 1 (IL-1R1) for IL-1β, Toll-like receptor 4 and Receptor for Advanced Glycation End product (TLR4 and RAGE) for High Mobility Group Box 1 (HMGB1) and Prostaglandin E2 receptor 2 (EP2 receptors) for Prostaglandin E2 (PGE2) (Jiang & Dingledine, 2013; Librizzi et al., 2012; N. Marchi et al., 2009; Noe et al., 2013; Rojas et al., 2015; Vezzani et al., 2015). All these pathways take active part in the increment of permeability of the BBB and extravasation of albumin that is observed hours or days after the beginning of SE both in the hippocampus and in the cortex too. In fact, in those models in which COX-2 is knockout, in wild-type mice treated with the EP2 antagonist (Jiang & Dingledine, 2013; Serrano et al., 2011) and by IL-1 receptor antagonist (IL-1ra, anakinra) (Librizzi et al., 2012; N. Marchi et al., 2009), extravasation of albumin is abolished. Moreover, granulocytes (and in particular neutrophils) begin to infiltrate cortex and hippocampus a few hours after SE onset in rats and mice, and are commonly observed in different SE models (Ravizza et al., 2005; Zattoni et al., 2011).

All these inflammatory pathways considered together could enhance neurodegeneration and molecular and structural changes of neuronal network leading to epileptogenesis.

Thus, the block of these important inflammatory pathways on one hand could reduce the intensity, duration and the severity of SE but even if this does not happen, it nevertheless produces important neuroprotection in terms of reduction of gliosis and prevention of epileptogenesis.

These evidences support the idea of more specific treatments besides anti-seizures drugs, targeting inflammatory pathways with a demonstrated pathologic role either in the acute phase of SE or toward its long-term consequences.

As regards humans, different case reports and case series reported evidence of an important role of neuroinflammation during and after SE too. Children with febrile SE compared to those

presenting with fever alone, have elevated serum levels of IL-1β, IL-6 and HMGB1 (Choi et al., 2011) and Interleukin 8 (IL8) and Epidermal Growth Factor (EGF) (Gallentine et al., 2017). Moreover, a lower ratio of Interleukin 1 Receptor Antagonist/ Inetrleukin 6 (IL-1RA/IL-6) was found to be strongly predictive of hippocampal T2 hyperintensity after FSE thus a potential biomarker of acute hippocampal injury after SE (Gallentine et al., 2017).

Studies on febrile infection-related epilepsy syndrome (FIRES) demonstrated increased Cerebrospinal fluid (CSF) levels of IL-6, Interleukin 8 (IL-8) and C–X–C motif chemokine 10 (CXCL10) when compared with CSF from patients with other inflammatory neurological diseases (Sakuma et al., 2015).

Massive opening of the BBB leads to an extensive extravasation of albumin into the brain and has been reported in patients who died in SE (van Vliet et al., 2007). Moreover, pronounced focal inflammation characterized by intense gliosis and strong IL-1β expression but sparse lymphocytic infiltration has been shown in patients with focal refractory SE treated with surgery (Juhász et al., 2013).

4.3.2. Neuronal loss, gliosis, and epileptogenesis

Many studies on animal model of SE and human patients with SE demonstrate signs of neurodegeneration following SE.

In the pilocarpine adult mice models of SE, do Nascimento et al (do Nascimento et al., 2012) demonstrate that neuronal degeneration and gliosis happened in specific and more susceptible brain areas hours to weeks following SE. In particular, they found that the maximum neuronal damage score and the Fluoro-Jade B-positive (FJB) neurons peak were found in the hilus of dentate gyrus of the hippocampus three and 12 h after SE onset. At one week after SE onset, the greatest neuronal damage score was detected in the Cornu Ammonis 1 (CA1) pyramidal cell

layer and the greatest numbers of FJB-positive neurons were found both in the CA1 and CA3 pyramidal cell layers of the hippocampus. The molecular Cornu Ammonis 3 (CA3) and CA1 pyramidal cell layers of the hippocampus expressed the highest presence of GFAP (Glial Fibrillary Acid Protein) immunoreaction (meaning gliosis) at one and three weeks after SE onset. Similarly, in an adult rat model of kainate-induced status epilepticus, Ebert et al (Ebert et al., 2002) demonstrated the presence of a typical massive neurodegeneration reminiscent of sclerosis in human temporal lobe epilepsy in several limbic structures of both hemispheres (particularly in the piriform cortex). These alterations localized mainly in temporal lobe circuits, could occur also after the induction of brief self-limiting seizures but after SE they are more pronounced and severe.

Moreover, spontaneous seizures recurrence occurred only after SE and not after brief seizures induction (Cardoso et al., 2011). Since SE both kills neurons and also leads to chronic epilepsy, neuronal death has been proposed to be an integral part of acquired epileptogenesis. The loss of synaptic input from the dying neurons is considered a critical signal to induce axonal sprouting and synaptic-circuit reorganization finally leading to epileptogenesis. Nevertheless, there is not an absolute link between epileptogenesis and neuronal death and epileptogenesis could also happen when neuronal death is minimal or absent. In fact, neurons could generate biochemical pathways that are on the programmed neurodegeneration pathway, initiating epileptogenesis without the necessity of having neuronal death as the final outcome (Dingledine et al., 2014).

Neurodegeneration has not only been demonstrated after convulsive SE but also after non-convulsive forms of SE and this has important clinical implications also for humans. In the study of Avdic et al (Avdic et al., 2018) on rats models of electrically induced 2-hours NCSE (demonstrated by intracranial EEG monitoring) even though MRI (Diffusion Tensor Imaging) DTI sequences acquired seven weeks post-NCSE did not show robust long-lasting changes in

fractional anisotropy within the hippocampal epileptic focus, different pathophysiological changes have been shown to develop over time. Neurodegeneration was already evident at six and 24 hours post-NCSE (FJB reaction and minimal S100B increment). At one week post-NCSE, both excitatory (Post-synaptic density protein 95 [PSD-95], Neuroligin-1 [NL-1], N-cadherin) and inhibitory synaptic protein levels (gephyrin, Neuroligin-2 [NL-2], neurofascin [NF]), were decreased in hippocampus, concomitant with an excessive microglial and astrocytic activation (ionized calcium-binding adapter molecule 1 [Iba1] and GFAP increment) while at four weeks, a continuous immune response in the hippocampus was accompanied with neuronal loss and gliosis (increment of GFAP, Iba1, S100B).

As regards humans, there are case reports and case series reporting various degree of atrophy and gliosis following SE. Even though these processes could be widespread, there are areas that are more susceptible to seizures-induced damage such as hippocampus, entorhinal cortex, amygdala, thalamus and other limbic structures. The results from the "Consequences of Prolonged Febrile Seizures in Childhood" study (FEBSTAT) showed that in one year time after febrile SE in children, the majority of those who had acute MRI T2 hyperintensity and increased volume of the hippocampus developed hippocampal atrophy. Moreover, even those without acute hippocampal MRI modifications developed reduced hippocampal growth (Lewis et al., 2014; Shinnar et al., 2012).

Evidences of histopathological atrophy and gliosis following SE in humans have been reported too. Kumar et al (Kumar et al., 2013) reported a case report of an adult patient with a FIRES episode with glutamic acid decarboxylase (GAD) positivity and evidences of bilateral hippocampal swelling and the presence of perictal MRI abnormalities on the left fronto-temporal lobes. The patient underwent to epilepsy surgery to control SE. The specimen revealed extensive neuronal loss and acute necrosis with marked astrocytosis in the hippocampus, mainly

involving the CA1 part but with milder neuronal loss in other parts of the hippocampus and subiculum too; patchy mononuclear inflammation (in particular lymphocytes) forming perivascular cuffs and extending into the surrounding parenchyma. This is the first clear histopathological evidence that hippocampal atrophy following status epilepticus is the result of acute neuronal necrosis and cell loss.

More recently, an interesting study by Lee et al (Lee et al., 2022) showed that after SE even though there aren't evident morphological alterations such as hippocampal atrophy at visual inspection, structural volumes and intra-hippocampal structural co-variance networks were significantly different in patients with SE compared to healthy controls. Moreover, global connectivity was increased.

Even though temporal lobe structures are more susceptible to SE related damages, alterations could appear as more widespread too. Hocker et al (Hocker et al., 2016) reported a case series of 19 patients with SRSE who developed various degree of diffuse brain atrophy after the SE. In that study, atrophy was measured by visual inspection calculating the ventricles-brain ratio (VBR) and comparing its value between the first MRI acquired during SE (within 2 weeks, median of 2 days after SE onset) and the second MRI acquired after the SE resolution (within 6 months, median of 11 days after the resolution). The ΔVBR was of 23.3%. Moreover, there was a significant positive correlations between ΔVBR and the duration of anaesthetic agent use (ρ = 0.54; p = .02) and the duration of hospital stay (ρ = 0.64; p = .003) and a significant negative correlation between ΔVBR and age (ρ = −0.49; p = .04). No significant correlation was observed between the ΔVBR and the level of disability (masured by mRS score) taken at admission (ρ = 0.13; p = .59), discharge (ρ = 0.13; p = .61), and during follow-up (ρ = 0.13; p = .66).

4.4. Neuro-glial degeneration biomarkers in Status Epilepticus

A biomarker is a measurable indicator of a condition or a biological process. In recent years, brain biomarkers have increased interest in many neurological conditions and in epilepsy and in SE too. Nevertheless, despite a plethora of published research, both in basic science and in clinical research, there are still no clinically approved brain biomarkers for epilepsy and SE diagnosis and prognosis.

Biomarkers could potentially be measured in each biofluid but minimally invasive and peripheral (serum/plasma) biomarkers play an important role in the evaluation of brain diseases. The characteristics of an ideal biomarker in SE are the following: (1) It should represent molecular and cellular alterations occurring in SE: it exists in CSF, and has a higher concentration in the brain parenchyma than in plasma then it can exudate when the BBB is opened; (2) It should be stable in the body fluids and be readily available at the bedside; (3) Its biochemical assessment should be easy, quick, and reliable; (4) Its measurement in fluid should represent a test with high sensitivity and specificity meaning that it is low or even undetectable in normal conditions (Hanin et al., 2020; Simani et al., 2020).

When seizures appear and become sustained they could provoke a dysfunction of the BBB with increased permeability. Leukocytes and albumin could thus enter activating microglia and astrocytes. This determines neuroinflammation and oxidative stress that enhances neuronal and glial damage. In this environment, the excitability of neurons increase and the seizures threshold decrease thus sustaining seizures in a vicious cycle (Hanin et al., 2020; Simani et al., 2020).

Potentially *diagnostic biomarkers* reflecting the occurrence of the process within the brain could facilitate the clinical translation of treatments such as anti-inflammatory or antioxidant treatments that could modify long term sequelae in SE besides anti-seizure medications. This

would allow stratifying patients who might best benefit from the treatment and also provide a pharmacodynamic measure of drug's efficacy.

Moreover, the identification of *prognostic biomarkers* predicting worse outcomes in patients with SE, including mortality, morbidity, cognitive and behavioral sequelae and epilepsy development would be extremely helpful for preventative interventions in patients at high risk (Terrone et al., 2019). Among these potentially prognostic biomarkers a major role is played by *biomarkers of neuro-glial degeneration*.

4.4.1. Biomarkers of neural degeneration and damage

Neuron-specific enolase

One of the first and most studied neurodegeneration biomarker is *Neuron-specific enolase (NSE)*. NSE is considered a biomarker of neuronal damage/injury.

NSE is a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. It exists in three dimeric forms, a, ß, and y. The y fraction is exclusive to neurons and neuroectodermal tissue and thus is termed NSE. Neuronal enolase is composed primarily of two gamma – gamma subunits, whereas glial cells contain two alpha – alpha subunits. NSE is released as a result of leakage across an injured neuronal membrane, and elevations of serum NSE are due to neuronal injury and increased permeability of the BBB. Thus, it is considered a biomarker of neuronal injury in different neurological conditions. In clinical practice, it has been validated for the prognostication of post-anoxic encephalopathy.

As regards to SE, there are numerous pre-clinical evidences. In a lithium-pilocarpine rat model of SE a significant elevation of serum NSE was evidenced after SE compared to age-matched controls. Increases in s-NSE were accompanied by histologic evidence of neuronal damage and such elevations of s-NSE appear to be roughly proportional to the extent of irreversible histologic

damage (Sankar et al., 1997). In a Kainik acid (KA) rat model of SE the mechanism of NSE elevation was explored. NSE elevation appeared not related to an elevation of NSE messenger ribonucleic acid (mRNA) expression but to post-transcriptional and/or post-translational changes. Thus, the amount of NSE released from damaged CNS neurons into CSF and serum may correlate with the degree of neuronal injury as well as BBB permeability (Schreiber et al., 1999). The majority of clinical studies on NSE in patients with SE goes back up to the mid-nineties. The studies of De Giorgio et al (DeGiorgio et al., 1995, 1996, 1999) showed that serum NSE was found to be elevated in the major subtypes of SE (generalized convulsive, complex partial, absence, and subclinical SE) and NSE levels correlate with outcome and duration of SE. NSE peaked an average of 41 hours after the onset of SE (41% of subjects peaked within 24 hours, and 33% peaked within 24 to 48 hours). Moreover, the presence of very high NSE levels in complex partial SE (CPSE) and absence SE provided evidences that brain injury occurs even in the absence of an epileptic encephalopathy or an acute brain injury. This means that CPSE and ASE themselves could cause brain injury (DeGiorgio et al., 1996). These results point to a need for an earlier treatment even in these forms of SE.

In the study of Correale et al (Correale et al., 1998) an increased in CSF NSE content in patients with cryptogenic/remote SE compared to control subjects was found within 24 hours from SE, together with the evidence of an alteration of BBB integrity. Furthermore, CSF levels of NSE were significantly higher than serum levels.

In a more recent study from the group of Hanin (Hanin, Denis, et al., 2022) s-NSE levels were confirmed to be higher in SE compared to those found in epileptic group and CSF-NSE levels were higher in SE than in controls. No differences in relation to SE refractoriness, semeiology and etiology was found. Nevertheless, when the diagnostic value of serum levels of NSE was compared to that of S100B and Progranulin, NSE showed a lower discrimination power.

In eleven patients, admitted to the intensive care unit for RSE, who underwent a minimum of three days of continuous EEG monitoring concomitantly with daily serum NSE and S100B assays, the relation of these two biomarkers and EEG scores able to monitor the seizure risk were investigated. Only NSE levels positively correlated with EEG scores, underlining the potential of NSE as a biomarker of EEG activity and of risk of seizure recurrence. In fact, NSE levels above 17 ng/ml were found to be associated with seizure in 71% of patients and an increase of more than 15% of NSE levels was associated with seizure recurrence in 80% of patients (Hanin, Denis, et al., 2022).

UCHL-1

Ubiquitin C-terminal hydrolase (UCH-L1) is a neuron specific cytoplasmic enzyme. There are three relevant enzymes in this class, including UCHL-1, 2, and 3. Only UCHL-1 is expressed abundantly in neurons and forms 1–5 % of total brain protein. Outside the central nervous system (CNS), much smaller amounts are found, e.g. in ovaries and testes.

Its main function is to remove oxidized or increased proteins in the normal or pathologic situations by joining the ubiquitination process.

It has been established as a reliable and potential biomarker of neuronal damage, and its CSF levels raise soon after acute neurologic insults such as ischemic stroke, subarachnoid hemorrhage, and traumatic brain injury. This neuronal protein can be readily detected in CSF and blood very early after injury (3 hours) and it reaches the peak levels within 24 hours which provides a valuable time-window for potential neuroprotective strategies.

Pre-clinical evidences showed that in kindled rats with intra-peritoneal pentylenetetrazole injections there were increased plasma UCHL-1 levels compared to controls, and levels were proportionally elevated in relation to the number of injections (Chmielewska et al., 2019). In

addition, downregulation of UCHL-1 following SE appears deleterious to neuronal survival, interfers with seizure termination contributing to hyperexcitability and exacerbates seizure-induced cell death (Reynolds et al., 2017).

As regards to human studies, Mondello et al (Mondello et al., 2012) measured UCH-L1 in 52 patients within 24 hours from a single or recurrent epileptic seizures and in 19 healthy controls. UCH-L1 concentrations were significantly higher in patients within 48 hours after an epileptic seizure in CSF and within 12 hours in serum compared to controls. Moreover, patients with recurrent seizures (n = 4) had significantly higher plasma UCH-L1 concentrations than patients with one or two seizures. The same results were observed by Asadollahi et al and in the study of Yasak et al (Asadollahi & Simani, 2019; Yasak et al., 2020) in serum. Elhady et al (Elhady et al., 2021) confirmed the great elevation of UCHL-1 serum levels in children with epilepsy compared to healthy controls and found a correlation with seizures severity thus hypothizing the possibility to use it as a biomarker of neuronal damage to monitor disease progression and severity for early identification of those with drug-resistant epilepsy and those who are in need for epilepsy surgery.

In addition, in the study by Li et al (Li et al., 2013) CSF levels appeared more elevated in patients than in controls, and among patients in those with generalized seizures than in those with focal seizures, in those with repetitive seizures and there was a correlation with seizures duration and severity too.

TAU/p-TAU

Tau protein is a microtubule-associated protein (MAPT), principally localized at neuronal level in the CNS where it participates to microtubular assembly and it maintains the structure of the axon.

High total-tau (t-TAU) and phosphorylated-tau (p-TAU) CSF levels are considered to index both axonal and neuronal damage in many different neurological disorders such as Alzheimer disease (AD), traumatic brain injury (TBI), acute ischemic stroke (AIS), viral encephalitis, and Creutzfeldt–Jacob disease (CJD), suggesting that tau CSF levels reflect the extent of axonal damage and neuronal degeneration.

In the intra-amygdala KA mouse model of SE, Alves et al. (Alves et al., 2019) found, in the hippocampus ipsilateral to the injection, neurodegeneration at 24 hours after the injection, mainly located at CA3 subfield; an increase in t-TAU mainly located at CA3 and an increase in p-TAU at CA1 and CA3 subfields respectively starting from four hours post KA injection.

Human evidences on the role of TAU as a biomarker of neuronal and axonal injuries after SE are controverse. In fact, in the study by Palmio et al (Palmio et al., 2009) t-TAU and p-TAU levels were analyzed in 54 patients after single seizures, repeated seizures or SE (9 patients) divided in accordance to the etiologies. The authors concluded that abnormal CSF TAU levels may be found in patients with seizures but there was no correlation between the presence of SE and t-TAU or p-TAU levels and finding increase CSF TAU content in a patient with an epileptic seizure just increases the probability of a symptomatic cause of the event.

In 2015 our group analyzed t-TAU and p-TAU CSF levels in 28 adult patients with a SE episode not symptomatic of an acute cause or progressive cause finding that t-TAU protein was the most sensitive marker of neuronal damage related to SE and it correlated with the duration of SE, refractoriness and disability development.

Nevertheless, more recently Sood et al (Sood et al., 2021) investigated the role of CSF t-TAU in children with SE. They found t-TAU levels significantly lower in SE patients than in controls without significant correlations with type, duration, etiology and response to antiseizure

medications, poor sensorium, outcome of SE and critical care needs. Thus, these authors concluded that CSF t-TAU was not a useful diagnostic or prognostic biomarker in pediatric SE.

Neurofilaments

Neurofilaments (Nf) are considered biomarkers of axonal damage/injury. Nf (A. Yuan et al., 2017) are members of the family of intermediate filaments (IFs) that have a diameter of 10 nm that is "intermediate" in diameter between actin filaments (6 nm) and myosin filaments (15 nm) found in muscle cells. Six major classes of IF proteins are known, and Nf constitutes the class IV. The most important function of Nfs is to maintain the structure of the cytoskeleton of the axons establishing cross-bridging with other filaments and they are abundant in the myelinated axons. Nfs are essential for the radial growth and structural stability of myelinated axons and for achieving the optimal propagation speed (conduction velocity) of electrical impulses along the axons. Synapses have long been considered only the degradative sites for Nfs reaching terminals by axonal transport but Nf could also have a role even in synapses beyond static structural support of the axon caliber: they maintain synaptic plasticity and dendritic spines and they take active part in the process of long term potentiation in the hippocampus. Moreover, Nfs form cellular scaffolds for docking and organization of synaptic vesicles, endosomes, and the endoplasmic reticulum.

From the structural point of view, all Nf subunits have a tripartite structure: a conserved central α-helical rod region (310 amino acids), a short variable head domain at the amino-terminal end (98 amino acids), and a tail of highly variable length at the carboxy-terminal end (63 – 618 amino acids). The difference in the length of the tail mainly determine the differences between the three major classes of Nf: Neurofilament light (NfL), medium (NfM) and heavy (NfH) chains (**Figure 4**).

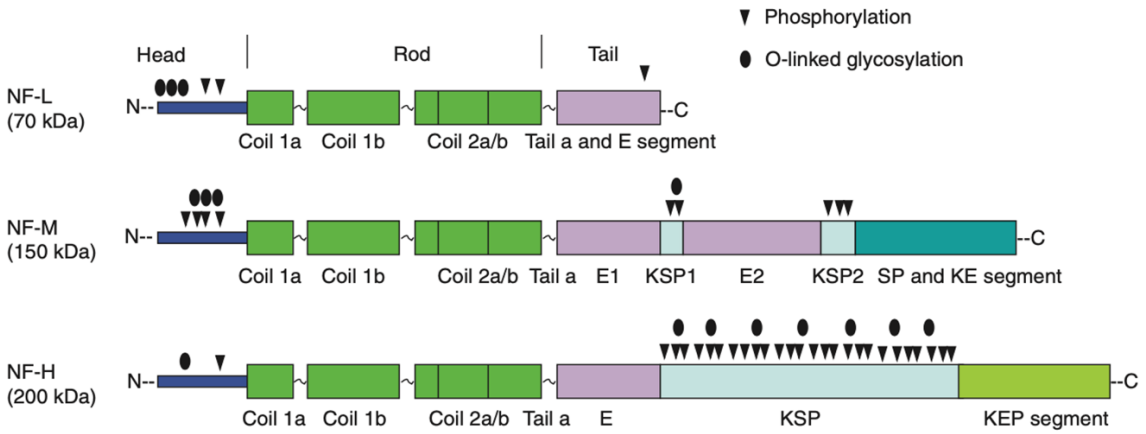

Figure 4: Domain structure and post-translational modifications of neurofilament (NF) subunits. (Yuan et al Cold Spring Harb Perspect Biol 2017)

The formation of Nfs, like that of other IFs, is believed to begin when two monomers associate into polar coiled-coil dimers (45 – 50 nm in length), which is the basic and essential building block of Nfs. Two dimers then form staggered antiparallel tetramers. The lateral association of eight tetramers (a total of 32 monomers) results in the formation of cylindrical structures known as unit-length filaments (ULFs), with an approximate diameter of 16 nm. Gradual end-to-end annealing of these ULFs in the longitudinal direction results in filament elongation, which is followed by radial compaction to ultimately achieve the final Nf of 10 nm diameter (**Figure 5**).

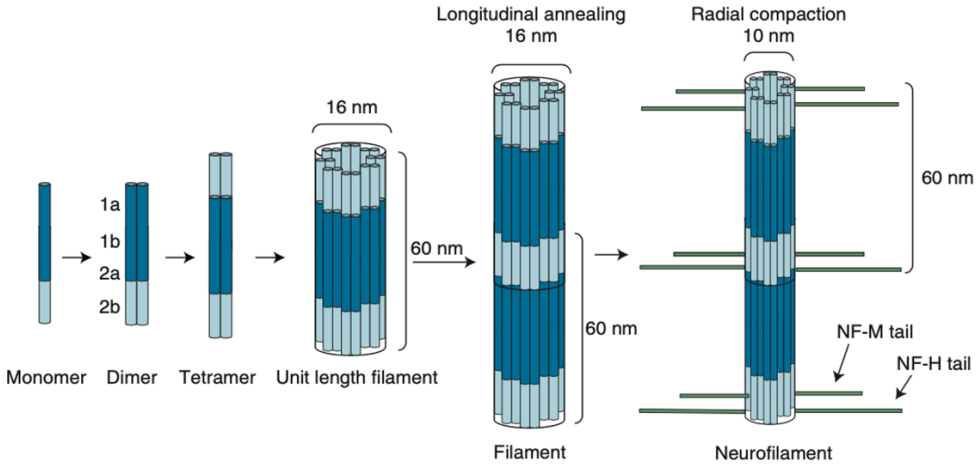

Figure 5: Neurofilament (NF) assembly. (Yuan et al Cold Spring Harb Perspect Biol 2017)

Nfs are among the most highly phosphorylated proteins in brain tissue. All subunits are phosphorylated at their amino-terminal head domain, whereas only the high-molecular-mass subunits (NfH and NfM) are extensively phosphorylated along their carboxy-terminal tail

domains. Neurofilament heavy chain protein (NfH) is the most extensively phosphorylated protein.

Nf proteins are substrates of multiple proteolytic systems, including calpains, the proteasome, and autophagy. NfH and NfM subunits are highly sensitive to proteases once they are dephosphorylated, suggesting that phosphate groups on these subunits confer resistance to protease cleavage and, hence, stability to the Nf cytoskeleton in axons.

Therefore, Nf represent a group of proteins specifically expressed in the axons of neurons, and they have emerged as promising biomarkers for neurodegeneration (markers of axonal injury, axonal loss, and neuronal death) experimentally and clinically in a range of neurologic disorders such as different forms of Charcot – Marie – Tooth disease (CMT), amyotrophic lateral sclerosis (ALS), familial forms of Parkinson's disease (PD), atypical parkinsonian syndromes and Parkinson's disease, multiple sclerosis (MS), spinal cord injury, Alzheimer's disease, frontotemporal dementia (FTD), and vascular dementia.

When axons are damaged or diseased, Nf might be released in significant amounts into CSF and then they pass into the blood stream where they can be measured.

In healthy people, sNfL levels show a u-shaped distribution in relation to age: high levels are found in newborns (probably related to the high neurons turnover of the developing brain), decreasing until late childhood reaching a nadir between the age of 10 and 15 years then beyond youth, sNfL levels increase in a linear fashion until the age of about 60 years, then steadily increasing (representing neuronal loss, which is associated with normal aging). On the contrary, lack of unique evidences exists about the relationship between sNFL and gender.

As regard to epilepsy and SE, in adult rat hippocampus, it has been demonstrated that degradation of Nf and changes in their phosphorylation occur within days after KA SE induction (Sherry & Turner, n.d.; Yang et al., 1996) and this happens even after brief seizures, lasting less

than one hour. On the contrary, in the hippocampus of immature rats the degradation of Nf seems not to happen suggesting that immature brain exhibits a remarkable resistance to neuronal death even exposed to severe, continuous seizures lasting less than one hour. In fact, after SE, a fast and transient increase in the expression of phosphorylation-independent NfH, NfM, and NfL epitopes happens (increment of about 40% from the control levels) and it is already evident 30 minutes after KA injection; it remains elevated up to six hours then their expression returns to the control levels one day after the treatment and remained thereafter unchanged up to seven days. The phosphorylation increase the stability of Nf. Moreover, there is a lack of mossy fibers sprouting and neuronal death in KA-treated immature rats. These findings are in contrast to those found in adult rats (Lopezpicon et al., 2004).

In recent years, interest has increased for Nf in humans with epilepsy and SE. In the study by Rejdak et al (Rejdak et al., 2012) in 41 patients (20 with a single seizure, 11 with repetitive seizures and 10 with SE) without acute symptomatic etiology and 18 controls, CSF content of NfH and heat shock protein 70 (HSP-70) were measured within 48 hours from the cessation of seizures. CSF NfH was increased in single seizures compared to controls and in SE compared to single seizures. NfH and HSP-70 showed a tendency for an inverse correlation only in SE and, in addition, high levels of NfH but low levels of HSP-70 seemed to be related with worse outcome. These results indicate that NfH seems to be a reliable marker of neuronal damage in patients who experience repetitive seizures or SE.

Nevertheless, since NfH have an high molecular weight, they are not suitable to be measured in the serum. For this reason more recent studies have focused their attention on NfL. In the study of Lybeck et al (Lybeck et al., 2021) sNfL levels were measured at 24-48 and 72 hours after cardiac arrest in patients with electrographic status epilepticus (ESE) and compared with those measured in patients without ESE. Moreover, serum levels of GFAP where measured at the same

time-points. After cardiac arrest, ESE appears associated with higher levels of serum NfL which may indicate a potential secondary neuronal injury caused by ESE itself. On the contrary, the association with GFAP, a marker of glial injury, appeared less clear.

4.4.2. Biomarkers of astroglial injury

S100B

S100B is part of the S100 protein family that, at present, comprises 25 Ca^{2+}-binding proteins that have different functions but that exhibit structural similarities. S100B refers to a protein identified in the mid-1960s, characterized by its solubility in a 100% saturated solution with ammonium sulfate. It is an homodimer (two β subunits) with a structure composed by two α-helix-loop-helix and in the loop there is the binding site for Ca^{2+} even though Zn could bind too (**Figure 6**).

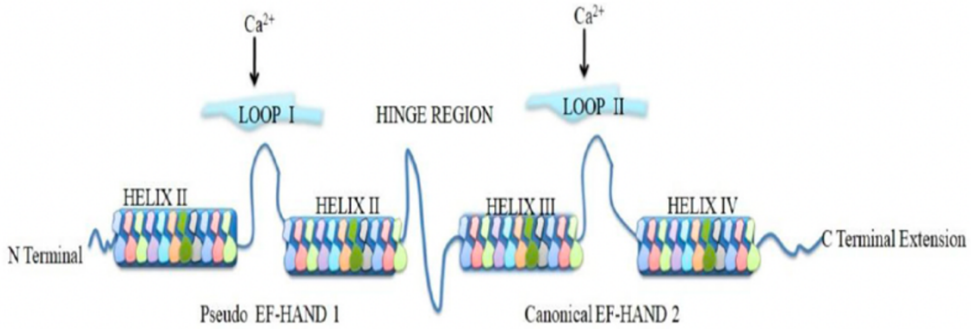

Figure 6: Structure of S100B protein. (Langeh et al, Current Neuropharmacology, 2021

S100B is expressed primarily in astrocytes but also in other glial cell types (such as oligodendrocytes, Schwann cells, ependymal cells, retinal Muller cells, enteric glial cells) and it has also been reported to be located in definite neuron subpopulations. Outside the nervous system it is mainly expressed in the adipocytes.

Intracellularly, S100B as a calcium-sensor protein, plays multiple functions: it transfers signals from second messengers, interacts with different molecules in different cell types, intervenes in

cell proliferation, survival and differentiation, participates in the regulation of cellular calcium homeostasis and enzyme activities and even interacts with the cytoskeleton.

At the beginning S100B was thought to be a biomarker of glial injury because its detection was simply interpreted as a consequence of its leakage from damaged cells. Now it is evident that S100B actively participate in the processes accompanying neural injury. The extracellular biological activity of S100B is strictly related to its extracellular concentrations. At low (nanomolar) concentrations that are the concentrations present in physiological conditions, it has a neurotrophic effect by promoting neurite extension, modulating the long-term potentiation process, protecting neuron survival, counteracting neurotoxicant insults and increasing scavenger activity of reactive oxygen species (ROS). At high (micromolar) concentrations it has a neurotoxic/pro-inflammatory effect by interacting with RAGE receptor and TLR-4 receptor that are present on different cells. It increases oxygen radicals production, proinflammatory cytokines and cellular adhesion molecules such as COX2 release and glutamate-induced neuronal death thus inducing mitochondrial dysfunction and apoptosis.

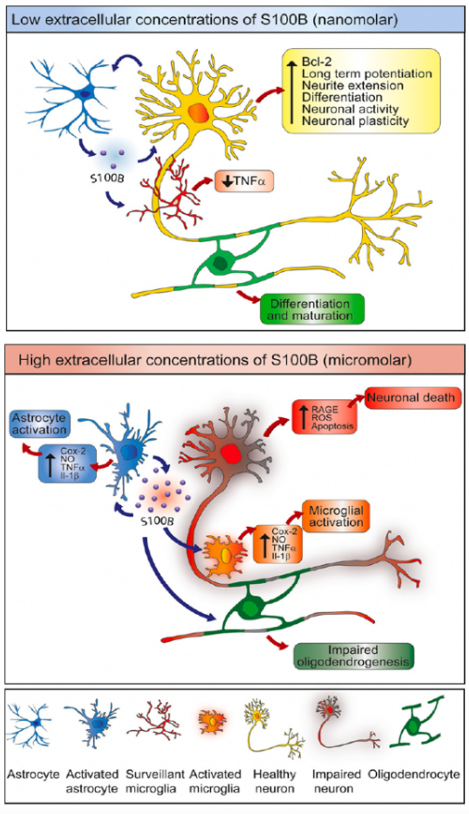

Figure 7: Biological activity of extracellular S100B in the different cell types of the CNS at low and high concentrations. (Michetti et al J of Neurochemistry 2019)

Therefore, S100B is primarily released by astrocytes and at high concentrations it turns astrocytes into a pro-inflammatory/neurodegenerative phenotype capable of inducing neuronal death and neuroinflammation by both a direct and an indirect pathway (**Figure 7**).

As clinical use, S100B was first found high in the CSF of MS patients during the acute phases of the disease while it was low during the stationary phase. Then its elevation was found in other brain pathologies too such as after acute brain injury (e.g. Traumatic Brain Injuries, Cerebrovascular Diseases [CVD]), neurodegenerative conditions (e.g. AD, PD, ALS), congenital and perinatal disorders (21 chromosome trisomy, asphyxiated newborns) and psychiatric disorders (schizophrenia). Thus, S100B is recognized as a sensitive but with a very low specificity biomarker of active astrocytes distress.

When BBB is disrupted, S100B from CSF is released into the serum (where its concentrations are lower).

Interestingly in animal model of different neurological conditions, the administration of anti-S100B neutralizing antibodies or the suppression of S100B production (by the administration of arundic acid or in S100B knockout mice models) or the prevention of S100B interaction with transcription factor p53 (by the administration of pentamidine or TRTK12 peptide) determine an improvement of the condition while the administration of S100B or its genetic overexpression determine the worsening of the condition. This could play an important role as possible therapeutic perspectives because S100B could become a therapeutic target.

As it is for Nf, the expression of S100B could be affected, even in healthy controls, by aging: some authors have suggested an increase in the expression of S100B in the brain cortex and hippocampus by activating astrocytes with age (Langeh & Singh, 2020; Michetti et al., 2019).

As regards to SE, in a Lithium-Pilocarpine rat model of SE, increased levels of S100B in the CSF was found parallel to the presence of evidences of neurodegeneration in dentate gyrus and CA1 hippocampal subfield and an increase in mossy fiber sprouting 24 hours after SE induction (de Oliveira et al., 2008).

In chronic epilepsy, many different studies showed that epileptic patients have increased serum levels of S100B versus healthy controls and its concentrations may reflect epilepsy severity thus it could have a prognostic value (Calik et al., 2013, 2014; Langeh & Singh, 2020; Liang et al., 2019; Meguid et al., 2018; Simani et al., 2020).

Fewer studies have focused the attention on SE. An observational study on children with SE found a significant difference between the S100B mean value of SE compared to the control group (single simple febrile seizure) and a strong positive correlation between S100B levels and degree of encephalopathy defined by degree of acute alterations defined on MRI in SE patients. Thus, the authors concluded that S100B levels can help defining which acute MRI alteration could be irreversible (Gunawan et al., 2019).

In a more recent paper by Wang et al (Wang et al., 2022) significantly increased serum levels of NSE, S100B and Vascular-Endothelial Growth Factor (VEGF) within one week in children with CSE were found compared to controls and thus they conclude that S100B could serve as a diagnostic marker. In the study of Hanin et al (Hanin, Denis, et al., 2022) serum S100B levels were found to be higher in SE patients compared with those measured in patients with epilepsy and in controls; while no significant differences were found for CSF S100B levels. No differences in relation to SE refractoriness, semeiology and etiology was found. Considering a cut off of 0.09 ng/mL the diagnostic value toward SE of serum levels of S100B was found to be good (AUC 0.748, Sensitivity: 59.7%, Specificity: 90.2%, PPV: 83.6%; NPV: 72.8%).

GFAP

Glial fibrillary acidic protein (GFAP) is the principal intermediary filament of astrocytes (class III of intermediate filaments). It is considered a known biomarker of astro-glial proliferation and reactive astrogliosis.

Animal models of SE have shown SE-induced alteration in GFAP expression. Signs of gliosis after SE have been shown in different studies. In the study by do Nascimento et al (do Nascimento et al., 2012) on a mice model of pilocarpine-induced SE, immunohistochemistry for GFAP showed that the molecular, CA3 and CA1 pyramidal cell layers of hippocampus expressed the highest presence of GFAP immunoreaction at one and three weeks after SE onset. In the rats models of electrically induced NCSE, Avdic et al (Avdic et al., 2018) found that, at one week after SE, in hippocampus, excessive microglial and astrocytic activation appeared (Iba1 and GFAP increment) and continues at four weeks accompanied by neuronal loss (increment of GFAP, Iba1, S100B).

Nevertheless, there are also evidences of astrocyte degeneration early after SE. In the study by Borges et al (Borges et al., 2006) in a mouse model of pilocarpine-induced SE, dentate hilus, immunoreactivity for GFAP was decreased, and the number of healthy appearing GFAP- or S100β-positive cells was significantly reduced (≥ 65%) one and three days after SE induction while 10 days after SE, hilar GFAP immunoreactivity had returned indicating astrocyte degeneration followed by astrogenesis leading to hilar repopulation. The alteration of GFAP expression did not involve only the hippocampus, but in a pilocarpine rat model of SE, Schmidt-Kastner et al (Schmidt-Kastner & Ingvar, 1994) found that at three days after SE, immunohistochemistry for GFAP was severely reduced both in substantia nigra pars reticulata and in basal cortical areas. The alterations in GFAP expression with reduction in GFAP positive astrocytes in hippocampus of rats pups exposed to hyperthermia and experiencing prolonged febrile seizures was also linked to a reduction in the magnitude of long term potentiation thus leading to impairment of learning and memory in developing brain (Griflyuk et al., 2022).

Evidences in humans showed that CSF levels of GFAP were found to be significantly increased within 24 hours after a single seizure in children compared to those measured in controls.

Seizure duration was positively correlated with CSF GFAP level and levels measured in patients with SE were higher compared to those achieved in patients without SE. In these patients no differences in NSE levels were found suggesting the possibility that GFAP may be a more sensitive marker of brain injury in some cases than NSE (Gurnett et al., 2003).

This result was confirmed in the study by Wang et al on a cohort of children with CSE in which serum levels of GFAP were found to be significantly increased at the fourth day after SE compared to healthy controls (Wang et al., 2022).

In a different cohort of adult patients with post-cardiac arrest electrographic status epilepticus higher levels of serum NfL were found while the association with GFAP appeared less clear (Lybeck et al., 2021). On the contrary, a transient but marked increase was evidenced immediately after (2 hours) a tonic clonic (TC) seizure. GFAP increase was more prominent than that of TAU, NFL and UCHL-1 indicating a possible major involvement of glial cells than neurons (Nass et al., 2021). Moreover, elevated GFAP levels in children with epilepsy were found to be a predictor for active seizures and they could be used to monitor disease progression and severity for early identification of those with drug-resistant epilepsy and those who are in need for epilepsy surgery (Elhady et al., 2021).

4.5. Short and long term prognosis and prognostic scores

Assess the patient's prognosis after a SE episode, as early as possible, is of paramount importance in SE management either to avoid over-treatment and its harmful possible consequences in patients with a probable good outcome (this is especially true for NCSE without coma in which the risk of further neurologic consequences related to sustained epileptic activity is not completely assessed while they could be at high risk of worsening due to therapeutic coma and its related complications) (N. A. Marchi et al., 2015) or to avoid under-detection and under-

treatment in patients with a possible bad outcome. For these reasons the majority of the existing literature is mainly focused on the short term prognostication in terms of in-hospital and 30-days mortality and disability assessment. Thus, data on long term prognosis in term of mortality and disability, but also of recurrence of SE and epilepsy development, cognitive and behavioral deficits and assessment of quality of life are scarce (Sculier et al., 2018).

4.5.1. Short term prognosis

Mortality

With differences among existing studies, mortality at discharge after an episode of SE varies between 9 and 61% while at 30 days between 19 and 65%.

To assess short term mortality, three different clinical prognostic scores have been proposed (González-Cuevas et al., 2016; Leitinger, Höller, et al., 2015; Rossetti et al., 2006; F. Yuan et al., 2018). They are based on different factors, which are well-known to play a decisive role in the determination of the outcome of SE: age, aetiology and history of epilepsy, level of disability before SE, level of consciousness before treatment, semiology of SE, comorbidities and characteristics of EEG. The aetiology of SE is unquestionably the most important determinant of SE prognosis in terms of mortality and disability (Neligan & Shorvon, 2010, 2011) with the worst outcome for those patients with acute symptomatic aetiologies related to structural acute brain damages (Logroscino et al., 1997; Neligan & Shorvon, 2010, 2011; Rossetti, 2006).

The existing clinical scores are: Status Epilepticus Severity Score (STESS) (Rossetti et al., 2006), modified STESS (mSTESS) (González-Cuevas et al., 2016) and Epidemiology-based Mortality score in Status Epilepticus (EMSE) (Leitinger, Höller, et al., 2015).

The STESS was the very first created score to assess in-hospital mortality after SE. It was firstly introduced and applied to a small (35 patients), prospective collected, SE population in 2006

(Rossetti, 2006) and subsequently validated in a larger multicenter one (154 patients) in 2008 (Rossetti et al., 2008). Thereafter it received some internal (Rossetti et al., 2013; Tsetsou et al., 2015) and external validations (Goyal et al., 2015; Sutter, Kaplan, et al., 2013) too even in populations of RSE (Madžar et al., 2016). STESS is a simple and quickly assessed score based on four parameters: level of consciousness before treatment administration; worst seizure type; age and history of previous seizures. The suggested cut off point was first set at 3 points (STESS-3) and then increased to 4 points (STESS-4) (Sutter, Kaplan, et al., 2013).

The mSTESS score was proposed in 2016. This score takes into account the four parameters of STESS (age with a cut off of 70 years instead of 65) and it adds to them the baseline disability measured by mRS and divided in three different levels. The mSTESS showed an increased accuracy and positive Predictive Value (PPV) while it showed a lower ceiling effect compared to STESS 3 (González-Cuevas et al., 2016).

The EMSE was first proposed in 2015 (Leitinger et al., 2016; Leitinger, Höller, et al., 2015). This is the first score based on available epidemiological data, coming from the real world. It is based on four parameters (so called EMSE-EACE): aetiology; age; comorbidity and worst EEG pattern. The score was firstly validated in a retrospective population and the cut-off was set at 64 points. EMSE was found to be superior to both STESS-3 and STESS-4 (Leitinger, Kalss, et al., 2015). Then this score received some external validations (Giovannini et al., 2017; Kang et al., 2015) for the short term mortality and disability assessment.

Overall, even though they may be useful in different clinical situations, all these scores showed a similarly high negative predictive value for short term survival after the SE episode but low positive predictive value for short term death after the SE episode. This implies that they should perfectly identify patients who will survive to SE episode while they have low reliability in the identification of those who will die due to SE episode. As a consequence, they should be used to

avoid over-treatment of patients who will go in anyway well while they could not be used to withdraw treatment in patients with high scores because a great part of them will even survive. This aspect was recently confirmed by machine learning models of validation of STESS and EMSE too (Brigo et al., 2022a, 2022b, 2022c).

Functional outcome and disability assessment

After an episode of SE, in 21-76% of patients a worsening of the clinical condition is recognized. Similarly to short term mortality, many different clinical aspects can influence short term functional outcome after an episode of SE. Among them age, aetiology, level of consciousness, semiology and duration/response to treatment of SE, comorbidities and characteristics of EEG could play an important role. Compared to mortality prediction, there are fewer studies that have tried to predict short term functional outcome.

The "Encephalitis, Nonconvulsive Status Epilepticus, Diazepam Resistance, Imaging, Tracheal Intubation" score (END-IT) was proposed in 2016 to determine the three month functional outcome after an episode of convulsive SE. This score showed a higher accuracy compared to STESS and EMSE. Nevertheless, it was applied on a deeply different population (composed by younger patients and with a high prevalence of encephalitis) thus it is not possible to directly extend the obtained results (F. Yuan et al., 2018).

Moreover, in 2016, Kang et al (Kang et al., 2015) used STESS and a modified version of EMSE score (with aetiology, comorbidities, level of consciousness and EEG or age, level of consciousness, duration and EEG) to predict functional outcome at discharge finding the superiority of the used versions of EMSE on the STESS-4.

Finally, in 2017 we found that on a population of adult patients with SE, EMSE appeared to be superior to STESS 3 and 4 predicting both 30 days mortality and functional outcome (Giovannini et al., 2017).

4.5.2. Long term prognosis

Possible long term consequences of SE are mortality and disability, but also recurrence of SE and epilepsy development, cognitive and behavioural deficits thus leading to worsening in the quality of life. Few evidences and studies have addressed the problem of long term prognosis after a SE episode. At present none of the aforementioned clinical scores has been validated to determine long term prognosis after SE; just a single score has been proposed to define long term mortality risk while there are no clinical scores able to determine the long term risk of recurrence of SE, the epilepsy development, the risk of functional disability and cognitive/behavioral consequences.

A precise definition of the individual long term prognosis should let to define better the caregivers' and healthcare system management burden. This could let to adopt the most appropriate and tailored strategy for each patient.

Long term mortality

A recent retrospective, multicenter cohort study analyzed 2-years mortality in adult patients diagnosed with SE. Overall 2 years mortality was 47.1%. The authors proposed a score, named ACD score (Age, Non-convulsive Status Epilepticus in coma, Duration), that takes into consideration the age at SE onset, the level of consciousness at admission, and the duration of status epilepticus.

This study found that age, long duration of SE, and nonconvulsive type of status epilepticus in coma (the higher points of the score) were associated with the development of new neurological deficits at discharge (defined as an increase of at least 5 points in National Institue of Health Stroke Scale

[NIHSS]) which were predictors of 2 years mortality especially in those patients with etiologies that were not damaging or were less damaging to the brain (Roberg et al., 2022).

Recurrence of SE

Recurrence rate of SE is estimated to reach 10.4-32% (Gasparini et al., 2021; Hesdorffer et al., 2007; Orlandi et al., 2022) within 4 years from SE. In a recent study by our research group (Orlandi et al., 2022) the recurrence rate of SE was 20.5% at 2 years with the highest rate observed in the first 6 month from the index event (7.9%). The independent risk factors for SE recurrence were: remote and progressive aetiologies, super-refractoriness (Orlandi et al., 2022) and the SE' length (Hesdorffer et al., 2007; Orlandi et al., 2022; Tsetsou et al., 2015).

Development of epilepsy

The risk of epilepsy development appeared to be increased in patients with a structural brain lesion (Hesdorffer et al., 1998b). In the study by Santamarina et al (Santamarina et al., 2015) 58.7% of adult patients with SE developed epilepsy at 10 months after SE. Toxic-metabolic etiologies developed epilepsy less frequently compared to other etiologies. At the multivariate analysis, the only significant association was with SE duration (considered cut off time 24 hours) and this was in particular true for acute symptomatic SE, while in the chronic, progressive symptomatic or cryptogenic SE, seizures developed regardless of the SE duration.

5. Aims and thesis overview

The main thrust of this thesis is the development of a multimodal approach to status epilepticus that comeprehends not only clinical and EEG variables, but also the use of neuroimaging techniques and biomarkers on biological fluids, with finality for diagnosis, stratification of SE severtiy, and short-term prognosis for the individual patient.

We therefore report in the next 4 chapters (chapters 6 to 9) four studies we conducted in this area of research in patients with SE.

In *chapter 6* we present the results of a monocentric retrospective study that focused on the evaluation of the diagnostic usefulness of qualitative and quantitative evaluation of PCT to support the diagnosis in suspected cases of NCSE. For each patient, the diagnostic EEG was reviewed and classified according to the Salzburg Criteria for NCSE as definite (D-NCSE) or possible (P-NCSE) and the relationship between PCT and EEG patterns were analysed.

In *chapter 7,* in a retrospective cross-sectional pilot study, we investigated the role of serum neurofilament light chain levels as a biomarker of seizure-related neuronal damage. To this aim, we excluded all the patients with an acute symptomatic etiology of the SE episode in which the elevaltion of sNfL could have been related to the underlying etiology too. The role of s-NfL as a prognostic biomarker in terms of short-term mortality, disability and refractoriness development in a cohort of adult patients with SE (30 subjects) was evaluated. The comparator groups were formed by patients with drug-resistant epilepsy (PWE, 30 subjects) and healthy controls (HC, 30 subjects).

In *chapter 8* in a cohort of 87 adult patients with SE of different aetiologies, we measured biomarkers of neuro-glial injury in patients with SE focusing on the role of the serum levels of both NfL and S100B. All samples were acquired during SE within 72 hours from SE diagnosis. The comparison groups were formed by healthy controls (HC, n = 27) and patients with epilepsy (PWE,

n = 30). Demographic, clinical and therapeutical information and thirty-days follow-up information regarding disability development were acquired for every included patient and analysed in relation to NfL and S100B values.

Chapter 9, is dedicated to the evaluation of the role of serum measurement of NfL and S100B as seizures-induced neuro-glial injury biomarkers to define 30-days mortality and treatment refractoriness development. Finally, we evaluate the performance of a new clinical-biochemical prognostic score to predict 30 days mortality after SE and we compared it to the that of the existing clinical prognostic scores.

6. Results 1 - Cortical and thalamic hyper-perfusion in non-convulsive status epilepticus. Relationship between perfusion CT patterns and Salzburg EEG criteria

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Abstract

Introduction: Status epilepticus (SE) is a neurological emergency and in particular nonconvulsive SE (NCSE) represents a diagnostic challenge. To improve clinical decision-making, cerebral perfusion-computed tomography (PCT) has been shown as a helpful tool to support the diagnosis of focal NCSE.

Materials and methods: This is a monocentric retrospective study. Among the 602 cases of SE observed between September 2013 to April 2020 we included 21 patients that studied with PCT. The perfusion maps were first visually analysed then a quantitative analysis (by regions of interest, ROI) was obtained. For each patient, the diagnostic EEG was reviewed and classified according to the Salzburg Criteria for NCSE (SCC) as definite (D-NCSE) and possible (P-NCSE). Finally, we analysed the relationship between PCT and EEG patterns.

Results: Hyper-perfusion was observed in 18 patients (86%), while in the remaining 3 (14%) a normo-perfused pattern was present. Hyper-perfusion was observed in 14 of the D-NCSE group (88%) and in the two patients with a P-NCSE (100%). No one among the patients with a P-NCSE had a thalamic hyper-perfusion, while among the 6 patients with continuous sustained epileptiform discharges > 2.5 Hz (pattern 1 of SCC), 4 (67%) had contemporary cortical and thalamic hyper-perfusion.

Conclusions: PCT could facilitate the differential diagnosis and speed-up the diagnostic process of NCSE in emergency situation. Finding cortical multi-lobar hyper-perfusion, especially if present together with homolateral thalamic hyper-perfusion in a patient with an acute-onset of motor/sensory/language deficits is highly suggestive for the presence of NCSE and is particularly related to continuous/sustained ictal patterns.

Key words: NCSE; perfusion CT; status epilepticus; EEG; Salzburg Criteria; LPDs.

Introduction

Status epilepticus (SE) is a common neurological emergency, with an incidence of 10-41 cases per 100 000 population (Logroscino et al., 2005; Trinka et al., 2012) and a mortality rate of around 20% (Trinka et al., 2012). Nonconvulsive seizures, nonconvulsive status epilepticus (NCSE), and the postictal state, in particular, may masquerade as a stroke and often represent a diagnostic challenge (Gugger et al., 2020; Manganotti et al., 2019). Failure in recognizing SE can have important negative consequences leading to incorrect treatment, diagnostic delay and potential treatment adverse effects (Pauletto et al., 2020). Furthermore, delay in antiepileptic treatment contributes to lengthen SE duration which in turns increases the probability of brain damage and unfavourable prognosis (González-Cuevas et al., 2019; Quigg et al., 2001). In particular, the risk of misdiagnosing NCSE, delaying the appropriate antiepileptic treatment, is a significant clinical problem in the elderly (Kubiak-Balcerewicz et al., 2017).

The gold standard for the diagnosis of NCSE is the EEG. However, in many hospitals, EEG acquisition is not always feasible 24/7 and whenever available the diagnosis of NCSE is not always straightforward. In recent years, the Salzburg Criteria for the EEG diagnosis of NCSE (SCC) have been theorized (Leitinger, Beniczky, et al., 2015; Leitinger et al., 2016) but there is still debate among experts, especially for those cases in which the diagnosis is that of a “possible SE” (Othman et al., 2020) and in cases belonging to the so called ictal-interictal continuum (Kapinos et al., 2018).

For these reasons, to improve clinical decision-making, cerebral perfusion-computed tomography (PCT) has been shown as a helpful tool to support the diagnosis of NCSE and to distinguish stroke from seizures/SE (Pauletto et al., 2020). CT perfusion has the advantages of routine availability in the emergency department, short acquisition time, and quantitative results (Guerrero et al., 2012). Indeed, PCT showed a sensitivity of 75-80% and specificity of 90% to diagnose focal SE and it is

particularly useful to support the diagnosis of focal NCSE (González-Cuevas et al., 2019; Hauf et al., 2009).

Cerebral blood flow and local metabolic requirement are usually tightly coupled. It is known that epileptic activity causes an increase in the metabolic demand of the affected cerebral cortex that is accompanied by a transient increase in blood perfusion (González-Cuevas et al., 2019; Meletti et al., 2018). This happens either in the seizure onset zone or in the areas involved by the propagation of epileptiform activity (Duncan, 1992). Thus, during seizures and status epilepticus the most frequently encountered pattern is hyper-perfusion characterised, by increased cerebral blood flow (rCBF) and cerebral blood volume (rCBV) and a reduced mean transit time (MTT) involving cerebral cortex and also deep structures such as the thalamus (González-Cuevas et al., 2019; Hauf et al., 2009; Ohtomo et al., 2021).

In this study we evaluate PCT findings in a series of NCSE with the aim to analyse the relationship between PCT results and the EEG patterns recorded soon after imaging acquisition. Especially, we evaluated PCT in the light of the Salzburg Criteria for the EEG diagnosis of NCSE.

Methods

Type of study

This is a retrospective analysis of our cohort of adult SE episodes prospectively collected from September 2013 to April 2020 at the Ospedale Civile Baggiovara (OCB) of Modena, Italy. At our institution a prospective collection of SE episodes (in patients > 14 years) is made with a dedicated case report form and modalities that we have already reported elsewhere (Giovannini et al., 2015).

Patients selection and adopted definitions

All NCSE studied in the emergency department with a PCT and an EEG were finally considered for this study. These patients were firstly evaluated as suspected stroke-codes due to the presentation with an acute onset of a focal neurologic deficit with or without a quantitative alteration of consciousness. All these patients finally turned to be a NCSE as confirmed by EEG and/or clinical features/evolution. The patient's demographic data, clinical characteristics, SE aetiology, semeiology and response to treatment were recorded and analysed.

In our centre a neurologist is on duty 24 h/day for seven days/week. PCT can be acquired 24 h/day for seven days/week too, as these examinations are part of the diagnostic process for reperfusion therapies in acute ischemic stroke.

Status epilepticus was defined accordance to the latest ILAE definition (Trinka et al., 2015) as a continuous seizure or two or more discrete seizures between which there is no complete recovery of consciousness that lasts 5 minutes for convulsive SE (CSE) and 10 minutes for NCSE.

Patients with a final diagnosis of SE were included in this study if: 1) they received a PCT study in the acute diagnostic phase and, 2) they received an EEG close in time after the PCT.

Thus, we excluded: 1) all those patients with SE who were not studied with a PCT; 2) those in which the PCT and or the EEG were subjected to artifact that compromised exam's interpretation; and 3) those SE in which the imaging study let us diagnose an ischemic stroke due to a large vessel occlusion.

EEG acquisition and diagnosis of NCSE

All the EEGs were acquired by the same neurophysiology staff in accordance to the 10-20 international system. The diagnosis of NCSE was done on clinical and EEG ground by the neurologist who took care of the patient. Afterward, a retrospective reclassification of the EEG patterns in

accordance to the Salzburg Criteria for NCSE was done for all the included NCSE. Two epileptologists expert in SCC (GG and GT) independently reviewed all the first acquired EEG for each patient to confirm the diagnosis of SE and to classify the EEG pattern in accordance to SCC classification in definite NCSE (D-NCSE) and possible NCSE (P-NCSE). The concordance between the two raters was measured by Cohen's kappa coefficient (k) and was excellent (k = 0.9). Discrepancies were resolved by the evaluation of the EEG by a third rater expert in SCC (SM) and blind to the evaluation of the first two raters.

Moreover, we subdivided the EEG ictal patterns between those characterised by the presence of continuous paroxysmal activity (continuous patterns) and patterns with repeated, discrete focal seizures arising from an interictal pattern and characterised by a clear spatio-temporal evolution of epileptiform activity (waxing and waning pattern).

We classified also the EEG patterns in accordance to the presence or absence of lateralized periodic discharges (LPDs).

Acquisition of perfusion imaging and evaluation

The imaging examinations were performed on a 128-slice system (Somatom Definition Siemens Healthcare). The protocol began with a lateral scout view to plan a conventional cerebral examination to detect structural abnormalities (120 kv, 320 mAs with reconstructions every 4 and 1 mm). For the PCT study, the technique included dynamic acquisition (70 kV and 150 mAs) with injection of 50 mL of non-ionic iodinated contrast material (Iomeron) at a concentration of 350 mg/mL by antecubital venous infusion using an injection pump to obtain a constant flow, with subsequent administration of 20 mL of NaCl. The plan scanning was from the middle fossa to the convexity to cover the supratentorial structures (total of 80 mm). For all the patients, the acquisition conditions were as follows: 5-mm thickness x 16i, rotation time 0.28 seconds, matrix 512 to the

convexity and collimation 32-1.2 mm. For each examination a total of 700 images were obtained and processed at a workstation (Syngo.via CT Neuro Perfusion, Siemens Healthineers, Erlangen, Germany) to analyse the following standard parameters: regional cerebral blood volume, regional cerebral blood flow and mean transient time.

Visual analysis was performed by a neuroradiologist (MM), blind to the patients' clinical information. Hyper-perfusion was defined as focally increase in the rCBF and rCBV and a decrease in the MTT respect to the unaffected contralateral cortex; hypo-perfusion was defined as focally decreased rCBF and increased in MTT possibly accompanied by decreased rCBV. When no perfusion alterations in rCBV, rCBF and MTT maps were detectable, the case was defined as normally perfused. As in other recent studies (González-Cuevas et al., 2019; Strambo et al., 2018; Van Cauwenberge et al., 2018) we chose qualitative visual analysis to define hyper-perfusion and hypo-perfusion because it is easily applicable to routine clinical practice and because of the lack of a precise quantitative definition (Strambo et al., 2018; Van Cauwenberge et al., 2018).

For quantitative analysis of PCT, Regions of Interest (ROIs) were identified and placed by an experienced neuroradiologist (MM). The pathological hemisphere was defined as the one contralateral to the affected body side showing the motor/sensory deficit, or the left-hemisphere in cases with isolated language deficit. For each patient, ROIs were placed in those areas of the affected hemisphere (either cortical-subcortical) in which the visual analysis revealed an increase or decrease of perfusion. For each ROI a corresponding one was automatically drawn in the unaffected hemisphere ("mirror ROI function") thanks to a mirror line previously placed along the midline (used landmarks: septum pellucidum and falx cerebri). Moreover, for each hemisphere 11 ROIs at fixed locations were placed (3 in frontal, 3 in temporal, 3 in parietal, 2 in occipital lobe) plus 2 in the thalamus. For all the defined ROIs the mean rCBV, rCBF and MTT values were extracted. Asymmetry indices (AIs) were calculated for the rCBV, rCBF and MTT as follows (Hauf et al., 2009):

AI = [(ROI pathological – ROI reference)/(ROI pathological + ROI reference)] × 100

An AI of rCBF > 10 was considered indicative of hyper-perfusion, an AI of rCBF < -10 was considered indicative of hypoperfusion as in previous studies (Hauf et al., 2009). **Supplementary Figure 2** shows the whole process.

Statistical analysis

Descriptive and frequency statistics were applied to describe the cohort. Parametric and non-parametric tests were used whenever required. For the comparisons Chi square test and Fisher exact test were used for categorical variables. Correlation analysis using Spearman's ρ and Kendall's τ correlation coefficient were run to define relation between rCBF values and clinical parameters. Paired-samples t tests and Wilcoxon signed rank test were used to compare the rCBF, rCBV, and MTT values between the affected and unaffected hemispheres. The p value was set at < 0.05. IBM SPSS Statistics software, version 26.0 was used.

Standard Protocol Approvals, Registrations, and Patient Consents

The scientific advisory boards of our institution approved the research report. Written informed consent has been obtained from patients included in the study.

Data availability

Anonymised data will be shared by request of any qualified investigator.

Results

Clinical characteristics of the included patients

Between September 2013 and April 2020, we observed 602 SE episodes. After the application of inclusion and exclusion criteria 21 NCSE (observed in 21 different adult patients) with out-of-hospital onset entered the study. The diagnosis of NCSE was based on the clinical presentation of an acute onset of a qualitative and/or quantitative alteration of consciousness not otherwise explained. The clinical suspicion was then confirmed by an EEG tracing showing a Definite or Possible NCSE in accordance to the SCC for 18 patients. Moreover, we included three patients for which the EEG, acquired after treatment, showed a focal slowing with interictal discharges with a perfect clinical-electroencephalographic concordance (tracings defined No-NonConvulsive Status Epilepticus, N-NCSE). All these three patients received antiepileptic treatment before the EEG acquisition due to the appearance of motor manifestations (two patients with an acute onset of a focal neurological deficit and subtle myoclonic jerks and one patient with an acute onset of a focal

neurological deficit and a subsequent convulsion). **Supplementary Figure 1** reported the selection process. **Table 1** reports the characteristics of the cohort.

Our population was composed by 11 men (52%), mean age 74±9.2 (median 72) ranging from 56 to 90 years. All the patients presented with a focal NCSE without coma: five patients had focal NCSE without impairment of consciousness (24%), one patient (5%) experienced a focal NCSE with impaired consciousness and 15 (71%) had an aphasic NCSE.

During the diagnostic process, thirteen patients (62%) had eventually some type of motor manifestation after the acquisition of PCT imaging, either in form of subtle myoclonic jerks (6 cases, 29%) or secondary evolution to a bilateral tonic-clonic seizure (6 cases, 29%) or in one case (5%) both were observed. Twenty patients (95%) had a SE that was responsive to anti-seizure medicines (ASMs) and the most frequent aetiology was the remote symptomatic vascular one (7 patients, 33%).

Table 1
Clinical Characteristics of the studied population.

	N	%
Considered population	21	100%
Age	Mean 74 Median 72 Range (56–90)	
Gender		
Male	11	52%
Type of SE		
Focal NCSE	21	100%
Symptom at presentation		
Aphasia	15	72%
Hemianopia	13	62%
Hypoesthesia	10	47%
Hemiparesis	14	67%
Visual or tactile extinction	6	29%
Evolution to motor manifestation	13	62%
Subtle motor manifestation	6	
Bilateral tonic-clonic	6	
Both	1	
Level of consciousness		
Alert	15	72%
Somnolent	4	19%
Stupor	2	9%
Coma	0	0%
GCS	Mean 11	
ILAE Semeiological classification of SE		
Aphasic SE	15	72%
Focal NCSE with impairment of consciousness	1	5%
Focal NCSE without impairment of consciousness	5	23%
Etiological classification		
Remote symptomatic	8	38%
Acute symptomatic	7	34%
Progressive symptomatic	5	23%
Cryptogenic	1	5%
Etiology		
Cerebrovascular disease	10	47%
Cerebral neoplasm	4	19%
Cerebral infection	1	5%
Precipitating factors in epileptic patient	1	5%
Inflammatory/paraneoplastic limbic encephalitis	1	5%
Toxic	1	5%
Unknown	1	5%
Others (MELAS and Remote CNS surgery)	2	9%
Treatment response		
Responsive	20	95%
RSE/SRSE	1	5%
EEG characteristics		
Ictal EEG	18	86%
Post-ictal EEG	3	14%
SCC Classification		
D-NCSE	16/18	89%
Pattern 1	6/16	38%
Pattern 2a	1/16	6%
Pattern 2b	1/16	6%
Pattern 2c	8/16	50%
P-NCSE	2/18	11%
Pattern 2e	2/2	100%

GCS: Glasgow Coma Scale; SE: Status Epilepticus; NCSE: Non Convulsive Status Epilepticus; MELAS: Mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes; CNS: Central Nervous System. RSE: Refractory Status Epilepticus; SRSE: Super Refractory Status Epilepticus; SCC: Salzburg Criteria for Non-Convulsive Status Epilepticus. D-NCSE: Definite Non Convulsive Status Epilepticus; P-NCSE: Possible Non Convulsive Status Epilepticus.
Patterns: 1: epileptiform discharges (ED) at > 2.5 Hz; 2a: ED ≤ 2.5 Hz with complete disappearance and clinical improvement after treatment; 2b: ED ≤ 2.5 Hz with associated subtle ictal phenomena; 2c: ED ≤ 2.5 Hz with typical spatio-temporal evolution; 2e: ED ≤ 2.5 Hz with fluctuation in frequency without a definite spatio-temporal evolution.

Perfusion CT characteristics

In 16 patients, the PCT was acquired within seven hours from the beginning of symptoms, while five patients had wake-up symptoms, therefore in these latter patients the SE onset-to-PCT time was undetermined. The median symptoms onset to PCT time was three hours.

Quantitative analysis of the ROIs showed a significant increase in the rCBF ($p < 0.001$) and rCBV ($p < 0.001$), and a decrease in MTT ($p = 0.001$) values in the affected hemisphere relative to those in the corresponding contralateral region (**Figure 1**).

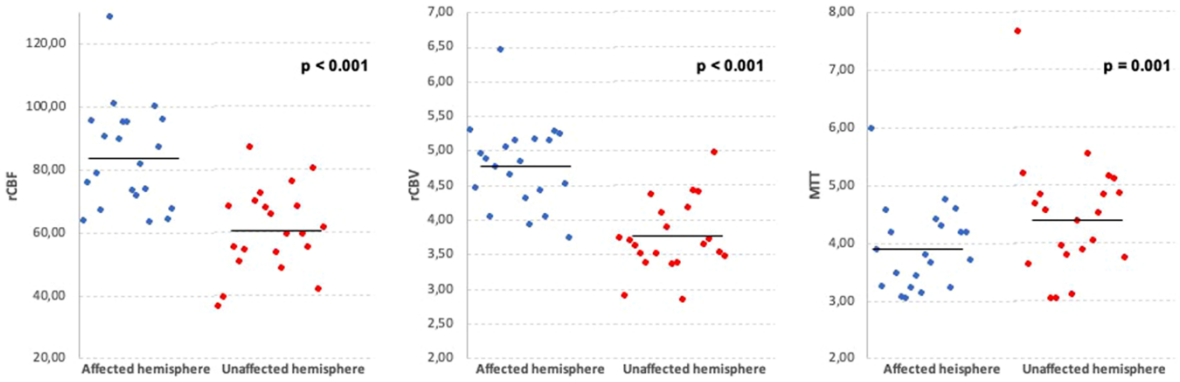

Fig. 1. Comparison of the values of rCBF, rCBV and MTT of the affected and unaffected hemisphere.

The evaluation of PCT allowed us to subdivide our cohort into the following two groups.

Hyper-perfused group. In 18 out of 21 patients (86%) a hyper-perfusion pattern was identified. The mean rCBF values in the affected hemisphere of these patients was 86 ml/100g/min and the mean of the AI of rCBF was 19.

Whenever at the visual analysis the pattern was judged to be hyper-perfused at the quantitative analysis there was always a rCBF AI > 10.

The hyper-perfusion involved cortical areas in 17 patients (81%). The homolateral thalamus was hyperperfused in eight patients (38%).

Normo-perfused group. Three patients showed a pattern of normal perfusion (14%). The mean rCBF values in the affected hemisphere of these patients was 68 ml/100g/min and the mean of AI of rCBF was -0,1. Whenever the visual analysis identified a normo-perfused pattern, the rCBF AI was always between $\pm$ 10.

We did not find hypo-perfusion patterns (no episode shown a rCBF AI < -10; also considering a less stringent criteria of AI < -7 allowed to define hypo-perfusion in any case).

The presence of PCT abnormalities was independent of SE duration (ρ = -0.17, p = 0.54), gender (τ = -0.03, p = 0.89) and age at SE presentation (ρ = -0.34, p = 0.13).

EEG findings

The first EEG was acquired within three hours from the PCT in 15 patients (71%; on average within one hour) while for six patients the EEG was acquired afterward (4-12 hours from the PCT, details for each patient are reported in **Supplementary table**). The recorded EEG showed an ictal pattern in 18 patients (86%), while a post-ictal focal slowing with or without interictal epileptic discharges was observed in the remaining three patients (14%). All these latter patients received antiseizures drug treatment for the sudden appearance of motor manifestation before the EEG acquisition. Overall, seven patients presented a bilateral tonic-clonic (TC) evolution after the acquisition of the PCT. In six of them the EEG acquired after the tonic-clonic seizure still documented a focal defined NCSE, while for one a focal post-ictal EEG was recorded. Going further into details: four of them had an acute onset aphasia with or without right hemiparesis, right hemianopsia right hemianesthesia, the PCT showed a focal left parieto-temporo-occipital cortical +/- thalamic hyper-perfusion and the EEG acquired after the bilateral TC evolution showed a D-NCSE with a parieto-temporo-occipital left focus; one patient had an acute onset left hemiparesis and left visual extinction, the PCT showed a right parieto-temporal hyper-perfusion and the EEG acquired after the bilateral TC evolution showed a focal D-NCSE with a right parieto-temporal focus; one patient had an acute onset aphasia, right hemianopsia, right hemiparesis and right hemianesthesia, the PCT was normo-perfused and the EEG acquired after the bilateral TC evolution showed a definite NCSE with a parieto-temporo-occipital left focus. Finally, one patient had an acute onset of aphasia and right hemiparesis, the PCT was normo-perfused and the EEG acquired after the bilateral TC evolution showed a post-ictal focal fronto-centro-temporal slowing with LPD.

The classification of the ictal EEGs following the application of SCC for all patients (Leitinger et al., 2016) is reported in **Table 1**. Finally, 12 patients presented a continuous pattern, while six patients had discrete focal seizures. As far as lateralized periodic discharges, six patients had LPDs, while in 12 they were absent.

Among the 18 patients presenting with a hyper-perfusion pattern,16 (89%) had an ictal EEG, while in the remaining two patients the EEG showed a post-ictal focal slowing with or without interictal epileptiform activity. All these latter patients received benzodiazepines treatment after PCT but before EEG acquisition due to motor manifestations' appearance. In all patients the lobe/s showing hyper-perfusion matched the spatial EEG location of ictal activities.

According to the SCC, 14 of the 16 patients (88%) had a pattern that reached the criteria for a "Definite-SE", while in two (12%) a pattern of ED ≤ 2.5 Hz with fluctuation without a clear evolution was observed thus a diagnosis of "Possible-SE" was made.

Considering the presence or absence of discrete seizures, 12 out of 16 (75%) showed a continuous pattern, while 4 (25%) had a waxing and waning pattern with focal seizures. LPDs were observed in four patients (25%) while for the other 12 they were absent.

Figure 2 showed an example of a typical hyper-perfusion pattern and EEG findings (PCT – EEG findings of all the cases are reported in online **Supplementary table**).

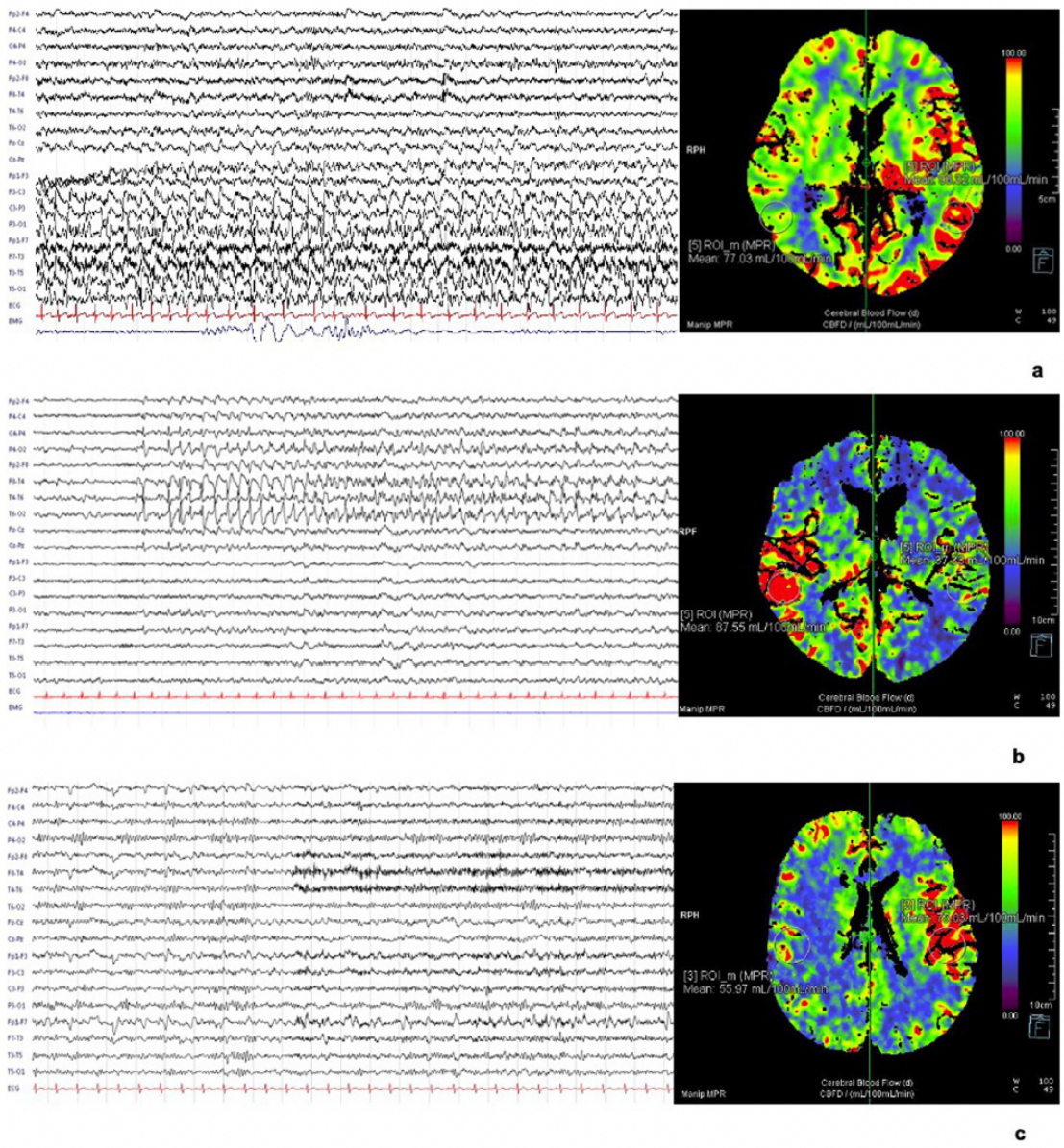

Fig. 2. Examples of hyper-perfusion patterns and corresponding EEG patterns.
EEG: D-NCSE type 1 pattern characterised by the presence of spike and wave at 2.5-3 Hz located in the left parieto-temporo-occipital leads. PCT: left parieto-temporo-occipital cortical and thalamic hyper-perfusion.
EEG: D-NCSE type 2c pattern characterised by right parieto-temporo-occipital ED evolving in space, frequency and morphology and generating discrete seizures separated by the persistence of LPDs occupying the same leads. PCT: right parieto-temporal cortical hyper-perfusion.
EEG: P-NCSE type 2 pattern characterised by the presence of ED at 1 Hz with frequency fluctuation in time located in the right fronto-temporal leads. PCT: right temporal cortical hyper-perfusion.

Table 2 shows the details of PCT and EEG findings.

Table 2
PCT patterns in relation to EEG patterns.

	Visual analysis	Mean rCBF values	Mean AI rCBF values	Mean rCBV values	Mean AI rCBV values	Mean MTT values	Mean AI MTT values	Quantitative analysis		SCC				CP Vs WWP	LPDs
Hyper-perfusion pattern	18 (86%)	86.28	18.87	4.89	14.15	3.85	-7.35	18 (100%)	Ictal EEG 16 (89%)	D-NCSE	14 (87.5%)	1 2a 2b 2c	6 (43%) 1 (7%) 1 (7%) 6 (43%)	CP: 12 (75%) WWP: 4 (25%)	Presence of LPDs: 4 (25%)
										P-NCSE	2 (12.5%)	2e	2 (100%)		
									Post-ictal EEG 2 (11%)						
Normo-perfusion pattern	3 (14%)	68.12	-0.10	4.07	-0.03	4.24	1.79	3 (100%)	Ictal EEG 2 (67%)	D-NCSE	2 (100%)	2c	2 (100%)	CP: 0 (0%) WWP: 2 (100%)	Presence of LPDs: 2 (100%)
									Post-ictal EEG 1 (33%)						

CBF: Cerebral Blood Flow; CBV: Cerebral Blood Volume; MTT: Mean Transit Time; SCC: Salzburg Criteria for non-Convulsive Status Epilepticus; NCSE: Non Convulsive Status Epilepticus; D-NCSE: Definite Non Convulsive Status Epilepticus; P-NCSE: Possible Non Convulsive Status Epilepticus; N-NCSE: No Non Convulsive Status Epilepticus.
Patterns: 1: epileptiform discharges (ED) at > 2.5 Hz; 2a: ED ≤ 2.5 Hz with complete disappearance and clinical improvement after treatment; 2b: ED ≤ 2.5 Hz with associated subtle ictal phenomena; 2c: ED ≤ 2.5 Hz with typical spatio-temporal evolution; 2e: ED ≤ 2.5 Hz with fluctuation in frequency without a definite spatio-temporal evolution. CP: Continuous patterns; WWP: waxing and waning patterns; LPDs: Lateralized Periodic Discharges.

Among the three patients with a normo-perfusion pattern, two (67%) had an ictal EEG and one (treated before EEG acquisition) had a post-ictal focal slowing. The two patients with an ictal EEG had a pattern that reached the criteria for a “Definite-SE”. Both of them had a waxing and waning pattern (100%) and LPDs were observed in all three cases.

Figure 3 showed an example of a normo-perfusion pattern and the corresponding EEG findings.

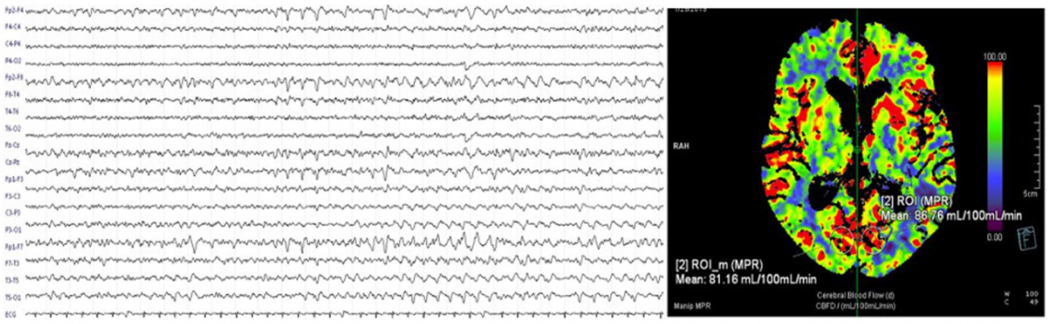

Fig. 3. Example of normo-perfusion pattern and the corresponding EEG pattern. EEG: D-NCSE type 2c pattern characterised by the presence of left posterior temporal ED and RDA at 1.5–2 Hz generating discrete seizures showing spatio-temporal evolution. PCT: normo-perfusion pattern.

Cortical and subcortical hyper-perfusion according to EEG patterns

Considering the application of SCC, an hyper-perfusion pattern was present in 14 out of 16 patients with a D-NCSE (88%) and in the two patients with a P-NCSE (100%). Notably no one among the patients with a P-NCSE had a thalamic hyper-perfusion while seven out of 16 (44%) patients with a D-NCSE had a thalamic hyper-perfusion. However, these differences did not reach the statistical significance (p = 0.45). Moreover, the two groups showed a similar cortical perfusion pattern (p = 1).

Table 3
Cortical and thalamic perfusion characteristics in patients with definite or possible NCSE in accordance to Salzburg EEG criteria.

	Hyper-perfusion pattern		Cortical hyper-perfusion		Thalamic-hyper-perfusion	
	N (%)	Sign.	*N* (%)	Sign.	*N* (%)	Sign.
SCC type and pattern						
D-NCSE (16)	14 (88%)	$p = 1$	13 (81%)	$p = 1$	7 (44%)	$p =$ 0.45
P-NCSE (2)	2 (100%)		2 (100%)		0	
Pattern 1 (6)	6 (100%)		6 (100%)		4 (67%)	
Pattern 2a (1)	1 (100%)		1 (100%)		0	
Pattern 2b (1)	1 (100%)		1 (100%)		0	
Pattern 2c (8)	6 (75%)		5 (63%)		3 (38%)	
Pattern 2e	2 (100%)		2 (100%)		0	
Continuous or discrete ictal pattern						
CP (12)	12 (100%)	$p =$ 0.1	12 (100%)	$p =$ 0.03	5 (42%)	$p = 1$
WWP (6)	4 (67%)		3 (50%)		2 (33%)	
Lateralized periodic discharges						
Presence of LPDs (6)	4 (67%)	$p =$ 0.1	4 (67%)	$p =$ 0.25	1 (17%)	$p =$ 0.32
Absence of LPDs (12)	12 (100%)		11 (92%)		6 (50%)	

NCSE: Non Convulsive Status Epilepticus; D-NCSE: Definite Non Convulsive Status Epilepticus; P-NCSE: Possible Non Convulsive Status Epilepticus; N-NCSE: No Non Convulsive Status Epilepticus.
Patterns: 1: epileptiform discharges (ED) at > 2.5 Hz; 2a: ED ≤ 2.5 Hz with complete disappearance and clinical improvement after treatment; 2b: ED ≤ 2.5 Hz with associated subtle ictal phenomena; 2c: ED ≤ 2.5 Hz with typical spatio-temporal evolution; 2e: ED ≤ 2.5 Hz with fluctuation in frequency without a definite spatio-temporal evolution. CP: Continuous patterns; WWP: waxing and waning patterns; LPDs: Lateralized Periodic Discharges.

All the patients with continuous pattern showed cortical hyper-perfusion while this was the case only for three out of six patients with waxing and waning pattern. This difference reached the statistical significance (p = 0.03). No differences in thalamic perfusion were found between continuous and waxing and waning patterns (p = 1).

Regarding the presence or absence of LPDs on ictal EEGs, we did find not find any difference in perfusion patterns (p = 0.10), neither considering cortical perfusion (p = 0.25) nor thalamic perfusion (p = 0.32). **Table 3** showed cortical and thalamic perfusion characteristics related to the observed EEG patterns.

Finally, considering impairment of consciousness and thalamic involvement we did not find any perfusion difference comparing patients with and without quantitative alteration of consciousness (p = 1). Thalamic hyper-perfusion was present in 40% of awake patients (See **Supplementary table**: patients n° 1, 4, 9, 13, 19, 20) and in 33% of patients with somnolence or stupor (See **Supplementary table**: patients n° 3, 11).

Discussion

Recently, the study of brain perfusion techniques to assess the presence of status epilepticus has increased considerably. Few studies, however, have investigated with these methods specifically

non-convulsive status epilepticus, and the relation between the EEG patterns and the characteristics of cerebral perfusion during NCSE. In this study we tried to provide an overview of perfusion patterns using perfusion CT in a series of NCSE with extra-hospital onset. CT perfusion is a technique approved by the international guidelines in the selection process of stroke patients for reperfusion intra-arterial procedures. Up to now, even though there are small studies that underline the usefulness of PCT to support the diagnosis of NCSE, there are no guidelines that approve the PCT study in the evaluation of a suspected NCSE. PCT is indeed an invasive technique (IV contrast and radiation exposure) but it is highly available and rapidly acquired in the emergency context, is faster and requires less patient's collaboration respect to the MRI. Moreover, in the last years the use of whole brain perfusion let a complete brain coverage to be possible. Finally, patients with SE have frequently some degree of alteration of consciousness and relatives are not always present in the emergency department setting, thus the lack of information about past medical condition frequently prevent them to be safely admitted to the MRI scan.

The main results of the study confirm that PCT is an imaging method that in an emergency context can provide useful indications for the diagnosis of NCSE. Although this is a retrospective study, it is evident that EEG pictures that meet the Salzburg diagnostic criteria for the definition of NCSE are associated with cortical hyper-perfusion with a good spatial/localization concordance. Furthermore, in our study we confirmed the cut off value of 10 to for CBF AI to distinguish between hyper- and normo-perfusion patterns.

To the best of our knowledge, this is the first study in which the relationship between PCT and EEG in NCSE is explored. Only in a recent study by Ohtomo et al (Ohtomo et al., 2021) this issue has been addressed but using MRI arterial spin labelling (ASL) technique and in a different clinical setting of comatose patients in the intensive care unit.

In our population, focal NCSE was mostly associated to the presence of a multi-lobar cortical hyper-perfusion located in the area of maximum EEG activity (81%) and in some cases associated with a hyper-perfusion in the homolateral thalamus (38%). It is well known that thalamus, and in particular pulvinar nucleus, is tightly connected to the cortex of temporal and parietal lobes and it is involved in the propagation of epileptic discharges. Diffusion Weighted Imaging (DWI) hyperintensities of cortex and homolateral thalamus have been described in the acute phase of SE (Giovannini et al., 2018) and Ohtomo et al. found that thalamic hyper-perfusion was significantly associated with NCSE in comatose patients (Ohtomo et al., 2021).

Comparing perfusion patterns in relationships with Salzburg EEG criteria for non-convulsive status, we did not find any statistical difference between Definite-NCSE and Possible-NCSE, either concerning cortical perfusion (81% and 100% respectively) or thalamic perfusion. This is not surprising due to the low numbers of studied cases. We noted however that both the two patients with Possible-NCSE did not show thalamic hyper-perfusion, while this was present in 44% of D-NCSE. Notably, in the study of Ohtomo et al. (Ohtomo et al., 2021) all the 21 patients with NCSE (both D-NCSE and P-NCSE) had cortical hyper-perfusion, while thalamic hyper-perfusion was present in 15 out of 19 (79%) of Definite-NCSE and in one out of two patients with Possible-NCSE (50%). This finding is of interest because the coexistence of thalamic and cortical hyper-perfusion seems to be a very specific pattern of NCSE. It follows that the finding of a PCT pattern with cortical plus thalamic hyper-perfusion in a patient with a doubtful NCSE on the clinical level is highly diagnostic for NCSE. Of course, prospective studies are needed to confirm these preliminary findings from retrospective case-series.

In our cohort, patients presenting with continuous epileptiform discharges had more frequently cortical hyper-perfusion compared to those with discreate seizures. Indeed, the two patients with a non-concordant PCT pattern versus EEG – normal perfusion with definite-NCSE – presented with a waxing and waning of discrete focal seizures. This finding obviously needs future confirmations but could suggest that hyper-perfusion is a condition establishing especially when the brain generate continuous/sustained ictal patterns.

Finally, regarding the presence of LPDs Ohtomo et al. found that the presence of periodic discharges was associated with both cortical and thalamic hyper-perfusion. In our population, we did not find differences between patients with and without LPDs. Several factors may explain this discordance. First, LPDs are a dynamic phenomenon, and in this study their presence was judged in the EEG tracing performed after PCT, which generally had a duration between 20 and 60 minutes. Therefore, there might have been cases with LPDs during the acquisition of PCT that were not revealed during the subsequent EEG. Moreover, it is possible that patients classified as being without LPDs would have afterwards developed them in an ictal-interictal continuum evolution. Second, the case histories observed in this study and in that of Ohtomo et al. are different. Indeed, in our study all patients had an out-of-hospital SE onset due in the majority of the cases to a remote symptomatic etiology. On the contrary in the study by Ohtomo et al, the majority of patients has an acute symptomatic etiology (like subarachnoid hemorrhage) and SE onset occurred after hours or days from admission. Moreover, at the time of PCT-EEG study none of our patient was in coma, while this was the case in the majority of the patients studied by Ohtomo et. Indeed, the fact that similar findings were observed in two different populations with different imaging methods is an interesting finding.

Study limitations

This is a monocentric study based on a small cohort of patients. This has had a great impact on the determination of statistical significance. Therefore, the finding of this study must be considered with caution and exploratory. The EEGs were not acquired at a fixed time interval after the PCT and patients did not undergo continuous EEG monitoring but only 20-60 minutes standard EEG. Thus, it is not possible to fully understand the evolution of the EEG patterns.

Since SE is a highly dynamic process, the most important strength of the present study is that EEG and PCT were acquired relatively close in time in the majority of the patients thus the observed perfusion pattern had a high probability of being the neuroimaging pattern correspondent to the observed EEG pattern. Moreover, treatment administration between PCT and EEG acquisition could have been potentially determined a bias in the analysis of the relationship between EEG-PCT findings. Actually, in our population five patients received treatment between PCT and EEG. For three of them the treatment resolved the SE (N-NCSE pattern in the subsequent EEG). These three patients were excluded from the analysis of the relationship between EEG-PCT findings.

For the other two patients the EEG revealed a Definite NCSE (for one the pattern was that of continuous ED > 2.5 Hz while the other had ED < 2.5 Hz with spatio-temporal evolution). Since SE is a clear dynamic condition and it is not possible to exclude that the treatment administration determined a change in the EEG pattern (thus creating an EEG-PCT decoupling) we reanalysed all the comparison analysis excluding those two cases. The presented results did not change after those patients' exclusion.

Conclusions

PCT could facilitate the differential diagnosis and speed-up the diagnostic process of NCSE in emergency situation. Finding cortical multi-lobar hyper-perfusion, especially if present together

with homolateral thalamic hyper-perfusion in a patient with an acute-onset and enduring alteration of consciousness is highly suggestive for the presence of NCSE.

Our results suggest that hyper-perfusion is a condition establishing especially when the brain generate abnormal continuous/sustained ictal patterns. Future studies confirming the relationship between EEG patterns and perfusion characteristics are needed to shed lights on the high dynamicity that characterise Status Epilepticus and the ictal-interictal continuum.

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Disclosure statement

G. Giovannini, M. Malagoli, A. Miani, G. Turchi, N Orlandi, AE Vaudano report no disclosures.

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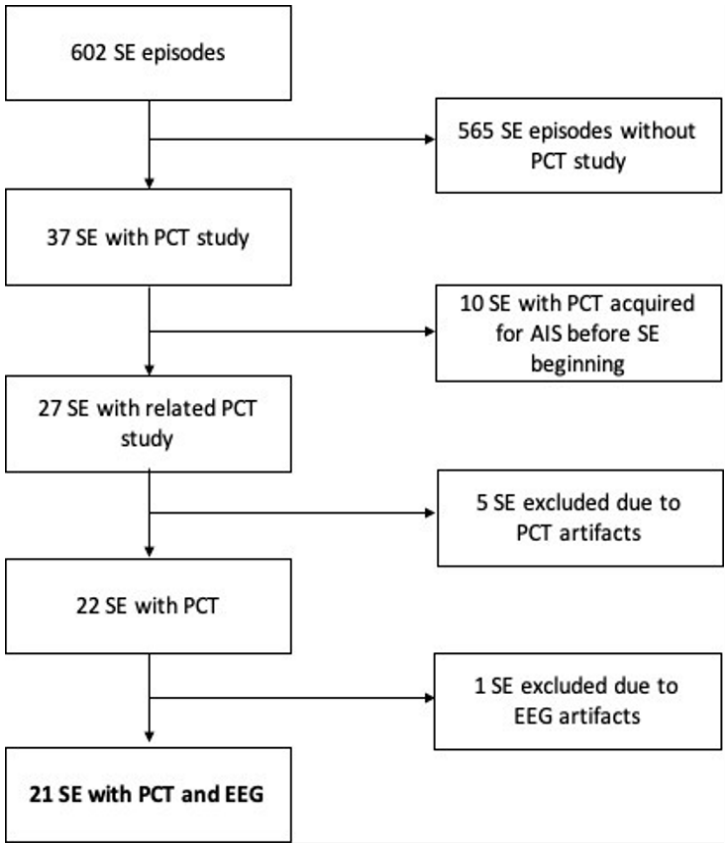
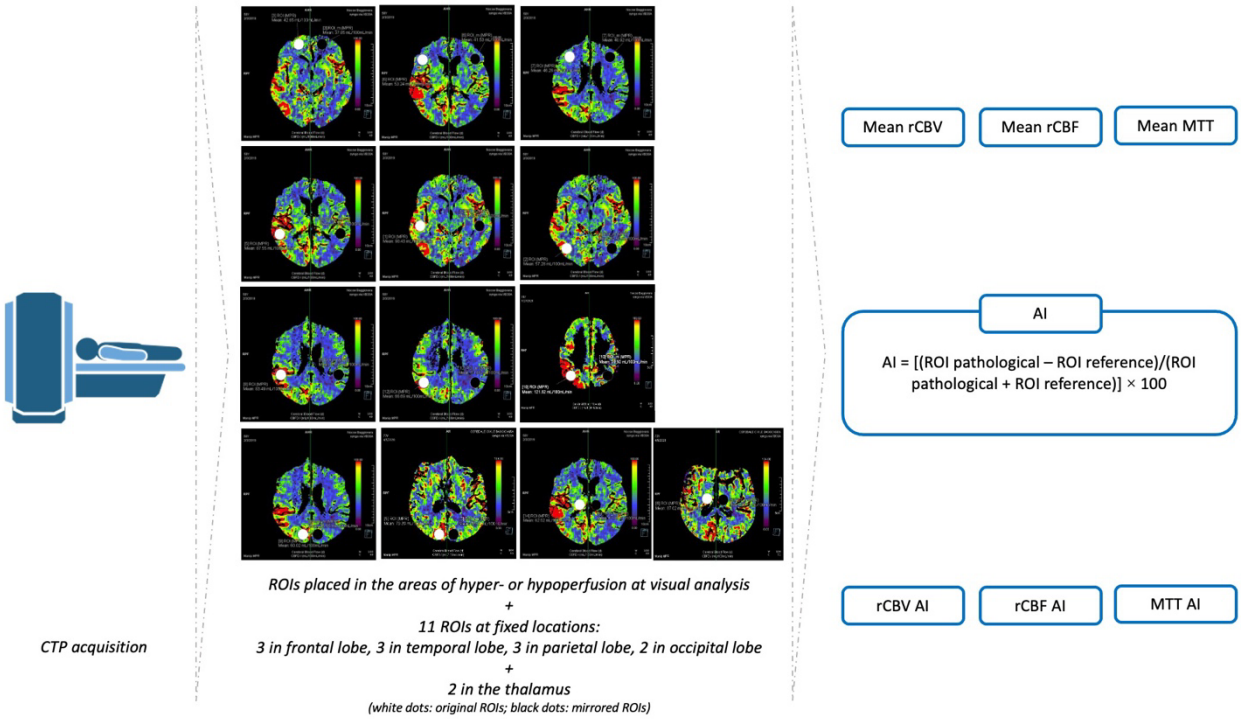
Supplementary material

Supplementary Figure 1: flow chart for population selection



AIS: acute ischemic stroke.

Supplementary Figure 2: imaging acquisition and elaboration process



Supplementary Table: Clinical, PCT and EEG characteristics of the examined population

Num	Clinical characteristics					PCT			EEG				
	Gender	Age	Type of Focal NCSE	Motor manifestations	Aetiology	Mean CBF affected hemisphere (ml/100ml/min)	Mean CBF AI	Location	T between PCT and EEG (h:min)	Areas involved	Type of SCC pattern	Continuous (CP) vs Discontinuous (WWP)	LPD
1	M	85	Aphasic	Subtle + bil TC ev	L T HGG	63.65	25.43	L PTO + L thalamus	00:35	L PTO	D-NCSE 2c	CP	No
2 (fig 2b)	F	58	w/o consciousness impairment	Bil TC ev	MELAS	75.96	31.71	R PT	08:37	R PT	D-NCSE 2c	WWP	Yes
3	F	68	Aphasic	Bil TC ev	Previous neurosurgery for L T meningioma	95.28	16.47	L PO + L thalamus	00:11	L PO	D-NCSE 1	CP	No
4	M	72	w/o consciousness impairment	Subtle	Remote R T ischemic stroke	78.81	17.66	R O + R thalamus	06:48	R TO	N-NCSE	-	-
5	F	78	Aphasic	Subtle	Remote ischemic strokes	67.12	14.11	L FP	05:27	L F	D-NCSE 2c	CP	No
6	M	72	Aphasic	No	Acute L PO ischemic stroke	90.46	24.60	L PO	01:50	L PO	D-NCSE 2a	CP	No
7	F	77	w/o consciousness impairment	Subtle	Remote R PT ischemic stroke	128.23	19.11	R PO	00:19	R PO	D-NCSE 2b	CP	No
8	M	56	Aphasic	No	Focal symptomatic epilepsy (R HS)	100.77	17.83	R FT	03:00	R FT	P-NCSE 2e	CP	No
9	M	67	Aphasic	Bil TC ev	SESA Syndrome	89.72	10.72	L thalamus	11:47	L PTO	D-NCSE 2c	WWP	No
10	M	88	Aphasic	No	Metastatic L TO lesion	94.79	16.86	L O	00:10	L PTO	D-NCSE 2c	WWP	Yes
11 (fig 2a)	F	82	Aphasic	Subtle	Acute embolic ischemic L FP stroke	95.16	18.77	L PTO + L thalamus	02:29	L PTO	D-NCSE 1	CP	No
12 (fig 2c)	M	66	Aphasic	Subtle	Anti-Ma2 encephalitis	73.20	15.51	L PT	02:47	L T	P-NCSE 2e	CP	Yes
13	F	82	Aphasic	No	Remote ischemic strokes	71.49	19.40	L PTO + L thalamus	00:14	L PTO	D-NCSE 1	CP	No
14	M	80	Aphasic	Bil TC ev	Cryptogenic	81.63	16.10	L PTO	00:28	L PTO	D-NCSE 1	CP	No
15 (fig 3)	F	90	Aphasic	Bil TC ev	Viral encephalitis	73.77	-1.28	-	00:25	L PTO	D-NCSE 2c	WWP	Yes
16	M	80	Aphasic	No	Remote L P ischemic stroke	63.22	-3.48	-	02:32	L CP	D-NCSE 2c	WWP	Yes
17	F	71	w/o consciousness impairment	Subtle	Remote R PTO ischemic stroke	99.88	25.44	R PO	08:00	R P	N-NCSE	-	-
18	M	68	Aphasic	No	Remote L PO haemorrhagic stroke	86.98	22.65	L PTO	00:16	L PO	D-NCSE 1	CP	No
19	M	64	w/o consciousness impairment	No	R T LGG in NF	95.82	10.97	R FT + R thalamus	10:30	R FT	D-NCSE 2c	WWP	Yes
20	F	71	with consciousness impairment	No	Acute LT intra-parenchymal haemorrhage	64.11	16.39	L TO + L thalamus	00:23	L PTO	D-NCSE 1	CP	No
21	F	75	Aphasic	Bil TC ev	L F neoplasia	67.37	4.45	-	00:10	L FC	N-NCSE	-	-

NCSE: Non Convulsive Status Epilepticus, PCT: Perfusion Computer Tomography, CBF: Cerebral Blood Flow, AI: Asymmetry Index, SCC: Salzburg EEG Criteria for NCSE, LPD: Lateralized Periodic Discharges, CP: continuous pattern, WWP: waxing and waning pattern, bit TC ev: bilateral tonic-clonic evolution, L: left , R: right, F: frontal, T: temporal, C: central, P: parietal, O: occipital, D-NCSE: Definite Non Convulsive Status Epilepticus, P-NCSE: Possible Non Convulsive Status Epilepticus, N-NCSE: No-Non Convulsive Status Epilepticus, HGG: high grade glioma, LGG: low grade glioma, MELAS: Mitochondrial Encephalopathy with lactic acidosis and stroke-like episodes, HS: hippocampal sclerosis, SESA: Subacute encephalopathy with seizures in alcoholics, NF: neurofibromatosis.

7. Results 2 - Serum neurofilament light as biomarker of seizure-related neuronal injury in status epilepticus

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Abstract

Biomarkers of neuronal damage in status epilepticus (SE) would be of great relevance for clinical and research purposes.

In a retrospective cross-sectional study, serum neurofilament light chain (NfL) levels were measured in patients with SE (30 subjects), with drug-resistant epilepsy (30 subjects) and healthy controls (30 subjects).

Serum NfL levels were higher in patients with SE (median: 26.15 pg/ml) compared to both epilepsy patients (median: 7.35 pg/ml) and healthy controls (median: 6.81 pg/ml) ($p < 0.001$). In patients with SE serum NfL levels showed a high correlation with CSF NfL ($\tau = 0.68$, $p < 0.001$) as well as with CSF t-tau levels ($\tau = 0.627$, $p < 0.001$), they were higher in SE lasting > 24 hours ($p = 0.013$), in refractory/super-refractory SE ($p = 0.004$), and in patients who died within 30-days or who presented a worsening of clinical conditions ($p = 0.001$). Values above 28.8 pg/ml predicted 30-days clinical worsening or death (OR 10.83; 95% CI 1.96-59.83; $p = 0.006$) and SE refractoriness (OR 9.33; 95% CI 1.51-57.65; $p = 0.016$).

In conclusion, serum NfL are increased in SE and correlate with SE treatment response, duration, and outcomes, representing therefore a promising biomarker of seizure-related neuronal damage.

Keywords: Status Epilepticus, neurofilaments, biomarkers.

Introduction

In 2015 a new operational definition of Status Epilepticus (SE) was proposed, emphasizing that SE is a time-dependent neurologic emergency requiring prompt treatment (Trinka et al., 2015). In approximately 30% of cases, SE is refractory to benzodiazepines and antiseizure medicines (ASM) and may lead to brain injury with cellular and molecular alterations (inflammation, or neuronal and astroglial injury) that could induce subsequent irreversible neurological impairment and further development of epilepsy, with a mortality rate ranging from 7% to 39% (Leitinger et al., 2019). For a better stratification of patients in terms of response to treatment, severity of damage and possible sequelae, having a diagnostic and prognostic biomarker would be of great support for both clinical practice and research. In SE, although cerebrospinal fluid (CSF) biomarkers have been proposed, none have yet been validated in clinical use to diagnose SE or to predict its clinical outcome (Hanin et al., 2020; Rejdak et al., 2012).

In the last decade neurofilaments (Nf) have been the subject of numerous preclinical and clinical studies, proving to be biomarkers of degeneration or acute damage in several neurological disorders (Gafson et al., 2020; A. Yuan et al., 2017). Neurofilaments, further distinguished in neurofilament light (NfL), middle (NfM) and heavy (NfH) based on their relative apparent molecular masses, are particularly expressed in the myelinated axons of neurons where they have a structural function in the cytoskeleton establishing cross-bridging with other filaments. Importantly, Nf light might be released in significant amounts into blood when neuroaxonal damaged is present, representing therefore a promising and minimally invasive biomarker of neuronal damage (Rejdak et al., 2012; A. Yuan et al., 2017). Notably, although Nf have been viewed traditionally as structural components primarily of axons and dendrites, besides structural functions, recent evidence has shown that distinctive assemblies of Nf subunits are also integral components of synapses (A. Yuan et al., 2017, 2018).

To date, no study has investigated whether NfL in peripheral blood may represent a biomarker of neuronal damage due to SE. For this aim we measured and compared serum NfL (sNfL) levels in adult patients with SE, chronic epilepsy and healthy controls.

Methods

This is a retrospective, monocentric, cross-sectional study. We analysed serum and CSF samples of patients and healthy controls collected at the OCB hospital of Modena, Italy, and stored (at -80°C in polypropylene storage tubes) in our biobank starting from October 2018.

Status Epilepticus group. Patients with SE in whom a blood sample was collected soon after SE diagnosis were included. We excluded all SE patients with conditions known to cause Nf elevation per se such as acute structural brain lesion (e.g. acute strokes, hemorrhages, post-anoxic encephalopathy, autoimmune or infectious encephalitis) and inflammatory/neurodegenerative diseases (e.g. multiple sclerosis, dementias, Parkinson disease) as reported in **Figure S1**. Specific etiologies retained in our study are detailed in the notes of **Table 1**.

TABLE 1 Clinical and demographic features and biomarkers in the different groups

Feature/biomarker	SE, $n = 30$	Epilepsy, $n = 30$	Healthy controls, $n = 30$	Significance
Gender, *n* (%)				
Male	16 (54%)	17 (57%)	10 (30%)	$p = .147$
Female	14 (46%)	13 (43%)	20 (60%)	
Age, years				
Mean (±SD)	45 (±19.9)	39 (±13.6)	40 (±14.7)	$p = .817$
Range	11–79	20–65	17–75	
sNfL, pg/ml				
Mean	101.14	8.54	13.14	
Median	26.15	7.35	6.81	$p < .001$[a]
IQR	97.07	6.41	9.29	
CSF NfL, pg/ml, $n = 17$				
Mean	2410	–	–	
Median	752	–	–	
IQR	4392	–	–	
t-tau, pg/ml				
Mean	21 997	–	–	
Median	512	–	–	
IQR	2217	–	–	
Duration of SE, *n* (%)				
≤24 h	18 (60%)	–	–	
>24 h	12 (40%)	–	–	
Time between SE onset and sample, *n* (%)				
≤24 h	14 (47%)	–	–	
>24 h	16 (53%)	–	–	
Etiology classification, *n* (%)[a]				
Acute symptomatic	10 (34%)	–	–	
Remote symptomatic	6 (20%)	–	–	
Progressive symptomatic	4 (13%)	–	–	
Unknown	6 (20%)	–	–	
In definite epileptic syndrome	4 (13%)	–	–	
Clinical manifestations, *n* (%)				
Motor symptoms	21 (70%)	–	–	
GCSE	7	–	–	
GCSE-NCSE	4	–	–	
FCSE	8	–	–	
FCSE-NCSE	1	–	–	
MSE-NCSE	1	–	–	
Nonconvulsive symptoms only	9 (30%)	–	–	
Treatment response, *n* (%)				
Responsive SE	20 (66%)	–	–	
RSE	5 (17%)	–	–	
SRSE	5 (17%)	–	–	
30-day outcome, *n* (%)				
Return to baseline conditions	17 (57%)	–	–	
Worsening of clinical conditions or death	13 (43%)	–	–	

Note: RSE was defined as failure of first-line therapy with benzodiazepines and one second-line treatment with antiseizure medications. In SRSE, SE continued or recurred despite the use of anesthetics for >24 h.

Abbreviations: CSF, cerebrospinal fluid; FCSE, focal convulsive SE; GCSE, generalized convulsive SE; IQR, interquartile range; MSE, myoclonic SE; NCSE, nonconvulsive SE; RSE, refractory SE; SE, status epilepticus; sNfL, serum neurofilament light; SRSE, superrefractory SE; t-tau, total tau.

[a]Statistically significant.

bRegarding the specific etiologies, the acute symptomatic group included four with toxic etiology, two with metabolic etiology due to hyponatremia, and four with precipitating factors in epilepsy; the remote symptomatic group included four with vascular etiology and two epileptic patients without an acute precipitating cause; the progressive symptomatic group included four low-grade tumors; the specific epilepsy syndrome group included three cases of genetic generalized epilepsy and one case of sleep-related hypermotor epilepsy.

At our center, all consecutive patients with SE have been prospectively collected (since 2013) using a specific status epilepticus form to collect demographic and clinical information, including age, gender, semiology, etiology, and dosage of anti-seizure medications (ASMs), anesthetic drugs and other therapies used. All SE episodes were reviewed by two Authors (SM and GG), and met the ILAE diagnostic criteria (Trinka et al., 2015). Treatment responsiveness was defined as SE cessation after first-line therapy with benzodiazepines alone or followed by second-line treatment with one ASM administered intravenously. Refractory SE (RSE) was defined as a failure of first-line therapy with benzodiazepines and one second-line treatment with ASMs. In super-refractory SE (SRSE), status continued or recurred despite the use of anesthetics for longer than 24 hours. The 30 days functional outcome was assessed using mRS and we defined worsening of the clinical condition an increase of at least one point in mRS compared to its baseline score before SE development. We chose an increment of 1 point as compared to baseline and not the absolute Rankin score as it allows to better define the worsening of clinical conditions (e.g. a patient with mRS of 3 at baseline who had 3 at 30 days from SE onset is defined as recovered, thus with a good outcome, independently from the absolute mRS value).

Epilepsy group. This group consisted of patients with drug-resistant epilepsy. Drug-resistance was considered as the failure of adequate trials of two tolerated and appropriately chosen and used ASM (either as monotherapies or in combination) to achieve sustained seizure freedom (Kwan et al., 2009). In these patients, blood samples were collected during hospitalization in the Epilepsy Monitoring Unit where they presented isolated seizures. None of them had an episode of SE during the hospital stay, neither experienced a SE before. Blood sample was collected < 24 hours from the last recorded seizure in all patients, the median delay was 3 hours. Clinical details and etiologies are reported in **Table S1**.

Healthy controls. This group consisted of age-matched healthy volunteers without any neuropsychiatric condition, and a negative family history for neurodegenerative diseases.

The local Ethics Committee approved the study (prot. 967/2017 and 556/2018 AOUMO) and informed consent was obtained from all participants included in the study. Anonymized data will be shared upon request by any qualified investigator.

Procedures

Serum NfL concentrations and, whenever available, CSF NfL were determined on Simple Plex™ NfL Assay (ProteinSimple, CA, USA) on Ella™ instrument, according to the manufacturers' instructions. Ella™ was calibrated using the in-cartridge factory standard curve. The Ella™ instrument allows rapid and ultra-sensitive measurement of biomarkers. This platform allows quantification of an analyte from 72 samples in a single disposable microfluidic cartridge, within 90 minutes.

Statistical analysis

Comparison of clinical data and sNfL between groups were performed using parametric or not parametric statistic as appropriate. Correlations analysis (Spearman's and Kendall's correlation) were applied to study the relationship between sNfL and demographic, clinical characteristics of SE as well as levels of t-tau.

A receiver operating characteristic (ROC) curve was plotted to calculate the cut-off point for sNfL level with the best sensitivity and specificity in predicting clinical outcome and treatment refractoriness (using the maximum value of Youden's index).

A univariable analysis was performed to identify factors associated with SE-related clinical worsening and drug refractoriness. For all the analyses the p value was set at < 0.05. All analyses were conducted using IBM SPSS Statistics 26.

Results

Ninety subjects were included in the study: 30 patients with SE (median age 39 yrs), 30 patients with epilepsy (median age 36 yrs) and 30 healthy controls (median age 41 yrs) (One way ANOVA with Bonferroni post-hoc test, $p = 0.817$). Clinical characteristics of patients with SE are reported in **Table 1**.

Levels of sNfL in patients with SE showed great variability, from values in the range of the control groups up to 50-fold. Kruskal-Wallis test with Dunn post-hoc test showed that serum NfL levels were markedly higher ($p < 0.001$) in patients with SE (median: 26.15 pg/ml) compared to both epilepsy patients (median: 7.35 pg/ml) and healthy controls (median: 6.81 pg/ml), while no differences were found between sNfL levels in patients with epilepsy and in healthy controls ($p = 0.91$) (**Table 1; Figure 1A**).

For 17 SE patients CSF was also available (collected at the same time of blood sampling) and a high correlation was found between sNfL and CSF NfL levels ($\tau = 0.68$, $p < 0.001$) (**Figure 1B**). For these SE cases a high correlation was also observed between sNfL and CSF t-tau levels ($\tau = 0.63$, $p < 0.001$; not shown).

When analyzing serum NfL in relation to treatment outcomes, sNfL levels were higher in patients with refractory and super-refractory SE (Responsive SE: median 13.35 pg/ml, RSE/SRSE: median 89.7 pg/ml; $p = 0.004$) (**Figure 1D**). Moreover, considering the duration of SE we observed that sNfL were increased in SE lasting > 24 hours respect with SE lasting < 24h ($p = 0.013$) (**Figure 1C**). Notably, we did not find any correlation between the serum levels of NfL and the time elapsed between the

estimated SE onset and samples collection ($\tau = 0.13$, $p = 0.352$). Patients presenting worsening of clinical conditions (increment of at least 1 point of mRS respect to baseline condition) had higher levels of sNfL compared to those who recovered (median: 102 pg/ml and 11.70 pg/ml respectively; $p = 0.001$) (**Figure 1E**).

As regards to the clinical presentation of SE, we did not find any differences in sNfL levels between motor and non motor forms of SE ($p = 0.859$). This result suggest the possibility that non convulsive forms of SE could damage the brain similarly as motor forms do thus sustaining a more aggressive treatment even for the non motor forms of SE.

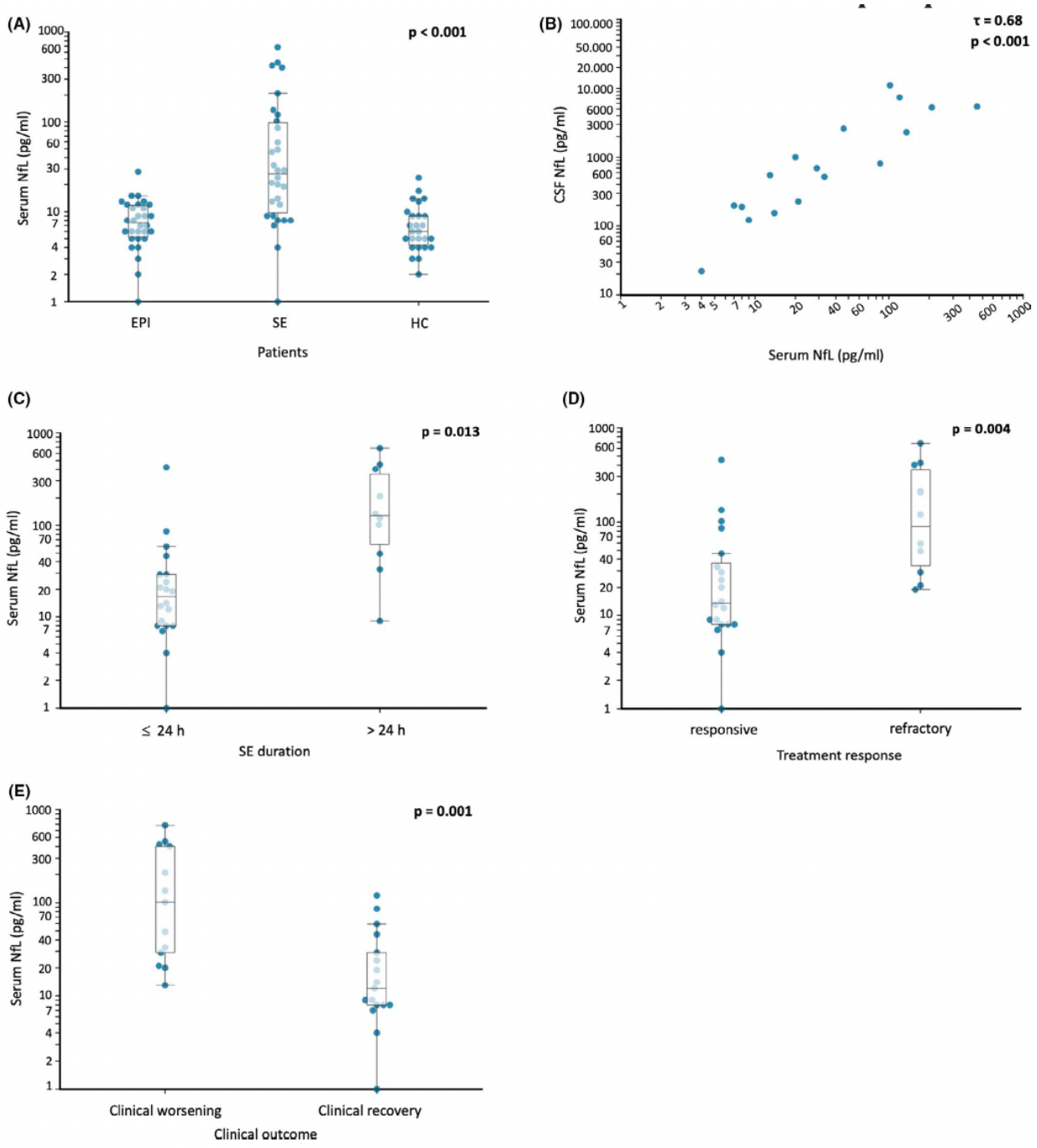

FIGURE 1 Neurofilaments across groups and in status epilepticus. (A) Log scale values of serum neurofilament light (NfL) across groups. EPI, epilepsy group; HC, healthy controls; SE, status epilepticus group. See text for groups details. (B) Serum–cerebrospinal fluid (CSF) correlation of NfL levels (log scale; $p < .001$). (C) Serum NfL levels in patients with status epilepticus resolved within 24 h (≤24 h) or with more prolonged duration (>24 h). (D) Serum NfL levels in patients with responsive or refractory status epilepticus. (E) Serum NfL in patients returning to baseline clinical condition and in patients who died or showed a clinical worsening after SE. Functional outcome was measured using the modified Rankin Scale

Finally, in patients with SE, we evaluated sNfL values to predict the development of treatment refractoriness (RSE and SRSE) and the 30-day clinical worsening. To find the best cut-off point we calculated ROC curves with Youden index for treatment response and for the 30-day outcome. The value of 28.80 pg/ml was the best cut-off both for treatment refractoriness (sensitivity: 80% and

specificity: 70%) and for 30-day clinical worsening (sensitivity: 77% and specificity: 76%). Serum NfL values above 28.8 pg/ml predicted a 30-day clinical worsening (OR 10.83; 95% CI 1.96-59.83; p = 0.006) and SE refractoriness (OR 9.33; 95% CI 1.51-57.65; p = 0.016) (**Table S2**).

Discussion

In this study, we found that in patients with SE, sNfL levels were, on average, higher compared with both healthy subjects and patients with chronic epilepsy. Having excluded from the study all episodes of SE caused by acute structural damage, it is conceivable that sNfL values for the most part reflect seizure-induced neuronal damage. The strict correlation observed between peripheral and central compartment (CSF) in patients with SE where both samples were available, although expected, confirms the reliability of the measurement of NfL on serum in SE. Such a high correlation may be a consequence of the frequent blood-brain barrier damage present during SE. Importantly, sNfL showed a high correlation with CSF total-tau that represent an established biomarker of neural damage already demonstrated to be elevated in patients with SE (Monti et al., 2015). NfL levels correlated also with duration of SE, response to first/second-line treatments, and functional outcomes. On the contrary, we did not observe a correlation between the serum levels of NfL and the time elapsed between the estimated SE onset and samples collection.

The samples were collected within the first 48 hours in all patients. Our explanation for this lack of correlation is that in this context probably the level of serum NfL primarily reflects the intrinsic severity of the SE. Nevertheless, we strong believe that this important aspect deserves further studies with a strict ad-hoc design (eg. multiple repeated samplings at different defined timepoints in the same patient both during SE and after its end). Notably, electrographic status epilepticus in patients with post-anoxic encephalopathy was recently found to be an independent predictor of high serum NfL levels at 72 hours after cardiac arrest (Lybeck et al., 2021).

As far as NfL in epilepsy patients, we observed sNfL levels comparable to those of healthy volunteers, supporting the idea that only repetitive seizure activity results in neuronal damage that can be indexed by serum NfL levels. In SE blood-brain-barrier disruption may contribute to an increased release of CSF NfL into blood making sNfL a particularly efficient biomarker in indexing neuroaxonal damage in this context. A few recent studies investigated serum NfL levels in drug-resistant epilepsy patients and after a single afebrile or febrile seizures, reporting NfL levels in the range observed in this study although obtained with a different method (SIMOA) (Evers et al., 2020; Nass et al., 2021; Ouédraogo et al., 2021). In the only study that explored the variation of serum NfL levels longitudinally after a single seizure, only a very small increment in sNfL sampled immediately after the seizure (7.9 pg/ml) compared to baseline values (7.2 pg/ml) and compared to values obtained thereafter was found (Evers et al., 2020).

Limitations ad conclusions

In conclusion, although the retrospective design and limited study cohort does not allow the prediction of short-term disability and cannot, therefore, drive therapeutic decisions, we believe that our preliminary results advocate a possible role for blood NfL as promising biomarker in SE, deserving further studies for both clinical and research purposes. Finally, although speculative, these results could support the role and importance that in SE neurofilaments play at the synaptic level and in particular at the level of glutamatergic synapses, which certainly have a fundamental role in the genesis and maintenance of ictal activity during SE (A. Yuan et al., 2018).

There is the need for future studies on the prognostic role of NfL in SE through the evaluation of multicenter cohorts including also different etiologies and the pediatric population too.

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Authors contribution

GG: conception and design of the study, collected and interpreted the data, performed statistical analysis.

RB: major role in the acquisition of data, revised the manuscript for intellectual content.

DF: conception and design of the study, performed statistical analysis.

AEV: Interpreted the data; revised the manuscript for intellectual content.

JM: Design and conceptualized study; revised the manuscript for intellectual content, performed statistical analysis.

SM: Design and conceptualized study; drafted and revised the manuscript for intellectual content.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflicts of interest

GG, RB, DF, AEV, JM, SM have no conflicts of interests to declare.

Supplementary material

Figure S1. Study flow-chart reporting the inclusion of status epilepticus patients.

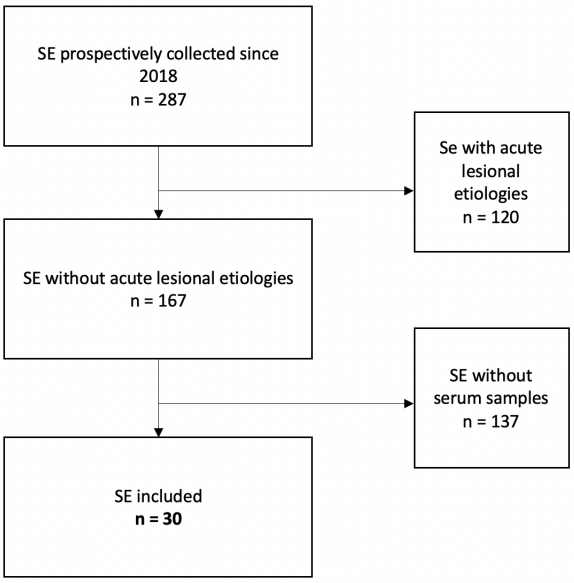

Table S1. Clinical data and etiology of the patients included in the epilepsy group.

Legend:
Unk: unknown, AED: antiepileptic drugs. GGE: genetic generalized epilepsy.

	Epilepsy group n = 30
Gender, n (%)	
female	13 (43%)
male	17 (57%)
Age, years (range)	39 (20-65)
Age at epilepsy onset, years (range)	24 (2-63)
Duration of epilepsy, years (range)	16 (6 months-50 years)
Seizure frequency, n/month (range)	7 (0-60)
Etiology, n (%)	
Hippocampal sclerosis	5 (17%)
Focal cortical dysplasia	7 (23%)
Cavernoma	2 (7%)
Low grade tumours	5 (17%)
Nodular heterotopia	1 (3%)
Complex malformation of cortical development	2 (7%)
Encephalocele	1 (3%)
MRI negative	6 (20%)
GGE	1 (3%)
Side, n (%)	
Right	17 (57%)
Left	9 (30%)
Bilateral	3 (10%)
Unk	1 (3%)
Lobe, n (%)	
Temporal	19 (64%)
Frontal	8 (27%)
Parietal	1 (3%)
Insular	1 (3%)
GGE	1 (3%)
Actual AEDs therapy, n (%)	
Single AED	10 (33%)
Two AEDs	11 (37%)
AEDs polytherapy	9 (30%)

Legend:
Unk: unknown, AED: antiepileptic drugs. GGE: genetic generalized epilepsy.

Table S2. Association between patient baseline variables and risk of SE refractoriness and worsening of clinical condition.

	OR	95% CI	p-value
Univariate analysis for Status epilepticus refractoriness			
Age	1.01	0.97-1.05	. 663
Gender	0.67	0.14-3.11	. 606
Clinical semeiology	0.46	0.08-2.81	. 404
Etiology	1.5	0.33-6.92	. 603
sNfL levels	9.33	1.51-57.65	**. 016**
Univariate analysis for clinical worsening*			
Age	1.03	0.99-1.08	. 109
Gender	0.96	0.23-4.10	. 961
Clinical semeiology	2.03	0.42-9.89	. 380
Etiology	0.71	0.16-3.05	. 638
Response to treatment	5.44	1.04-28.53	**. 045**
sNfL levels	10.83	1.96-59.83	**. 006**

Clinical semeiology: convulsive vs non-convulsive forms; etiology: structural vs non-structural; response to treatment: responsive vs refractory and super-refractory forms; sNfL: dichotomized at 28.80 pg/ml.
*Worsening of clinical condition: an increase of at least one point in mRS compared to baseline value before SE development or death.

8. Results 3 - Neuro-glial degeneration in Status Epilepticus: exploring the role of serum levels of Neurofilament Light Chains and S100B as a prognostic biomarker for short term functional outcome.

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Abstract

Background: The last ILAE definition of Status Epilepticus (SE) highlights that the persistence of the epileptic activity per se could determine irreversible brain damages that could be responsible for long term consequences. The measurement of neuro-glial injury biomarkers could help in the identification of those patients who will eventually develop short- and long-term consequences of SE. At present none of the already studied biomarkers has been validated to be used in everyday clinical practice. In this study, we explore the role of NfL and S100B as a prognostic biomarker to identify patients who will develop short term disability after an episode of SE.

Methods: this is a retrospective assessment of the serum levels of both NfL and S100B in a cohort of 87 adult patients with SE prospectively collected in our SE registry (Modena Status Epilepticus Registry – MoSER –) at Baggiovara Civil Hospital (Modena, Italy). All samples were acquired during SE within 72 hours from SE diagnosis. The comparison groups were: healthy controls (HC, n = 27) and patients with epilepsy (PWE, n = 30). Demographic, clinical and therapeutical information and thirty-days follow-up information regarding disability development were acquired for every included patient and analysed in relation to NfL and S100B values.

Results: Serum levels of NfL were significantly higher in SE compared to those of PWE (median 7.35 pg/ml, IQR 6.4, $p < 0.001$) and HC (median 6.57 pg/ml, IQR 9.1, $p < 0.001$); S100B serum levels were higher in SE (median 0.11 ug/L, IQR 0.18) compared to PWE (median 0.03 ug/L, IQR 0.03, $p < 0.001$) and HC (median 0.02 ug/L, IQR 0.008, $p < 0.001$). However, only NfL serum levels were found to be an independent predictor of 30 days functional outcome whereas S100B levels did not.

Conclusions: Our results suggest that NfL measurement in serum during SE could help predicting the short-term functional outcome.

Keywords: Status Epilepticus, prognosis, biomarkers, S100B, Neurofilament, disability

1. Introduction

Status Epilepticus (SE) is a common neurological emergency characterized by high short-term morbidity and mortality. The last ILAE definition of Status Epilepticus considers SE as "a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms which lead to abnormally prolonged seizures (after time point t1); It is a condition that can have long-term consequences (after time point t2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures" (Trinka et al., 2015). From this definition it is evident that the persistence of the epileptic activity per se could determine brain damage that could be responsible for long-term consequences such as epilepsy development, cognitive and behavioural deficits.

Thus, besides clinical-electroencephalographic evaluation, a multimodal approach, based on the measurement of neuro-glial injury biomarkers could help in the identification of those patients who will develop short- and long-term consequences of SE.

Many different potential biomarkers of neural and glial degeneration have been studied so far both in serum and CSF of patients with epilepsy and SE (Hanin et al., 2020; Hanin, Denis, et al., 2022; Simani et al., 2020) but, at present none of those has been validated to be used in everyday clinical practice.

Among them, Neurofilament light chains (NfL) are proteins especially present in the axons of the myelinated neurons were they play a structural role establishing cross-bridging with other filaments and at the synaptic level were they maintain synaptic plasticity. Their role as a marker of neurodegeneration has been established in many different neurological diseases (Abu-Rumeileh et al., 2020; Disanto et al., 2019; S. Engel et al., 2020; A. Yuan et al., 2017, 2018) and has been already evaluated in a previous exploratory study in SE by our group (Giovannini et al., 2022).

S100B is a protein particularly expressed by glial cells and by astrocytes where it plays the role of a calcium-sensor protein intervening in the regulation of many different processes involving proliferation, survival and differentiation (Arcuri et al., 2005; Shimamoto et al., 2014). Many different studies have recognized it as a sensitive biomarker of active astrocytes distress and at high extra-cellular concentrations it actively participates in the processes accompanying neural injury (Michetti et al., 2012, 2019; Villarreal et al., 2014). Previous studies demonstrated elevated serum levels both in patients with epilepsy (Calik et al., 2013, 2014; Langeh & Singh, 2020; Liang et al., 2019; Meguid et al., 2018; Simani et al., 2020) and SE (Gunawan et al., 2019; Wang et al., 2022).

In this study, we explore the role of NfL and S100B as a prognostic biomarker to identify those who will develop short term disabilities after an episode of SE.

2. Materials and methods

2.1 Study design, setting, and patients

We retrospectively measured the serum levels of NfL and S100B in a cohort of 87 adult patients with SE prospectively collected in our SE registry (Modena Status Epilepticus Registry – MoSER –) at Baggiovara Civil Hospital (Modena, Italy) from September 1, 2013 to December 31, 2021. All samples were acquired during SE within 72 hours from the diagnosis. All aetiologies were included but patients with post-anoxic encephalopathy.

Before 2015, SE was considered a continuous seizure that lasts 5 min or longer or two or more discrete seizures without complete recovery of consciousness between them (Lowenstein et al., 1999). After 2015, the definition given by the International League Against Epilepsy (ILAE) was systematically adopted and prospectively applied (Trinka et al., 2015) and patients were included if they presented an episode of SE lasting more than 5 min for tonic-clonic SE, 10 min for focal SE with impaired consciousness, and 10–15 min for absence SE. All cases of SE that occurred before 2015

were reviewed by two authors (SM and GG) to ensure that all met the ILAE diagnostic criteria. The cases of non-convulsive SE were diagnosed according to the Salzburg electroencephalographic (EEG) criteria (Leitinger, Beniczky, et al., 2015; Leitinger et al., 2016).

Demographic, clinical and treatment information were acquired for every included patient examining hospital informatic database. A SE episode was defined as responsive to treatment

If it was controlled by the infusion of a benzodiazepine alone or followed by a single anti-seizure medication. Cases not controlled were considered refractory to treatment (RSE). A quote of these cases needed the admission to the intensive care unit (ICU) and the administration of third lines/anaesthetic drugs. Those cases persisting despite the application of third lines drugs for 24 hours or reappearing at the weaning of anaesthesia were considered Super-Refractory (SRSE).

Thirty-days follow-up information were acquired through hospital informatic database examination, clinical visits as well as telephone interviews. Disability's level was measured using the Modified Rankin Scale (mRs). A worsening of clinical condition was defined as an increase of at least one point in mRS at 30 days follow-up compared to the level of disability present before SE.

Serum levels of investigated biomarkers were compared to those of two comparison groups: healthy controls (HC, n = 27) without any neuropsychiatric condition and a negative family history for neurodegenerative diseases whose blood samples have been donated and stored in the "Modena NeuroBiobank" (a biobank dedicated to research for neurological disorders active since 2018) and patients with epilepsy (PWE, n = 30) whose blood samples were collected after isolates seizures during the stay in the Epilepsy Monitoring Units. Both groups were age and sex-matched with SE group.

2.2 Biomarkers measurements

Serum NfL concentrations were determined through immune-enzymatic test on Simple Plex NfL Assay (ProteinSimple) on an Ella instrument, according to the manufacturer instructions and calibrated using the in-cartridge factory standard curve.

S-100B serum levels were measured through chemiluminescence immunoassay (CLIA, LIASON, DiaSorin).

2.3 Statistical analysis

IBM SPSS Statistics Version 26 was used for statistical analysis. We described categorical variables as percentage and proportions, and continuous variables as mean and standard deviation (SD) or as median and interquartile range (IQR), depending on the underlying distribution. Univariate comparisons were performed with Fisher's Exact test, the chi-square test and the Mann–Whitney test. Linear regression analysis was used to determine the predictors of serum levels of Nfl and S100B. Logistic regression analysis was used to determine predictors of 30 days functional outcome. To find the best cut-off for these biomarkers for the analysed outcome, ROC curves were calculated. The statistical threshold for all the analysis was set at a p level of 0.05.

3. Results

We measured the serum levels of NfL chains and S100B in an adult population of 87 patients (female 54 -62%- median age 70) with a clinical or electro-clinical diagnosis of SE. The clinical characteristics of the analysed population are presented in **Supplementary material (Table S1)**.

SE patients showed a great variability in serum levels of NfL (median 64.7 pg/ml, IQR 139.2). The levels of NfL were independently influenced by gender (B = 0.634 95% CI 0.072-1.195, p = 0.027),

level of patient's consciousness before treatment (B = 0.815 95% CI 0.249-1.382, p = 0.005) and refractoriness to treatment (B = 1.015 95% CI 0.359-1.671, p = 0.003) when adjusted for the other variables (duration of SE and age). Significantly higher levels were found in females (median 90.85 pg/ml, IQR 151.8, p = 0.012), stuporous and comatose patients (median 163 pg/ml, IQR 392.8, p = 0.001) and in those with refractory and super-refractory forms of SE (median 113, IQR 204.1, p < 0.001) **(Table1).**

Table 1: Variables influencing the levels of NfL

Variables	B coefficent	95% CI	p
SE duration > 24 h	-0.068	-0.743 – 0.606	0.841
Gender (Female)	**0.634**	**0.072 – 1.195**	**0.027**
Stupor/Coma before treatment	**0.815**	**0.249 – 1.382**	**0.005**
Age	0.271	-0.008 – 0.550	0.057
Refractoriness to treatment	**1.015**	**0.359 – 1.671**	**0.003**

The levels of S100B showed a median value of 0.11 ug/L with an IQR of 0.18. They were influenced by level of patient's consciousness before treatment (B = 0.913 95% CI 0.365-1.461, p = 0.001) independently from the other variables (aetiology, age and gender). In particular, levels of S100B were significantly higher in stuporous and comatose patients compared to alert or somnolent ones (median 0.19 ug/L, IQR 0.50 p = 0.001) **(Table 2).**

Table 2: Variables influencing the levels of S100B

Variables	B coefficent	95% CI	p
Gender	0.519	-0.029 – 1.067	0.063
Aetiology	-0.412	-0.939 – 0.116	0.124
Stupor/Coma before treatment	**0.913**	**0.365- 1.461**	**0.001**
Age	0.222	-0.058 – 0.502	0.119

3.1 Biomarkers in SE compared to controls' populations

The clinical characteristics of the PWE and HC populations are presented in **supplementary material (Table S2 and S3)**. Since age and gender could influence NfL and S100B levels and the comparison

groups were younger compared to SE patients, a case control matching analysis to balance group in relation to age and gender was performed. In SE group, serum levels of NfL were significantly higher compared to those of PWE (median 7.35 pg/ml, IQR 6.4, $p < 0.001$) and HC (median 6.57 pg/ml, IQR 9.1, $p < 0.001$). No differences were found between PWE and HC ($p = 0.936$). The same was observed for the S100B serum levels: SE (median 0.11 ug/L, IQR 0.18), PWE (median 0.03 ug/L, IQR 0.03, $p < 0.001$), HC (median 0.02 ug/L, IQR 0.008, $p < 0.001$). On the contrary, serum levels of S100B were significantly higher in PWE compared to HC ($p = 0.002$).

3.2 Serum biomarkers and 30 days disability

The median value of mRS pre-SE was 1. An overall worsening of the clinical conditions was observed in 57 patients (66%) and the median mRs value at hospital discharge and at 30 days was 4 points. Serum levels of NfL were significantly higher in patients who worsened after SE compared to those who completely recovered (median 97.20 pg/ml, IQR 167.80 vs median 26.15 pg/ml, IQR 90.74, $p = 0.001$). The same results were observed for serum levels of S100B (median 0.14 pg/ml, IQR 0.32 vs 0.09 pg/ml, IQR 0.10, $p = 0.018$) (**Figure 1**).

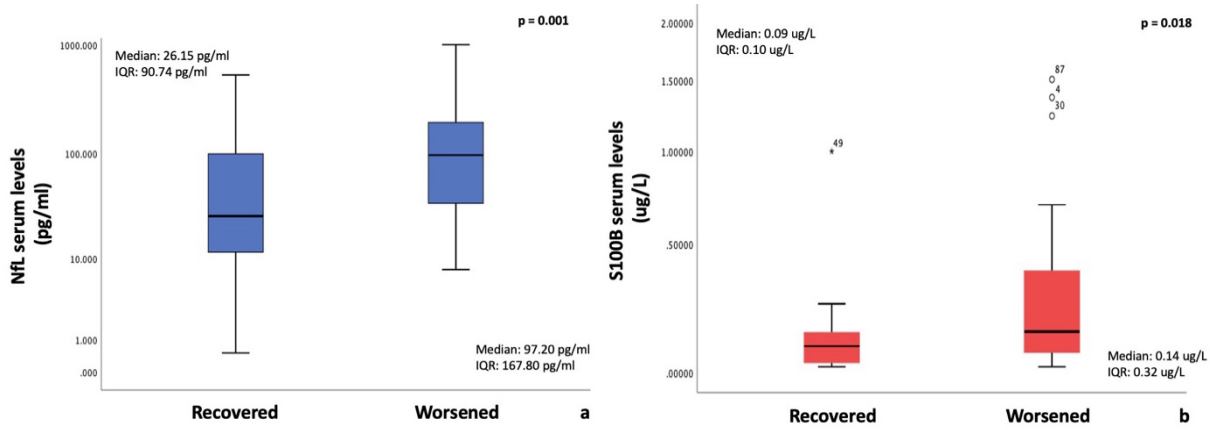

Figure 1. a) Comparison of serum levels of NfL between patients who completely recovered after SE and those who presented a worsening of clinical conditions. b) Comparison of serum levels of S100B between patients who completely recovered after SE and those who presented a worsening of clinical conditions.

At the univariate analysis only the NfL serum levels were found to be a predictor of 30 days functional outcome whereas S100B levels did not. Through the ROC curve an accuracy of 71.8% (95% CI 60-84) for NfL in predicting 30 days disability was found. The Youden index calculation, showed a value of 33.4 pg/ml as the best cut off for 30 days disability prediction (Sensitivity 77%, Specificity 60%). The multivariate analysis showed that NfL with the abovementioned cut off, were an independent predictor of 30 days disability after correction for age (OR 3.9 95% CI 1.41-10.64 p = 0.009) (**Figure 2**).

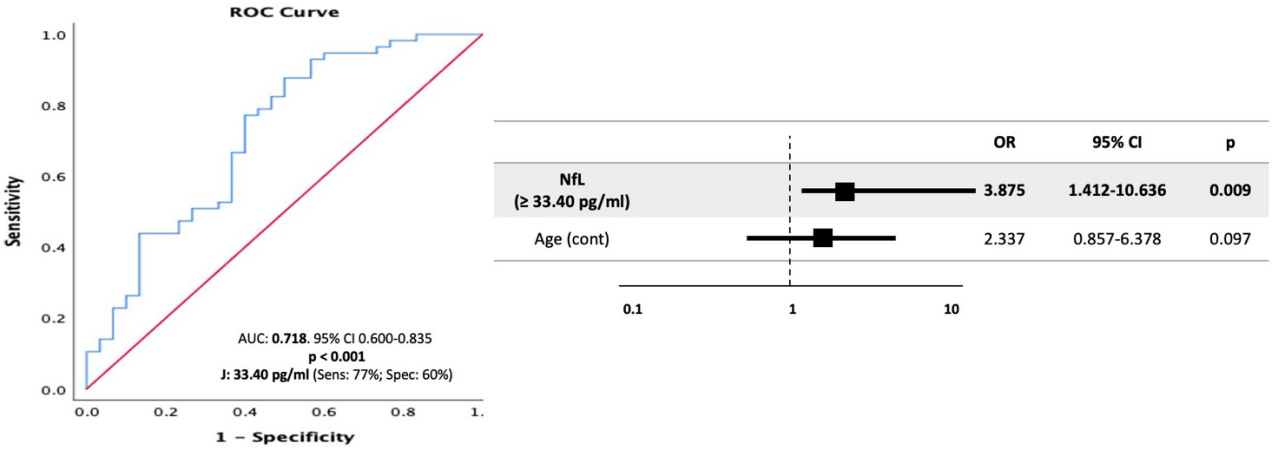

Figure 2. ROC curve for 30 days disability development. The AUC for NfL was 0.718. The Jouden index calculation show the best cut off at 33.40 pg/ml giving a sensitivity of 77% and a specificity of 60%. Multiple regression analysis showed that NfL ≥ 33.40 pg/ml are an independent predictor of 30 days disability development.

4. Discussion

In this study we confirmed that the measurement of serum levels of S100B and NfL in the first hours from SE diagnosis could give insights into the neuro-glial degeneration related to the presence of sustained seizures. Pre-clinical evidences from animal models of SE in favour of this aspect are well defined (Sherry & Turner, n.d.; Yang et al., 1996). In humans, this important point is still poor studied. As it has been previously highlighted by another previous study (Lybeck et al., 2021), electrographic SE after cardiac arrest is associated with higher levels of serum NfL compared to those found in post-anoxic patients without SE, which may indicate a potential secondary neuronal injury caused by electrographic SE.

In the present study, we found that both biomarkers were significantly elevated in SE patients compared to levels found after a single epileptic seizure in PWE and to those found in healthy controls. This result confirm our previous findings (Giovannini et al., 2022) and the results of a recent study on children with convulsive SE (Wang et al., 2022) in which serum S100B levels measured in the first 24 hours from SE diagnosis were found to be significantly elevated compared to those found in healthy children. Moreover, as regard to isolated seizures in PWE, in line with previous findings, we found that only serum S100B levels appeared increased compared to levels found in healthy controls (Calik et al., 2013, 2014; Liang et al., 2019; Meguid et al., 2018; Simani et al., 2020) serum NfL levels did not differ (Evers et al., 2020; Nass et al., 2021). This result suggests that single seizures could determine a more prominent glial perturbation rather than neuronal damage.

We already know that SE is a condition that could determine a bad short-term prognosis both in terms of death and disability. Many patients who survived a SE episode will never regain the performance status present before the SE episode. Thus, it is particularly important to predict the patient functional outcome. Literature lacks studies addressing specifically this point and no potentially serum biomarker has been studied so far trying to answer this question. Moreover, the majority of existing clinical prognostic scores for SE are directed toward the determination of the risk of death after a SE episode (Gao et al., 2016; Leitinger, Beniczky, et al., 2015; Rossetti et al., 2008) and not toward the functional outcome. In our study, 66% of patients worsened after SE. Worsening patients showed increased serum levels of both studied neuro-glial degenerative biomarkers compared to those found in patients with a full recovery. In line with these results, a recent study showed that S100B was higher in those SE episodes with an high degree of MRI, potentially irreversible abnormalities (Gunawan et al., 2019). However, our results suggest that only serum levels of NfL could predict disability development after SE, while S100B levels did not. To the best of our knowledge, this is the first study to address this question in SE. In accordance with our

results, in the BITSTRO study serum S100B levels after a single epileptic event appeared poorly predictive for seizures recurrence, mortality, re-hospitalization within seven days from the first event (Freund et al., 2015).

5. Conclusions and future perspectives

Our results suggest that NfL measurement in serum during the first hours of SE could help predicting the short-term functional outcome. For the future it will be important to try to replicate these results in other different population and to try to incorporate these results in clinical prognostic scoring systems.

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Supplementary material

Table S1: Clinical characteristics of patients with SE (SE)

Variable		n	%
Total population		87	100
Gender (F)		54	62
Age	< 65 y	35	40
	≥ 65 y	52	60
	Median	70	
	IQR	25	
Etiology	Acute Symptomatic	47	54
	Others	40	46
Clinical presentation	w/o motor phenomena	44	51
	w motor phenomena	43	49
STESS	0-2 pt	36	41
	3-6 pt	51	59
	Median	3	
	IQR	2	
EMSE	< 64 pt	58	67
	≥ 64 pt	29	33
	Median	55	
	IQR	48	
Pre-existing history of epilepsy		29	33
Treatment Response	Responsive	36	41
	RSE/SRSE	51	59
30 d mortality		14	16
30 d functional outcome	Recovery	30	34
	Clinical worsening	57	66
mRs pre SE	Median	1	
	IQR	3	
mRs at hospital discharge	Median	4	
	IQR	4	
mRs at 30 d	Median	4	
	IQR	4	
Epilepsy development after SE (n = 46)		14	30
SE recurrence (n = 46)		5	11

Table S2: Clinical characteristics of patients with epilepsy (PWE)

Variable		n	%
Total population		30	100
Gender (F)		13	43
Age	Median	36	
	IQR	24	
Etiology	MCD	10	33
	HS	5	17
	Cavernoma	2	7
	LEAT	5	17
	Encephalocele	1	3
	GGE	1	3
	MRI negative	6	20
N of ASM at the sampling time	Median	2	
	IQR	2	
Years of epilepsy	Median	11	
	IQR	19	

MCD: Malformations of cortical development; HS: hippocampal sclerosis; LEAT: Long-term epilepsy-associated tumors; GGE: Genetic Generalized Epilepsy; ASM: anti-seizure medications.

Table S3: Characteristics of healthy controls (HC)

Variable		n	%
Total population		27	100
Gender (F)		18	67
Age	Median	40	
	IQR	19	

9. Results 4 - A Multimodal approach to predict short term outcome after Status Epilepticus: the ADiReN score

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Abstract

Background: Short-term prognosis after SE has been already explored mainly in terms of mortality and disability development. To assess short term mortality, three different prognostic scores have been proposed so far: STESS (Status Epilepticus Severity Score), mSTESS (modified STESS) and EMSE (Epidemiology-based Mortality score in Status Epilepticus).

We evaluated the role of serum levels of Neurofilament light chains (NfL) and S100B to predict SE short-term outcome in terms of 30-days mortality and refractoriness to treatment. Then, the performance of a new score, based on a multimodal (clinical-electroencephalographic-biochemical) approach, to determine short-term mortality prognostication and its performance compared to the existing prognostic scores was assessed.

Methods: Serum levels of NfL and S100B in adult patients with SE prospectively collected in our SE registry (Modena, Italy) from 2018 to 2021 were evaluated. All samples were acquired within 72 hours from the diagnosis. The predictive power of these biomarkers toward short-term mortality and refractoriness to treatment was assessed.

Results: The serum NfL and S100B were measured in 87 patients (female 62%; median age 70 years). Serum levels of NfL were significantly higher in refractory SE compared to responsive SE episodes, while no differences were found for S100B. NfL (cut-off 19.75 pg/ml) appeared as an independent predictor of refractoriness development (OR 8.2 95% CI 2.08-32.58 p = 0.003) and an independent predictor of 30-days mortality. Values ≥ 70.25 pg/ml were associated to a significantly lower cumulative survival (log rank test: 0.002) adjusted for age (HR 5.26, 95% CI 1.16-23.78, p = 0.031). Finally, we proposed a new multimodal prognostic score for short-term mortality, so called ADiReN score (**A**ge, **Di**sability, **Re**fractoriness, **N**eurofilament), that appeared to have an higher discriminative power (AUC of 87.5%; 95% CI 79%-95.9%) and especially an increment of the ability to correctly identify those patients at higher risk of death compared to existing clinical scores (increment of PPV).

Conclusions: Our results suggest that serum NfL measurement within 72 hours from SE diagnosis could help predicting treatment refractoriness and short-term mortality. We propose a new prognostic score based on a multimodal evaluation that outperforms already established scores.

Keywords: Status Epilepticus, prognosis, biomarkers, S100B, Neurofilament, mortality

Introduction

Status Epilepticus (SE) is a common neurological emergency characterized by high short-term morbidity and mortality. With differences among existing studies, short-term mortality after an episode of SE varies between 9 and 61% at discharge, and between 19 and 65% at 30-day. To assess short term mortality, three different clinical prognostic scores have been proposed so far (González-Cuevas et al., 2016; Leitinger, Höller, et al., 2015; Rossetti et al., 2006; F. Yuan et al., 2018): STESS (Status Epilepticus Severity Score) (Rossetti et al., 2006), mSTESS (modified STESS) (González-Cuevas et al., 2016) and EMSE (Epidemiology-based Mortality score in Status Epilepticus) (Leitinger, Höller, et al., 2015). These scores are based on different clinical factors, which are well-known to play a decisive role in the determination of SE outcome: age, aetiology and history of epilepsy, level of disability before SE, level of consciousness before treatment, semiology of SE, comorbidities and characteristics of EEG.

The STESS was the very first created score to assess in-hospital mortality after SE. It was firstly introduced and applied to a small (35 patients), prospectively collected, SE population in 2006 (Rossetti et al., 2006) and subsequently validated in a larger multicenter one (154 patients) in 2008 (Rossetti et al., 2008). Thereafter it received some internal (Rossetti et al., 2013; Tsetsou et al., 2015) and external validations (Goyal et al., 2015; Sutter, Kaplan, et al., 2013) also in selected populations with refractory SE (RSE) (Madžar et al., 2016). STESS is a simple and quick score based on four parameters: level of consciousness before treatment administration; worst seizure type; age and history of previous seizures. The suggested cut off point was first set at 3 points (STESS-3) and then increased to 4 points (STESS-4) (Sutter, Kaplan, et al., 2013). The mSTESS score was proposed in 2016. This score takes into account the four parameters of STESS (age with a cut off of 70 years instead of 65) adding to them the baseline disability level measured by modified Ranking scale (mRS) and divided in three different categories. The mSTESS showed an increased accuracy and positive

predictive value (PPV) while it showed a lower ceiling effect compared to STESS 3 (González-Cuevas et al., 2016).

The EMSE was first proposed in 2015 (Leitinger, Höller, et al., 2015; Leitinger, Kalss, et al., 2015). This is the first score based on available epidemiological data, coming from the real world. It is based on four parameters (so called EMSE-EACE): aetiology; age; comorbidity and worst EEG pattern. The score was firstly validated in a retrospective population and the cut-off was set at 64 points. EMSE was found to be superior to both STESS-3 and STESS-4 (Leitinger, Kalss, et al., 2015). Then this score received some external validations (Giovannini et al., 2017; Kang et al., 2015) for the short-term mortality and disability assessment.

Overall, even though they may be useful in different clinical situations, all these scores showed a similarly high negative predictive value for short-term survival after the SE episode but low positive predictive value for short-term death after the SE episode. This implies that they should perfectly identify patients who will survive to SE episode while they have low reliability in the identification of those who will die due to SE episode. Therefore, they should be used to avoid over-treatment of patients who will go in anyway well, while they should not be used to withdraw treatment in patients with high scores because a great part of them will even survive. This aspect was recently confirmed by machine learning models of validation of STESS and EMSE too (Brigo et al., 2022a, 2022b, 2022c).

All these abovementioned scores are based only on clinical and electroencephalographic parameters. Recently, many different potential neuro-glial injury biomarkers have been studied in SE and these could potentially help identifying patients who will develop short- and long-term consequences of SE (Giovannini et al., 2022; Gunawan et al., 2019; Hanin et al., 2020; Hanin, Denis, et al., 2022; Simani et al., 2020; Wang et al., 2022).

In this study we explored the role of serum levels of two already studied neuro-glial injury biomarkers in SE, Neurofilament light chains (NfL) and S100B to predict short-term outcome of SE in terms of 30-days mortality and refractoriness to treatment development. Then, we explored the performance of a new score to determine short-term prognostication of death in SE based on a multimodal (clinical-electroencephalographic-biochemical) approach and we compared it to the existing prognostic scores.

Materials and methods

Study design, setting and adopted definitions

At Baggiovara Civil Hospital (Modena, Italy) a prospective SE registry (Modena Status Epilepticus Registry – MoSER –) exists since 2013. Patients' body fluids could be stored in our Biobank since 2018. We measured the serum levels of NfL and S100B in adult patients with SE from October 2018 to December 2021. All samples were acquired during SE within 72 hours from the diagnosis. All aetiologies were included but patients with post-anoxic encephalopathy.

The definition of SE given by the International League Against Epilepsy (ILAE) was systematically adopted (Trinka et al., 2015): SE was diagnosed if it lasted more than 5 minutes for tonic-clonic SE, 10 minutes for focal SE with impaired consciousness, and 10–15 minutes for absence SE. For cases of non-convulsive SE, they were diagnosed according to the Salzburg electroencephalographic (EEG) criteria (Leitinger et al., 2016; Leitinger, Höller, et al., 2015).

Through the examination of the hospital informatic database, demographic, clinical and treatment information were retrieved for every included patient.

To assess disability before SE episode, the mRS was applied as follows: 0, no symptoms; 1, no significant disability despite symptoms (i.e. able to carry out all usual duties and activities); 2, slight disability (i.e. unable to carry out all previous activities but able to look after own affairs without assistance); 3, moderate disability (i.e. requiring some help but able to walk without assistance); 4, moderately severe disability (i.e. unable to walk without assistance and unable to attend to own bodily needs without assistance); 5, severe disability (i.e. bedridden, incontinent and requiring constant nursing care and attention).

The response to treatment was defined as follows: a SE episode was defined as responsive to treatment If it was controlled by the infusion of a benzodiazepine alone or followed by a single anti-seizure medication; cases not controlled by this therapy were considered refractory to treatment

(RSE). A quote of these last cases needed the admission to the intensive care unit (ICU) and the administration of third lines/anaesthetic drugs. Those cases persisting despite the application of third lines drugs for 24 hours or reappearing at the weaning of anaesthesia were considered Super-Refractory (SRSE). Treatment followed an internal protocol (publicly available at http://salute.regione.emilia-romagna.it/percorso-epilessia/PDTASE_AOU.pdf) based on the recommendations of international guidelines. (Glauser et al., 2016; Minicucci et al., 2020; Neurocritical Care Society Status Epilepticus Guideline Writing Committee et al., 2012).

Follow-up information regarding mortality and recurrence of seizures and SE episodes were acquired through hospital informatic database examination, clinical visits, as well as telephone interviews.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the local ethical committee (approval number 793/2022/SPER/AOUMO SIRER ID 5221 – SE-BIO) and was conducted according to the ethical principles for medical research involving human subjects in the Declaration of Helsinki.

Biomarkers measurements

Serum NfL concentrations were determined through a Single Plex Assay on the Ella Platform (Bio-Tchne), according to the manufacturer instructions and previous publication from our group (Giovannini et al., 2022).

S-100B serum levels were measured through a quantitative automated chemiluminescent immunoassay (CLIA), LIAISON, DiaSorin.

Outcome measures

We considered treatment refractoriness (as previously defined) and 30-days mortality as the two main outcome measures.

Statistical analysis

IBM SPSS Statistics Version 26 was used for statistical analysis. We described categorical variables as percentage and proportions, and continuous variables as mean and standard deviation (SD) or as median and interquartile range (IQR), depending on the underlying distribution. Univariate comparisons were performed with Fisher's Exact test, the chi-square test and the Mann–Whitney test. Multiple logistic regression analysis was used to determine predictors of 30-days mortality and refractoriness development. To find the best cut-off for these biomarkers for the analysed outcomes, ROC curves and Youden index were calculated. A survival analysis through the Kaplan-Meier analysis was performed and the Cox regression allowed us to find those variables independently associated to the 30-days mortality risk.

Logistic Regression and ROC analysis with Youden index calculation were used to create the ADiReN (Age Disability Refractoriness Neurofilament) score and to validate it in our population. The performance of the new score was then compared to that of the existing clinical scores (STESS, mSTESS and EMSE). For all the above-mentioned analysis, the statistical threshold was set at a p level < 0.05.

Data availability

Upon request from qualified investigators, we will share anonymized data.

Results

We measured the serum levels of NfL chains and S100B in an adult population of 87 patients (female 54 -62%- median age 70) with a clinical or electro-clinical diagnosis of SE. In all the patients the serum sample was acquired during SE and within 72 hours from SE diagnosis. The clinical characteristics of the studied population are reported in **Table 1**.

Table 1: Clinical characteristics of patients with SE (SE)

Variable		n	%
Total population		87	100
Gender (F)		54	62
Age	< 65 y	35	40
	≥ 65 y	52	60
	Median	70	
	IQR	25	
Etiology	Acute Symptomatic	47	55
	Remote Symptomatic	12	14
	Progressive Symptomatic	19	22
	In defined epileptic syndrome	3	3
	Multifactorial	3	3
	Unknown	3	3
Clinical presentation	w/o motor phenomena	44	51
	w motor phenomena	43	49
Duration of SE	Median	3 days	
	IQR	4	
Time from SE beginning to sample	Median	1 day	
	IQR	2	
STESS	0-2 pt	36	41
	3-6 pt	51	59
	Median	3	
	IQR	2	
EMSE	< 64 pt	58	67
	≥ 64 pt	29	33
	Median	55	
	IQR	48	
Pre-existing history of epilepsy		29	33
Treatment Response	Responsive	36	41
	RSE/SRSE	51	59
30 d mortality		14	16
30 d functional outcome	Recovery	30	34
	Clinical worsening	57	66

mRs pre SE	Median	1	
	IQR	3	
mRs at hospital discharge	Median	4	
	IQR	4	
mRs at 30 d	Median	4	
	IQR	4	
Epilepsy development after SE (n = 46)		14	30
SE recurrence (n = 46)		5	11

Treatment refractoriness

Fifty-one patients (59%) had SE episodes classified as refractory to first-line and one second-line treatment. Serum levels of NfL were significantly higher in refractory SE compared to responsive SE (median 113 pg/ml, IQR 204.10 vs median 32.50 pg/ml, IQR 69.33, p < 0.001). On the contrary, no significant differences were found in serum levels of S100B between refractory and responsive SE (p = 0.107) **(Figure 1)**.

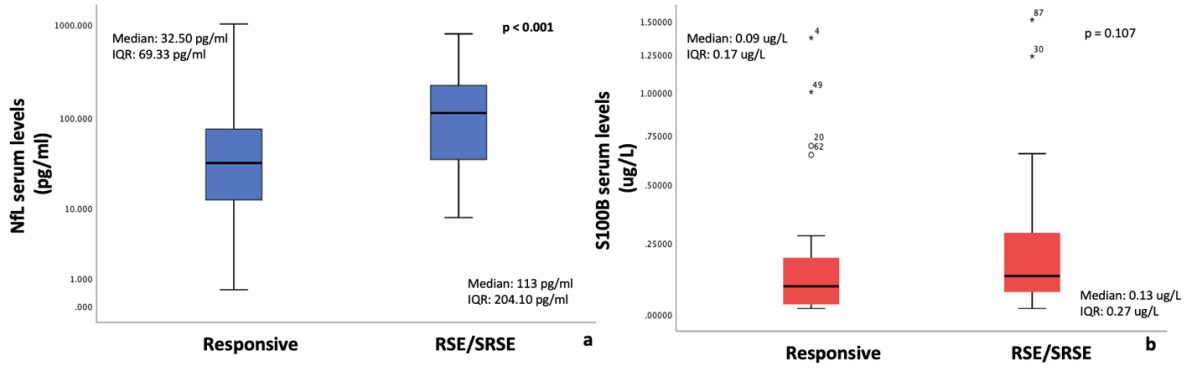


Figure 1. a) Comparison of serum levels of NfL between patients with RSE/SRSE and those with Responsive SE.

b) Comparison of serum levels of S100B between patients with RSE/SRSE and those with Responsive SE.

At the univariate analysis serum NfL levels were found to be a predictor of refractoriness development while S100B did not.

Through the ROC curve an accuracy of 73.8% (95% CI 63-85) for serum NfL levels in predicting refractoriness development was found. The Youden index calculation, showed a value of 19.75

pg/ml as the best cut-off NfL value for refractoriness development prediction (Sensitivity 94%, Specificity 39%). At the multivariate analysis, NfL with the considered cut-off, appeared as an independent predictor of refractoriness development (OR 8.2 95% CI 2.08-32.58 p = 0.003) **(Table 2).**

Table 2: Univariate and Multivariate logistic regression analysis for treatment refractoriness development.

Univariate logistic regression analysis			
	OR	**95% CI**	**p**
NfL (cut off 19.75 pg/ml)	**10.182**	**2.652-39.087**	**0.001**
S100B	1.398	0.707-2.762	0.335
Age	1.003	0.980-1.027	0.774
Gender	1.072	0.445-2.579	0.877
Aetiologies other than acute symptomatic	0.759	0.322-1.787	0.527
With motor manifestations	0.795	0.338-1.870	0.599
STESS	1.507	0.634-3.583	0.353
EMSE	**3.143**	**1.163-8.493**	**0.024**
mRS pre-SE	1.211	0.952-1.539	0.119
Presence of LPDs	1.207	0.481-3.034	0.688
Presence of PMA	**9**	**1.011-80.130**	**0.049**
Multiple regression analysis			
	OR	**95% CI**	**p**
NfL (≥ 19.75 pg/ml)	**8.230**	**2.079-32.583**	**0.003**
EMSE (cut off 64 pt)	2.059	0.717-5.914	0.180

Thirty-days mortality

In the analysed cohort, we observed a 30-days mortality of 17%. Serum levels of NfL were significantly higher in patients who died within 30 days from the beginning of SE compared to those who survived (median 117 pg/ml, IQR 356 vs median 46 pg/ml, IQR 133.25, p = 0.009). The same was observed for serum levels of S100B (median 0.20 μg/L for dead, IQR 0.27 vs median 0.10 μg/L for survivors, IQR 0.13, p = 0.007) (**Figure 2**).

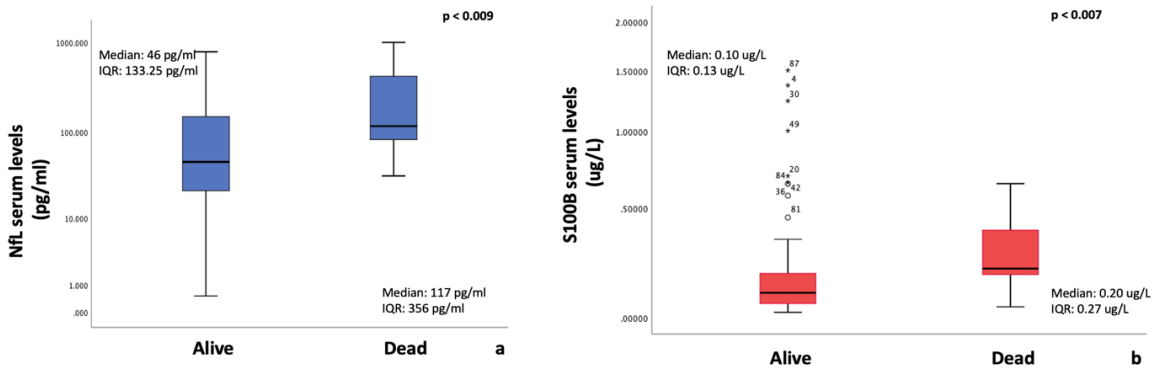


Figure 2. a) Comparison of serum levels of NfL between patients died at 30 days and those survived.

b) Comparison of serum levels of S100B between patients died at 30 days and those survived.

At the univariate analysis, only NfL serum levels were found to be a predictor of 30-days mortality whereas S100B levels were not.

Through the ROC curve an accuracy of 72% (95% CI 60-84) for NfL in predicting 30-days mortality was found. The Youden index calculation, showed a value of 70.25 pg/ml as the best cut-off in our population for 30 days mortality prediction (Sensitivity 86%, Specificity 59%). The multivariate analysis showed that NfL with the abovementioned cut off, was an independent predictor of 30-days mortality after correction for age (OR 5.71 95% CI 1.09-29.98 p = 0.04) **(Table 3).**

Table 3: Univariate and Multivariate logistic regression analysis for 30 days mortality.

Univariate logistic regression analysis			
	OR	**95% CI**	**p**
NfL (cut off 70.25 pg/ml)	**8.6**	**1.793-41.249**	**0.007**
S100B	0.955	0.637-1.432	0.823
Age	**1.106**	**1.040-1.177**	**0.001**
Gender	0.783	0.245-2.497	0.679
Aetiologies other than acute symptomatic	0.265	0.068-1.030	0.055
With motor manifestations	0.512	0.156-1.675	0.268
STESS	**5.231**	**1.093-25.042**	**0.038**
EMSE	**3.302**	**1.021-10.676**	**0.046**
Treatment refractoriness	1.951	0.560-6.799	0.294
Need for anaesthetic agents	**4.173**	**1.254-13.883**	**0.020**
mRS pre-SE	**1.617**	**1.180-2.214**	**0.003**
Presence of LPDs	0.889	0.248-3.183	0.856
Presence of PMA	3.222	0.183-56.883	0.424
Multiple logistic regression analysis			
	OR	**95% CI**	**p**
Age (cont)	**1.099**	**1.030-1.173**	**0.005**
NfL (≥ 70.25)	**5.714**	**1.089-29.980**	**0.039**

The survival analysis showed that values ≥ 70.25 pg/ml were associated to a significantly lower cumulative survival when compared to values < 70.25 pg/ml (log rank test: 0.002) and through the Cox regression analysis, the 30 days mortality rate appeared to be significantly higher in patients with NfL values ≥ 70.25 pg/ml than in patients with NfL values < 70.25 pg/ml when adjusted for age (HR 5.26, 95% CI 1.16-23.78, p = 0.031) **(Figure 3).**

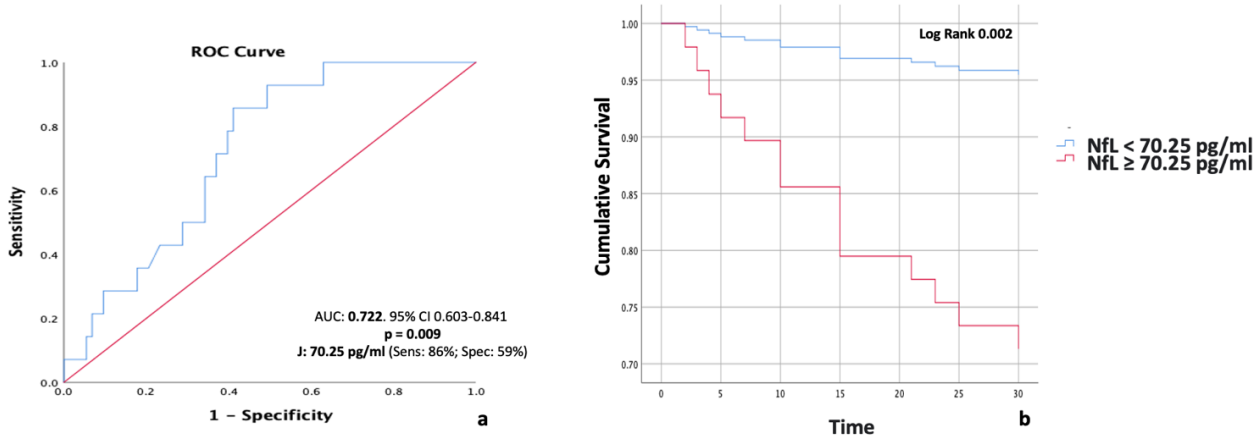


Figure 3. a) ROC curve showing the accuracy of NfL in predicting 30 days mortality.

b) Survival analysis showing that values of NfL ≥ 70.25 pg/ml were associated to a significantly lower cumulative survival when compared to values < 70.25 pg/ml (log rank test: 0.002); mortality rate appeared to be significantly higher in patients with NfL values ≥ 70.25 pg/ml than in patients with NfL values < 70.25 pg/ml when adjusted for age (HR 5.26, 95% CI 1.16-23.78, p = 0.031).

Thirty-days mortality prediction scores

STESS, mSTESS and EMSE scores performance in the studied population

We evaluated in our population, the performance of the three most important existing short term-mortality predictive scores: STESS, mSTESS, and EMSE.

The STESS, considered with the original cut-off of 3 points (STESS 0-2 and STESS 3-6), showed a sensitivity of 86%, a specificity of 47%, a positive predictive value of 24% and a negative predictive value of 94%. The overall accuracy of the STESS was of 53%. The AUC of the ROC curve showed a value of 63.4% (95% CI 49.4%-77.3%, p = 0.115).

The mSTESS, considered with the original cut-off of 4 points (mSTESS 0-4 and 5-8), showed a sensitivity of 64%, a specificity of 74%, a positive predictive value of 32% and a negative predictive value of 92%. The overall accuracy of the STESS was of 72%. The AUC of the ROC curve showed a value of 73.4% (95% CI 59.9%-86.9%, p = 0.006).

The EMSE, with the original cut off of 64 points (EMSE < 64 and ≥ 64), showed a sensitivity of 57%, a specificity of 71%, a positive predictive value of 28% and a negative predictive value of 90%. The overall accuracy of the EMSE was of 69%. The AUC of the ROC curve showed a value of 67.9% (95% CI 55.1%-80.6%, p = 0.035).

A multimodal new score for 30-days mortality prediction: The ADiReN score

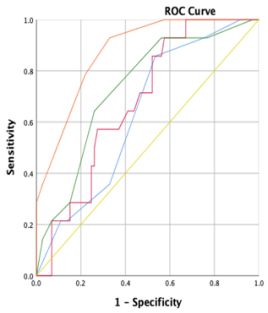
With a multimodal approach, combining clinical and laboratory variables, we created a new score to predict 30-days mortality after an episode of SE. We choose those clinical and biochemical parameters that, in our population, resulted as independent predictors of 30-days mortality in multivariate analysis: *i)* age, *ii)* pre-SE disability level measured by mean of mRS, *iii)* the need to recur to the anaesthetic drugs to control the SE, and *iv)* the serum levels of NfL during SE. For each parameter the best cut-off for 30-days mortality was calculated through the ROC curve and the Youden index statistics. These cut-off were as follows: NfL: 70.25 pg/ml, age: 77 years, mRS pre-SE: 2 points. Through a multiple logistic regression model comprehensive of all the considered variables, we attributed values to each of them based on each β score value.

The new score, ADiReN score (Age, Disability, Refractoriness, Neurofilament) varies between 0 and 6 points. Thereafter, we tested the performance of the new score in our population. We first calculated the score for each patient, then through the ROC analysis an AUC of 87.5% (95% CI 79%-95.9%) was found. The Youden index calculation showed 3 points as the best cut-off (0-2 and 3-6). With this cut-off we found a sensitivity of 79%, a specificity of 78%, a positive predictive value of 41% and a negative predictive value of 95%. The overall accuracy of the ADiReN was of 78%.

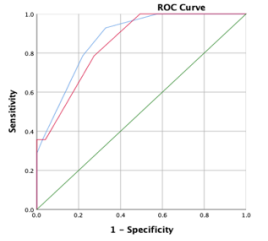
Finally, to assess the contribution given by NfL to the score performance, using the same methodology, we calculated the score without NfL (so called ADiRe). The best cut-off for this score in the examined population was of 2 points. The AUC reduced to 85% (95% CI 75.7%-94.4%).

Compared to the previous one there was an increase of sensitivity and negative predictive value to 100% but an important decrease in specificity (51%) and positive predictive value (28%). The overall accuracy lowered too (59%). The ADiReN score and the comparison of the ROC curves of all the prognostic scores is reported in **Figure 4**.

ADiReN score		
		Score
Age (years)	< 77	0
	≥ 77	2
mRS pre-SE	< 2	0
	≥ 2	1
Need of third line therapies	no	0
	yes	2
sNfL during SE	< 70.25 pg/ml	0
	≥ 70.25 pg/ml	1
Total		0-6



Area Under the Curve					
Test Result Variable(s)	Area	Std. Error	p	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
STESS	0,634	0,071	0,115	0,494	0,773
mSTESS	0,734	0,069	0,006	0,599	0,869
EMSE	0,679	0,065	0,035	0,551	0,806
ADiReN	**0,875**	**0,043**	**0,000**	**0,790**	**0,959**



Area Under the Curve					
Test Result Variable(s)	Area	Std. Error	p	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
ADiReN	**0,875**	**0,043**	**0,000**	**0,790**	**0,959**
ADiRe	0,850	0,048	0,000	0,757	0,944

Figure 4. The new ADiReN score (Age, Disability, Refractoriness, Neurofilaments) and the comparison of the ROC curve for 30 days mortality of the ADiReN/ADiRe, STESS, mSTESS and EMSE.

Discussion

In this study, we evaluated two known biomarkers of neuro-glial degeneration in the prediction of short-term outcome of SE in terms of refractoriness to treatment and 30 days mortality.

Acute (< 72 hours) serum levels of NfL were confirmed to be an independent predictor of refractoriness development as we have already reported in a previous pilot study (Giovannini et al., 2022), even though we found a different cut-off that is attributable to the different characteristics of the two studied population (in the pilot study we excluded all the acute symptomatic aetiologies). There are no other previous studies trying to define the role of these biomarkers in refractoriness development prediction.

In this larger and aetiologically variable cohort, we confirmed the predictive power of acute serum levels of NfL on short-term mortality prediction. Short-term mortality prediction in SE is an extremely important issue and its clinical predictors have been already extensively studied and established in literature.

In this study, we tried to explore a new and multimodal approach to mortality prognostication in SE: we created a simple clinical tool for predicting individual outcome based on parameters that could be available soon before obtaining the results of diagnostic tests. For this reason, even if aetiology is a well-known factor influencing SE prognosis, it was not chosen as a parameter since in many cases could be available neither upon admission and nor in the subsequent days (i.e. SE related to autoimmune etiology, or in cryptogenic cases).

Thus, the parameters that compose the proposed score consist of four variables that are available at patient's presentation or can be available early during patient's management: age and mRS (that reflects the presence of comorbidities and is widely used in other neurological emergencies, such as acute stroke) are directly available upon the patient' s presentation; refractoriness can be determined early after response to first- second-line treatment. Regarding serum NfL measurement, this biomarker is still assessed only for research purposes. Nevertheless, since it represents a well-established biomarker of neuronal/axonal damage predictive of evolution/prognosis in many different neurological conditions, in the near future it could enter in everyday clinical practice. Thus in near future the results of serum neurofilament could be rapidly available after some hours from the sampling.

From our results, the ADiReN score seems to have an high prognostic accuracy and in particular to especially increase the PPV, thus the ability of the score to identify correctly those who are at high risk of short-term mortality. Moreover, the improvement of this weak point for all the existing

scores seems to derive from adding to clinical variables the value of the NfL thus from the multimodal evaluation.

Recently, the group of Hanin et al tried to combine clinical-biological variables to predict short-term and long-term outcome after SE finding that the produced models have a stronger discrimination power compared to STESS and mSTESS (Hanin, Demeret, et al., 2022).

Study limitations

The first limitation of our study is that it is a monocentric study. Even though our center is the reference center for neurological emergencies and at the same time, it has the reference center for epilepsy for our district thus making possible to have a high degree of variability in aetiologies, we believe it will be important to try to replicate our results in different centers. Moreover, it will be important to design prospective studies to let the biomarkers' measurement to be performed at different timepoints in the same patient thus creating a curve. Finally, it is necessary to apply the new score in different settings and in different populations.

Conclusion

This study attempt to introduce a biological biomarker into a clinical prognostic score in SE. For other conditions serum biomarkers has already been well established in clinical practice (e.g post-anoxic encephalopathy prognostication). Of course, our study needs external validations in other different population and in bigger multicentre studies but has to be pursued as a roadmap for the future.

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10. Summary, Conclusion and Future Perspectives

Status epilepticus is an heterogeneous condition whose severity and consequences need an urgent improvement in our knowledge in the following areas, which are the subject of this doctoral dissertation:

1) In the diagnostic field, to ensure early identification and treatment of patients. Although EEG remains the gold-standard, this tool has limitations, and a multimodal approach including the use of neuroimaging methods we believe will have increasing relevance in the near future. In this thesis we evaluated the diagnostic power of CT perfusion in an emergency setting (emergency department) for out-of-hospital onset SE demonstrating how it is an accurate tool in detecting SE-related hyperperfusion and therefore of clinical utility.

2) In the prognostication, identifying biomarkers of neuronal/glial damage that can predict the severity of SE. This goal was pursued in the studies reported in Chapters 7 to 9 of this thesis. The data presented indicate that light neurofilaments, that can be obtained from a blood sample as early as the first few hours after SE onset, are a promising and reliable biomarker. Serum NfL levels also predict both response to treatment and short-term mortality, as well as post-SE disability. Therefore, we believe that if the results obtained in our cohort will be replicated and confirmed on external populations, they can certainly become a biomaker of SE severity in clinical practice.

Unlike the NfLs, the results obtained on the S100B protein were not able to stratify SE severity. Indeed, although S100B levels are increased in patients with SE compared with control populations, they cannot predict SE severity and short-term clinical outcomes at least in the population investigated.

In the near future, it is desirable to further develop the integration of imaging methods and fluid biomarkers of SE severity and prognosis. This multimodal approach could optimize the acute treatment of this condition, and taylor it addressing neuroprotection and/or counteracting the development of epileptogenesis.

In particular, two aspects, which are not covered in this thesis but are closely related to, concern the development of chronic epilepsy and brain atrophy after a first episode of SE. In this regard, the multimodal approach (clinical-EEG-imaging-biomarkers) we believe is the most appropriate to investigate these two serious consequences of SE. To date, in fact, we do not know precisely which patients will develop chronic epilepsy, which atrophy (whether static or progressive), which functional alterations of brain circuits after an episode of SE.

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