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CICLO XXIX

THE ROLE OF CEREBROSPINAL FLUID AND BRAIN IMAGING BIOMARKERS IN STATUS EPILEPTICUS

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At first, there's just darkness and silence.

"Are my eyes open? Hello?"

I can't tell if I'm moving my mouth or if there's even anyone to ask. It's too dark to see. I blink once, twice, three times. There is a dull foreboding in the pit of my stomach. That, I recognize. My thoughts translate only slowly into language, as if emerging from a pot of molasses. Word by word the questions come: Where am I? Why does my scalp itch? Where is everyone? Then the world around me comes gradually into view, beginning as a pinhole, its diameter steadily expanding. Objects emerge from the murk and sharpen into focus.

I know immediately that I need to get out of here.

FROM THE PREFACE OF "BRAIN ON FIRE" [1]

ABSTRACT

My field of research concern epilepsy and in particular patients with status epilepticus (SE), a neurological emergency and a possible life-threating condition due to recurrence of seizures. In these three years of PhD route, I focus my studies on possible predictors of neurological and epileptic outcomes in patients with SE, to improve knowledge and management. We started with prospective collection of all cases of SE in our district (follow literature criteria of definition and treatment). Then we select those patients that undergone to simultaneous serum and CSF detection, and neuroradiological brain investigation.

We divided our research in three categories:

1) Analysis of possible CSF biomarkers of neuronal damage in patients with SE and possible correlation with timing from SE's onset and SE evolution (refractory or responsive SE).
2) Analysis of serum and CSF endogenous molecules (neurosteroids) with a direct influence in neuronal activity. In animals, Allopregnanolone seems to have an anticonvulsant effect, due to GABAergic activity close to benzodiazepines' one; on the contrary, Pregnanolone sulfate seems to have a proconvulsant activity. Evaluation of these molecules in healthy subject and in patients with SE could expand knowledge of neurosteroids panel and address new therapeutic approaches for SE, especially for resistant/refractory cases.
3) Identification of possible neuro-radiological markers of refractoriness, through serial brain MRI in cases of new onset refractory SE with early aggressive course and potential immuno-mediated aetiology (NORSE). Bilateral claustrum hyperintensity seems to represent a transient radiological markers in these cases in which unfortunately autoantibodies are not yet discovered.

SOMMARIO

Il mio ambito di ricerca ha da sempre riguardato l'epilessia e in particolare i pazienti con stato epilettico (SE), un'emergenza neurologica con possibili ripercussioni cliniche a distanza, a causa del ripetersi di crisi epilettiche, potenzialmente dannose. Durante questi tre anni di dottorato, la mia ricerca è stata incentrata sull'individuazione di possibili biomarker, in grado di predire l'outcome neurologico ed epilettologico nei pazienti con SE, al fine di migliorare la conoscenza e la gestione di questa patologia. Abbiamo iniziato con uno studio prospettico di raccolta di tutti i casi di SE afferenti al nostro distretto (seguendo i criteri di definizione e trattamento della letteratura). In seguito, abbiamo selezionato i pazienti sottoposti a simultaneo prelievo di siero e liquor, oltre alle indagini neuroradiologici cerebrali.

Abbiamo suddiviso la nostra ricerca in tre ambiti:

1) Analisi di possibili biomarcatori liquorali di danno neuronale in pazienti con SE e possibile correlazione con il timing rispetto all'esordio dello SE e alla sua evoluzione (SE refrattario o responsivo).

2) Analisi su siero e liquor di molecole endogene (neurosteroidi), con una documentata influenza diretta sull'attività neuronale. Negli animali, infatti, l'allopregnanolone sembra avere un effetto anticonvulsivante, mediante un'azione GABAergica, simile alle benzodiazepine; al contrario, il pregnenolone solfato sembra avere un'attività proconvulsivante. La valutazione di queste molecole in soggetti sani e in pazienti con SE potrebbe espandere la conoscenza sui diversi neurosteroidi e rendere disponibili nuovi approcci terapeutici per lo SE, soprattutto per i casi resistenti / refrattari.

3) Individuazione di possibili marcatori neuro-radiologici di refrattarietà, attraverso lo studio di risonanza magnetica cerebrale nei casi di SE refrattario di nuova insorgenza con decorso aggressivo a possibile eziologia immuno-mediata (NORSE). Il riscontro di transitoria iperdensità bilaterale del claustrum sembra rappresentare un marcatore radiologico in questi casi, in cui purtroppo un autoanticorpo non è ancora stato scoperto.

ABBREVIATIONS

Aβ 1-42	Beta-amyloid 1-42
AED	Anti-epileptic drug
AP	Allopregnanolone
CSF	Cerebrospinal fluid
CNS	Central nervous system
DBS	Deep brain stimulation
ECT	Electroconvulsive therapy
EEG	Electroencephalography
EMSE	Epidemiology-Based Mortality Score in Status Epilepticus
FIRES	Febrile infection related epileptic syndrome
GOS	Glasgow Outcome Sale
ICU	Intensive unit care
IGIV	Immuno-globuline
mRS	modified Rankin scale
MRI	Magnetic resonance imaging
NORSE	New onset refractory status epilepticus
NSE	neuron-specific enolase
PEX	Plasma-exchange
PS	Pregnenolone sulfate
p-TAU	phosphorylated-TAU
RSE	Refractory status epilepticus
SE	Status epilepticus
SRSE	Super-refractory status epilepticus
STESS	Status Epilepticus Severity Score
TMS	Transcranial magnetic stimulation
t-TAU	Total-TAU
VNS	Vagal nerve stimulation (VNS)

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CHAPTER 1. INTRODUCTION

1. STATUS EPILEPTICUS

1.1 Definition

The Status Epilepticus (SE) is a neurological emergency and a possible life-threating condition due to recurrence of seizures. Its definition changed many times through the years. 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"[2]. 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"[3].

In the Glossary of Descriptive terms (2001) the SE was defined 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" [4].

Since the very first definition, two different conditions appeared as the core of the SE: the first one is a unique prolonged seizure and the second one is the frequently repetition of single seizures; both without spontaneous resolution that imply the need for a pharmacological intervention. These first definitions do not precisely define the length of time that has to pass since the beginning of this condition to classify it as SE. Given the lack of a unique and precise definition of SE, different operational definitions have been used in different textbooks, papers and clinical studies. In the majority of them a SE is defined as a seizure that lasts for at least 30 minutes.[5] 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.[6] in 1999 defined GCSE as "...≥5 minutes of continuous seizure or two or more discrete seizures between which there is incomplete recovery of consciousness".

Recently (2015), the ILAE Task Force on Classification of Status Epilepticus[7] 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 either the time after which a seizure become a SE (t1) and so when a treatment must be started; or the time after which the prolonged seizure activity start to cause neuronal damage (t2) defining how aggressively a treatment should be. These time points are clearly defined for GCSE while for the other forms of SE there is lack of evidences and they are only indicative.

1.2 Pathophysiology and basis for treatment refractoriness

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". In the very first milliseconds to seconds, protein phosphorylation appears. This induces ionic channels to open or close, neurotransmitters and modulators release, 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 $GABA_A$ receptors that are more and more internalized, while, at the same time, AMPA-R and NMDA-R move to the synaptic membranes. 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 anti-NMDA-R antagonist 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[8].

In the minutes to hours' time range, plastic 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, there are long-term changes in gene expression. Many changes in gene expression are the result of seizure-induced neuronal death, and of the resulting neuronal re-organization[9]. All these changes can produce long term brain damage among which the most commonly recognize alteration acquired after SE is mesial temporal sclerosis. 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[10].

Besides these mechanisms that determine and sustain the SE, GCSE itself develop systemic effects that need immediate recognition and treatment in the general management of SE. 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. Given that, 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 part in the cell damage associated with GCSE. Respiratory and metabolic acidosis, electrolyte imbalance (for example, hyperkaliemia), hyperthermia, and rhabdomyolysis may all occur. All these systemic alterations can potentially worsen the brain damage.

In this view, although the outcome of SE is mainly due to the aetiology 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.

2.3 Classification

The classification of SE has changed many times too. In the first classifications[2,3] the only distinction was between partial and generalized SE.

In 2001 ILAE classification[4] more details were added reproducing the seizures classification. So Generalized Status Epilepticus (GSE) was divided in Generalized tonic-clonic SE (GCSE), Clonic SE, Absence SE, Tonic SE, Myoclonic SE (MSE); while Focal Status Epilepticus (FSE) recognized Epilepsia partialis continua, Aura continua, Limbic SE and Hemiconvulsive SE with hemiparesis.

The 2006 ILAE classification[11] outlined nine different possible types of SE: Epilepsia partialis continua (EPC) 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 last classification of SE[7] introduces four axis of classification according to which every episode of SE is defined: 1) Semiology; 2) Etiology; 3) EEG correlates; 4) Age.

1) 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).

2) 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 syndrome when an episodes of SE appeared it is always triggered by something, so that it could be classified as Acute Symptomatic.

3) 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), absolute and 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.

4) 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)

1.4 Epidemiology

Incidence of SE

The annual incidence of SE varies significantly across studies, e.g. from 9.9 to 41 per 100.000/year. It has been found higher in the United States studies, e.g. 21 per 100.000/year in the Rochester population[12], 41 per 100.000/year (20 per 100.000/year in whites) in Richmond population[13], than in the central Europe studies e.g. 11.6 per 100.000/year in Lugo area[14], 10.7 per 100,000/year in Bologna area[15], 17.1 per 100.000/year in Marborg in the German study[16], 15.5 per 100.000/year in Geneva population[17] and 9.9 per 100.000/year in the Swiss study of Coeyteaux et al.[18]. The only exception was the Ferrara study in which an incidence of 27.2 per 100.000/year has

been reported[19]. In Modena population, if we excluded "area nord", the incidence is about 15-16 cases per 100.000/year (personal data).

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 appears particularly difficult.

According to the age of onset, the incidence of SE shows a bi-modal, U-shaped age distribution. The highest incidence of SE occurred in the first year of life. The second highest incidence of SE occurred in the elderly population, representing the largest group of patients at risk for developing SE[13,14,17,18,19,20]. The recurrence rate of SE showed a similar behaviour. The incidence of SE was found to be grater (till two-times higher) in males than in females at all ages[14,17,18,19]. This is probably in part related to a different males/females distribution of the underlying aetiologies but in part it is possibly related to a different gender seizures threshold under the influence of the steroid hormones. An exception to these findings is represented by the higher incidence in women compared to men previously found in the Bologna's, Lugo's and Modena's study[14,15,22]. The aetiologies of SE differs among adults and children too: among children fever and low levels of AED are the most frequently found[13] while among adults, acute symptomatic aetiology is the most frequently encountered, among which cerebrovascular diseases are the most frequently reported etiology[13,14,15,19]. Overall, a positive history of epilepsy before SE development is found in nearly a half of patients[14,15].

Mortality

The mortality, measured in the majority of the studies as 30-days case fatality and in some other at hospital discharge, is overall high after an episode of SE[21].

It varies from 5% to 46%[13,14,15,16,17,18,19,22,23] of SE episodes in the different studies. This variance is partly related to different inclusion criteria (e.g. inclusion or exclusion of post- anoxic SE condition related to a known high mortality, inclusion or exclusion of paediatric population, age group with a lower mortality) and partly related to different aetiologies composition of the studied population, 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 AEDs and withdrawal of alcohol[13,15,19,21] and in patients with remote symptomatic or idiopathic aetiologies.

Probably related to the different distribution of aetiologies, mortality appears also to be related to age: it increases with increasing age, the lowest in children (3% in Richmond study) and the highest in the elderly (38% in Richmond study)[13, 23].

Moreover, mortality appears to be higher in white population when compared to non-white population[13], this finding could be explained by different aetiologies distribution among different races. Mortality appears to increase increasing the length of SE too[19].

In the study of Logroscino et al.[12] incidence and mortality rates of SE have been found to have increased in the period 1935-1984 with a stable case fatality. The increased incidence and mortality are attributed by the authors to the increased survival over time of patients after an episode of cardiac arrest and to the improved recognition of myoclonic SE after cardiac arrest. Excluding that group, the authors found that the overall case-fatality decreased over time[12]. In a recent study by Neligan et al.[24] on the SE of England and Wales from 2001 to 2013 a decrease in the SE mortality rate was found besides a possible increase in the number of SE due to the introduction of the 5-minutes length of time new definition of SE[6] . The authors suggest, as a possible explanation, that a policy of early and aggressive treatment could have improved the SE prognosis.

Refractoriness to treatment.

Among SE, the Refractory Status Epilepticus (RSE) is a particular critical conditions characterized by seizures that continues despite the use of a first and a second line therapies. 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 antiepileptic drugs (2 or 3 drugs) and on the need of an anesthetic therapy. Refractory SE develops in 23% to 43% of patients with SE, with a mortality rate of 17% to 65%[22,25,26,27,28,29,30] . The most frequent aetiologies in RSE are brain tumours and post-anoxic encephalopathy[30].

1.5 Treatment

SE could be classified upon the response to treatment. SE is generally divided in four stages/phases even if it is well known that stages can merge one into the other.
The most used SE therapy approach is based on the four steps way of treatment:

1) 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[31,32,33]. 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 (iv administration is considered off label for SE treatment in Italy) and Clonazepam (iv 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[35]) whenever an IV line is not available. A recent Cochrane review[34] on anticonvulsant therapy for SE, found that in a pre-hospital setting, when an IV line is not possible to find, 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[35].

2) 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 AED is established. The most important international guidelines[31,32,33] suggest the use, as a first line therapy for this stage, of IV Phenytoin/Fosphenytoin. The efficacy of Phenytoin 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, and 'purple glove syndrome' and tissue necrosis)[35].

Thus, whenever there is any contraindication to use Phenytoin, IV Valproic Acid or Phenobarbital should be chosen. Valproic Acid 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[36]. The major concern is about the possibility of developing hyper-ammonaemia and the related hepatic encephalopathy[37,38]. A recent review[36] shows that Valproic Acid is as effective as phenytoin in the treatment of established SE with a lower profile of adverse events than phenytoin. Given the presence of few, small RCTs, international guidelines consider Valproate as an alternative first line therapy to Phenytoin in the treatment of established SE.

IV Phenobarbital is found to be at least as effective as a combination of Diazepam and Phenytoin[39] and it was showed not inferior to Lorazepam in the initial treatment of SE[40]. Phenobarbital carries a high risk of CNS depression (sedation, hypotension and respiratory failure) especially if administered after benzodiazepines used and for this reason its used is nowadays mostly avoided.

There are emerging evidences in favour of the use of new antiepileptic drugs (e.g. Levetiracetam, Lacosamide) in SE treatment even though randomized, controlled trials are not still available in the literature and so they have not been introduced in the international guidelines yet.

Levetiracetam acts with a not completely understood antiepileptic mechanism, based mostly on SV2A proteins and, to a less extent, on Ca2+ calcium channels and 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. At present, since the majority of studies on its use in stage II SE are small, retrospective or prospective and not randomized, Levetiracetam has now a level of evidence IIA and its use is recommended in GCSE after BDZs failures if there are contraindications to Phenytoin or Valproic Acid or as the first choice, in FMSE or NCSE35[41,42].

At last, it is still ongoing a multicenter prospective randomized double blind study (ESETT) comparing the administration of Levetiracetam, Phenytoin and Valproic Acid in SE stage II[43,44].

Lacosamide acts as enhancer of slow inactivation of Na+ channels and reduces the activity of CRMP2. It has few drug to drug interactions, hepatic metabolism and it carries few side effects (the most worrying of which is III degree AV conduction block, always reported in patients with known cardiovascular risk factors[45,46]). 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 or valproate or when these drugs are contraindicated[35].

3) Refractory Status Epilepticus (RSE) or Stage III

If seizures continue despite the treatment with at least two trials of intravenous antiepileptic drugs the SE is defined as refractory and the patient is admitted to the Intensive Care Unit (ICU) and anesthetic therapy is needed. It has been suggested to titrate the anesthetic therapy to a dose sufficient to maintain a burst-suppression (BS) EEG pattern (level at which all electrographic seizures are usually controlled). The level of anesthesia has to be monitored using either repetitive EEG, at least once every 24 hours, or, if there is the possibility, starting an EEG monitoring (c-EEG).

There are no randomized control trials for this stage and guidelines are based upon retrospective case series. The anesthetics most 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[47,48]. Moreover, there are Inhalation halogenated anesthetics (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 RSE[35]. 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[49] .

During this phase it is also recommended to continue AEDs 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 be sometimes, per se, the SE treatment).

4) Super-Refractory Status Epilepticus (SRSE)

Super-refractory status epilepticus can be defined as SE that has continued or recurred despite 24 hours of general anesthesia[49]. It generally develops in patients with severe acute brain injuries or as a de novo condition in patients with no history of previous epilepsy in the context of a suspicious autoimmune encephalopathy (NORSE/FIRES). This stage represents a "terra incognita" in which any clinical evidence is completely lacking and treatment is totally based on results from small case series in which patients at this stage, are frequently treated with multiple drugs and it is extremely difficult to find out evidence of efficacy for each of them.

The backbone of the therapy in this stage is still anesthetic 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 anesthetic therapy has to be reestablished and consequently institution/withdrawal patterns should be every 24-48 hours initially and then at 5-7 days-cycles[49]. Besides anesthesia, antiepileptic drugs should always be administered throughout the course of anesthesia, so that when the anesthetic agent is withdrawn there should be an adequate antiepileptic coverage.

Even if there are few evidences an incredible number of different therapies could be tried at this stage: Ketamine, Lidocaine, Magnesium sulphate, Pyridoxine, Steroids and Immunotherapy, Ketogenic diet, Hypothermia and in extreme cases respective neurosurgery. Moreover, as a "last resort" therapy in long lasting super-RSE, there are therapies for which there are no or scarce evidences. Among them we recognize:

Transcranial magnetic stimulation (TMS), Vagal nerve stimulation (VNS), Deep brain stimulation (DBS), Electroconvulsive therapy (ECT), CSF drainage and even classical music [35,48,49,50].

1.6 Prognosis

Assess the patient's prognosis in SE episodes, as early as possible, appears to be of paramount importance in SE management either to avoid over-treatment and its harmful possible consequences in patients with probable good outcome (this is especially true for NCSE without coma in which the further neurologic consequences of risk related to SE is not completely assessed while they could be at high risk of worsening due to therapeutic coma and its related complications[51]) or to avoid under-detection and under-treatment in patients with a possible bad outcome.

Moreover, among predictors of bad outcomes, the identification of those modifiable could lead to a possible outcome improvement.

Besides these considerations, predicting refractoriness of SE should be important for the correct management of SE itself (e.g. using c-EEG monitoring, referral to an ICU).

1.6.1 Predictors of mortality

Different studies have analyzed the issue of mortality predictors in SE:

• Etiology. The majority of studies find that the etiology of SE is the most important predictor of SE related mortality[52]. In particular, acute symptomatic etiologies[23] and, among them, the so called "potentially fatal etiologies" (that means the presence of etiologies that are judge to be related to death independently from the presence of SE if left untreated) [27,51,53,54] are particularly predictive of mortality after SE. Thus, anoxic encephalopathy[55] and acute CNS infections[56] are conditions at high mortality risk.

Even if etiology has been found to determine the mortality risk, it is not the only factor as it is suggested by the increased mortality risk in patients with cerebrovascular disease with SE compared to patients with cerebrovascular disease without SE (32% vs 12%[13]) suggesting an interaction between injured brain and epileptic activity. Moreover, Rossetti et al.[57] found that the development of SE in post-anoxic patients increased the mortality risk when compared to post-anoxic patients without SE.

• Duration of SE episode. In literature there are some contrasting evidences; some authors found that, even if with different cut-off time, the longest is the duration of SE the highest is the mortality[56,58,59,60]. Nevertheless, given that some patients with very prolonged SE episodes survive, prolonged SE does not have to be considered hopeless on the sole basis of the duration[61]. However, in some other studies, duration of SE was not found to be a risk factor for 30-days mortality[23,29,62,63].

• Type of SE. In the Rochester study[23] the type of SE was not a risk factor for increased short-term mortality. This result was confirmed later on by Hui et al.[64].

• Gender. Logroscino et al.[23] reported a lower risk of death in women than it was in men.

• Time to treatment. Literature data are somehow contrasting: in different studies[56,64] a delay in treatment administration (> 30 min or > 1 hour form SE onset) has been found to be associated to a poor outcome; while in some other studies[54,62] a treatment latency of > 1 hour or 120 minutes was not found to be associated to a poorer outcome.

• Intra/extra-hospital onset. In some previous studies[62,63] the intra-hospital onset was found to be predictive of mortality and this finding is supposed to be an expression of a preponderance of acute etiologies among intra-hospital onset SE episodes.

• Adherence to guidelines treatment. Contrasting data in literature: Vignatelli et al.[62] reported that treatment non adherence to guidelines was independently related to a worse outcome while in the study of Rossetti et al.[54] this was not predictive for increased mortality (either over-dosed, under-dosed or wrong drugs sequence).

• Age. Increased age (with different cut-off) is found to be related to increased mortality[23,51,53,54,56,58,65,66].

• Level of consciousness at first clinical evaluation. Coma on the first clinical presentation reflects the severity of the SE and the underlying etiology. A deeply reduced level of consciousness (stupor/coma) at first medical presentation before any treatment, appears to be related to increased mortality[61,66].

• Comorbidity. Contrasting data: Alvarez et al.[67] and Rossetti et al.[54] found that the presence of comorbities was not independently related to outcome, while Marchi et al.[51] found an increased mortality increasing the CCI (Charlson Comorbidity Index) score.

• Hospitalization complications. Hospitalization complications have been found to be independently associated to mortality after SE[68]. Among hospital related complications, the development of infections during SE (and in particular respiratory tracts infections) is related to and increase the risk of mortality[69,73]; but the authors suggest that it is not possible to drawn causality a cause of the presence of possible unmeasured confounders.

• Response to treatment. It seems to be related to a decrease in the mortality probability[55].

• Need of ventilation and therapeutic coma. The need of intubation was significantly related to poor outcome[56]. Marchi et al.[51] found that therapeutic coma (without

differences among the anesthetics chosen) is independently related to increased risk for mortality.

• EEG feature. In the study of Jaitly et al.[70], the presence of burst suppression (BS), after status epilepticus ictal discharges (ASIDs) or periodic epileptiform discharges on the EEG (PEDs) were found to be independently predictive of bad outcome. On the other side, Garzon et al.[71] found that the outcome was not related to any specific patterns independently from etiology and age. Overall the clinical significance of PLEDs (PEDs) or persistent evidence of electrographic seizures post SE remains controversial. However, their prognostic predictive value may be related more to the underlying etiology rather than to any independent influence[52].

1.6.2 Predictors of morbidity

The functional outcome after an episode of SE has been addressed many times. In literature the level of disability after SE has been measured in different ways (e.g. Glasgow Outcome Sale, GOS or mRs) comparing the score after SE (at discharge, at 30-days, at 3 months etc.) to the score before SE.

The usual predictors are the following:

• Etiology. The most relevant independent predictor of a functional decline is the acute symptomatic etiology[53,64,65,67] and in particular the potentially fatal etiologies[51,65,67,87]. Among etiologies, SE developed in the context of an epilepsy history was found to be protective against clinical worsening after the SE episode[29], while SE in the context of lack of previous seizures was independently related to a worse prognosis[51]. SE without an evident brain lesion is at low risk of functional deterioration[72].

• Type of SE. Generalized form of SE (generalized discharges on EEG) appears to be predictive of a bad outcome[29]. Other two subsequent studies[64,65] do not find any association of type of SE with morbidity.

• Age. Advanced age at presentation[51,65] is a risk factor for increased morbidity.

• Comorbidity. Comorbidities do not seem to be related to increased morbidity in a study conducted by Alvarez et al.[67].

• Intra/extra-hospital SE development. SE present on admission (that means that develops out of hospital) is found to be related to a better functional outcome when compared with SE that develops during hospitalization[72]. The authors attribute that to the in-hospital development of SE in the context of more pronounced systemic complications and to a treatment delay.

• Level of consciousness at first clinical evaluation. Coma on presentation is considered to be a risk factor for increased morbidity[29,66]. Overall, a low level of consciousness at the SE onset has been clearly and consistently associated with a poor prognosis, although this probably reflects the underlying etiology, in particular the anoxic damage[52].

• Hospitalization complications. In a study of Sutter et al.[69], the development of infections during SE relates to and increases the risk of worsening of the clinical conditions after the SE. In a recent study, Semmlack et al.[73] found a strong association between infections development and bad outcome in SE but the authors suggest that, even after multivariate analysis, it is not possible to drawn causality a cause of the presence of possible unmeasured confounders. In RSE, the development of sepsis was found to be independently related to decline of functional outcome[74].

Moreover, an increased length of hospitalization[53] is considered to be a risk factor for a bad outcome.

• Duration of SE episode. A study conducted by Drislane et al.[29] addresses the role of the duration of SE in predicting bad outcome. The authors conclude that duration of SE shorter than 10 hours was associated with a better outcome, but this was not significant when combining duration, etiology, presentation in coma and type of SE in a multivariate analysis.

In another study analyzing RSE only, longer duration of SE episode (more than 10 days) was independently associated with poor functional outcome[74].

• Adherence to guidelines treatment. In a study comparing two centers (one large academic center where adherence to treatment guidelines was higher and a small peripheral center with a lower adherence to treatment guidelines) with a non-different SE severity between them, Rossetti et al.[75] found a non-statistically different outcome. In a subsequent study[54] they confirmed the previous results (either overdosed, under-dosed or wrong drugs sequence). However, these results are influenced by different definitions of treatment appropriateness and different treatment protocols.

• Therapeutic coma. Marchi et al.[51] found that therapeutic coma (without differences among the anesthetics chosen) is independently related to increased risk for worsening of clinical conditions after SE.

1.6.3 Predictors of refractoriness

• Gender. Some studies have reported a female predominance among RSE[76,77]. In particular, in a Turkish population of patients with RSE76 the female gender was found to be an independent predictor for refractoriness. Authors gave a possible explanation of that, based on a probable hormonal differences in female/male seizures threshold even if a hospital referral bias could not be completely ruled out in that study.

• Age. Sutter et al.[78] found that younger age was associated with RSE development in the univariate analysis but lost the association in the multivariate analysis. On the contrary, Agan et al.[76] found that patients with RSE were significantly older than patients with responsive SE. Moreover, some others authors[26,28] found that there is no clear correlation between age and refractoriness development.

• Type of SE. Sutter et al.[78] found that NCSE in coma was associated with RSE development in the univariate analysis but lost the association in the multivariate analysis. In the study of Mayer et al.[25] the development of NCSE or FMSE was found to be related to the development of refractoriness.

• Etiology. The presence of acute symptomatic etiologies is found to be an independent predictor of refractoriness development[76,78] and among them, the presence of acute symptomatic encephalitis were found to be particularly predictive of refractoriness development[26,79]. SE that develops as a de novo episode in patients without previous history of epilepsy is at high risk of refractoriness[28]. Cryptogenic etiology is also found to be predictive of refractoriness development in a recent study[80] while a SE due to low levels of AEDs in epileptic patients is frequently not-refractory26.

• Level of consciousness at first clinical evaluation. Coma/stupor at the beginning of SE is found to be predictive of refractoriness[28,66,78.]

• Hospitalization complications. Sutter et al. [69] and Semmlack et al. [73], found that patients that have infections during the SE episode have a higher risk of developing RSE compared to patients without infections, and the probability increases with the numbers of infections. Nevertheless, the authors suggest that it is not possible to drawn causality a cause of the presence of possible unmeasured confounders.

• Laboratory findings. Sutter et al.[78] found that decreased serum albumin levels (< 35 g/l) at the SE onset is an independent refractoriness predictors. Previous studies have found association of increase of acute phase proteins (procalcitonin PCT) and development of refractoriness and mortality and this is supposed to reflect the interrelations of systemic inflammatory reactions and the epileptic activity[81.]

• Time to treatment. Different studies have found no relation before time to treatment and the development of refractoriness [23,28,66.]

Referring to RSE, predictors of mortality and increased morbidity are:

• Presence of periodic epileptiform discharges on the EEG (PEDs)[77]

• Development of fever as a complication of mechanical ventilation during hospitalization[79].

• High levels of CSF proteins and white cells (as an expression of increased inflammation)[82].

• Longer duration of mechanical ventilation[82].

• Need of a suppression-burst pattern (SB) or isoelectric pattern to control seizures[82] .

Among RSE, acute symptomatic etiology appears to be very frequent[23], and in particular, viral CNS encephalitis[79.] Dealing with treatment of RSE, there are no differences in terms of mortality and return to baseline conditions between monotherapy and politherapy with anesthetics agents and between reaching or not EEG burst suppression patterns[27]. A presence of history of epilepsy and the SE types do not influence the outcome[82]. The advanced age was found to be associated to unfavorable outcome in some studies[23,77] whereas this association was not found in some others[82].

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2. Biomarkers: definitions and applications

The term "biomarker", a portmanteau of "biological marker", refers to a broad subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly. Medical signs stand in contrast to medical symptoms, which are limited to those indications of health or illness perceived by patients themselves. There are several more precise definitions of biomarkers in the literature, and they overlap considerably. In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention." [83]

World Health Organization (WHO) has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [84].

An even broader definition takes into account not just incidence and outcome of disease, but also the effects of treatments, interventions, and even unintended environmental exposure, such as to chemicals or nutrients. In their report on the validity of biomarkers in environment risk assessment, the WHO has stated that a true definition of biomarkers includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" [85].

Biomarkers are merely the most objective, quantifiable medical signs modern laboratory science allows us to measure reproducibly. The use of biomarkers, and in particular laboratory-measured biomarkers, in clinical research is somewhat newer, and the best approaches to this practice are still being developed and refined. The key issue at hand is determining the relationship between any given measurable biomarker and relevant clinical endpoints[86].

FDA defines a biomarker as "Any measurable diagnostic indicator that is used to assess the risk or presence of disease." [87]

The use of biomarkers in basic and clinical research as well as in clinical practice has become so commonplace that their presence is considered the primary endpoints in clinical trial. In the case of specific biomarkers that have been well characterized and

repeatedly shown to correctly predict relevant clinical outcomes across a variety of treatments and populations, this use is entirely justified and appropriate. In many cases, however, the "validity" of biomarkers is assumed where, in fact, it should continue to be evaluated and re-evaluated.

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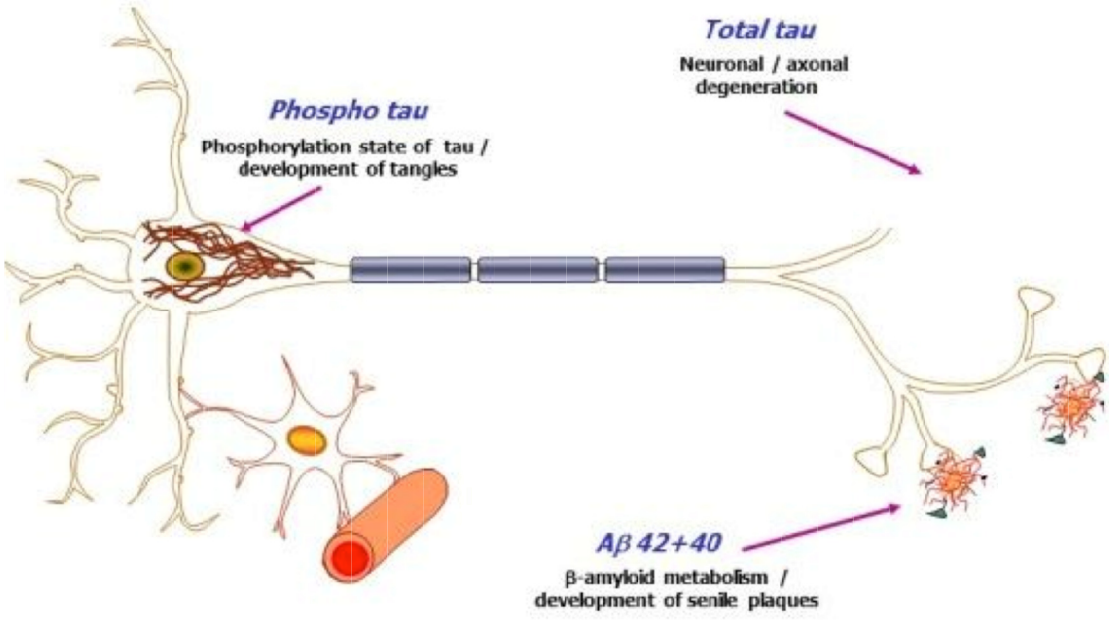
CHAPTER 2. EXPERIMENTAL

2.1 CSF BIOMARKERS OF NEURONAL DAMAGE TO PREDICT OUTCOME IN STATUS EPILEPTICUS

A. Motivation

As illustrate in the paragraphs related to the pathophysiology of the status epilepticus and in the previous studies about prognosis, refractoriness of SE seems to determine a worst outcome. Probably, it depends on the direct damage that repeated seizures caused on the brain. Unfortunately, nowadays, we can't predict the evolution of the status at the early beginning, neither we can predict a neurological impairment. We can use only clinical features and empirical scales, in ex. STESS [88] and EMSE [89], as a measure of outcome, but reliable biomarkers of brain damage induced directly by SE are lacking. Few studies evaluate some molecules of neuronal damage (NSE and S100 protein) with controversial results.

Tau protein is located in neurons of central nervous system (CNS) and is available and measurable in CSF. In particular high values of tau protein in CSF are considered as index of axonal and neuronal damage in several disease.



Our laboratory uses t-tau protein, p-tau protein and Aβ 1-42 CSF dosage to differentiate the subtypes of degenerative cognitive impairment. In literature, no study has specifically evaluated the CSF levels of tau proteins in patients with SE, to predict neurological and epileptic outcomes. We consider very useful to have biomarker of SE prognosis, available with less invasive procedure and relatively low costs for kit test, since the first stage of the disease. We supposed that RSE and SRSE cases had the highest risk of neuronal damage and that is probably time related. Obviously we excluded all cases with acute brain damage such as acute head trauma, viral encephalitis, and acute stroke in which tau protein could increase regardless of status.

Interesting, we tried also to evaluate the role of anaesthetic drugs on the brain; several evidence support the role of neuro-protection due to burst suppression, on the other side some studies consider a possible role in brain damage, especially for propofol.

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B: Study 1. CSF TAU PROTEINS IN STATUS EPILEPTICUS

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Abstract

Tau protein is a phosphorilated microtubule-associated protein, principally localized at neuronal and axonal level in central nervous system (CNS). Tau levels in the cerebrospinal fluid (CSF) are considered to index both axonal and neuronal damage. To date, however, no study has specifically evaluated the CSF levels of tau proteins in patients with status epilepticus (SE). We evaluated the levels of these established biomarkers of neuronal damage in patients with SE who received a lumbar puncture during SE between 2007 and 2014. SE cases due to acute structural brain damage, including CNS infection, as aetiology were excluded. Clinical, biological, therapeutic, and follow- up data were collected. Group comparison between patients stratified according to SE response to antiepileptic drugs (AED), disability, and epilepsy outcomes were performed. Twenty-eight patients were considered for the analyses (mean age 56 years): 14 patients had abnormally high CSF t-tau level, six patients had abnormally high CSF p-tau level, and only three patients had abnormally low Aβ 1-42 level. CSF t-tau value was higher in patients who developed a refractory SE compared to patients with seizures controlled by AED. CSF t-tau values were positively correlated with SE duration and were higher in patients treated with propofol anaesthesia compared to patients that did not received this treatment. Patients with higher CSF t-tau had higher risk of developing disability (OR=32.5, p=0.004) and chronic epilepsy (OR=12; p=0.016) in comparison with patients with lower CSF t-tau level. Our results suggest that CSF t-tau

level might be proposed as biomarker of SE severity and prognosis. Prospective studies are needed to evaluate the effects of propofol on tau pathology in this setting.

Keywords: status epilepticus; tau; refractory status epilepticus; epilepsy; seizures; biomarkers.

1. Introduction

According to the WHO Dictionary of Epilepsy status epilepticus (SE) occurs "when a seizure persists for a sufficient length of time or is repeated frequently enough to produce a fixed or enduring epileptic condition" [1]. Clinical and experimental studies have shown that SE may induce neuronal damage [2, 3] and is associated with high mortality and poor neurological outcome [4, 5]. The prediction of outcome can be difficult, and is mainly based on clinical features and aetiology [6-8]. To date no reliable biomarker exists to predict SE outcomes. Markers of brain injury, such as neuron-specific enolase (NSE) or S-100b protein (S-100), have been measured in patients with seizures or SE with controversial findings [9-12]. Increased serum and CSF levels of NSE have been reported in a subset of epileptic patients with SE [13].

Tau protein is a phosphorilated microtubule-associated protein, principally localized at neuronal and axonal level in central nervous system (CNS). High total-tau (t-tau) and phosphorilated-tau (p-tau) CSF levels are considered to index both axonal and neuronal damage and are currently used in clinical research for early Alzheimer's disease diagnosis together with the determination of amyloid precursor protein cleavage peptides such as A-beta 1-42 (Aβ 1-42) [14, 15]. Increased levels of CSF tau proteins are also found in patients with various neurological diseases, such as traumatic brain injury, acute ischemic stroke, viral encephalitis, Creutzfeldt-Jacob disease, suggesting that tau CSF levels reflect the extent of axonal damage and neuronal degeneration [16-20].

In relation to seizures and epilepsy, only few studies have evaluated tau proteins as potential biomarkers of seizures-related neuronal damage [21, 22]. A recent study evaluated t-tau, p-tau and Aβ 1-42 proteins in groups of patients with different seizures type and aetiologies founding no evidence of increased t-tau and p-tau levels compared with control groups [23].

To date, however, no study has specifically evaluated the CSF levels of tau proteins in patients with SE, and the potential prognostic utility of these biomarkers in this clinical context.

2. Material And Methods

A retrospective observational study was performed between 2007 and 2014 at the NOCSAE Hospital, Modena – Italy, including all patients admitted with SE who received a lumbar puncture during SE in the suspect of a CNS infection. SE was defined as ongoing seizures, or repetitive seizures without intercurrent normalization of consciousness or return to baseline for at least 30 min [24].

In order to avoid interpretation bias in biomarkers analyses due to causes of neuronal injury other than seizures the following exclusion criteria were considered: (i) evidence (by head CT or MRI) of an acute brain insult as aetiology of SE; (ii) evidence of viral or bacterial CNS infection; (iii) evidence of cognitive decline in the context of a possible neurodegenerative disorder; (iv) evidence of a progressive CNS disorder (i.e. brain tumour). According to the ILAE criteria for the clinical classification of SE we excluded cases due to an "acute symptomatic" as well as "progressive" CNS disease [25].

The final study was conducted on a patients' cohort of 28 subjects fulfilling our inclusion/exclusion criteria for whom information about demographic data, clinical features, diagnostic findings, therapeutic interventions, and clinical outcomes were acquired (*Supplementary Table 1*). All these patients were examined for bacterial and viral infections including: HSV 1 and 2, varicella-zoster virus, HHV 6, cytomegalovirus, Epstein–Barr virus, rubella, parvovirus B19, enterovirus, HIV, and mumps. All virological studies were performed using DNA polymerase chain reaction (PCR). Each patient also had a head CT and/or brain MRI at hospital admission or < 24 hour to exclude an acute brain damage as causative event of SE. All included subjects had a EEG monitoring of variable duration confirming SE diagnosis. EEG was defined as positive if showing an ictal or periodic pattern or, if performed postictally, showing focal slowing (with or without interictal discharges) unexplained by other causes, or generalized slowing with interictal epileptiform activity [8].

CSF samples were acquired from a few hours after admission to a maximum of 20 days (median of 72 hours)(*Supplementary Table 2*). Standard CSF chemical analysis was performed to determine blood cell count and total protein concentration values. Isoelectro-focusing (IEF) on agarose gel support was performed to check the presence of oligoclonal IgG bands (OB). For CSF Aβ 1-42, t-tau, and p-tau181 dosage, analysis was performed on CSF samples stored at -80°C in polypropylene storage tubes. CSF Aβ 1-42, t-tau, and p-tau181 were measured with the ELISA method following manufacturer instructions (Innogenetics, Gent, Belgium). All tests were performed in the same laboratory by the same biologist in accordance with recent guidelines [26]. Cut-off values were established according to literature and to our laboratory data [27, 28]: 350 pg/ml for t-tau; 60 pg/ml for p-tau; and 500 pg/ml for Aβ 1-42.

Statistical analysis was performed using STATA software and parametric or not parametric tests were used as appropriate. Patients were classified in groups according to the following variables: (i) response to AED; responsive SE versus refractory/super-refractory SE [29]; (ii) disability outcome; worsening in disability was measured as a change ≥ 1 point in modified Ranking score (mRS) score at 6 months follow-up; (iii) epilepsy outcome; we considered patients who developed (or not) spontaneous seizures after six months of follow-up. Post-SE epilepsy was defined as the occurrence of at least one unprovoked epileptic seizure not earlier than four weeks after termination of SE.

The treatment protocol for SE was similar in all the patients and followed the guidelines of the Italian League Against Epilepsy [30] and of the European Federation of Neurological Societies [31]. Benzodiazepines were used as first line therapy (i.v. lorazepam) followed by treatment with phenytoin or valproate i.v. at appropriate dose as second line AEDs, in mono or polytherapy: phenytoin was used in 21 patients, valproate in 13. Levetiracetam (i.v.) was used in 10 patients (in three as monotherapy); i.v. lacosamide was use in one subjects in polytherapy. If seizures persisted after AEDs treatment the SE was considered refractory and the patient was treated with general anesthesia in intensive care unit (ICU)(12 patients): propofol was used in 10 patients, midazolam in five, and thiopental in one. If seizures persisted or recurred after more than 24 hours of general anesthesia the SE was considered super-refractory.

Independent t-tests or Mann-Whitney-U test were used to compare the groups on different variables as appropriate. Correlation analyses were performed to study the relationship between SE duration, time interval between the SE onset/admission and

lumbar puncture, and CSF values. Univariate logistic regression analyses were performed to find the predictive value of each independent variable in discriminating disability and epilepsy outcome at six months; by using these results, we constructed multiple regression models adding only those variable with a p value<0.25 in order to find the best model of prediction. For these analyses, we treated CSF biomarkers values as dichotomous and the classification was based on the median value of the group. The accuracy of these models was assessed by area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Patients with previous history of epilepsy or seizure were not considered in logistic regression with epilepsy outcome as dependent variable.

The scientific advisory board of our institution approved the research protocol according to local regulations and informed written consent was obtained from patients.

3. Results

Table 1 reports the main demographic, electroclinical and CSF data of the patients (mean age 56 years; range 11-79 years; 18 females). According to outcomes, 11 patients (39%) worsened in functional outcome assessed by mRS score, 5 of which died; 11 patients (39%) had refractory/super-refractory SE. For those patients with available follow-up at six months (n=22), 10 (45.4%) developed chronic epilepsy. Considering cut-off values, 14 patients had abnormally high CSF t-tau level, six patients had abnormally high CSF p-tau level, and only three patients had abnormally low Aβ 1-42 level.

Statistical analyses demonstrated (*Supplementary Table 3*) that patients with refractory/super-refractory SE had higher CSF t-tau level (p=0.0005) compared to responsive SE (Figure 1a). Patients with mRS worsening showed higher CSF t-tau level (p=0.005), greater frequency of refractory SE (p=0.03), greater frequency of remote symptomatic SE (p=0.01), and greater use of anesthetic drugs (p=0.01). A sub-analysis in dead patients compared to all other subjects did not show any difference in demographic, electroclinical and CSF variables. Patients who developed chronic epilepsy at six months were characterized by greater frequency of remote symptomatic SE (p=0.03).

Correlation analyses performed between SE duration and CSF values showed that only t-tau level was positively correlated with SE duration (rs=0.47, p=0.01)(Figure 1b). The time interval between the SE onset/admission and CSF sample did not correlate with CSF biomarkers concentrations.

Considering SE treatment, higher t-tau values were found in patients who underwent lumbar puncture during general anesthesia with propofol compared to patients who were treated with other drugs or who underwent lumbar puncture before anesthesia (p=0.0019). The relative risk of having t-tau value higher than the median value in patients treated with propofol was 3.3 (p=0.0008).

Univariate logistic regressions (*Table 2*) performed with disability outcome (mRS) as dependent variable showed that refractory SE (OR=5.6, p=0.04), use of anesthetic drugs (OR=8.6, p=0.01), and higher level of CSF t-tau (OR=32.5, p=0.004) were significant predictors of disability development and/or worsening. Using several stepwise logistic regression analyses inclusive of all variables with $p<0.25$ in univariate logistic regression, we found that the best model for predicting disability outcome included CSF t-tau level, need of ICU, and AED refractoriness with 82.14% of cases correctly classified. Sensitivity and specificity of this model were 90.91% and 76.47%, respectively (AUC=0.89). In this model, only CSF t-tau reliably and independently predicted disability outcome (OR=21.2, p=0.013). Univariate logistic regressions performed with epilepsy outcome as dependent variable showed that remote symptomatic cause (OR=25.6, p=0.010) and higher level of CSF t-tau (OR=12, p=0.016) were significant predictors of development of epilepsy at six months.

Table 1. Demographics and clinical features of patients with SE (n=28).

Age, mean (sd)	56,10 (19)
Gender: M (%) – F (%)	10 (35) – 18 (65)
Previous history of epilepsy/seizure, yes –no (%)	3 (10,7) – 25 (89,3)
Prevalent type of seizure **Motor (%)** **Not motor (%)**	 14 (50) 14 (50)
SE presumed aetiology, **Symptomatic remote (%)** **Drug withdrawal, toxic-metabolic, autoimmune or unknown (%)**	 10 (35,7) 18 (64,3)
SE duration (days), median (range)	4 (1-30)
Days from SE onset and lumbar puncture, median (range)	3 (0-20)
SE outcome, **Responsive (%)** **Refractory and Super-refractory (%)**	 17 (61) 11 (39)
Anaesthetic drugs use, yes –no (%)	12 (43) – 16 (57)
CSF hyperproteinorrachia, yes –no (%)	5 (18) – 23 (82)
CSF Oligoclonal bands, yes –no (%)*	7 (25,9) – 20 (74,1)
CSF t-tau (pg/ml), median (range)	401 (68 – 195618)
CSF p-tau$_{181}$(pg/ml), median (range)	39,5 (6 – 132)
CSF Aβ $_{1-42}$(pg/ml), median (range)	934,5 (309 – 1504)
Development of chronic epilepsy, yes –no (%)**	10 (45,4) – 12 (54,6)
mRS worsening, yes –no (%)	11 (39,3) – 17 (60,7)

Table legend. SE: status epilepticus; F: female; M: male; CSF: cerebrospinal fluid; mRS: modified Rankin scale; sd: standard deviation
*CSF oligoclonal bands were not evaluated in 1 patient
**Evaluated in 22 patients; 6 patients were lost at follow-up or died before the final follow-up

Table 2. Univariate logistic regression results for disability and epilepsy outcome.

	mRS WORSENING			DEVELOPMENT OF EPILEPSY AT 6 MONTHS		
	OR	**p**	**95% CI**	**OR**	**p**	**95% CI**
Age (≥ or ≤ 56years)	1,01	0,358	0,97-1,06	1	1,000	0,18-5,35
Gender	2	0,390	0,4-9,7	0,5	0,578	0,10-3,53
Previous history of epilepsy/seizure	3,5	0,327	0,28-44,8	-	-	-
Prevalent type of seizure	1,35	0,699	0,29-6,18	0,7	0,696	0,13-3,86
SE response to AED	5,68	**0,040**	1,07-29,99	1,33	0,746	0,23-7,62
SE aetiology	8,16	**0,019**	1,41-47,01	25,66	**0,010**	2,2-298,4
SE duration (≥ or ≤ 4 days)	3,8	0,11	0,73-19,66	2,3	0,34	0,40-13,60
Days from SE onset and lumbar puncture (≥ or ≤ 3 days)	3,8	0,11	0,73-19,66	2,3	0,34	0,40-13,60
Anaesthetic drugs use	8,66	**0,015**	1,52-49,22	2	0,43	0,35-11,2
CSF hyperproteinorrachia,	2,81	0,307	0,30-20,45	0,5	0,654	0,04-7,21
CSF OB	1,12	0,895	0,19-6,43	4,5	0,135	0,63-32,29
CSF t-tau, median	**32,5**	**0,004**	3,12-337,81	12	**0,016**	1,58-91,08
CSF p-tau$_{181}$, median	1,35	0,699	0,29-6,18	0.6	0,640	1,12-3,63
CSF Aβ $_{1-42,}$ median	0,74	0,699	0,16-3,39	0,47	0,39	0,08-2,62

Table legend. SE: status epilepticus; CSF: cerebrospinal fluid; mRS: modified Rankin scale; OD: odds ratio; CI: confidence interval; OB: oligoclonal bands; AED: antiepileptic drugs. In bold significant values.

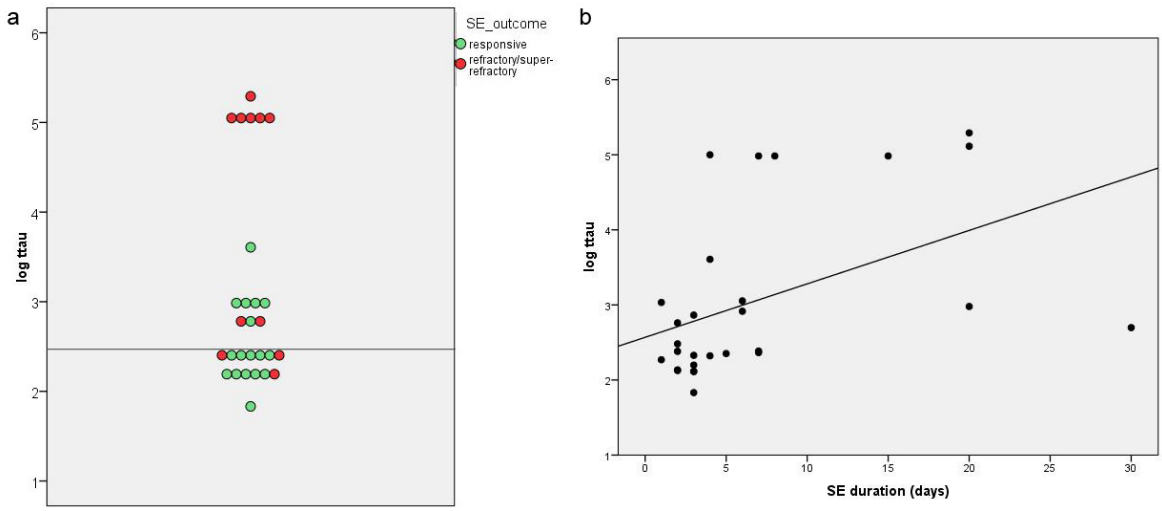

Figure 1. a). Distribution plot of t-tau in patients with status epilepticus (SE); red dots indicate patients with refractory or super-refractory SE, green dots represent cases with SE responsive to AED; horizontal line represents t-tau cut-off value (350 pg/ml); b). Positive correlation between status epilepticus duration (days) and CSF values, meaning that subjects with higher t-tau values had longer status epilepticus duration. t-tau value are represented as logarithmic value for display purpose.

Supplementary Table 1. Clinical data of the examined patients. Pts are numbered from the one with the highest t-Tau CSF level to the lowest.

Pt	Age/ gender	Previous history of seizures/ epilepsy	Prevalent type of seizures	SE outcome	SE duration (days)	Need of ICU	AED* used	Other drugs	Presumed aetiology of SE	Trigger condition	Cognitive/ neurological outcome	Development of chronic epilepsy
1	38/F	No	Tonic clonic	Super-Refractory	20	Yes	lev, pht	Propofol	Remote symptomatic	Infection/ Fever	Severe encephalopathy	Yes
2	30/F	No	Myoclonic	Super-Refractory	20	Yes	lev, pht, lcm	Propofol, Midazolam, Thiopental	Unknown	Infection /Fever	Normal	No
3	47/F	No	Complex partial	Refractory	4	Yes	lev, pht	Propofol	Metabolic	HypoNa+	Severe encephalopathy	No
4	72/F	No	Focal motor	Super-Refractory	7	Yes	lev, pht, vpa	Propofol	Remote symptomatic	Infection/ Fever	Severe encephalopathy	Yes
5	77/M	No	Complex partial	Super-Refractory	8	Yes	lev, pht, vpa	Propofol, Midazolam	Remote symptomatic	-	Death	n.a.
6	69/M	No	Focal motor	Refractory	15	Yes	pht, ltg, oxc, pb	Propofol	Remote symptomatic	-	Severe encephalopathy	Yes
7	72/F	No	Complex partial	Responsive	4	No	pht	-	Remote symptomatic	Infection/ Fever	Normal	Yes
8	79/F	No	Focal motor	Responsive	6	No	pht, vpa	-	Unknown	-	Normal	Yes

9	39/M	No	Complex partial	Responsive	1	No	vpa	-	Remote symptomatic	-	Language deficit	Yes
10	76/M	No	Focal motor	Responsive	20	No	vpa, pht	-	Remote symptomatic	Infection/ Fever	Language deficit	No
11	77/F	No	Focal motor	Responsive	6	No	pht	-	Unknown	-	Death	n.a.
12	65/M	No	Myoclonic	Refractory	3	Yes	pht	Propofol	Unknown	-	Death	n.a.
13	16/F	Yes	Focal sensory	Responsive	2	Yes	lev	Propofol	Remote symptomatic	-	Mild cognitive deficit	Yes
14	68/M	Yes	Complex partial	Super-Refractory	30	Yes	pht, lev, vpa, pb	Propofol	Remote symptomatic	-	Death	Yes
15	43/M	No	Focal motor	Responsive	2	No	vpa, pht	-	BDZ withdrawal in substance abuse disorder	-	Normal	No
16	70/F	No	Complex partial	Responsive	7	No	vpa	-	Unknown	-	Normal	No
17	78/M	No	Tonic clonic	Responsive	2	No	lev	-	Unknown	Infection/ Fever	Normal	No
18	58/F	No	Tonic clonic	Refractory	5	Yes	vpa	Midazolam	Lithium overdosage	-	Normal	No

19	51/F	No	Complex parital	Responsive	4	No	pht, vpa	-	Unknown	-	Normal	Yes
20	52/M	No	Focal motor	Responsive	3	No	lev	-	BDZ withdrawal	-	Normal	No
21	27/F	No	Focal motor	Responsive	2	No	pht	-	NMDAr encephalitis	-	Normal	Yes
22	79/F	No	Dyscognitive	Responsive	2	No	pht, vpa	-	Unknown	Infection/ Fever	Normal	No
23	50/M	No	Dyscognitive	Responsive	3	No	pht	-	Unknown	Infection/ Fever	Normal	No
24	59/F	No	Dyscognitive	Responsive	1	No	vpa	-	Multidrug withdrawal for neurpathic pain	-	Death	n.a.
25	11/F	No	Focal sensory	Refractory	7	Yes	pht, lev	Midazolam	NMDAr encephalitis	-	Normal	No
26	64/F	No	Focal motor	Responsive	3	No	pht	-	Unknown	Infection/ Fever	Normal	No
27	37/F	Yes	Focal sensory	Refractory	3	Yes	pht	Midazolam, Propofol	Remote symptomatic	-	Normal	n.a.
28	67/F	No	Dyscognitive	Responsive	3	No	pht, vpa	-	BDZ withdrawal in Bipolar disorder	-	Normal	n.a.

Table legend: F: female; M: male;CBZ: carbamazepine; CLB: clobazam; PHT: phenytoin; LCM: Lacosamide; LEV: levetiracetam; LTG: lamotrigine; MDZ: midazolam; PRO: propofol;OXC: oxcarbazepine; THIO: thiopentale; TPM: topiramate; VPA: valproate. n.a.: not available.

* The first line treatment was i.v. lorazepam or diazepam in every patient.

Supplementary Table 2. Cerebrospinal fluid data of the examined patients. Patients are numbered from the one with the highest t-Tau CSF level to the lowest.

Pt	Lumbar puncture (days from SE onset)	Cells	Proteins	Oligo Clonal bands	Total Tau (pg/ml)	P Tau (pg/ml)	B amyloid (pg/ml)
1	5	No	Yes	Yes	195618*	127*	453*
2	9	No	No	No	130000*	69*	1118
3	1	No	No	No	99955*	132*	940
4	13	No	No	No	96401*	6	409*
5	4	No	Yes	No	96401*	83*	309*
6	8	No	No	No	96401*	42	1091
7	2	No	No	Yes	4050*	64*	1504
8	3	No	No	No	1131*	51	909
9	2	No	No	Yes	1080*	12	604
10	12	No	No	No	953*	35	1137
11	1	No	No	No	823*	55	819
12	0	No	Yes	No	732*	74*	983
13	13	No	No	Yes	576*	38	951
14	3	No	No	Yes	499*	19	568
15	5	No	No	No	303	41	1411

16	8	No	No	No	243	47	1189
17	0	No	No	No	241	49	1050
18	1	No	Yes	No	225	38	776
19	4	No	No	No	210	30	929
20	2	No	No	No	158	14	863
21	2	No	No	No	136	27	958
22	1	No	No	Yes	134	25	909
23	5	No	Yes	No	68	9	762
24	20	No	No	No	186	36	896
25	3	No	No	Yes	232	46	1090
26	1	No	No	n.a.	130	34	698
27	1	No	No	No	130	34	950
28	1	No	No	No	213	46	1123

	RESPONSE TO AED			mRS WORSENING			DEVELOPMENT OF EPILEPSY at 6 months **		
	AED Responsive (n=17)	**AED not responsive (n=11)**	**Group comparison**	**Not disability worsening (n=17)**	**Disability worsening (n=11)**	**Group comparison**	**No (n=12)**	**Yes (n=10)**	**Group comparison**
Age, mean (years)	58,76	52	ns	53,3	60,3	ns	54,8	59,5	ns
Gender: M – F (n)	6-11	4-7	ns	5-6	5-12	ns	5-7	3-7	ns
Previous history of epilepsy/seizure, yes –no (n)	1-16	2-9	ns	1-16	2-9	ns	-	-	ns
Prevalent seizure type (n) motor not motor	 8 9	 6 5	ns	 8 9	 5 6	ns	 7 5	 5 5	ns
SE aetiology (n) Symptomatic remote Other/unknown (n)	 4 13	 6 5	ns	 3 14	 7 4	p=0,01	 1 11	 7 3	p=0,03
SE mean duration (days), median	3	7	p=0,003	3	7	ns	3,5	5	ns
Days from SE onset and lumbar puncture, median	2	3	ns	2	5	ns	2,5	3,5	ns
SE evolution Responsive Refractory/super-refractory	-	-	-	13 4	7 4	p=0,03	8 4	6 4	ns
Anaesthetic drugs, yes –no (n)	1-16	11-0	p=0,000	4-13	8-3	p=0,01	4-8	5-5	ns
CSF hyperproteinorrachia, yes –no (n)	1-16	4-7	p=0,04	2-15	3-8	ns	2-10	1-9	ns
CSF Oligoclonal bands*, yes –no (n)	4-12	3-8	ns	4-12	3-8	ns	2 -9	5-5	ns
CSF t-tau, median	241	96401	p=0,0005	225	953	p=0,005	236	1105,5	ns
CSF p-tau$_{181}$, median	36	46	ns	38	42	ns	39	51	ns
CSF Aβ $_{1-42}$, median	929	940	ns	950	896	ns	995	919	ns

Supplementary Table 3. Group comparison results according to response to AED, disability, and epilepsy outcome

Table legend. n: numbers of subjects; SE: status epilepticus; F: female; M: male; CSF: cerebrospinal fluid; mRS: modified Rankin scale; sd: standard deviation
*CSF oligoclonal bands were not evaluated in 1 patient
**Evaluated in 22 patients; 6 patients were lost at follow-up or died before the final follow-up

4. Discussion

In this study we evaluated if established biomarkers of brain damage were increased during status epilepticus. The hypothesis tested was to find out if repeated seizures induce a neuronal damage that can be indexed by cerebrospinal fluid tau proteins; therefore, we have excluded from the study all SE cases with a symptomatic aetiology due to an acute or progressive CNS insult.

A first finding concerns the different patterns of CSF values for t-tau, p-tau, and Aβ 1-42 proteins. Indeed, only a few patients showed pathological levels of p-tau (21%) and Aβ 1-42 (11%), whereas 50% of the patients had pathologically high t-tau values. In particular, t-tau values showed great variability, from normal values (< 350 pg/ml) to dramatically high levels (> 90.000 pg/ml in six patients). Therefore t-tau protein was the more sensible marker of neuronal damage in our sample of SE. Moreover, we found that only t-tau correlated with the duration of SE. These findings are consistent with results of tau pathology in other brain disorders. P-tau is considered to reflect the phosphorylation state of tau, being a more direct signature of Alzheimer Disease. Consistent with this, in patients with ischemic stroke, CSF t-tau but not p-tau levels were increased and showed correlation with the size of the infarct, indicating that CSF t-tau reflects the degree of neuronal damage [32]. Also in Creutzfeldt–Jacob disease marked elevation of t-tau but not p-tau levels has been reported [19].

We found that t-tau was higher (i) in patients with refractory and super-refractory SE compared to patients with SE responsive to AED and (ii) in patients who developed a clinical and functional worsening in disability. These two results support the hypothesis that t-tau might index the severity of status epilepticus and the concept that repeated seizures in this clinical context might induce neuronal damage. Notably, our multiple stepwise logistic regression analysis found that the best model for predicting disability outcome included CSF t-tau level, need of ICU, and AED refractoriness with 82.14% of cases correctly classified.

Only two previous studies have addressed CSF tau proteins as markers of seizures-related neuronal damage [22, 23]. Both studies investigated mixed cohorts of patients after single or repetitive seizures finding no increase in CSF biomarkers. High t-tau high level was observed only in cases with acute symptomatic seizures due to CNS damage [22]. The authors concluded that abnormal CSF tau levels can be found in patients with

seizures. However, isolated or repetitive seizure per se does not seem to lead to the elevation of CSF tau. Moreover, in both studies a small subset of patients was categorized as status epilepticus: t-tau levels were not reported to be high in these cases and did not correlate with the number of seizures. It should be noted however, that the SE cases included in these studies were responsive to first line therapy (i.v. benzodiazepine) or second line AED (i.v. valproate). In our study, we observed that the majority of patients with SE classified to responsive to AED showed normal t-tau levels (11 out of 17 patients). Taken together, these evidences are concordant in showing that isolated o repeated seizures which respond promptly to first or second line AED treatment does not induce t-tau pathology and neuronal damage. Conversely, our study demonstrates that severe, refractory/super-refractory SE cases, are associated to a marked increase in t-tau levels possibly reflecting neuronal damage.

Another interesting finding was that t-tau levels were significantly higher in patients for whom CSF samples were collected during general anaesthesia with propofol in ICU. These patients who presented refractory SE received propofol treatment for at least one cycle of 24-72 hour (or more in super-refractory SE cases). The use of general anaesthetics and ICU staying were also associated to worse functional outcome and disability. These findings raise the question of a role of general anaesthesia and propofol in inducing tau pathology and subsequent cognitive worsening. Of course, the need of ICU and anaesthetics drugs are related to the intrinsic severity of the status epilepticus and therefore it is difficult to disentangle the effects of different variables on outcomes. Previous studies in both adults and children have reported that the need and the duration of anaesthetic treatment were related to worse functional outcome and namely worse cognitive outcome [6] [6, 33]. The evidence that patients with SE treated with propofol had high or dramatically high level of total tau CSF proteins deserve attention and need to be verified in future prospective studies. Indeed, recent researches focused on the effects of general anaesthesia on tau CSF levels showed that several anaesthetics drugs can affect tau pathology in different and complex ways [34, 35]. Anaesthetics have been previously shown to induce tau hyperphosphorylation through a mechanism involving hypothermia-induced inhibition of protein phosphatase 2A activity [36, 37]. However, also the effect of propofol on tau phosphorylation under normothermic conditions has been recently demonstrated in the mouse [38] and brief exposure to different anaesthetics in human surgery procedures has resulted in t-tau increase [39].

These evidences support the urgent need of studies to assess the role on anaesthetics in a severe brain disorder as status epilepticus.

Of course our study has several limitations, which are related to the retrospective design. An important one concerns the lack of precise information about the exact time of onset of seizures respect to CSF sampling. For SE cases that started during hospitalization this information was available, for cases in whom seizures begun out of hospital the timing was approximate in the majority and we assumed the hospital admission as reference point. Another limitation concerns the exact number of seizures that occurred before and after CSF sampling. Finally, in the present cohort no case of SE in idiopathic or genetic epilepsy syndromes was present, therefore no data on these disorders were acquired. However we believe that despite some limits this study provided interesting results that can have clinical relevance.

In conclusion, we provide for the first time evidence of tau pathology during status epilepticus. Total tau proteins showed a marked increase in refractory and super-refractory SE cases and t-tau was a strong predictor of status severity, development of disability, and subsequent epilepsy. The use of propofol anaesthesia was also associated to high t-tau levels. These results deserve future verifications with prospective studies.

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C. Discussion & perspective

Our findings support new prospective in the comprehension of status epilepticus effects on CNS in humans. Possible biomarkers of neuronal damage since the early stages could improve and guide better treatments and approaches.

Refractory SE cases are related to higher percentage of mortality and morbidity and an early intervention is mandatory.

A recent paper published by our group (Giovannini et al. 2015)[22] reported 83 SE cases in one year of prospective analysis in Modena district; cases with RSE/super-RSE represent 31% of all SE cases (SRSE accounted for 17% of all SE episodes). We observed a similar incidence of cases with refractory SE that in previous studies, taking into account the possible differences in classification and population included (we included also post-anoxic aetiology). The overall short-term mortality was 37%. Comparing mortality in cases with RSE with cases with responsive SE, a striking difference was evident with a mortality of 54% in RSE compared with a mortality of 20% in responsive SE. Moreover, we confirm that RSE is related to high short-term disability. This is not the consequence of less aggressive treatment; rather it reflects the severity of the underlying conditions.

Moreover, stupor/coma at onset is the most relevant clinical factor associated with SE refractoriness. Probably is due to a delay in correct diagnosis of SE (often after 24 hours) and consequently in the treatment delay.

SE needs to be considered a neurological emergency supported by several different aetiologies with very different evolutions. We need to improve our knowledge in this field, especially in humans. We collected a lot of evidence that refractory cases often evolves in neurological disabilities and chronic epilepsy, sometimes independently by the underline conditions but primary as a consequence of repeated seizures.

This hypothesis is supported by our demonstration of high t-tau protein levels in CSF only in refractory cases.

These preliminary data were published in May 2015 in the special issue of Epilepsy & Behaviour on “Status epilepticus”, following the poster presentation during the London-Innsbruck Colloquium on Status epilepticus and acute seizures, held in London on April 9-11 2015.

Starting from these evidences, in the last 2 years we collected other 25 patients with SE that undergone to CSF analyses, for a total of 53 cases; we decide to reach at least 60 patients to repeat statistical analysis. We hope to reach the purpose in the next few months.

Our intention is to propose CSF collection in patients with SE, not only to exclude CNS infection, but also to analyze prognostic factors of possible brain damage.

2.2 SERUM AND CSF NEUROSTEROIDS IN STATUS EPILEPTICUS

A. Motivation

Starting from the awareness that SE refractoriness is the principal reason for complications and mortality, we evaluate possible new therapeutic strategies to reduce refractory cases. A new molecule derived from steroids family, called allopregnanolone (AP), seems to have antiepileptic properties, due to the effects on GABAergic current. In addition respect Benzodiazepine mechanism, AP modulate also extra-synaptic $GABA_A$ receptors with possible application as second line SE therapy. Several studies on animals support this hypothesis.

AP has a unique particularity that differentiates it from other AED; it's an endogenous molecule, a hormone derivate from progesterone, with a direct activity in CNS. For this reason is one of the called neuro-steroids.

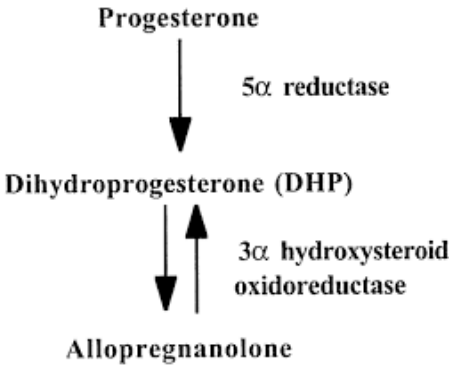

Figure showed the steps for the composition of AP starting from progesterone and respective enzymes involved.

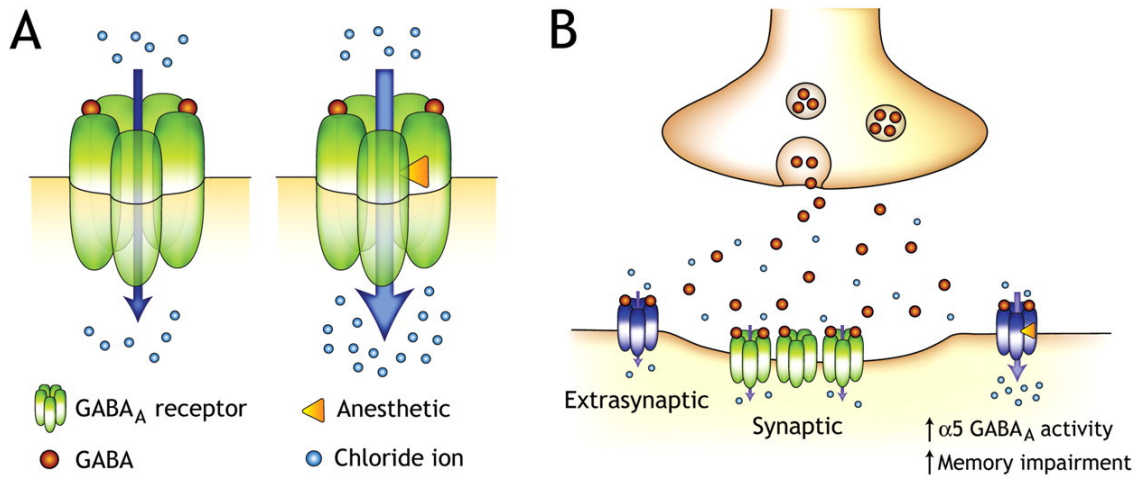

Figure showed GABA$_A$ receptors with sub-unite site for ligands. In post-synaptic membranes are located the two sub-types of GABA receptors: synaptic and extra-synaptic.

Other neuro-steroids are considered to influence neuronal activity; in particular pregnenolone sulphate seems to have a proconvulsant mechanism.

In literature, no study analysed physiological values of such neurosteroids in humans, considering gender and age, nor in epileptic patients.

We started from our laboratory CSF storage to select normal CSF in normal patients, in which lumbar puncture was done to exclude neurological disease.

Then we considered our cohort of patients with SE and we selected those that undergone to simultaneously CNS and serum detection to evaluate the dosage of the principal neurosteroids (PS and AP).

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B. Study 2. DECREASED ALLOPREGNANOLONE LEVELS IN CSF DURING STATUS EPILEPTICUS

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Short title: Neurosteroids in *status epilepticus*

KEY WORDS: Allopregnanolone; Cerebrospinal fluid; LC-MS/MS; Pregnenolone sulfate; Status epilepticus.

Summary

Neuroactive steroids are increasingly considered as relevant modulators of neuronal activity. Especially allopregnanolone (AP) and pregnenolone sulfate (PS) have been shown to possess, respectively, anticonvulsant or proconvulsant properties. In view of the potential role of these steroids, we aimed at evaluating AP and PS levels in cerebrospinal fluid (CSF) and blood samples obtained from patients with *status epilepticus* (SE). To this purpose, we enrolled 41 patients affected by SE and 41 subjects investigated for non-epileptic neurological disorders. Liquid chromatographic procedures coupled with electrospray tandem mass spectrometry and routine laboratory investigations were performed. Significantly lower AP levels were found in CSF of patients affected by SE (-30%; $p < 0.05$, Mann-Whitney test). Notably, AP was not detectable in 28 out of 41 patients affected by SE ($p < 0.01$ vs controls, Fisher's exact test). In serum, AP levels did not differ in the two considered groups. Conversely, PS was present at similar levels in the investigated groups. Finally, differences in AP levels could not be explained by a variation in CSF albumin content. These findings indicate that AP is defective in the CSF of patients affected by SE. This phenomenon was not dependent on carriers for steroids, such as albumin.

INTRODUCTION

In the central nervous system (CNS), allopregnanolone (AP) behaves as a positive modulator of γ-aminobutyric acid type A ($GABA_A$) receptor and it definitely enhances both tonic and phasic $GABA_A$-dependent inhibitory currents.[1-3] This biological property has been investigated in different models of epilepsy, in which it was consistently demonstrated that AP is neuroprotective and anticonvulsant.[4] In contrast, sulfated neurosteroids such as pregnenolone sulfate (PS) negatively modulate the $GABA_A$-dependent inhibition.[2] Specifically, PS was shown to induce seizures when injected into the brain.[5]

In spite of this evidence, very few data are currently available on the possible role of AP and PS in human epileptic disorders.[6] One of the reasons for this still incomplete knowledge is the difficulty to quantify with high specificity the neuroactive steroids by currently available methods.[7] To address this question, Rustichelli et al.[8] have recently set a method based on the removal of interfering phospholipids from samples. By this way, PS was clearly detected and measured. On the contrary, AP was probably incompletely separated from other steroids, such as the cognate molecule pregnanolone (PREG), which was not previously considered as a possible co-eluting isomer. Although PREG is also a positive modulator of $GABA_A$ currents, with slightly lower potency in comparison to AP, a correct identification of PREG and AP is required to clarify which step in their processing could be definitely altered.[1]

For these reasons, we aimed at further improving our protocol by setting a procedure to clearly separate PREG from AP. Then, we applied the new procedure to evaluate the role of AP and PS in a serious neurological disorder such as *status epilepticus* (SE). The role of neurosteroids appears to be particularly relevant since, recently, administration of AP has been proposed as possible therapeutic innovation to overcome the early refractoriness to benzodiazepines observed during SE.[9,10]

MATERIALS AND METHODS

Chemicals and reagents

Amplifex™-Keto Reagent Kit, human albumin (≥ 96%), AP (3α-hydroxy-5α-pregnan-20-one), PREG (3α-hydroxy-5β-pregnan-20-one), and PS (5-pregnen-3β-ol-20-one sulfate) were by Sigma-Fluka (St. Louis, MO). The internal standards (ISs) sodium pregnenolone-17α,21,21,21-D_4 sulfate (PS-D_4) and 5α-pregnan-3α-ol-20-one-17α,21,21,21-D_4 (AP-D_4)

were from CDN Isotopes (Quebec, Canada). The 3β isomers of AP and PREG, respectively, epiallopregnanolone (epiAP) and epipregnanolone (epiPREG) (Steraloids Inc., Newport, RI), were used in order to monitor their retention times. Liquid chromatography-mass spectrometry (LC-MS) purity grade acetonitrile, methanol, formic acid and ammonium formate were from Sigma-Fluka; ultra-pure water was purified by a Milli-Q Plus185 system from Millipore (Milford, MA, USA). Phree Phospholipid Removal Tubes (1.0 mL) were supplied by Phenomenex (Torrance, CA).

High-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Preparation of standard solutions

A standard stock solution containing PS, AP and PREG was prepared and serially diluted with methanol to obtain working solutions at 10 different concentration levels, in the range between 0.6-600 ng/mL for PS and 0.04-40 ng/mL for AP and PREG. The internal standard calibration solution (IS) was prepared by diluting a stock solution of PS-D_4 and AP-D_4 with methanol up to the concentration of 20 and 10 ng/mL for PS-D_4 and AP-D_4, respectively. All solutions were stored at -20 °C until use.

LC-MS/MS conditions

The HPLC system consisted of an Agilent 1200 Series Binary Pump equipped with a vacuum degasser, an autosampler and a thermostatted column compartment (Agilent, Waldbronn, Germany). The chromatographic separation was performed on a Kinetex XB-C18 column (75x2.1 mm; 2.6 µm particle size; Phenomenex) preceded by a UHPLC C18 SecurityGuard cartridge (2.1 mm; Phenomenex). The column temperature was set at 40 °C and the autosampler was maintained at 4 °C. Gradient elution was performed at a flow-rate of 0.4 mL/min with a mobile phase of water/acetonitrile 90/10 added with 3 mM ammonium formate and 0.1% formic acid (mobile phase A) and acetonitrile/water 90/10 added with 3 mM ammonium formate and 0.1% formic acid (mobile phase B) using the following program: 0 to 1 min, isocratic at 30% (B); 1 to 10 min, linear gradient from 30 to 40% (B); 10 to 13 min, linear gradient from 40 to 100% (B); 13 to 18 min, isocratic at 100% (B), 18 to 20 min, linear gradient from 100 to 30% (B). A pre-equilibration period of 10 min was used between each run. The injection volume was 10 µL and the injector needle was washed with methanol. Mass spectrometric detection was performed using an Agilent QQQ-MS/MS (6410B) triple quadrupole mass analyzer equipped with an ESI ion

source (Agilent, Waldbronn, Germany) operating in the positive mode. The ESI source parameters were set as follows: the capillary voltage was 3500 V, the nebulizer (N_2) pressure was 35 psi, the drying gas (N_2) temperature was 350 °C, the drying gas flow was 10 L/min and the electron multiplier voltage was 700. The mass spectrometer operated in multiple reaction monitoring (MRM) mode with a dwell time of 100 msec. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas (15 psi). The Agilent MassHunter Workstation Acquisition software version B.05.00 (B2043) was used for instrument control, data acquisition, qualitative and quantitative data analysis. MS/MS parameters were optimized by direct infusion of each derivatized analyte, including the ISs, at 50 ng/mL in the initial LC mobile phase at a flow of 10 µL/min. For each analyte, one precursor ion and three MRM transitions were set up; the validity of the chosen MRM transitions was verified by LC-MS/MS analyses of human albumin solutions spiked with the derivatized analytes and ISs (50 ng/mL) and processed as described below. The mass spectrometer was calibrated to <2 mDa mass error prior to each batch analysis.

Calibration and quality control samples

Since the target analytes are endogenous compounds, 5% human albumin in phosphate buffered saline was used as blank matrix for calibration and quality control samples. Calibration samples were prepared by spiking 200 µL of human albumin solution with 50 µL of the IS solution and 50 µL of the working solutions; the samples were then subjected to the sample processing described above. The concentration range in the obtained calibration samples (n = 10) was 0.15 to 150 ng/mL for PS and 0.01 to 10 ng/mL for AP and PREG; calibrator concentrations were chosen to give optimal coverage in the range of clinical interest. The concentration of the deuterated internal standards in the calibration samples was 5 and 2.5 ng/mL for PS-D_4 and AP-D_4, respectively. Low, medium and high concentration quality control (QC) samples were prepared by spiking human albumin solution (200 µL) with 50 µL of the IS solution and 50 µL of the working solutions at three concentration levels (low, medium, high). These QC samples were processed as described above; final concentrations in the obtained samples were: 0.15, 45, 150 ng/mL for PS and 0.01, 3, 10 ng/mL for AP and PREG, respectively; n=5 for each level.

Calibration model

Each calibration sample was assayed in triplicate on three separate days. The regression lines were calculated using a weighed (1/x) linear regression model. Calibration curves were generated from the peak-area ratio of each analyte quantifier transition to the corresponding deuterated IS; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate the standard curves by the method of least squares using a weighed (1/x) linear regression model.

LOD and LOQ

The sensitivity of the developed procedure was evaluated by determining the limit of detection (LOD) and lower limit of quantification (LOQ). The LOQ is the lowest concentration of the analyte in a sample which can be quantified with an acceptable accuracy (±20%) and precision (RSD% < 20%). The LOD and LOQ values for each analyte were calculated from their corresponding calibration plots as 3 σ/S and 10 σ/S, respectively, where σ and S are the standard deviation and the slope of the regression line (International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Harmonized tripartite guideline: validation of analytical procedure: text and methodology Q2(R1),
http://www.ich.org/LOB/media/MEDIA417.pdf (Accessed November 2005).
The LOD and LOQ values for each analyte were then validated by analyzing blank albumin samples fortified with analytes at the calculated LOD and LOQ levels.

Selectivity

Aliquots (200 µL) of human albumin solution were processed as described and analyzed for the presence of any interference across the retention window of each analyte and IS. Absence of interfering components is accepted if the response is less than 20% of the LOQ values for the analytes and 5% for the internal standards.

Imprecision and accuracy

Method imprecision and accuracy were determined by replicate analyses of the QC samples spiked at low, medium and high levels (n=5, each). The within-run and the between-run precision values were evaluated as percent relative standard deviation (RSD%) of the "found" values. RSD % = (SD/mean) x 100. The obtained values should not exceed 15% for the QC samples, except for the LOQ (low level QC samples) which should

not exceed 20% (Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Evaluation, 2001). Accuracy of the method was evaluated by analyzing post-spiked QC samples, i.e. blank albumin samples (200 µL) spiked with the analytes and ISs after the SPE procedure, dried under nitrogen and derivatized; the found levels were compared with the nominal analyte concentration to calculate accuracy, expressed as percent of the estimated concentration. The mean concentration should be within 15% of the nominal values for the QC samples, except for the LOQ which should be within 20% of the nominal value (Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Evaluation, 2001).

Carry-over

Carry-over effect was evaluated by injecting processed blank albumin samples after analyses of calibration samples spiked at the upper limit of quantitation (150 ng/mL for PS and 10 ng/mL for AP and PREG). For acceptance, carry-over in the blank samples following the high concentration should not exceed 20% of the lower limit of quantitation (Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Evaluation, 2001).

Matrix effect and recovery

Matrix effect (ME) and recovery values were calculated for each analyte at low, medium and high concentrations. Matrix effect was determined by dividing the analyte peak area in the post-spiked QC samples to the peak area in absence of matrix (peak area of the neat standard at the same nominal concentration): 50 µL of the IS solution and 50 µL of the working solutions, dried under nitrogen and derivatized with Amplifex Keto Reagent as described. According to Matuszewski et al.,[21] the matrix effect should be between 75% and 125%. Recovery was determined by comparing the levels found in the QC samples spiked before the sample processing with the levels found in the post-spiked QC samples, i.e. with no nominal loss of the target analytes.

Method validation

The following parameters were evaluated according to the Center for Drug Evaluation and Research (CDER) Guidance for Industry (Bioanalytical Method Evaluation, 2001): calibration model, limit of detection (LOD), limit of quantification (LOQ), selectivity, imprecision, accuracy, carry-over, matrix effects, and recovery.

Sample processing

Serum and CSF samples (200 µL) were spiked with 50 µL of the IS solution, vortexed (90 sec) and added with 1000 µL of acetonitrile/methanol (70/30; +1.0% formic acid). The samples were then sonicated (10 min, 4 °C), centrifuged (15.000 rpm, 10 min, 10 °C), and the supernatants were purified on Phree-SPE cartridges to remove endogenous phospholipids. Eluates were evaporated (Concentrator Plus #5305, Eppendorf AG, Hamburg, Germany) at 35 °C and derivatized with 50 µL of Amplifex Keto Reagent for one hour at room temperature. Subsequently, samples were added with 150 µL methanol/water (70/30), centrifuged (15.000 rpm, 10 min, 10 °C) and transferred in autosampler vials for LC-MS/MS analysis. Injection volume: 10 µL.

Patients

We retrospectively considered records of patients admitted with SE between January 2007 and August 2015, who received lumbar puncture at SE onset or during SE (Table S1). Serum and CSF samples were extracted from our CSF bank by considering patients for which the clinical information and electroencephalography (EEG) were diagnostic for SE. SE was defined as ongoing seizures, or repetitive seizures without intercurrent normalization of consciousness or return to baseline for at least 30 min.

The treatment protocol for SE was similar in all the patients and followed the guidelines of the Italian League Against Epilepsy.[11] If seizures persisted after antiepileptic drug treatment, the SE was considered refractory or super-refractory and patients were treated with one or multiple third-line agents.[12] Serum and CSF samples were simultaneously acquired from a few hours after admission to a maximum of 20 days (median of four days).

The SE group was compared with patients that underwent a lumbar puncture for suspected idiopathic intracranial hypertension, CNS infection, or inflammatory disease between 2007 and 2015. Fifty subjects that had negative results at the end of the diagnostic work-up were considered as non-epileptic control population. Nine patients were excluded from this initial group due to pathological findings in CSF parameters (high intracranial pressure, high cell counts, and positivity for oligo-clonal bands). The final control group consisted of 41 subjects, all proved to be healthy people (Table S2). The mean age was 45.6 years (range 16-80), 28 were female (68%).

The health service (AUSL-Modena) Institutional Review Board approved the research protocol according to local regulations in accordance with the current revision of the Helsinki Declaration, and informed written consent was obtained from patients or their relatives.

Table S1. Demographic and clinical features of the patients with *status epilepticus* (SE). Each patient had a head CT and/or brain MRI at hospital admission or within 24 hours. All included subjects had EEG monitoring of variable duration confirming SE diagnosis.

	All (41)	
	n	**(%)**
Female gender	26	63%
Mean age, years (range)	55.8	(11-79)
Previous history of epilepsy°	1	2%
Electroclinical classification		
NCSE	10	24%
GCSE	4	10%
MSE	3	7%
PCSE	24	59%
Etiology Classification*		
Acute Symptomatic	20	49%
Progressive Symptomatic	5	12%
Remote Symptomatic	8	20%
Idiopathic	1	2%
Cryptogenic/*de novo*	7	17%
AED Treatment		
First line: Benzodiazepines	41	100%
Second line: Phenytoin	19	47%
Second line: Valproate	22	53%
Second line: Levetiracetam**	14	34%
Second line: Lacosamide**	4	10%
Third line agents/ICU	18	44%
Resolution	23	56%
Refractory SE (RSE)	18	44%
Return To Baseline Conditions	23	56%
30 days mRS ≥ 3	15	36%
30 days mortality	6	15%
Development of epilepsy***	27	66%

Table legend. NCSE: non convulsive status epilepticus; GCSE: generalized convulsive status epilepticus; MSE: myoclonic status epilepticus; PCSE: partial complex status epilepticus. ICU: intensive care unit; SE: status epilepticus; RSE: refractory status epilepticus. °None of the patients was on AED chronic therapy. *No case with anoxic etiology was included. **As polytherapy with valproate or phenytoin. ***Defined as the occurrence of at least one unprovoked epileptic seizure not earlier than four weeks after termination of SE.

Table S2. Demographic and clinical features of the control group. Each patient had a head CT and/or brain MRI.

	All (41)	
	n	**(%)**
Female gender	28	68%
Male gender	13	32%
Mean age, years (range)	45.6	(16-80)
Previous history of epilepsy°	0	0%
Reason for CSF tap		
Headache	13	32%
Acute confusion	9	22%
Suspected idiopathic intracranial hypertension	10	24%
Suspected normal pressure hydrocephalus	1	2.5%
Suspected optic neuritis	1	2.5%
Loss of consciousness	3	7.3%
Suspected CSF hypotension	4	9.7%

All these patients had normal CSF parameters (glucose; proteins; cell counts; no oligo-clonal bands). The final diagnostic work up excluded a neurological disorder in each subject.

Statistics

Data were compared using the Fisher's exact test or the Mann-Whitney test (Sigmaplot 11, Systat Software, San Jose, CA). Results are presented as median and interquartile range (IQR), and they were regarded significantly different at $p < 0.05$.

RESULTS

Steroids were derivatized as indicated in Fig. S1,[13] and calibration curves were obtained for each analyte (Fig. S2). Elution ensured the complete separation of PREG and AP and prevented any interference with other analytes (Fig. S3). Multiple reaction monitoring (MRM) ratios (Table S3) confirmed that interference from the biological matrix was prevented (Fig. S4). Our method was validated (Tables S4 and S5) and allowed an accurate and precise detection of AP, PREG, and PS. Each analyte level was determined via regression curve of the area ratios of the analyte to the corresponding IS.

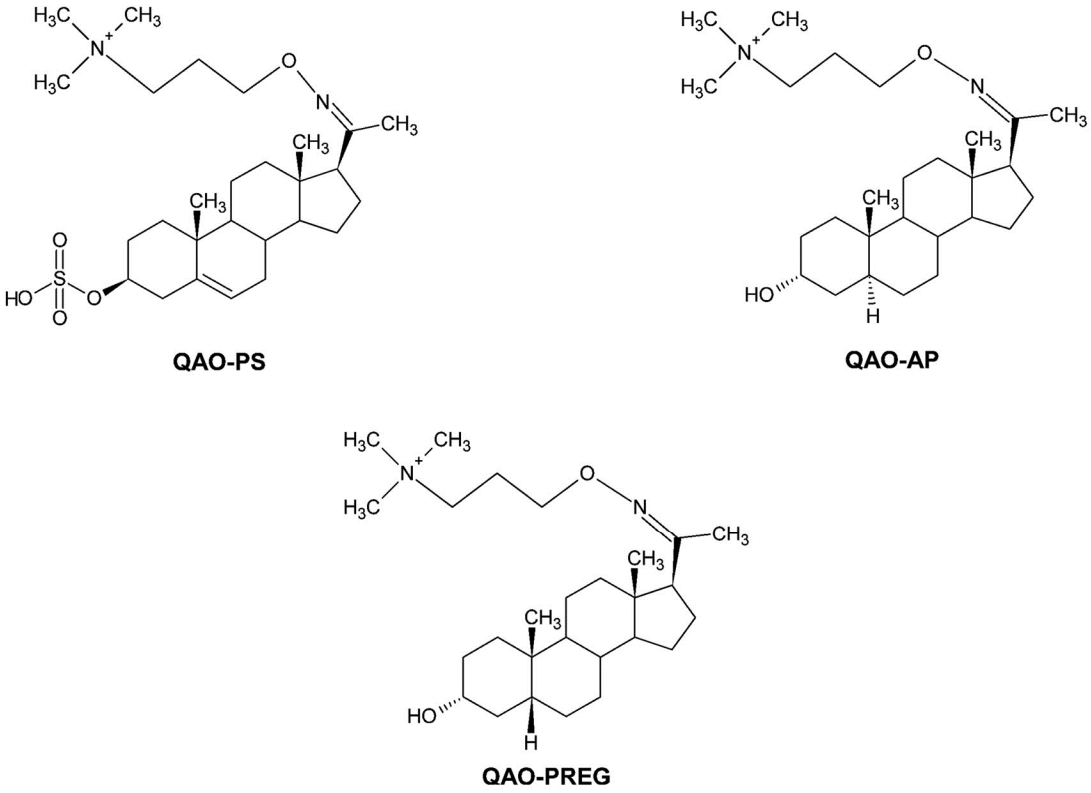

FIGURE S1

Chemical structures of the selected neurosteroids as quaternary aminooxy (QAO) derivatives. O-(3-trimethylammonium-propyl) hydroxylamine) bromide, commercially available as AmplifexTM Keto reagent**,** was used for derivatization**.** The QAO-derivatization at C-20 position leads to the formation of both *cis*- and *trans*-isomers, with the *trans*-isomer accounting for approximately 95% of the total derivatization products, since the formation of the *cis*-isomer would be unfavorable because of steric hindrance.[13] PS: pregnenolone sulfate; AP: allopregnanolone; PREG: pregnanolone.

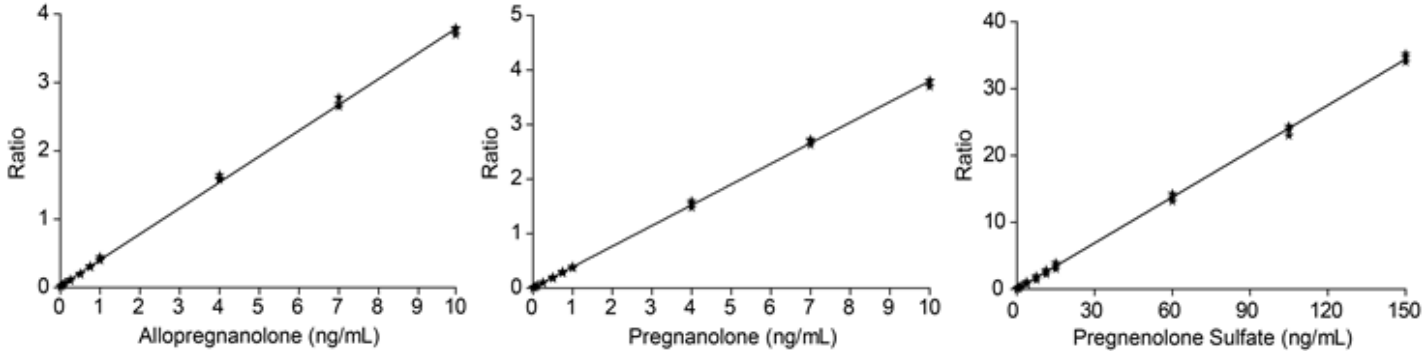

FIGURE S2

Calibration curves for quantification of pregnenolone sulfate, pregnanolone and allopregnanolone. Ratios were obtained by normalizing peak-area of the analyte quantifier transition with that of the corresponding internal standard (PS-D_4 for pregnenolone sulfate, and AP-D_4 for allopregnanolone and pregnanolone).

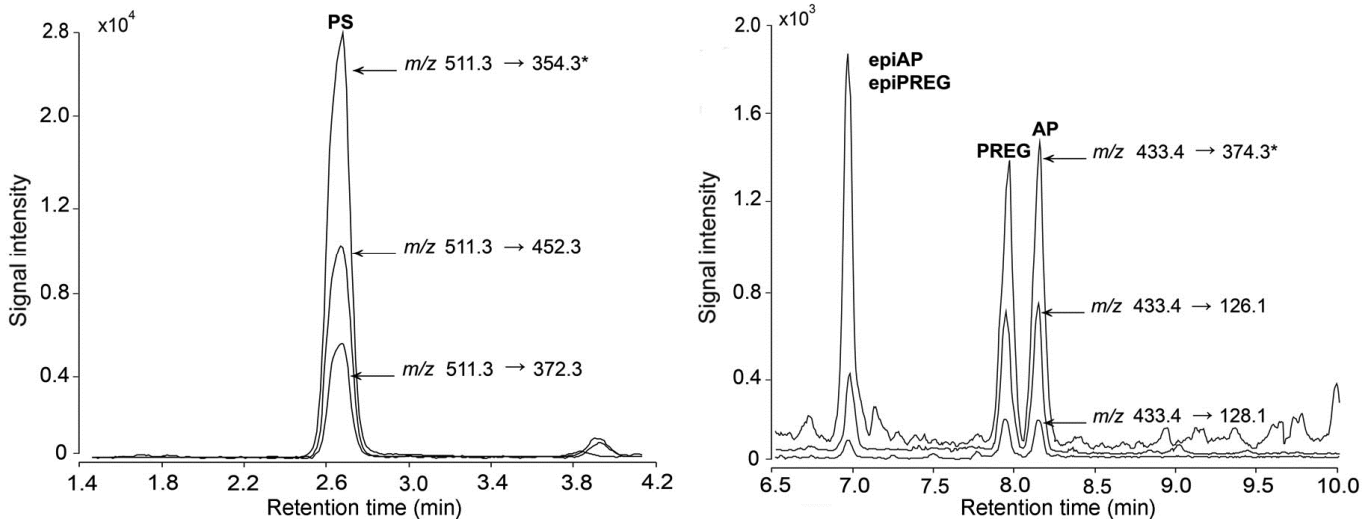

FIGURE S3

Representative MRM chromatogram of a calibration sample showing the monitored transitions for the selected neurosteroids as QAO-derivatives. The quantifier transitions are marked with an asterisk. The quantifier MRM transition of QAO-PS showed a 13-fold sensitivity enhancement compared to that of underivatized PS at the same concentration; in this case the derivative was analyzed in the positive mode and the underivatized analyte in the negative mode, due to its poor ESI ionization. The QAO-derivatization led to a marked sensitivity enhancement (≥ 40-fold) also for the quantifier transition of AP and PREG. For the deuterated internal standards (ISs) we monitored the same transitions of the target analytes with *m/z* values shifted to the corresponding mass values. The LC-MS/MS conditions were optimized by analyzing standard solutions and also spiked blank albumin extracts. This sample was also spiked with epiAP and epiPREG.

As the four reduced progesterone metabolites (AP, PREG, epiAP and epiPREG) have common precursor ion and MRM transitions, therefore a satisfactory chromatographic resolution is required for their accurate quantification. PS: pregnenolone sulfate (1.5 ng/mL); AP: allopregnanolone (0.1 ng/mL); PREG: pregnanolone (0.1 ng/mL). The sample was also spiked with epiallopregnanolone (epiAP) and epipregnanolone (epiPREG), both at 0.05 ng/mL.

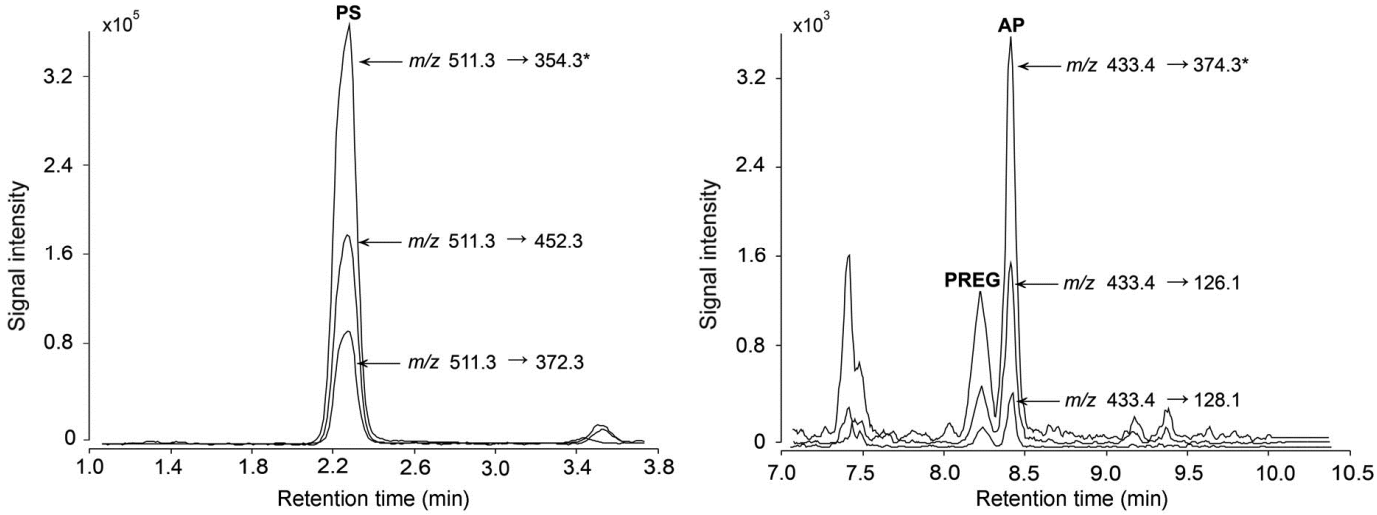

FIGURE S4

Representative MRM chromatogram showing the QAO-neurosteroids in serum of a control subject. The quantifier transitions are marked with an asterisk. To test the best solvent system for protein precipitation, human albumin aliquots (200 µL) were spiked with known amount of the target analytes, vortexed, and added with 1.0 mL of: pure ACN, pure MeOH and ACN/MeOH in different ratios (70/30, 50/50 and 30/70). All the mentioned solvents (and mixtures) were added with 1.0% formic acid, in order to comply with the requirements of the chosen SPE stationary phase. The obtained samples were processed and derivatized as described above. Best results in terms of recovery were achieved using ACN/MeOH (70/30; +1.0% formic acid); the procedure led to satisfactory results in terms of signal response and retention time reproducibility, confirming the adequate phospholipid removal. PS: pregnenolone sulfate, AP: allopregnanolone, PREG: pregnanolone.

Table S3. Experimental HPLC-MS/MS parameters for the target neurosteroids as QAO (quaternary aminooxy) derivatives. Best chromatographic results in terms of resolution and peak shape were achieved under the chromatographic conditions described above. The cis- and trans-isomers for each derivatized analyte were well separated and the ratio of the major to minor derivatization product was consistent from run to run and between the calibration samples. The experimental LC-MS/MS parameters for the derivatized neurosteroids are summarized; only the dominant trans-isomers were monitored for the quantification of the target analytes. For each analyte, one precursor ion and three MRM transitions were set up, monitoring the more abundant product ion for quantification and less abundant product ions as qualifier ions for confirmation. PS: pregnenolone sulfate, PREG: pregnanolone, AP: allopregnanolone.

	MRM transitions (m/z)[a]	MRM ratio[a]	Fragmentor Voltage (V)	CE (eV)	Dwell time (msec)	T_R (min)[b]	RSD% ^{TR}
QAO-PS	**511.3 → 354.3**		88	**28**	100	**2.78**	0.62
	→ 452.3	MRM1/MRM2: 2.0	88	20	100	3.89	
	→ 372.3	MRM1/MRM3: 3.2	88	32	100		
QAO-PS-D_4	**515.3 → 358.3**		88	**32**	100	**2.76**	0.61
	→ 456.3	MRM1/MRM2: 2.1	88	20	100	3.86	
	→ 376.3	MRM1/MRM3: 3.5	88	28	100		
QAO-PREG	**433.4 → 374.3**		132	**24**	100	**7.99**	0.57
	→ 126.1	MRM1/MRM2: 2.7	132	40	100	9.51	
	→ 128.1	MRM1/MRM3: 11.5	132	38	100		
QAO-AP	**433.4 → 374.3**		132	**24**	100	**8.37**	0.49
	→ 126.1	MRM1/MRM2: 2.6	132	40	100	9.82	
	→ 128.1	MRM1/MRM3: 10.2	132	38	100		
QAO-AP-D_4	**437.4 → 378.3**		88	**24**	100	**8.33**	0.50
	→ 130.1	MRM1/MRM2: 3.3	88	40	100	9.80	
	→ 132.1	MRM1/MRM3: 15.0	88	40	100		

[a]Bold masses depict the quantifier transition, while second (MRM2) and third (MRM3) transitions were used for confirmation purposes (qualifier transitions). [b] The retention time (T_R) was a mean of 30 analyses (10 calibration samples analyzed in triplicate); bold retention times are referred to the predominant *trans*-isomer

Table S4. Calibration curve parameters for the derivatized neurosteroids (as QAO derivatives). Calibration range: 0.15 – 150 ng/mL for PS; 0.01 – 10 ng/mL for AP and PREG. Linearity was adequate in the range 0.15-150 ng/ml for PS and 0.01-10 ng/mL for AP and PREG, with correlation coefficient values (r^2) of at least 0.994. The LOQ values were 10 pg/mL for PS and 5 pg/mL for AP and PREG, confirming the satisfactory sensitivity of the developed procedure. The LOQ values fulfilled the usually acceptance criteria, being the corresponding deviation from the expected concentration (% accuracy) less than 20%. Monitoring the MRM transitions shown in Table S2, no co-eluting peaks >20% of the analyte peak area at the LOQ level, and no co-eluting peaks >5% of the internal standard peak area were observed in albumin samples; therefore the developed procedure was found to be selective for the target analytes. PS: pregnenolone sulfate, PREG: pregnanolone, AP: allopregnanolone.

	Slope (± SD)	Intercept (± SD)	r^2	LOD (pg/mL)	LOQ (pg/mL)
QAO-PS	0.228 (± 0.006)	+ 0.041 (± 0.012)	0.994	3	10
QAO-PREG	0.381 (± 0.004)	+ 0.008 (± 0.001)	0.998	1.5	5
QAO-AP	0.379 (± 0.013)	+ 0.014 (± 0.001)	0.996	1.5	5

Table S5. Method validation data for the derivatized neurosteroids (as QAO derivatives): imprecision (relative standard deviation, RSD%), accuracy and recovery values at low, medium and high concentrations (0.15, 0.45, 150 ng/mL for PS and 0.01, 3, 10 ng/mL for AP and PREG, respectively) in quality control samples. PS: pregnenolone sulfate, PREG: pregnanolone, AP: allopregnanolone.

	Within-run RSD%			Between-run RSD%			Accuracy (%)			Recovery (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
QAO-PS	8.9	6.5	5.7	9.7	7.1	6.4	94.8	96.1	97.3	86.6	87.9	88.6
QAO-PREG	10.8	7.2	6.3	11.8	8.3	7.2	93.2	94.4	95.9	87.1	88.2	89.6
QAO-AP	10.2	6.7	5.9	11.5	8.1	6.8	94.3	95.2	96.1	87.4	88.1	90.8

In CSF obtained from both patients affected by SE and controls, AP was clearly detectable in most but not all samples (Fig. 1). Specifically, AP was below the limit of quantification in 15 out of 40 controls (one sample was missing), and in 28 out of 41 patients affected by SE ($p < 0.01$, Fisher's exact test). Then, we compared AP values by excluding subjects in which the analyte was undetectable, and found significantly lower levels (-30%; $p < 0.05$, Mann-Whitney test) in patients affected by SE (Fig. 1A). This difference was unaffected by excluding outliers from controls. At variance, AP was detectable in all serum samples, but no difference was found by comparing patients affected by SE with controls (Fig. 1B).

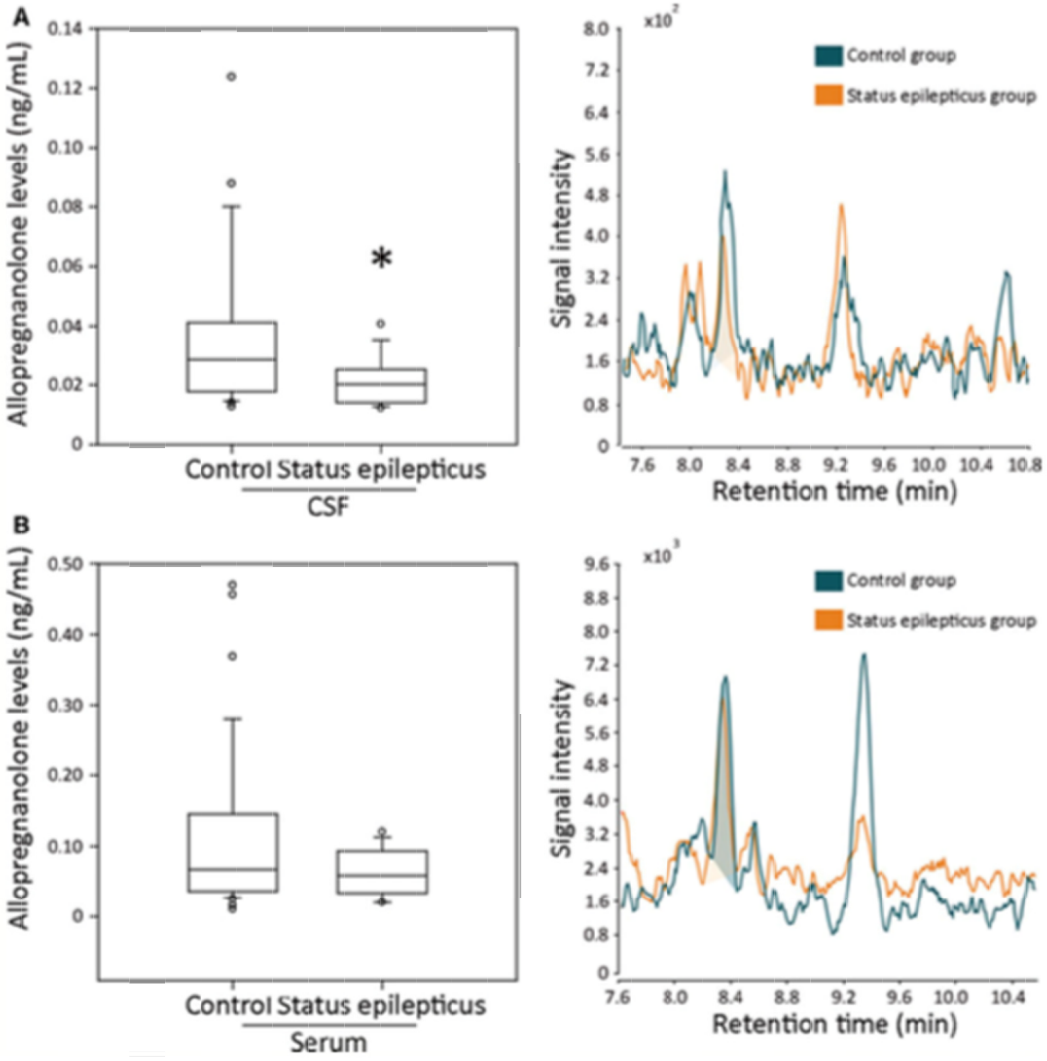

FIGURE 1

FIGURE 1. Allopregnanolone (AP) levels measured in cerebrospinal fluid (CSF) and serum in the course of *status epilepticus* (SE). In **A**, AP levels in CSF, illustrated in boxplot, were significantly lower (*p < 0.05, Mann-Whitney test) in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right (red, SE group; black, control group). In **B**, AP levels in serum were not significantly different in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right (red, SE group; black, control group).

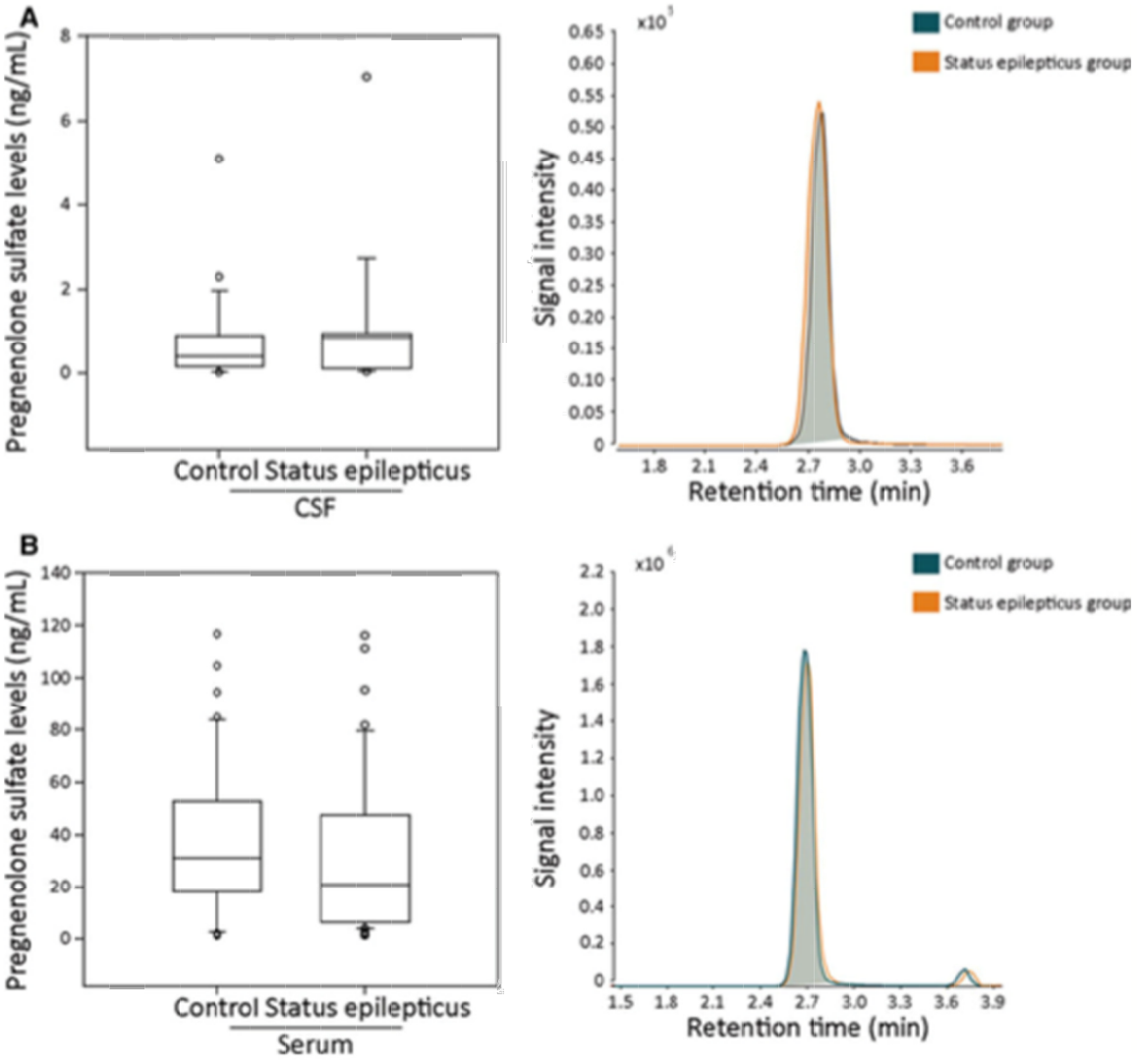

FIGURE 2
Pregnenolone sulfate (PS) levels measured in cerebrospinal fluid (CSF) and serum in the course of status epilepticus (SE). In **A**, PS levels in CSF, illustrated in boxplot, were significantly similar in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right (red, SE group; black, control group). In **B**, PS levels in serum were not significantly different in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right (red, SE group; black, control group).

Changes similar to those found for AP were demonstrated also for PREG (not shown). Specifically, PREG was measured in CSF of 32 controls (0.022 ng/mL, IQR 0.016 to 0.025) and 34 patients (0.018 ng/mL, IQR 0.014 to 0.020; $p < 0.05$). No differences were instead found for serum levels (controls: 0.030 ng/mL, IQR 0.018 to 0.064; patients: 0.028 ng/mL, IQR 0.018 to 0.063).

Peaks corresponding to PS were detected in all sera, whereas 34 out of 41 patients affected by SE and 36 out of 40 controls presented detectable PS in CSF. As shown in Fig. 2A, PS levels were not significantly different in CSF. Similarly, quantification of PS in serum of both groups did not reveal significant differences (Fig. 2B).

Finally, we considered the possibility that the alterations found in AP and PREG levels could be explained by CSF albumin content or, additionally, could be influenced by sex differences. However, albumin was present at similar concentration in CFS of both groups (SE patients: 19.1 mg/dL, interquartile range 12.8 to 25.1; controls: 17.9 mg/dL, interquartile range, 11.2 to 22.7), and similar levels were observed for all analytes in both males and females of controls (Table S6) and patients (Table S7).

Table S6. Allopregnanolone (AP), pregnanolone (PREG), and pregnenolone sulfate (PS) levels measured in cerebrospinal fluid (CSF) and serum of male and female control subjects. Data are reported as median and interquartile range. No significant differences were found.

	CSF		**SERUM**	
	Male	**Female**	**Male**	**Female**
AP	0.029 (0.018-0.049)	0.029 (0.017-0.041)	0.062 (0.033-0.105)	0.082 (0.036-0.185)
PREG	0.023 (0.017-0.026)	0.021 (0.015-0.024)	0.031 (0.017-0.064)	0.029 (0.021-0.065)
PS	1.379 (0.465-1.965)	0.626 (0.329-1.205)	62.220 (27.480-102.655)	40.930 (24.050-68.805)

Table S7. Allopregnanolone (AP), pregnanolone (PREG), and pregnenolone sulfate (PS) levels measured in cerebrospinal fluid (CSF) and serum of male and female patients affected by SE. Data are reported as median and interquartile range. No significant differences were found.

	CSF		SERUM	
	Male	**Female**	**Male**	**Female**
AP	0.020 (0.131-0.031)	0.020 (0.015-0.026)	0.039 (0.022-0.056)	0.037 (0.025-0.079)
PREG	0.018 (0.015-0.020)	0.017 (0.014-0.020)	0.018 (0.015-0.06)	0.036 (0.021-0.062)
PS	0.626 (0.297-1.228)	0.481 (0.270-1.260)	43.015 (16.23-108.59)	24.160 (6.855-55.350)

DISCUSSION

We evaluated AP, PREG and PS in CSF of patients affected by SE using a modified LC-MS/MS approach. Major achievements of this investigation were (i) the possibility to separate and quantify AP and PREG, and (ii) the observed significant reduction in CSF but not in serum of these steroids, in presence of (iii) stable levels of PS. These results suggest that synthesis of endogenous anticonvulsants such AP and PREG is defective or that, alternatively, their catabolism is enhanced in patients in which seizures evolved into SE. Moreover, reduction in their levels may result in enhanced modulation by proconvulsant steroids such as PS.

The possibility to characterize the role of neuroactive steroids in humans and animal models has been hampered by technical limitations, of which the "matrix effect" is the most important for LC-MS/MS.[14] Here we show that it is possible to overcome this specific problem by increasing the efficiency of phospholipid removal.[7] By this strategy we obtained a specific identification and quantification of AP, PREG and PS in human CSF and serum samples, leading to the observation of the selective reduction in CSF AP and PREG levels. As no other data are available on these steroids in SE, we cannot exclude that the observed changes might have preceded the SE. The few available studies on peripheral AP in epileptic patients suggest the presence of reduced interictal levels[15] and biphasic changes following a seizure.[6] However, almost all of our patients were seizure-free before to develop the SE.

Indeed, the role of AP has been investigated both in animal models and humans and inhibition of 5α-reductase by finasteride consistently resulted in increased epileptic

activity.[2,4,6,15] No information is instead available for 5β-reductase, which synthesizes PREG. Thus, it is possible that a reduced activity of 5α-reductase in SE could be responsible for lower AP availability in CSF. However, we found that also PREG was affected in a similar manner, suggesting that 5β-reductase and maybe other enzymes could be dysregulated in SE. Alternatively, a reduced availability of a common substrate of these enzymes, such as progesterone, may be the reason for the reduced availability of AP and PREG in SE. In such a case, restorative therapies could be taken into account.

Because of their lipophilic nature, steroids require a carrier to be solubilized in CSF. For this reason, we hypothesized that changes in CSF albumin could explain the differences observed in AP and PREG levels. However, albumin levels were similar in both groups of patients, suggesting that AP was less available under SE. Additionally, no differences were found for the proconvulsant neurosteroid PS, for which the number of available samples was similar to that of PREG, so that we exclude that differences in albumin or other carriers could have affected our results.

In conclusion, an important consequence of our study is that AP and PREG reduction may contribute to the epileptic activity observed in SE. In such a case, to reestablish levels of these steroids may be an important therapeutic target in patients affected by SE. Indeed, the limited, but encouraging evidence obtained in pediatric patients treated with AP to stop super-refractory SE is in agreement with this hypothesis.[10]

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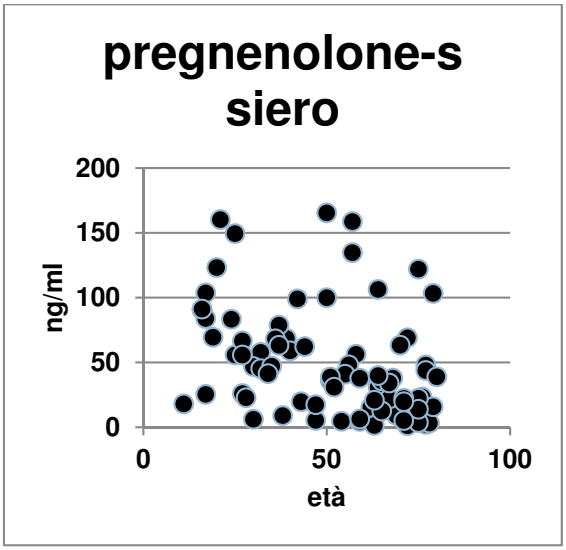
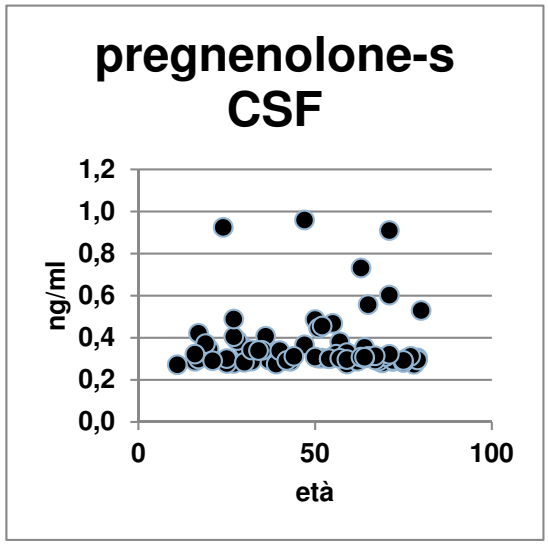
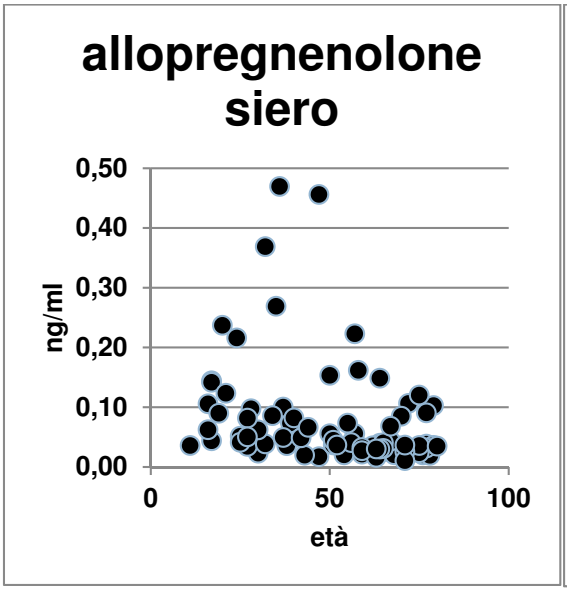
C. Discussion & perspective

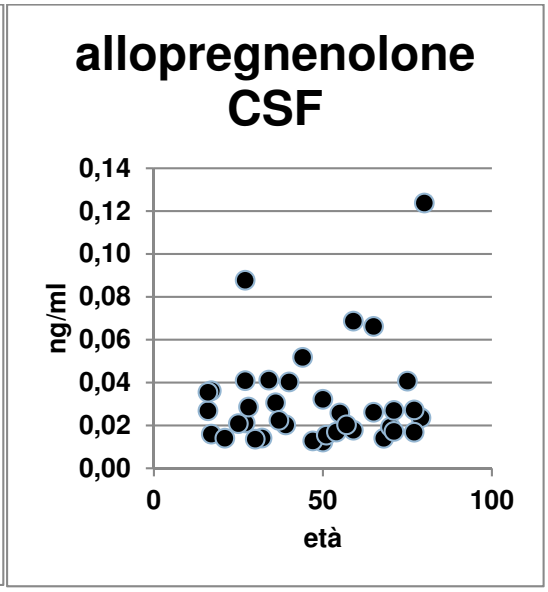
In addition to these published data, we evaluate also the possible influence of age on neurosteroids. We found that:

- Patients group has a median age slightly higher than controls.
- We found that age didn't influence serum and CSF values of AP and PS. Analysis in the control group confirm the data in the entire sample.

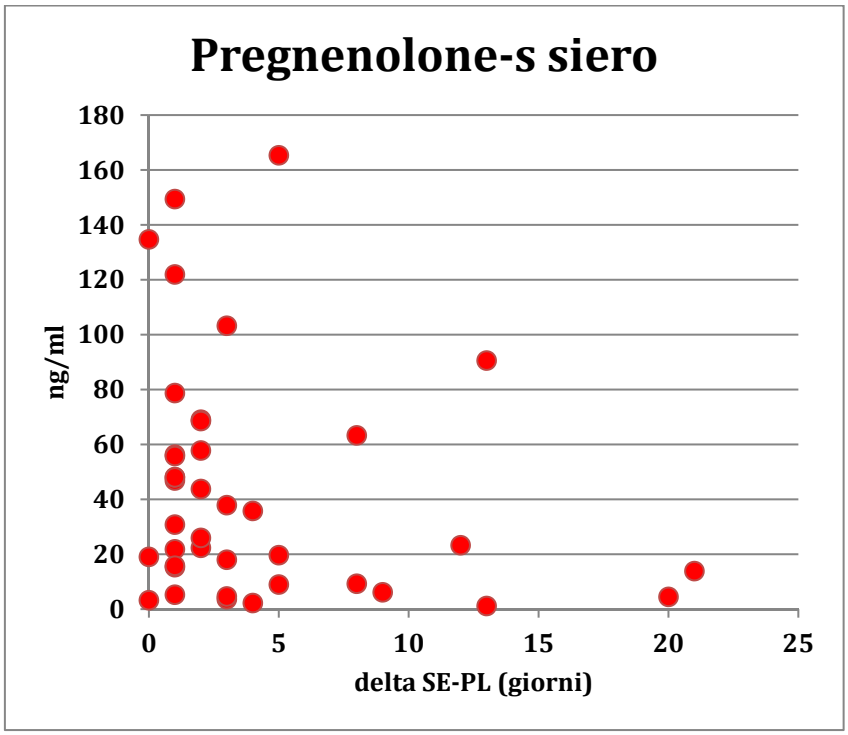


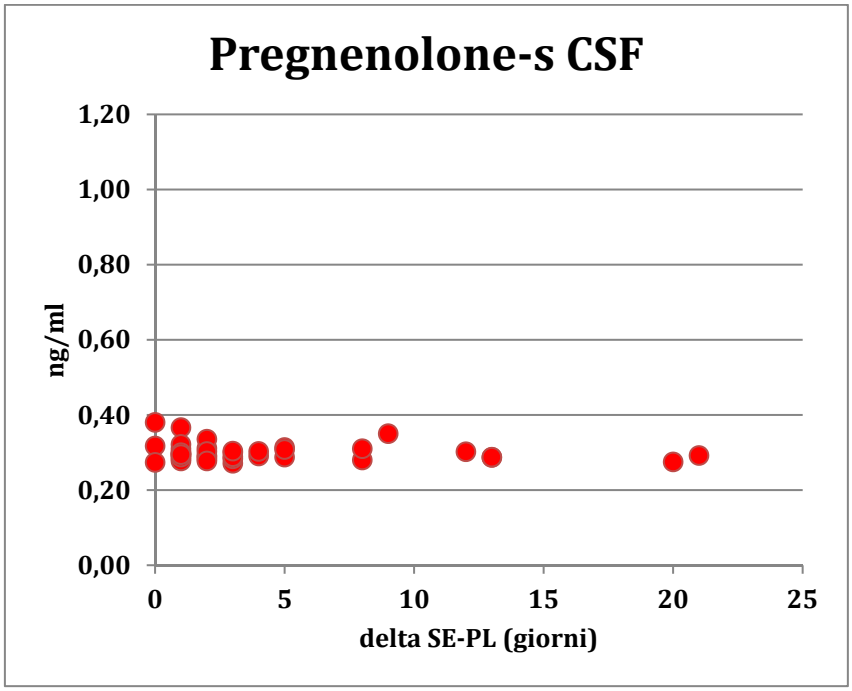


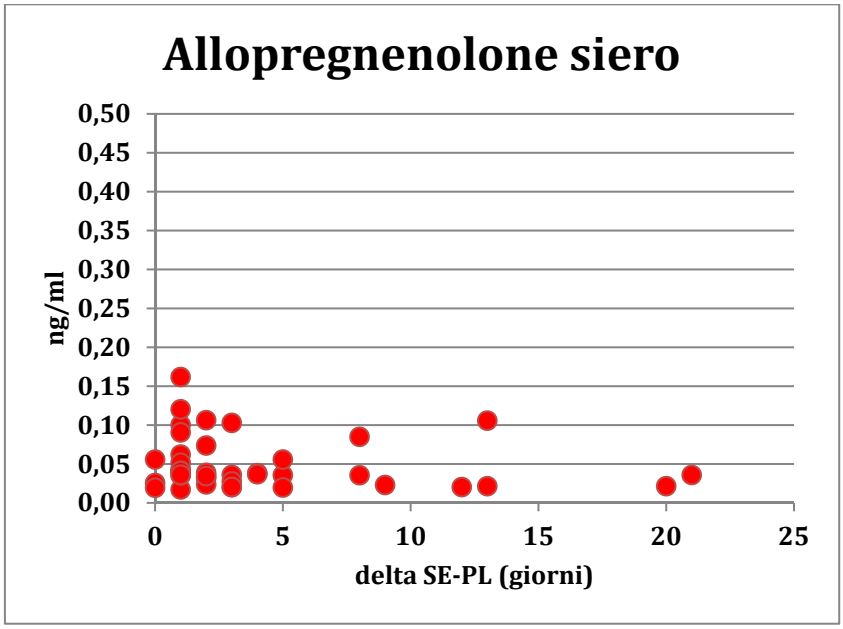


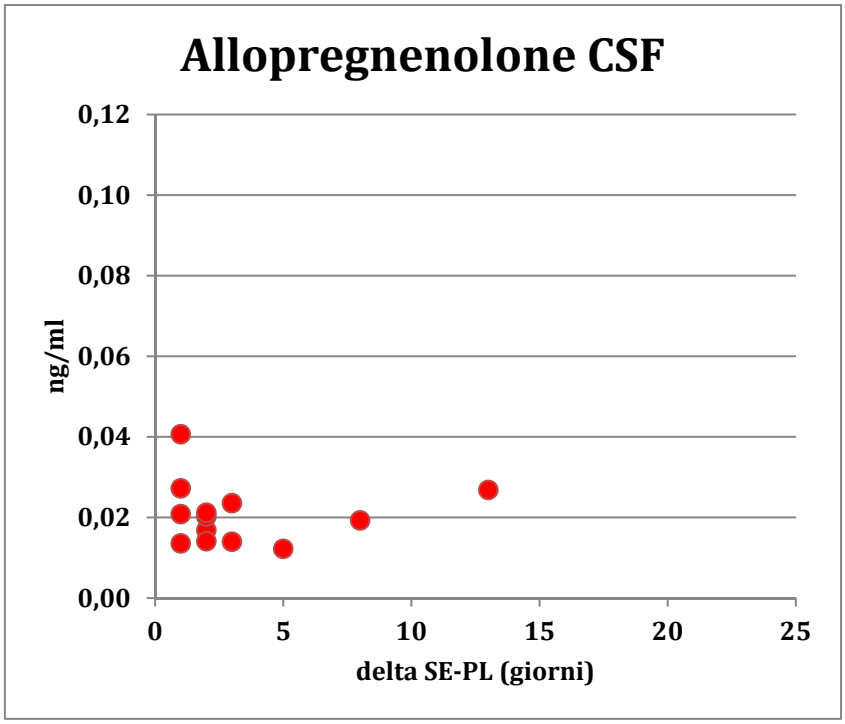


- Timing of lumbar puncture didn't influence the neurosteroids values, nor in serum nor in CSF. Limits are due to the small sample, in particular for CSF in which AP and PS are detected often with under threshold values.









In conclusion, we need to increase the number of patients and controls that undergo to simultaneous detection of serum and CSF to confirm our hypothesis on AP role in status epilepticus.

2.3 MRI FINDING AS POTENTIAL BIOMARKER IN STATUS EPILEPTICUS: THE ROLE OF CLAUSTRUM

A. Motivation

SE causes energy failure of the Na/K ATPase pump, which in turn causes sodium and water influx leading to cellular swelling (cytotoxic edema). Excessive release of excitatory amino acids such as glutamate and increased membrane ion permeability are postulated as other mechanisms that cause cytotoxic edema. The resultant cytotoxic edema is evident on imaging in various parts of the brain and is usually associated with mild mass effect.

Transient focal hyperintensity on diffusion-weighted MR images (DWI) with corresponding reduction of the apparent diffusion coefficient (ADC) is an increasingly recognized phenomenon in the peri-ictal phase of epileptic seizures. It has been described in experimental models of epilepsy as well as in patients, especially in association with prolonged seizures or status epilepticus (SE)[1,2,3]. These DWI alterations are thought to be associated with increased energy metabolism and hyper-perfusion, so DWI can be useful to locate the epileptogenic focus[4,5]. DWI seems to visualize pathophysiological aspects early after onset of seizure activity such as source of ictal activity, of seizure propagation and mechanisms of chronic tissue damage.

Principal MRI abnormalities were found in the focal cortex, hippocampus, thalamus, and splenium in focal SE patients[3,4,6,7,8,9,10,11,12]; in the generalized convulsive SE (GCSE) patients, these MRI abnormalities were usually widespread across the cortical areas and thalamus, accompanied with crossed cerebellar diaschisis sometimes[13,14,15]. These MRI changes were often reversible but in severe or prolonged seizures can become irreversible and lead to permanent brain damage such as brain atrophy, laminar necrosis, or medial temporal sclerosis.

In some cases of refractory SE, abnormal lesions were observed in the putamen, globus pallidus, and caudate nucleus. Although some speculate that the basal ganglia are a part of the modulatory control system in epileptic seizure[16], the exact mechanism behind the MRI changes in the basal ganglia is still not clear[17]. Moreover, Cianfoni et al. described that the presence of brain abnormalities on the MRI study obtained after a single seizure or epileptic status, is generally considered as the origin, the cause of epilepsy; nevertheless, MRI changes may be sometimes its consequence[18].

In literature, several papers described post-ictal MRI changes in patients with status epilepticus and some brain regions seem to be "typical" findings related to the seizures activity. Most of the authors try to explain the wide spectrum of seizure induced MRI abnormalities as a pure consequence of the repeated seizures. They did not consider that all those patterns were probably associated to an equally wide spectrum of undiagnosed, obscure underlying conditions[19].

Recently, the same author published a bibliographic search to address the quality of evidence in clinical reports supporting the assertion that brain MRI signal abnormalities are a direct consequence of seizures[20]. The search resulted in 91 publications corresponding to 413 cases. There was a wide range of clinical features and EEG and MRI abnormalities. Premorbid or comorbid conditions were present in many cases, and some of them are potential causes of MRI changes. Claimed evidence for MRI signal abnormalities as a direct consequence of ictal activity was mostly based on the similarity with previous reports, animal models, reversibility, congruent EEG, MRI changes not respecting vascular territories, and ruling out other etiologies. So, the author concluded that evidence supporting the notion of seizure-induced excitotoxicity is questionable in the studied reports of postictal MRI abnormalities.

This point of view stimulates the search for new etiologies in the context of de novo refractory SE. Especially, some MRI pattern could become the radiological marker of specific pathological entities. In this direction goes our research on patients with an acute de novo refractory status epilepticus, started after a febrile illness, that develop reversible, isolated, bilateral claustrum hyperintesity after few days from seizures onset.

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B. Study 3. CLAUSTRUM DAMAGE AND REFRACTORY STATUS EPILEPTICUS FOLLOWING FEBRILE ILLNESS

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Key words: status epilepticus; myoclonus; MRI; fever; NORSE; FIRES; claustrum.

ABSTRACT

Objective: to characterize the clinical, EEG, and brain imaging findings in an adult case-series of patients with de novo refractory status epilepticus (SE) occurring after a febrile illness. **Methods**: a retrospective study (2010-2013) was undertaken with the following inclusion criteria: *(a)* previously healthy adults with refractory SE; *(b)* seizures onset 0–21 days after a febrile illness, and *(c)* lacking evidence of infectious agents in CSF; (*d*) no previous history of seizures (febrile or afebrile), as well as previous or concomitant neurological disorder. **Results**: among 155 refractory SE cases observed in the study period, six (17 – 35 years-old) fulfilled the inclusion criteria. Confusion and stupor were the most common symptoms at disease onset, followed after a few days by acute repeated seizures that were uncountable in all but one. Seizures consisted of focal motor/myoclonic phenomena with subsequent generalization. Anti-epileptic drugs failed in every patient to control seizures, all subjects requiring intensive care unit admission. Barbiturate-coma with burst-suppression pattern was applied in four out of six patients for 5 – 14 days. One subject died in the acute phase. In each patient we

observed a reversible bilateral Claustrum MRI hyperintensity on T2 weighted sequences, without restricted diffusion, time-related with SE. All patients had negative multiple neural antibodies testing. Four out of five surviving patients developed chronic epilepsy.

Conclusions: this is a hypothesis-generating study of a preliminary nature supporting the role of the Claustrum in post-febrile de novo SE; future prospective studies are needed to delineate the specificity of this condition, its pathogenesis, and the aetiology.

INTRODUCTION

In the last two decades several authors described a series of syndromes characterized by the development of a difficult to treat status epilepticus (SE) in previously healthy children after a febrile illness.[1-7] The condition is characterized by a refractory status epilepticus and followed by drug resistant epilepsy, with often severe neuropsychiatric sequelae or death. These entities have been named with different acronyms but probably FIRES ("febrile infection related epilepsy syndrome") is the one that best underscores the main features of the disorder.[8] Cases with a similar clinical picture have been described also in adults and in these case-series the most frequently used definition is "new onset refractory status epilepticus" (NORSE).[9-13] Recently, it has been pointed out that probably different terms has been used to describe the same condition.[14-15] However, there is no consensus among investigators. Adult cases are more heterogeneous, some with clear similarities with FIRES cases (with only the age at onset as the main difference), while other representing probably different conditions.

We describe here a case-series and a literature review of young adults fulfilling the definition of FIRES, all showing in the early acute phase of the disease a striking alteration of the Claustrum on MRI, mainly bilaterally.

The Claustrum function has remained obscure for decades. In 1996 its involvement in a case of de novo refractory status was reported for the first time.[21] Only in recent years the scientific community has gained a strong interest in the function of the Claustrum, mainly with respect to its role in sensory integration and consciousness.[16-20]

METHODS

Patients and investigations

In a retrospective multicentric study (Gen 2010 – Dec 2013) information including demographic data, clinical features, diagnostic findings, therapeutic interventions, and clinical outcomes of patients fulfilling the following inclusion criteria were acquired: *(a)* previously healthy adults (> 16 years of age) with refractory SE [failed intravenous treatment with anti-epileptic drugs, requiring general anesthesia];[22] *(b)* onset of seizures 0–21 days after a febrile illness, and *(c)* lacking evidence of infectious agents in CSF. Exclusion criteria were a previous history of seizures (febrile or afebrile), as well as previous or concomitant neurological disorder. Data were extracted from the participating centers reviewing clinical charts, EEG (video-EEG) recordings and MRI imaging (when available). During the study period 155 cases of refractory SE were observed. Among these, six fulfilled our inclusion criteria.

No family history for febrile seizures or epilepsy was reported. No one had personal and familial history of immunological disorder.

All patients were examined for viral and bacterial infections. The following blood and cerebrospinal fluid (CSF) analyses were performed: viral tests including HSV 1 and 2, varicella-zoster virus, HHV 6, cytomegalovirus, Epstein–Barr virus, rubella, parvovirus B19, enterovirus, and mumps. All virological studies were performed using DNA polymerase chain reaction (PCR). The presence of oligoclonal IgG bands (OB) was checked with isoelectro-focusing (IEF), performed with agarose gel support. Antinuclear antibodies (ANA), anti-phospholipid antibodies, anti-DNA antibodies, anti-cardiolipin antibodies, anti-extractable nuclear antigen antibodies (anti-ENA), anti-thyroid antibodies were analyzed with immuno-enzymatic tests and indirect immuno-fluorescent staining.

Thorax-abdomen computed tomography was performed in all patients showing no occult neoplasm. An extensive blood analysis and testing of classical onco-neural antibodies (anti-GAD, anti-Yo, Ri, Hu, anti-Ma2) was negative in all subjects.

Standard Protocol Approvals, Registrations, and Patient Consents

The scientific advisory boards of participating institutions approved the research protocol according to local regulations and consent was obtained from patients or their relatives. This research is reported following the STROBE guidelines.

Literature Review

Searches for identification of studies were run in from 1990 to 2014 in MEDLINE and PubMed. Searches were limited from 1990 to the present day since studies carried out previously would necessarily have included participants without MRI. The search keywords were: “AERRPS”; “FIRES”; “NORSE”; “status epilepticus” AND “Claustrum”; “epilepsy” AND “Claustrum”; “status epilepticus” AND “neuroimaging”. For each citation considered, the abstract was read (when available). The bibliography of each of the retrieved papers was examined to identify relevant references that could have been missed by electronic search. Only peer-reviewed original articles providing images of the Claustrum involvement were accepted for inclusion in the review.

RESULTS

Clinical data, interventions, and outcomes of the six patients are summarized in **Table 1**. Age at onset was between 17 – 35 years; different ethnicities were represented. Fever, in the context of a flu-like condition, preceded the onset of neurologic symptoms in all patients. Confusion, stupor, sleepiness, and signs of consciousness alteration were the most common symptoms at disease onset, followed after a few days by acute repeated seizures that were uncountable in all but one patient. Seizures consisted in all subjects in focal motor/myoclonic events with alternating side involvement and subsequent generalization. In patients # 1 a dramatic generalized myoclonus was present through the entire acute phase of the disease. Anti-epileptic drugs (AED) failed in every patient to control seizures, all subjects requiring intensive care unit admission and treatment with midazolam and propofol. Barbiturate-coma with burst-suppression pattern was applied in four out of six patients for 5 – 14 days. Corticosteroid i.v. treatment was used as adjunctive therapy in all patients during the acute early phase, while intravenous immuno-globulins (IVIG) or plasma exchange (PEX) were used in four patients. One patient did not survive the acute phase (pt #3: sepsis during ICU staying three weeks after onset). One of the surviving patients was lost to follow-up since he returned to his home country (pt. #5). Four are on AED therapy: three have active chronic focal epilepsy despite adequate drug regimens, one presented seizure clusters for months despite AED poly-therapy before epilepsy control.

Table 1. Most important clinical data in the reported patients.

Pt.	Age Sex Ethnicity	Fever* (days)	Presenting symptoms	Seizures semiology	Frequency of seizures	Evolution of SE	Need of ICU	AED used	Other Drugs	Barbiturate Coma (Days)*	Immuno therapy	Clinical outcome	Seizure outcome
1	29 F Caucasian	Yes (5)	Confusion/ stupor	Staring/eye deviation and focal hemiclonic; then generalized myoclonic state	Uncountable	Super-refractory	Yes	PHT; LEV; LCM	MDZ; Propofol	Yes (10)	Steroids, IVIG.	Normal life; mild deficits in executive functions	Seizure-free on LEV, CLB
2	24 F Caucasian	Yes (7)	Confusion/ sleepiness	Focal hemiclonic, myoclonic; then generalized	Uncountable	Refractory	Yes	PHT, LEV, TPM, CLB	MDZ, Propofol	No	Steroids	Cognitive and behav. deficits	Focal epilepsy (2-3 s/m) on LEV, CBZ, CLB
3	29 F Asian	Yes (5)	Confusion/ stupor	Focal hemiclonic, myoclonic; then generalized	Uncountable	Super-refractory	Yes	VPA, PHT, TPM, LEV, LCM, CBZ	MDZ, Propofol	Yes (14)	Steroids	Death	-
4	35 M Caucasian	Yes (6)	Seizures	Eye deviation and focal hemiclonic, with alternating side; then generalized	Uncountable	Refractory	Yes	VPA, LEV, PHT	MDZ	No	Steroids, PEX	Normal life, slight attentional deficits	Focal epilepsy (1 s/m) on LTG, LEV
5	21 M Arabian	Yes (3)	Headache/ sleepiness	Focal hemiclonic, with alternating side; then generalized	Uncountable	Super-refractory	Yes	VPA, PHT, LEV, TPM	MDZ, Propofol	Yes (5)	Steroids, IVIG	Normal life.	Not available
6	17 F Caucasian	Yes (6)	Stupor/ severe sleep disruption	Focal motor, tonic, chewing and complex automatisms	Isolated seizures	Super-refractory	Yes	VPA; CBZ; PB	MDZ, Propofol	Yes (7)	Steroids, IVIG	Mild cognitive deficit, attention deficit	Seizure-free final follow-up

Table legend: F: female; M: male; IVIG: intravenous immuno-globulins; PEX: plasma exchange; CBZ: carbamazepine; CLB: clobazam; PHT: phenytoin; LCM: Lacosamide; LEV: levetiracetam; LTG: lamotrigine; MDZ: midazolam; OXC: oxcarbazepine; TPM: topiramate; VPA: valproate. * Fever: in brackets it is reported the number of days before neurological symptoms onset.

Brain Imaging

During the acute phase of the disorder brain MRI showed bilateral Claustrum involvement in all patients (**Figure 1**): these positive imaging studies were acquired with a time lag of 3 – 10 days from SE onset (**Table 2**). Notably, in all patients the first MRI study, undertaken in the few days preceding the development of seizures or in the first days (0 – 2 days) after the onset of refractory SE, was negative. Beyond high signal on FLAIR/T2 and diffusion weighted images [but with normal apparent diffusion coefficient (ADC)] (**Figure 2**), no other sequences revealed Claustrum abnormalities; no contrast enhancement was observed in any case. MRI studies undertaken after SE resolution no longer showed Claustrum alterations (**Figure 2**).

We did not observe signal alterations involving the medial temporal lobe, the splenium of corpus callosum, or other brain regions in any patient. MRI studies in the chronic phase were available for three patients and showed mild diffuse atrophy in one.

Notably, Claustrum alterations were not observed in any other case of status epilepticus, for which an MRI study was available during the acute phase (104 cases), considering any aetiology.

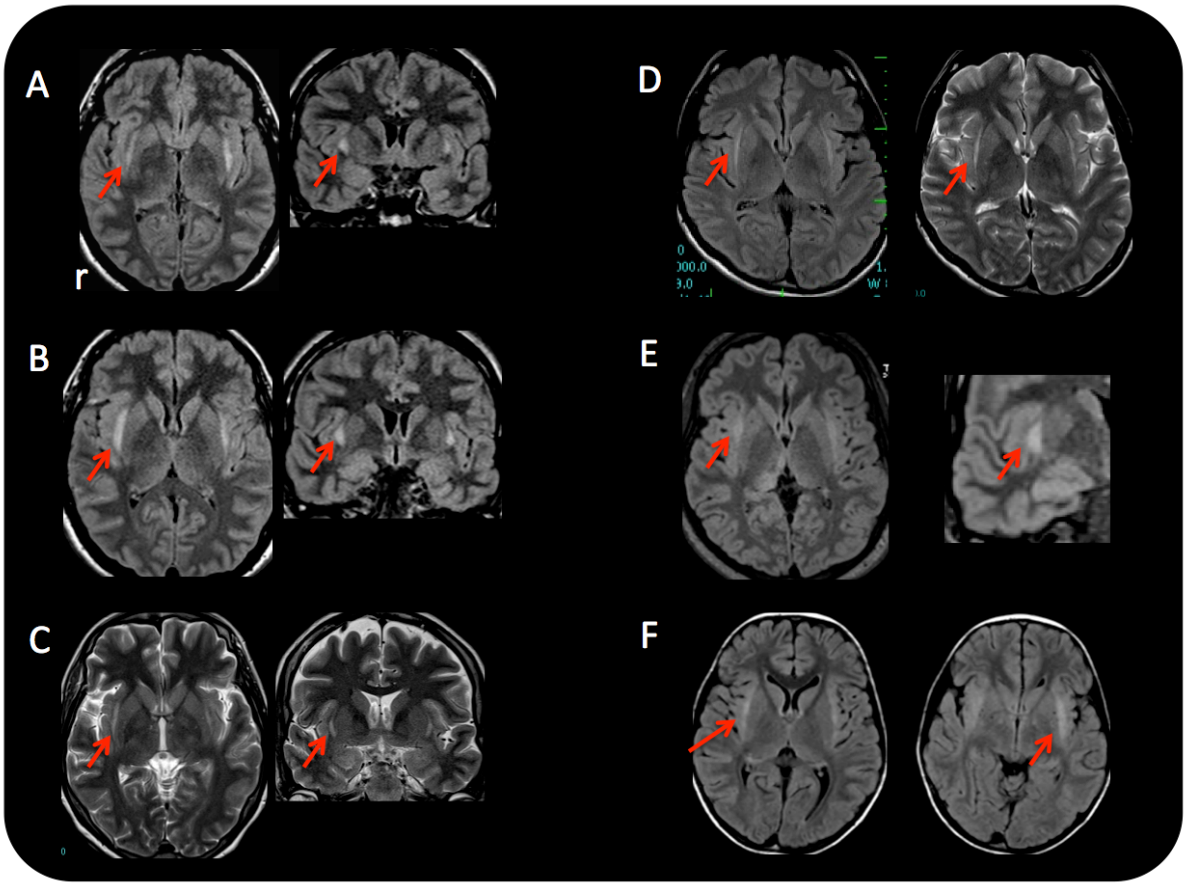


Figure 1. The Claustrum sign.
A-B: axial and coronal FLAIR images showing (red arrows) the Claustrum hyperintensity during the status epilepticus in Pt #1 and #2; 10 and 4 days from onset respectively. **C**: axial and coronal T2 images showing (red arrows) the Claustrum hyperintensity during the status epilepticus in Pt #3; 3 days from onset. **D**: axial FLAIR and axial T2 images showing (red arrows) the Claustrum hyperintensity during the status epilepticus in Pt #4; 9 days from onset. **E**: axial FLAIR images acquired during SE (left image) and coronal detail (right image) of the Claustrum hyperintensity in Pt #5 (red arrows); note also the posterior thalamus hyperintensity (the patient experienced also visual seizures). **F**: axial FLAIR images showing (red arrows) the Claustrum hyperintensity during the status epilepticus in Pt #6; 7 days from onset. r: right.

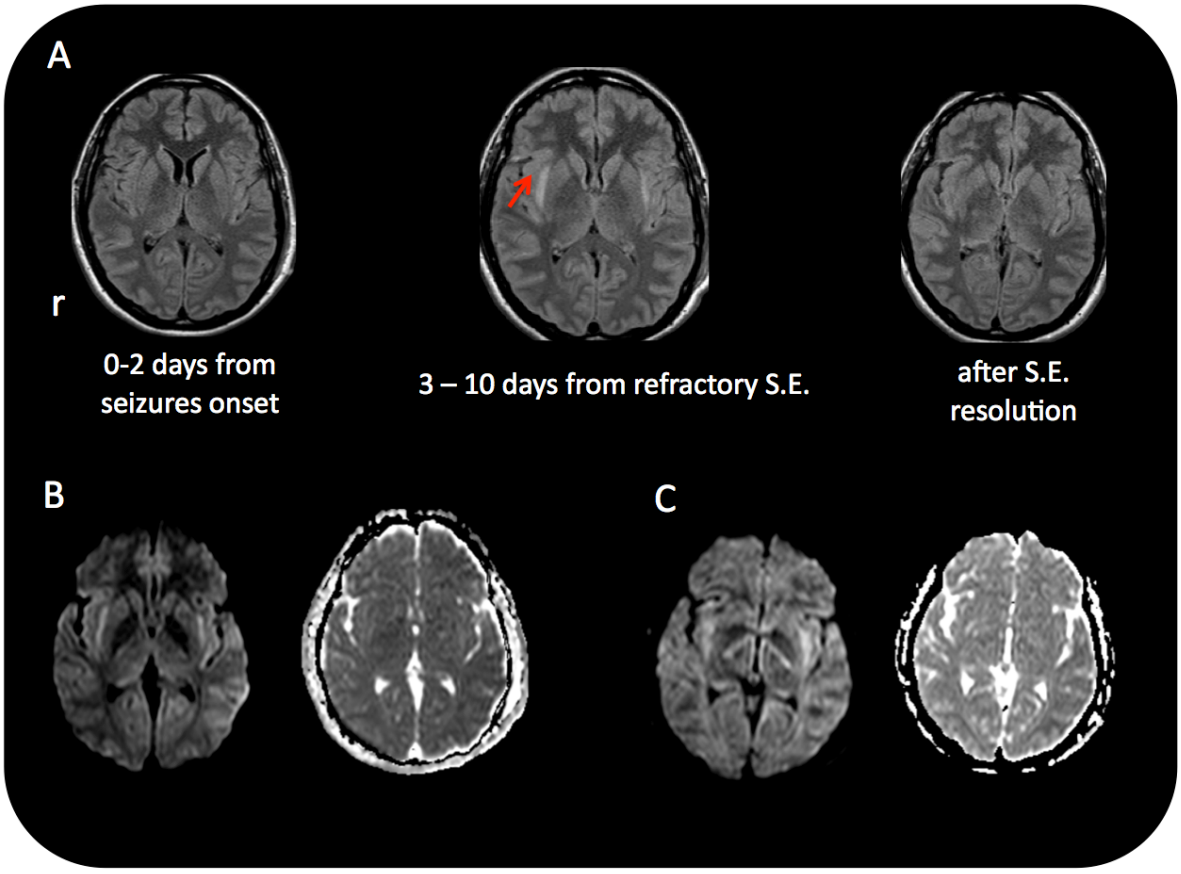


Figure 2. The typical temporal sequence of MRI alterations.
A: normal MRI (FLAIR sequences) before the full developed status epilepticus (left); bilateral hyperintensity of the Claustrum in FLAIR imaging during status epilepticus (central image); normal appearance of the Claustrum after the resolution of the status (right image). Images refer to Pt. # 2.
B-C: DWI (left) and ADC (right) images of patients # 3 and # 6 showing high signal in the region of the Claustrum in DWI with normal ADC. The same pattern was observed in other patients. DWI sequences were not acquired in patient # 1.

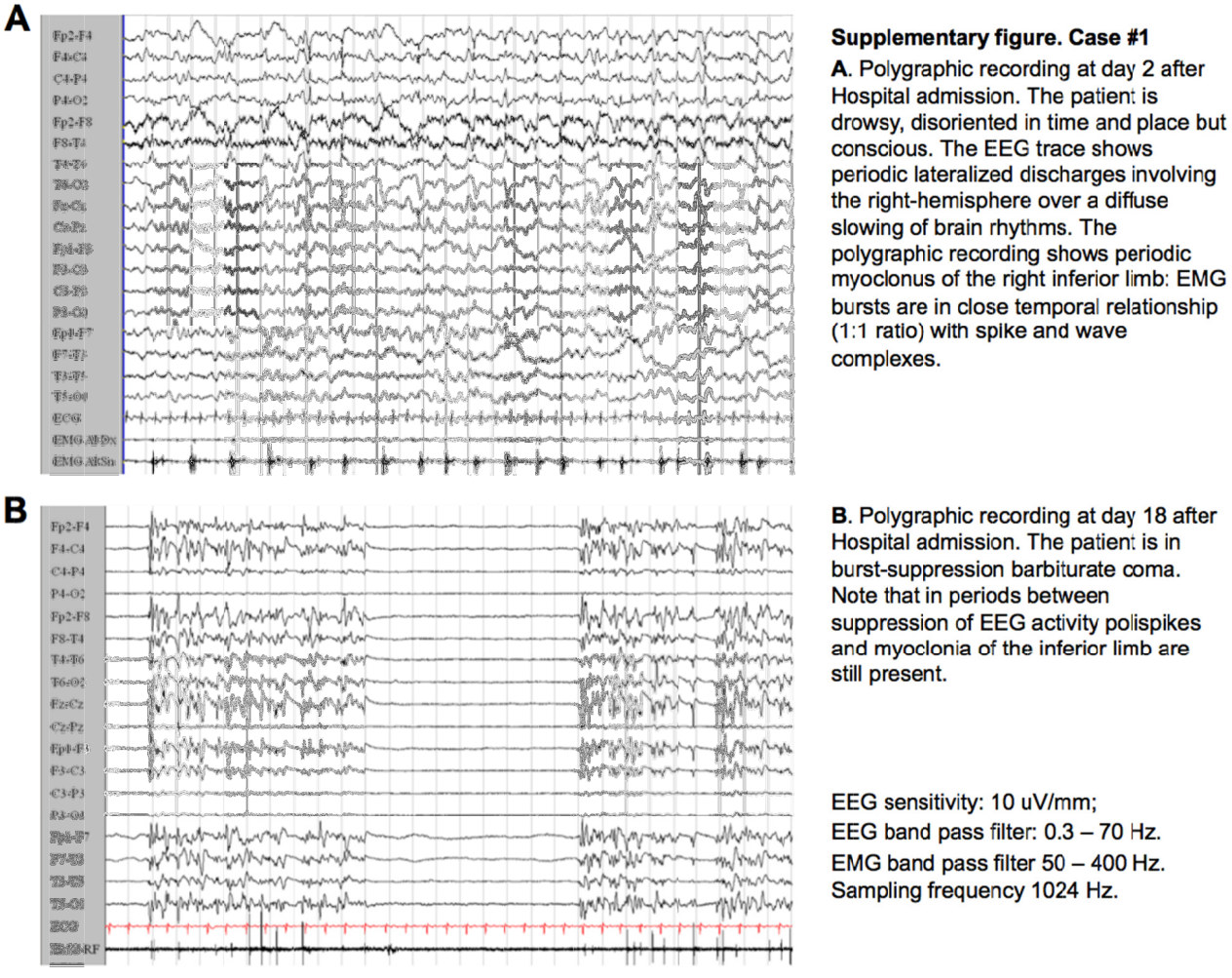
Table 2. Most important investigational data during the acute phase in the reported patients.

	CSF			Brain MRI in acute phase					
Pt.	Cells (ul)	Oligo. bands	EEG	Claustrum	Other brain regions	Timing from fever (days)	Timing from SE onset (days)	Vigilance	Other negative findings
1	3	Absent	G. SW; PD; centro-temporal SW	Bilateral	Insular cortex	17	10	Coma (on anaesthetic treatment)	LGI1, Caspr2 NMDAr, AMPAr, $GABA_{(B)}$, $GABA_{(A)}$, mGlu-R1-R3-R5, POLG1 mutations
2	0	Absent	G. SW; PD; centro-parietal SW	Bilateral	None	11	4	Coma (on anaesthetic treatment)	LGI1, Caspr2 NMDAr, AMPAr, $GABA_{(B)}$, mGlu-R3,
3	20	Absent	PD, SW with alternating side predominance (left> right)	Bilateral	None	9	3	Coma (on anaesthetic treatment)	LGI1, Caspr2 NMDAr,
4	19	Absent	PD, SW with alternating side predominance (left> right)	Bilateral	None	13	9	Stupor	LGI1, Caspr2 NMDAr, VGCC, mGlu-R3,
5	23-3	Absent	Multifocal with fronto-temporal, occipital SW	Bilateral	Right posterior thalamus	10	2	Coma (on anaesthetic treatment)	LGI1, Caspr2 NMDAr, AMPAr, $GABA_{(B)}$, mGlu-R3
6	10	Present	Fronto-temporal bilat theta, L or R fronto-temporal SW	Bilateral	None	10	4	Coma (on anaesthetic treatment)	LGI1, Caspr2 VGCC-Ab, NMDAr

Table Legend: G: generalized; SW: spikes and waves; PD: periodic discharges; Oligo: oligoclonal. FLAIR: fluid attenuated inversion recovery; DWI: diffusion weighted imaging; ADC: apparent diffusion coefficient. NMDAr: antibodies to N-methyl-D-aspartate receptor; LGI1: antibodies against leucine-rich glioma inactivated protein 1; Caspr2: contactin-associated protein-like 2; $GABA_{(A)}$, $GABA_{(B)}$: antibodies against the gamma-aminobutyric acid -A and -B receptor. AMPAr: antibodies against amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; VGCC: antibodies against voltage-gated calcium channel; mGLU-R3: antibodies against metabotropic glutamate receptor 3.

Other investigational data

EEG during the acute phase showed multifocal spikes and sharp waves over fronto-central regions in the majority, with periodic lateralized discharges (**Figure e-1**). Seizures arose independently from the two hemispheres with secondary generalization in every patient. All patient had negative multiple neural antibodies testing, including antibodies to N-methyl-D-aspartate receptor (NMDAR), leucine-rich glioma inactivated protein 1 (LGI1), and contactin-associated protein-like 2(Caspr2) (previously attributed to voltage-gated K channels) (see **Table 2)**.[23] One subject (pt. #1) had additional negative testing for CSF and serum antibodies against gamma-aminobutyric acid-A receptor ($GABA_A$) responsible for the recently described acute autoimmune encephalopathy with refractory seizures/status epilepticus.[24] This patient was also screened for mitochondrial disorders. The most common mutations in the POLG gene p.A467T, p.W748S, and p.G848S were tested and were negative;[25] muscle biopsy didn't show specific alterations, and no respiratory chain defects were observed on muscle tissue specimens.



Supplementary figure. Case #1

A. Polygraphic recording at day 2 after Hospital admission. The patient is drowsy, disoriented in time and place but conscious. The EEG trace shows periodic lateralized discharges involving the right-hemisphere over a diffuse slowing of brain rhythms. The polygraphic recording shows periodic myoclonus of the right inferior limb: EMG bursts are in close temporal relationship (1:1 ratio) with spike and wave complexes.

B. Polygraphic recording at day 18 after Hospital admission. The patient is in burst-suppression barbiturate coma. Note that in periods between suppression of EEG activity polispikes and myoclonia of the inferior limb are still present.

EEG sensitivity: 10 uV/mm;
EEG band pass filter: 0.3 – 70 Hz.
EMG band pass filter 50 – 400 Hz.
Sampling frequency 1024 Hz.

Literature review

Overall 16 cases were included in the review (14 studies, reported in **Table 3**). The online supplementary reference (**Ref e-1**) reports 53 studies on neuroimaging changes during SE that did not show any case with claustrum involvement. The patients' age range was 6 – 65 years (eight females). Fever, in the context of a flu-like condition, preceded the onset of neurologic symptoms in 15 out of 16. Drowsiness, confusion, and seizures were the presenting symptoms after a fever episode. In the acute phase seizures configured a SE in all cases, with a predominance of focal motor and secondarily generalized fits. In seven cases benzodiazepines and AED were successful in controlling SE, while in eight patients the SE was considered refractory or super-refractory with the need of drug-induced coma.[22] One patient died in SE.[27] MRI studies showing bilateral Claustrum alterations were acquired during the SE acute phase in stuporous/unconscious patients, with a time-lag of 3 to 20 days from SE onset. MRI alterations in the acute phase consisted in every patient in hyperintense appearance of the Claustrum on T2-weighted images. No case showed contrast enhancement. In two cases apparent diffusion coefficient (ADC) maps were reported with no evidence of decreased diffusion, as in our cases.[31, 37] In five cases medial temporal lobe hyperintensities were also present.[27-29, 31, 34]

Follow-up MRIs were reported for 11 out of 16 patients showing normal findings or mild atrophy. In the MRI studies undertaken after SE resolution, the Claustrum alterations were always reversed. Clinical follow-up data were available for 10 patients. Eight out of 10 developed chronic focal epilepsy despite AED treatment.

The neuropathological examination in the patient died during SE reported marked astrocytic reaction in the Sommer sector and end folium of both hippocampi and in the Claustrum bilaterally. No sign of encephalitis or global hypoxic-ischemic changes were observed.[27]

A presumed aetiology was lacking in 13 out of 16 cases. HSV1 encephalitis, mumps encephalitis, and VGKC antibodies-related encephalitis were the presumed causes for three patients. In seven patients (children-adolescents' cases) the authors considered the condition as belonging to the AERRPS/FIRES spectrum.

Table 3. Most relevant findings in reports describing Claustrum damage in patients with new onset status epilepticus.

Author, year [ref]	Name given to the condition	N of Pts	Age Sex	Presenting symptoms	Fever (days before onset)	Presumed aetiology	Seizure semiology	Evolution of SE	MRI from SE onset (days)	Immuno-therapy	Outcome/ Epilepsy
Kimura et al., 1994 [28]	None	1	7 M	Lethargy	Yes (7)	HSV1 (herpes stomatitis the week before)	Focal motor	Refractory	11	None	Behavioral impairment Chronic epilepsy
Sperner et al., 1996 [20]	None	1	12 F	Dizziness, fatigue	Yes (14)	None	Focal motor	Responsive	7	Steroids	Normal life No epilepsy
Shiihara et al., 2006 [29]	Acute enc. with refractory SE	1	12 F	Fluctuation of vigilance	Yes (7)	None	Focal motor	Super-refractory	20	None	Severe cognitive-deficit Chronic Epilepsy
Ishida et al., 2006 [30]	Non-herpetic acute limbic enc.	1	8 M	Seizures	Yes (und.)	None	Und.	Responsive	Und.	Und.	Und.
Saito et al., 2007 [31]	AERRPS	1*	10 M	Fluctuation of vigilance	Yrs (6)	None	Focal motor	Super-refractory	11	Steroids, IVIG	Mild cognitive deficit No epilepsy
Specchio et al., 2011 [7]	FIRES	3 *	6-14 3 M, 1 F	Confusion, stupor, agitation	Yes (4-8)	None	Focal motor	Responsive in 3/ Refractory in 1	Und.	Steroids, IVIG in 3 out of 4	Mild to severe cognitive deficit/ Chronic Epilepsy
Gujjar et al., 2011 [32]	None	1	39 F	Lethargy	Yes (4)	None	Focal motor	Super-refractory	14	None	Normal life Chronic Epilepsy
Ishii et al., 2011 [33]	None	1	21 M	Seizures	Yes (7)	Mumps (PCR serum)	Focal motor	Refractory	7	None	Und.
Serrano-Castro et al., 2013 [34]	FIRES	1	19 F	Seizures	Yes (7)	None	Focal motor	Responsive	3	Steroids	Normal life No epilepsy
Hiraga et al., 2014 [35]	None	1	65 F	Fluctuation of vigilance	Und.	Ab anti-VGKC	Focal motor	Responsive	3	Steroids	Memory loss Epilepsy outcome not reported
Mumoli et al., 2014 [36]	None, but possible FIRES	1	14 M	Drowsiness, confusion	Yes (10)	None	Focal occipital	Responsive	10	Steroids, IVIG	Normal life Chronic Epilepsy

Cartagena et. al 2014 [37]	None	1*	22 F	Und.	Und.	None	Focal motor	Refractory	7	Und.	Und.

Table legend: N: number; Pts: patients; SE: status epilepticus; M: male; F: female; AERRPS: acute encephalitis with refractory, repetitive partial seizures; FIRES: febrile infection-related epilepsy syndrome; IVIG: intravenous immuno-globulins; Und: undetermined. *cases extracted from group studies.

DISCUSSION

We reported clinical, EEG, and brain imaging findings of a homogeneous group of six adult patients that developed a new-onset refractory or super-refractory SE starting a few days after a febrile illness and showing a striking Claustrum alteration, mainly bilaterally. We ascertained through an extensive literature review that at least 16 other cases have been described from 1996 to nowadays. Some of these cases were observed in children and were attributed to FIRES.[7,30] Probably, Claustrum involvement in febrile-related new onset SE is more frequent than has been reported. Indeed, even for the cases included in the review several of the imaging findings were not identified as Claustrum alterations, but rather as peri-insular or external capsule lesions.[26,31,35] For these reasons, multicentric studies are needed to evaluate the actual Claustrum involvement in both children and adults with febrile-related new onset SE.

Our observations are important for several reasons. First, this imaging sign seems to be associated to a very aggressive form of SE, with focal motor seizures and myoclonus, often requiring ICU admission and anesthetic drugs. Indeed, considering our patients' group and the previously observed cases, refractory or super-refractory SE cases were observed in 14 out of 22 patients (64%), and chronic epilepsy was reported in 12 out of 14 cases for which a follow-up was available/possible (85%). Second, no definite aetiology was ascertained with the exception of three patients.[26,32,34] Considering the striking homogeneity of the observed cases, it seems highly probable that an unknown but specific aetiology accounts for febrile-related SE with Claustrum hyperintensity. From this point of view, the literature review, and the reported cases allow us to affirm that Claustrum involvement was not observed in the context of refractory SE secondary to acute structural brain damage or to other specific SE aetiologies. Importantly, no other case with Claustrum involvement was observed in our cohort of SE cases observed during the 4-year study period across the different participating Centers (104 cases with MRI peri-, post-SE). Moreover, we screened 56 publications on peri-ictal imaging changes in SE and only three cases were identified, notably all fulfilling our inclusion criteria. It seems therefore quite unlikely that claustrum lesions represent a consequence of refractory SE *per se*.

This retrospective uncontrolled study prevents us to make inferences about analysis of outcomes in relation to treatments. However, immune-modulatory treatment could have been relevant to outcomes.[12,13] Notably, in the reported six patients the four that received early IVIG/PEX (beyond i.v. steroids) had a good outcome also concerning

cognitive aspects, while the two patients that did not receive such treatment died or had severe cognitive deficits. This observation together with the reversibility of the Claustrum lesions, the preceding febrile illness, and the absence of any detectable infectious agent, supports a role of inflammation-mediated mechanisms as relevant in the determination of status epilepticus and encephalopathy in the presented cases.[15] The neuropathological findings in one previously reported patient who died during SE showing evidence of only reactive gliosis without signs of encephalitis support this hypothesis[27]

Our findings underscore the key-role of the Claustrum dysfunction in the pathophysiology of this form of refractory or super-refractory SE. Indeed, the time-course of the MRI alterations suggests that the SE was strictly time-related to Claustrum dysfunction/damage. Of course, it is not possible to affirm if Claustrum damage is the initial event leading to ictogenesis and SE, or if conversely it is the consequence of this peculiar form of febrile-related SE. Indeed fever, seizures, or both, could be relevant to induce Claustrum alterations. However, we suggest that Claustrum could have a relevant role in the maintenance of this very aggressive form of refractory SE.

Experimental evidences in animal models indicate that some subcortical structures and circuits act as critical modulators of seizure propagation and maintenance.[38] In recent years, the concept that during focal seizures activity, specific cortical-subcortical circuits can be critical in sustaining epileptic activity and ictogenesis has been supported by functional brain imaging studies.[39] Notably however, few observations pointed attention to the Claustrum within this framework, even in animal models.[40] This can be explained by the paucity of clinical data about the Claustrum involvement in epilepsy and by the unknown functions of this thin layer of gray matter. Recent advances in neuro-anatomy and physiology indicate this subcortical region as a highly relevant hub-centre in neural synchronization of several (and distant) cortical areas, and, as a consequence, a structure involved in integration of conscious perception.[16-20] Considering these physiological properties of the Claustrum, this structure could also be a key region in promoting the propagation and synchronization of *abnormal* epileptic activity from several cortical regions. To this point two properties of claustral neurons are relevant. First, claustro-cortical fibers connect the Claustrum with several cortical areas including the prefrontal, pre-postcentral, posterior parietal, orbitofrontal, and medial temporal cortex.[19, 20] Second, the Claustrum is constituted by densely packed and tightly interconnected GABAergic interneurons.[16, 17] Therefore, the Claustrum can potentially

bind together and modulate neural activities (epileptic activity in our case) from and to widespread cortical areas.

Interestingly, a recent EEG/fMRI study in patients with focal drug-resistant epilepsies found a common brain region with increased hemodynamic responses in relation to interictal epileptiform discharges, regardless of the localization of interictal and ictal activity.[39] This region was close to the frontal Piriform cortex, and its Talairach coordinates suggest that it corresponds to the Claustrum. Notably, $GABA_A$ receptor binding (measured by flumazenil PET) was reduced in patients with more frequent seizures.

Even if this report is a hypothesis-generating study of preliminary nature, our findings suggest that the Claustrum could be an attractive new target for epilepsy and status epilepticus therapy that deserves to be explored in future studies. Future prospective studies are needed to delineate the specificity of this imaging biomarker, its pathogenetic role in refractory status epilepticus, and the aetiology of the condition.

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C. Discussion and perspective

Our findings point the attention on the possible role of specific radiological brain pattern as a biomarker to identify specific clinical conditions.

We made a collection of several cases of de novo refractory SE that share most of the features:

- all started after a febrile illness
- all started with impairment of consciousness, confusion, stupor
- all rapidly develop in RSE/SRSE
- most present myoclonic seizures or motor emiclonic seizures
- all develop a bilateral, often isolated, hyperintensity in claustrum region
- all patients were treated for possible immune-mediated aetiology without specific autoantibodies positivity.

Claustrum hyperintensity is specifically time related; all patients had normal MRI at status onset, and develop this transient signal alteration between day 3 and 10 after seizures onset. We think that this neuroradiological pattern is specific of a subtype of immune-mediated encephalopathy in which the specific autoantibodies is not yet discovered. Prompt correct diagnosis, treatment and management, especially in ICU, could change the outcomes of this severe SE.

After this publication we collect several identical cases, one in our centre, other in several part of Italy, Europe and United States. We are working in this direction, to expand this casuistry to reinforce our hypothesis. We would evaluate similar cases in paediatric population because probably some cases of FIRES and NORSE had the same underline aetiology in different ages.

We collect also patient's follow up:

The principal evidence is that all survival patients develop chronic and often drug resistant epilepsy. None of the patients presented relapse or late brain MRI modifications.

CHAPTER 3. DISCUSSION

3.1 RELEVANCE OF POTENTIAL BIOMARKERS IN MANAGEMENT OF STATUS EPILEPTICUS

A. The role of CSF: outcome and implication for therapies

We demonstrated that several endogenous molecules could change in CSF of patients with status epilepticus, if we compare the levels with healthy subjects. Some of that changes could be a direct consequence of repeated seizures, and in particular of those conditions in with seizures became refractory to antiepileptic drugs (RSE) or even to anaesthetic therapies (SRSE). In the case of tau proteins, is well known that its increase in CSF is related to axonal and neuronal damage, especially in acute brain damage.

We demonstrated an increase in CSF t-tau protein only in patients with RSE compared with responsive ones, independently from aetiology (acute brain injury were a priori excluded). We found also a positive correlation with SE duration, suggesting a direct neuronal damage induced by persistence of seizures activities.

We need to expand the cohort of patients and try to relate these findings with the brain alterations documented by MRI, distinguish reversible cases from chronic/atrophy evolution ones. Early tau proteins dosage in CSF of patients with SE could predict neurological and epileptic outcome. It's necessary to take into account patients comorbidities and the underline aetiology of the SE that could per se evolve in a worse outcome. However these findings could direct and influence more appropriate therapies: prevent unnecessary treatment in good cases and making timely treatments for the most aggressive forms of SE.

Another point of view is the possibility to use new therapeutic molecules. Few evidences support that allopregnanolone, an endogenous neurosteroids, have an antiepileptic function, with a mechanism close to benzodiazepines. No standard values of this hormone are available in humans. Few reports tried to use this drug in treatment of patients with SRSE with great expectations, especially in children, without severe adverse events.

We demonstrated that patents with SE had decrease levels of allopregnanolone compared with healthy subjects. We can hypothesize that in patients with SE, a

consumption of this antiepileptic molecule occurs. Therefore, administration of intravenous allopregnanolone could be a new therapeutic strategy in refractory to benzodiazepine cases.

We recently participated to the SAGE trial, addressed to SRSE, with a good feeling. Expanding experience in more cases should be necessary to evaluate real efficacy of this drug.

B. The role of MRI: timing and considerations

Brain MRI in patients with status epilepticus is relevant for several reasons:

- to evaluate at onset possible SE' s aetiology (stroke, encephalitis, post-traumatic injury tumours, etc.)
- to follow SE's evolution with brain alteration that compare few days after repeated seizures and probably due to SE (ex. Hippocampal sclerosis after RSE). This radiological marker could predict the development of chronic epilepsy after SE, often drug-resistant.
- to diagnose rare and under recognize aetiologies starting from clinical data and identify common features. This is the case of new advance in definition of immuno-mediated encephalopathies with epilepsy and status epilepticus.

We identify a subcategory of NORSE that started after a febrile illness with stupor/confusion and prevalent myoclonic severe SE, with an isolated, bilateral hyperintensity of claustrum regions on MRI. This alteration is strictly time-related with SE because is found only between 3-10 days after SE's onset. This could represent a neuroradiological marker of a particular autoimmune encephalopathy that begins with RSE and often evolve in catastrophic neurological impairments. This finding could guide correct and timely treatment in immuno-mediate direction, despite the lack of specific antibodies.

3.2 CONCLUSIONS

In clinical practise, biomarkers are the principal instruments that lead to the correct diagnosis and treatment. This is valid for all the medical disciplines and for several, but not all the disease. Unfortunately, biological markers are not available for all the diseases, with an impact in outcome and prognosis.

Status epilepticus is a neurological and potentially dangerous condition that underlines a great number of causes. Identify the aetiology is the mandatory condition that guide the correct treatment. In fact SE treatment foresee antiepileptic and anaesthetic drugs, based on the different stages of SE, but often only the treatment of the specific underline aetiology should develop to complete resolution of the SE. This is true for metabolic causes (hyponatremia, hyperammoniemia, renal dysfunction, infections ecc…) but also for immune-mediate cases in which antibodies identification could support immunological therapies (in ex. steroids, IGIV, PEX ecc…). Some cases escape to our knowledge and improve the panel of potential biomarkers to identify aetiologies underline SE was the objective of our works.

We follow several ways:

a) Principally we use CSF, direct font of information in CNS changes. We discovered: a potential relationship between persistence of intractable seizures and direct neuronal damage; a potential new therapeutic approach with endogenous neurosteroid (AP) that seems to be consumed in CSF of status epilepticus patients.
b) Then we identify an MRI pattern that should be specific of acute encephalopathy associated with refractory SE started after a febrile illness; claustrum damage should represent a radiological marker of this condition and could justify the refractoriness of the status supported by the widespread interconnection of this deep thin line of grey matter with the cortex.

Obviously, our hypothesis needs to be confirmed with a large cohort of patients and probably with a multicentre collaboration.

What has driven and will guide us in the search is the challenge to improve treatments of patients with epilepsy and status epileticus. I hope to be able to continue research with the colleagues that help me in these years.

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