**Chapter 2**

**Prospective clinical trials to investigate clinical and molecular biomarkers**

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* To date, no validated molecular or cellular biomarker exists for any aspect of epilepsy
* Inflammatory biomarkers are under investigation in prospective clinical studies in epilepsy
* The search for clinically useful biomarkers needs to explore multiple biological systems

**Abstract**

Among clinical studies, randomized studies as well as well-designed observational studies are providing the highest quality data. These studies represent also a good opportunity to examine biomarkers of ictogenesis and epileptogenesis. To date, no validated molecular or cellular biomarker exists for any aspect of epilepsy. We provide an overview of the inflammatory biomarkers under investigation in prospective clinical studies in epilepsy: proinflammatory cytokines in prolonged febrile seizure; High Mobility Group Box 1 (HMGB1) as a prognosis biomarker in epilepsy and the interaction between inflammation and metabolism, in particular iron metabolism in epilepsy. The designs of the EU-EPISTOP project following prospectively Tuberous Sclerosis patients from birth to the start of the epilepsy and of the SANAD-II study illustrate how such studies can be used to find new inflammatory biomarkers of ictogenesis and epileptogenesis. If we want to bridge the current gap between having numerous biomarker candidates from preclinical studies and their selective use in clinical practice, we need to explore multiple biological systems, not just including inflammation. It is also crucial that those involved in the design and support of relevant clinical studies recognize this gap and act accordingly and in the interests of improving the diagnosis and prognosis for epilepsy.

**Keywords:** Biomarkers; Epilepsy; HMGB1; IL-1; Inflammation; Prospective study

**Introduction**

Clinical studies play a major role in driving advances in medical knowledge. The corresponding impact for clinical practice is dependent on the quality of the data, which are in turn influenced by the appropriateness of the methodology. In prospective clinical studies, participants are enrolled and then followed-up in order to identify the occurrence of events of interest, depending on the hypothesis. This approach is typically applied to cohort studies in which events occur relatively frequently. In retrospective studies, subjects are studied based on previous outcomes and the data are acquired *a posteriori*. The use of a prospective design decreases the risk of bias, particularly in the selection of subjects, and improves the completeness of the data.

It is often believed that only randomized controlled trials provide a sufficiently robust level of evidence because of their pre-eminent position in the hierarchy of Evidence Based Medicine. However, well-designed observational studies can often provide results comparable in quality to those from randomized trials, challenging the misconception that such studies are second-rate.

As in other fields of medicine, the search for reliable biomarkers has become a major effort in epilepsy. A biomarker is an objectively measured characteristic of a biologically normal or pathological condition. The best known examples are probably blood glucose in *diabetes mellitus* and prostate-specific antigen in prostate cancer. In the epilepsy field, biomarkers of ictogenesis and epileptogenesis would be helpful to predict the occurrence of seizures as well as the development of epilepsy itself or to measure the progression of the condition once established. At the individual level, this would change the patient experience if we could predict when a seizure might occur. Biomarkers of epileptogenesis are also required for the design of effective and affordable antiepileptogenesis clinical trials.1 To date, no validated molecular or cellular biomarker exists for any aspect of epilepsy.

Biomarkers of disease and treatment outcome in humans are most likely to be obtained from cohorts of patients that are followed prospectively from the point of diagnosis and the initiation of therapy. In terms of candidate inflammatory biomarkers, very few human studies have adopted this approach to date. Most of the work in this area is based on the knowledge acquired in preclinical studies.1

A summary and discussion of five illustrative examples are reported here.

**Proinflammatory cytokines in children with febrile status epilepticus: a possible biomarker of hippocampal injury?**

*(W Gallentine)*

The Consequences of Prolonged Febrile Seizures in Childhood (FEBSTAT) study is a prospective multicenter study designed to assess the potential relationship between febrile *status epilepticus* (FSE) and the subsequent development of temporal lobe epilepsy (TLE) that has been suggested by retrospective investigations.3 Febrile seizures (FS) are the most common cause of seizures in childhood, affecting 2-5% of all children. Of children with FS, 10% will have prolonged FSE, lasting more than 30 minutes in duration. The mechanisms by which FS and FSE occur, as well as their contribution to epileptogenesis, remains poorly understood. Although genetics is likely to have a substantial influence, animal models have suggested that inflammatory processes may also play an important role, particularly those mediated by IL1-β, IL-1RA, TNF- and IL-6.4,5

The FEBSTAT study has enrolled a large cohort of children with FSE and will follow these children for up to 15 years using MRI, EEG, and developmental testing. One of the study objectives is to identify biomarkers of hippocampal injury and subsequent development of mesial temporal sclerosis and TLE. In this context, MRI performed with 72 hours of FSE has revealed hippocampal T2 hyper-intensity with associated increases in hippocampal volume in approximately 10% (22 out of 226) of children.6 Follow up MRI (performed at 1 year on 14 of the 22 subjects with prior acute change) has shown development of hippocampal sclerosis (characterized by increased T2 signal and atrophy) in 71% (10 of 14) of cases and decreased hippocampal volumes in 86% (12 of 14).7 In contrast, only 1 out of 116 children without prior acute hippocampal changes showed hippocampal abnormality at 1 year. Based on these findings we have concluded that hippocampal T2 hyper-intensity following FSE likely represents acute brain injury, often evolving into radiographic mesial temporal sclerosis.7 In a substantial number of children, EEG within 72 hours of FSE revealed focal slowing (23.6%) and attenuation (12.6%) with maximal abnormality seen over the temporal region.8 Both of these EEG findings were associated with acute hippocampal T2 signal abnormality. As such, EEG may also be an easily obtainable marker of acute injury following FSE.8

A secondary study to FEBSTAT has been conducted to explore the association between plasma cytokines and the development of FSE in children.9 Cytokine analysis was performed on a pilot cohort of children with FSE (n=33) and compared to children with fever alone (n=17). IL-1β levels trended higher and IL-1RA trended lower following FSE, but did not reach statistical significance. As IL-1RA is typically induced by IL-1β, one might predict the FSE group to have higher IL-1RA levels, corresponding to the surge of IL-1β. However, this was not seen in most subjects. Furthermore, the FSE group had a significantly lower IL-RA to IL-1β ratio overall. Based on these findings, we hypothesize that in some children with FSE the IL-1RA response to the IL-1β surge is inadequate, which might result in a lack of regulatory response to suppress the proconvulsant effects of IL-1β, thus creating the propensity for FSE. These findings are consistent with those from animal models which have implicated the IL-1 system in the pathogenesis of FSE. It suggests that the balance between the pro-inflammatory and anti-inflammatory cytokine response should be studied in addition to the absolute level of cytokines. These observations are currently being explored further within the FEBSTAT cohort, with plasma cytokines under investigation as potential biomarkers of acute hippocampal injury.

**Molecular isoforms of HMGB1 as prognostic biomarkers in epilepsy**

*(L Walker)*

Experimental studies have established that the neuroinflammatory High Mobility Group Box-1/Toll-like Receptor 4 (HMGB1/TLR4) axis contributes to the generation of spontaneous seizures and is one of the first known mediators of sterile neuroinflammation evoked by epileptogenic injuries, as revealed by experimental studies.10,11 HMGB1 is a highly conserved non-histone nuclear protein that is expressed by most eukaryotic cells. It binds to chromatin and regulates gene transcription. *Non-acetylated* HMGB1 is an indicator of passive release from necrotic cells whereas *acetylation* of key lysine residues within the protein signifies active, inflammatory release.12-14 Redox modification of three key cysteine residues, C23, C45 and C106, determines the functional activity of HMGB1. *Disulfide* HMGB1, containing an intramolecular disulfide bond between C23 and C45 and a reduced C106, induces cytokine release in macrophages, microglia and astrocytes.15 *Reduced* HMGB1, in which all three cysteine residues are reduced, forms a heterocomplex with the C-X-C motif chemokine 12 (CXCL12) and binds CXCR4 to initiate chemotaxis.16 Finally, HMGB1 undergoes terminal oxidation, during which all cysteine residues acquire sulfonyl groups, and is rendered immunologically inert.17 Total HMGB1 has been shown to be increased in neurons and glia in epileptic foci from patients with drug-resistant epilepsy and in corresponding animal models.10,11 *Disulfide* HMGB1 is the isoform that promotes seizures and exacerbates cell loss through modulating Ca2+ permeability of NR2B-NMDAR.10,11,18

Novel mass spectrometry analysis is the only method able to determine the acetylation and redox states of HMGB1 and has been utilized to identify dynamic changes in the brain and blood of animals undergoing epileptogenesis following a precipitating insult. Notably, HMGB1 inflammatory isoforms increased in blood before disease onset and could prospectively identify animals that later developed epilepsy. These changes persisted during the active epilepsy phase in those animals (*Walker et al, submitted*). Persistence of the pathological disulfide isoform in animals that developed epilepsy may reflect failure of adequate resolution of inflammation, the biological ‘switch off’ from an initially neuroprotective inflammation, to a pathological state.

Pilot data from patients with newly diagnosed epilepsy were presented demonstrating an early expression of the inflammatory, pathological disulfide isoform of HMGB1, which may indicate likelihood of experiencing further seizures. This was also supported by blood analysis of patients with long-standing drug-refractory epilepsy, who invariably express the acetylated, disulfide isoforms in contrast to patients with well-controlled epilepsy, in whom these pathological isoforms were notably absent (*Walker et al, submitted*).

Finally, treatment of animals with anti-inflammatory drugs during epileptogenesis arrested disease progression and prevented the blood increase in HMGB1 isoforms. Blood HMGB1 isoforms may predict therapeutic response to disease-modifying drugs. The development of isoform-specific antagonists would seem the next logical goal, given that the disulfide isoform of HMGB1 seems to be the form most likely to promote seizure generation.18 Currently, isoform-specific antagonists are still lacking.19Taken together, HMGB1 isoforms are potentially novel prognostic, diagnostic and predictive biomarkers of drug-resistant epilepsy in humans, and highlight potential new targets for drug development.

Currently, prognostic models for seizure recurrence following a first seizure and for seizure remission following an epilepsy diagnosis can broadly stratify patients, but lack precision [c.f. MESS and SANAD models].20,21 These models are based on clinical factors including age, seizure type, EEG and MRI. Therefore, patients with a first seizure would greatly benefit from identification of circulating biomarkers that improve prediction of disease prognosis and treatment outcome. Based on these findings, further studies examining the utility of total HMGB1 and its isoforms as biomarkers in patients exposed to a potentially epileptogenic injury or with a first presentation of seizures, and as predictors of therapeutic effects of drugs with different mechanisms of action, are warranted. Further studies are also needed to identify if the changes in the blood HMGB1 levels may act in the epileptogenic zone though a blood-brain-barrier opening.

**Links between proinflammatory cytokines and iron metabolism in adult epilepsy patients?**

*(M Tombini)*

Proinflammatory cytokines are also established factors promoting neuronal hyperexcitability and seizure susceptibility in rodents.22 IL-1β has been identified as a key actor. However, other proinflammatory cytokines or regulatory mechanisms have also been identified to play a potential role in brain hyperexcitability and seizure occurrence.22 Furthermore, inflammation may affect other biological processes such as iron status and red blood cell (RBC) profile. Chronic inflammation is often associated with a decrease in blood serum iron, thereby lowering the bioavailability of iron in order to contrast uncontrolled cellular proliferation, and reduce the excessive production of reactive oxygen radicals and/or to withhold iron from pathogenic microorganisms.23 There are no clear data regarding the association between the decrease of blood serum iron and brain inflammation. Only one study in the past evidenced an increase of serum level of soluble transferrin receptor in non-anemic Multiple Sclerosis patients with active disease reflecting an increased iron turnover 24.The possible interaction between inflammation and iron metabolism has already been investigated in epilepsy by studying iron-deficiency anemia in febrile seizures, but the results were somewhat conflicting.23

A recent study evaluated the serum levels of cytokines and the markers of iron metabolism, including the hemoglobin concentration (Hgb), the hematocrit (Hct), the transferrin level (Tf) and the ratio of ceruloplasmin to transferrin (Cp/Tf), in 37 adults with focal epilepsy during the interictal period. A group of 43 healthy subjects served as controls.23 The patients with epilepsy had an increase in IL-6 (p = 0.026) and a decrease in TNF-α (p = 0.002). For the first time, a significant change in iron metabolism was found, with an increase in Cp/Tf (p = 0.011) and a decrease in Tf (p = 0.031). This is possibly driven by cytokine modifications. In fact, the proinflammatory cytokines have been shown to directly modulate these serum biomarkers. Ceruloplasmin (Cp), a multicopper enzyme carrying around 95% of circulating copper (Cu), is an acute phase protein, and cytokines (IL-6, IL-1 and TNF-α) have been shown to increase its biosynthesis 25. Transferrin (Tf), the iron transport protein that binds iron in the plasma and circulates it between the iron-containing compartments, is a negative acute phase protein, and as serum ferritin (SF) is reduced during immune stimulation 26, resulting in less iron being available for cellular processes. According to these data TNF-α positively correlated with Tf (p = 0.005). Finally, an inverse correlation between TNF-α and the duration of epilepsy (p = 0.021) was detected.23 These findings suggest an imbalance in inflammatory profiles in adult patients with focal epilepsy compared to healthy subjects; in particular, an increase in IL-6 and decrease in TNF-α during the interictal phase. In this group of patients, the duration of epilepsy inversely correlated with TNF-α, supporting the hypothesis that epilepsy and seizures can provoke an inflammatory response and cytokine alterations.

For the first time, a decrease of Tf and an increase of Cp/Tf system have been reported in sera of epilepsy patients compared to controls. Tf is known to be reduced during immune stimulation suggesting that, in these patients, the changes in cytokines in the plasma — the increase in IL-6 and decrease in TNF-α — could drive the reduction of Tf. In fact, inflammation and cytokines might directly influence iron metabolism and RBC status (through the mediation of hepcidin), leading to anemia in some cases.23

This study illustrates that changes in the inflammatory state could impact other biological processes such as iron metabolism thus highlighting that investigations for biomarkers should be conducted considering several biological systems such as inflammation and metabolism.

**EPISTOP: a prospective study evaluating clinical and molecular biomarkers of epileptogenesis in tuberous sclerosis complex**

*(S Jozwiak)*

Tuberous Sclerosis Complex (TSC) is a genetically determined neurocutaneous syndrome affecting 1 child in 6,000. This disease can be considered as a model of severe focal epilepsy, as 70 to 90% of patients are affected by epilepsy and in most cases the seizures are drug-resistant. In the majority of patients, epilepsy manifests in the first months of life. Moreover, half of the patients develop cognitive impairment, autism spectrum disorders or other neurodevelopmental disturbances. Previous studies demonstrated that antiepileptic drug treatment before the onset of seizures but after electroencephalographic (EEG) deterioration results in a significant decrease in seizures, the risk of drug-resistant epilepsy and the risk of neurodevelopmental impairment.27

 Due to progress in the management of TSC, an increasing number of patients are diagnosed prenatally or soon after birth, enabling follow-up to commence before the onset of epilepsy. The EPISTOP study is the first prospective comparative trial carried out in pediatric patients with TSC, with the aims: 1) to examine the risk factors for, and molecular and neuroimaging biomarkers of, epilepsy susceptibility, epileptogenesis and drug-resistant epilepsy, 2) to identify possible new therapeutic targets to block epileptogenesis in humans, and 3) to estimate the impact of preventative antiepileptic drug treatment on epileptogenesis.

One of the most expanded parts of the project is molecular analysis directed towards identification of peripheral blood biomarkers that indicate ongoing epileptogenesis. Samples are collected prospectively at set time-points, beginning at birth and extending through age 24 months, including extra samples when EEG abnormalities and seizures appear. These serial blood samples are then subjected to transcriptome, proteome and metabolome analysis and miRNA profiling. The correlation of these analyses with the electroclinical observations (i.e. presence of epilepsy, morphology of seizures, neurodevelopmental outcome, response to antiepileptic drug treatment, electroencephalographic findings, neuroimaging studies) will be performed to identify ictogenesis and epileptogenesis biomarkers in TSC.

 During epileptogenesis, changes in gene expression of several immune and inflammatory mediators have been reported. Inflammatory mediators are of particular interest since a persistent and complex activation of inflammatory pathways has been shown in TSC brain cortical tubers.28-30 IL-1β and HMGB1 seem of particular interest.28,31,32 Cortical tubers show an elevated level of proinflamatory IL-1β which is correlated with promoter hypomethylation at CpG and non-CpG site.31 This leads to the permanent activation of IL-1β mediated inflammatory signaling. Moreover, in experimental TSC models an increase in oxidative stress is observed.33-35 Indeed, in cortical tubers the expression of oxidative stress markers such as HO-1, Hsp70 and GCLC is also elevated.33 Furthermore, IL-1β mediated signalling and oxidative stress are known triggers leading to HMGB1 release.22,36 Finally, blood expression profiles of TSC patients reveal changed expression of cytokines, their receptors and other regulators of inflammatory pathway.37 Thus, in EPISTOP, potential peripheral biomarkers, with special attention to inflammatory mediators, their expression and epigenetic regulation, will be correlated with EEG abnormalities and seizure development. This project illustrates the design of a cohort study that might provide, in the near future, some biological markers in patients with TSC. Since the mTOR pathway is also involved in other causes of epilepsy, this would lead to the evaluation of such markers outside the group of TSC patients.

**Opportunity to collect biological samples from randomized clinical trials**

*(G Sills)*

The SANAD studies remain the largest ever randomized controlled trials in epilepsy and explored comparative effectiveness of standard and new AEDs in newly-diagnosed, previously untreated focal and generalized epilepsies.38,39 These studies have provided invaluable evidence about the efficacy and tolerability of anticonvulsant agents and have informed treatment decisions, driving regulations and cost-effectiveness analyses of AEDs. During these studies, a proportion of participants (n=1,000) provided a blood sample for genetic analysis. Those samples have since contributed to numerous international research efforts aimed at identifying causes of epilepsy (i.e. ILAE Consortium on Complex Epilepsies, Epi4K, Epi25). They have also been employed in studies of the genetic influences on treatment outcome in epilepsy, as a standalone cohort 40, as part of a strategic collaboration with University of Melbourne 41, and more recently as a key contributor to the EU-FP7 funded EpiPGX project ([www.epipgx.eu](http://www.epipgx.eu)). The original SANAD samples are also currently undergoing whole exome sequencing as a part of the CENet project in Canada, which aims to identify rare coding variants that segregate with response to drug treatment in non-acquired focal epilepsies.

In 2012, funding was secured for the SANAD-II study, which is designed to further investigate the effectiveness and cost-effectiveness of standard and new AEDs in newly-diagnosed epilepsy. A total of 1,510 participants, aged 5 years and above, from across the UK are being followed prospectively for a period of at least 2 years from initiation of the first ever AED. In Arm A of SANAD-II, lamotrigine (LTG) will be compared against levetiracetam (LEV) and zonisamide in people (n=990) for whom LTG is considered the standard treatment (predominantly focal epilepsies). In Arm B of SANAD-II, valproate (VPA) will be compared against LEV in people (n=520) for whom VPA is considered the standard treatment (predominantly generalised and unclassified epilepsies). Recruitment to SANAD-II began in April 2013 and will close in May 2017, with follow-up continuing until 2019 and reporting expected shortly thereafter. Once again, all participants in SANAD-II are asked to donate a blood sample for DNA extraction and analysis and, at the time of writing, over 1,000 samples have been obtained. These samples will contribute to future genomic and pharmacogenomic studies in epilepsy.

One of the major disappointments in the SANAD trials has been the failure to collect other biological samples (i.e. serum, urine) for use in non-genetic biomarker analyses. Prospectively collected samples, with matched clinical outcome data, would be hugely valuable in this respect. Unfortunately, despite several attempts to secure the necessary funding for additional sample collection in SANAD-II, all efforts were unsuccessful. This represents an enormous missed opportunity.

**Conclusions**

These summaries provide a good overview of the challenges in identification of biomarkers in the epilepsy field. A large number of candidates are coming from preclinical studies that have been key to establishing the role of inflammation in ictogenesis and epileptogenesis.2 The current knowledge gap is the relevance of these potential biomarkers in humans. Clinical studies are essential to evaluating whether any of these inflammatory mediators might function as effective diagnostic or prognostic biomarkers for epilepsy. This is a crucial step before any attempted translation into clinical practice.

In considering the potential of inflammatory biomarkers for epilepsy, it is important to remember that the relationship between inflammation and epilepsy should not be regarded as a single link between two distinct pathophysiological entities. When we think about the role of inflammation in epilepsy, it is first important to distinguish the type of epilepsy syndrome as well as the individual aspects of the immune response that might be involved. This is well illustrated by the investigation undertaken in children with FSE showing an increase in some proinflammatory cytokines and a lower ratio of IL-RA/IL-1β. This pilot study has been conducted in a well-defined clinical condition – i.e. FSE – which is known to be a risk factor for hippocampal injury and perhaps progression to TLE. Thanks to the availability of samples from the FEBSTAT study, we have the opportunity to mine inflammatory pathways for candidate biomarkers of hippocampal injury after FSE.

The EPISTOP study is also a good illustration. This study focuses its investigations on TSC, another well-characterised condition. TSC frequently leads to refractory focal seizures and its genetic basis and the consequences on the intracellular pathways are well-known. Moreover, there are already preliminary data showing changes in inflammatory pathways.28-30 The focus on relatively homogeneous clinical phenotypes is likely to be a productive approach to identifying a biomarker for ictogenesis or epileptogenesis which can then be evaluated in the context of all epilepsy types.

The use of more elaborate biomarkers might be another way to evaluate the relevance of inflammatory mechanisms in humans. In this respect, HMGB1 seems to be a good example. As explained above, the increase of the disulfide isoform of HMGB1 appears to predict seizure recurrence after a first episode. The acetylated disulfide isoforms are elevated in patients with refractory epilepsy but not in patients who are well-controlled. The disulfide isoform of HMGB1 is therefore a potential biomarker candidate for epileptogenesis in humans. This requires further investigations, and the understanding of whether this is true irrespective of epilepsy type and underlying aetiology.

The report by Tombini on the links between inflammation and the iron metabolism is also of importance in giving us a more global view of the changes that can result from the interaction between epilepsy and inflammation. This might equally apply to other endogenous biological pathways and processes.23 This consideration has been taken into account by the EPISTOP study, which plans to conduct transcriptome, proteome and metabolome analysis, plus miRNA profiling, in addition to the more focused study of inflammatory response in patients with TSC.

**Future directions**

In order to move forward, it is essential that future prospective studies in epilepsy make every effort to collect biological samples that will help to facilitate biomarker (including immunological and inflammatory markers) and systems biology analyses. The search for clinically useful biomarkers needs to explore multiple biological systems, not just including inflammation. In many respects, this approach is opposite to that of preclinical studies that often concentrate on just one pathway or one mechanism. This has to be understood if we want to bridge the current gap between having numerous biomarker candidates and their selective use in clinical practice. It is crucial that those involved in the design and support of relevant clinical studies recognize this gap and act accordingly in the interest of improving the diagnosis and prognosis for epilepsy.

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