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Clinical biomarkers in brain injury: a lesson from cardiac arrest

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TABLE OF CONTENTS

1. ABSTRACT

Cardiac arrest (CA) is the primary cause of death in industrialized countries. Successful resuscitation rate is estimated of about 40%, but a good neurological outcome remains difficult to achieve. The majority of resuscitated victims suffers of a pathophysiological entity termed as "post resuscitation disease". Today's efforts are mainly pointed to the chain of survival, often devoting less attention to post-resuscitation care. Resuscitated patients are often victims of nihilistic therapeutic approach, with clinicians failing to promptly institute strategies that mitigate the ischemia-reperfusion injury to vital organs. Only after 72 hours prognostication can be realistically attempted. Neurological evaluation relies on a combination of clinical, instrumental and laboratoristic parameters, since no one alone holds a specificity of 100%. Biochemical markers, such as neuron specific enolase and S-100b, may contribute to predict prognosis after CA. To the contrary, when used individually the necessary precision remains poorly characterized. Biochemical studies suffer from substantial methodological differences hampering attempts to summarize their findings. We review the information available on biochemical markers of brain damage for neurological prognostication after CA.

2. INTRODUCTION

Cardiac arrest (CA) is a major cause of death in industrialized countries (1) the leading cause of death in Europe, affecting about 700,000 individuals a year, while approximately 400,000-460,000 people in the USA experience CA each year (2-3). It has been evaluated that more than 275,000 CA/year are treated by emergency medical services in Europe (4). Patients who have been resuscitated from CA often remain unconscious for some time (5). This loss of consciousness may represent severe permanent brain damage or merely a reversible metabolic disturbance. Therefore, though the initial success of cerebral and cardiopulmonary resuscitation (CPR) is approximately 39% (range 13 to 59%), the functional survival rate is less than 5% (6-11). This is because patients who are successfully resuscitated following cardiac arrest often present with what is now termed as "post resuscitation disease" (12). The most prominent features are post resuscitation ischemic brain damage and myocardial failure. Although post-resuscitation myocardial dysfunction has been implicated as an important mechanism accounting for fatal outcomes after cardiac resuscitation (13-15), morbidity and mortality after successful cardiopulmonary resuscitation largely depend on

recovery of neurologic function. As many as 30% of survivors of cardiac arrest, in fact, manifest permanent brain damage (8, 16-17) and in some instances only 2–12% of resuscitated patients have been discharged from the hospital without neurological dysfunction (18). The rate of those resuscitated victims that die subsequently, is comparable around the world: in Ontario (Canada), 72% of primarily resuscitated patients die in the hospital (19); in Taipei (Taiwan), 75% of primarily resuscitated patients die in the hospital (20); in Goteborg (Sweden) and in Rochester (USA), 68% (21) and 65% (22) respectively, of primarily resuscitated victims die within the first month. Poor survival has been shown for patients with out-of-hospital (OOH)-CA (16, 23), and severe hypoxic brain damage is an important cause of morbidity and mortality in patients who initially undergo successful resuscitation (24). The overall neurological outcome after CPR and return of spontaneous circulation (ROSC) may be influenced by many factors related not only to the patient and his underlying diseases, but also to OOH and in-hospital standard of care (25-31). Despite these very disappointing survival rates, CA research has been mainly focused on approaches applied during CA in the OOH setting, also known as the four links of the "chain of survival" (early access, early cardiopulmonary resuscitation, early defibrillation, and early advanced care) (32), giving only little attention to therapies mitigating the so-called "post-resuscitation syndrome" (33). In the 2005 Guidelines of the European Resuscitation Council, consisting of 189 pages, only three pages are devoted to post-resuscitation care (34).

The main factor still limiting recovery after initially successful CPR is the brain's vulnerability to hypoxia. Reaching the endpoint of ROSC after a period of prolonged whole-body ischemia, it starts an unnatural condition in which the brain is the organ more affected. Consequently, survivors to CA are at risk of brain death or poor neurological outcome up to a persistent vegetative state.

3. FROM NEGOVSKY DATA TO ILCOR CONSENSUS STATEMENT

The patho-physiology of CA comprises not only the ischemic mechanism, but also the more complex reperfusion phenomenon. In the early 1970s, Negovsky recognized that the pathology caused by complete whole-body ischemia and reperfusion was unique in that it had a clearly definable etiology, time course, and constellation of pathological processes (33, 35-36). He named this state "post-resuscitation disease" and himself stated that a second, more complex phase of resuscitation begins when patients regain spontaneous circulation after CA (33). Recently an International Liaison Committee on Resuscitation (ILCOR) Consensus Statement has successfully purposed the new term of "post-cardiac arrest syndrome" (PCA-S) (37), to indicate the chain of events that happens in the body after the no-flow period. Since ROSC they indicate during the first 72 hours 3 equally important phases in which the targets of the medical team are: to limit the ongoing damage, to prevent the recurrence of CA and to support organ functions (37).

The first immediate phase occurs within the initial 20 minutes after ROSC, the second, named early, lasts 6-12 hours and the third (intermediate) persists till 72th hour. Only after these 3 phases, during the subsequent recovery period (fourth phase), prognostication is feasible. Most of patients who are still comatose 48h after ROSC (in absence of protocol of Mild Therapeutic Hypothermia, MTH) do not regain consciousness (38). In the victims of CA, not only survival is a target to achieve, but great importance is obviously given to the neurological recovery of the patient, since post-CA brain injury is one of the four characterizing key components of PCA-S (together with post resuscitation myocardial dysfunction, systemic ischemia/reperfusion response and persistent precipitating events) (37).

Predicting survival and especially the neurological outcome after ROSC in victims of CA still remains a difficult issue, and a procedure used to support non-treatment decisions should ideally have no false-positive results (39-40) reaching a specificity of 100% for poor neurological outcome. Accurate prediction of the neurologic outcome of comatose patients following CA is essential to establish the model of care (41). The American Medical Association (42-44), the American Academy of Neurology (45), and the Society of Critical Care Medicine - American College of Chest Physicians (46) have focused the importance for developing guidelines on prevention and management of CA, pointing attention on the opportunity to stop/reduce treatment in case of spread injury; from clinical point of view is necessary to perform periodical audit. The decision to continue, limit or stop intensive care is a major problem for deep ethical implications and also for resources allocation (47). A false prediction of a bad outcome may cause the patient to be denied life supporting treatment. On the other hand, a falsely optimistic prediction, although less serious from an ethical point of view, may lead to unnecessary prolongation of costly therapy (48). Early assessment of patients brain damage remains quite difficult in the intensive care unit, and could be actually reachable only trough the union of the answer given by clinical examination, neuro-functional and neuro-imaging evaluation, and biochemical markers dosing.

The present review is aimed at investigating the role as prognostic indicator of biochemical markers of brain damage. Two brain-derived proteins, neuron specific enolase (NSE) and protein S-100 beta (S-100b) could be used early in predicting of outcome in unconscious victims of CA. However there is no defined cut-off level for prognostic purposes. Moreover, the introduction of MTH in daily clinical practice imposes a critical reevaluation of the validity of common prognostic indicators. We analyze the usefulness of these markers, summarizing also the confounding factors may delay a widespread clinical application for life support decision making.

4. PATHOPHYSIOLOGY OF BRAIN DAMAGE FOLLOWING CA

Brain is highly dependent on sustained blood flow, due to the lack of its own energy and nutrient

reserves. During Hypoxic-Ischemic insult (H-I), several cellular mechanisms are set in motion that trigger cell damage (49). The severity, intensity and timing of asphyxia, as well as selective ischemic vulnerability, determines the extension and degree of severity of the ensuing damage (50-52). There are many circumstances, which render the brain especially vulnerable to ischemia (53-56). Increasing the knowledge on the pathogenesis of brain damage is essential in order to implement effective prevention strategies. The ischemic theory focuses on the role of H-I as a determinant of breakdown of neuronal metabolism and the subsequent cerebral damage (57, 58, 59). Evidence supporting this model stems from several studies which demonstrated that: a) a severe and acute loss of oxygen in cerebral tissue leads to a reduced protein synthesis and neuronal death within minutes from the insult (60) b) anoxia also acts as a triggering factor for an uncontrolled and elevated release of excitatory neurotransmitters, which contribute to brain damage even with long-time kinesis (61) c) apoptosis could influence the result of ischemia when this is not sufficiently severe to determine a tissue necrosis (62). These evidences are based on the results of several experiments using animal models: the reduction of glucose supply and the lower availability of ATP in neuronal cells produce a Ca^{++} overload in the citoplasmatic fluids that activates several lythic enzymes and, at the same time, reduce the production of antioxidant molecules and structural proteins which are useful for cellular homeostasis (63-65). One of the consequences of the "calcium overflow", provoked by anoxia in affected cells, is represented by a release of excitatory neurotransmitters which, in turn, hyperstimulate postsynaptic neurons and oligodendroglia through the opening of specific receptors, so allowing a further entrance of calcium within these cells (66). Cell damage is enhanced by the production of free radicals and nitric oxide that attack the structural components of the neurons (67, 68). Free radicals, together with other toxic factors such as histamine and serotonin, could be produced by the activation of mast cells in the brain. The role of these cells in brain damage has been supported by the observation that IL-9, a cytokine that binds to the receptor of these cells, exacerbates the excitotoxic damage induced by ibotenate in mice (69). Apoptosis as a result of ischemia has been hypothesized on the basis of the apoptotic bodies found in the brain of asphyxiated animals. Apoptosis is thought to be provoked by mild or moderate ischemia sufficient to determine the lesion of vital components, such as mitochondria, that release pro-apoptotic molecules (i.e. cytochrome c) in the cytosol (70).

5. NSE AND S-100 ASSESSMENT IN EXPERIMENTAL ANIMAL MODELS

Experimental models of cardiac arrest have shown that cerebral ischemic injuries are evident after only a 5 minute interval of ischemia, and necrosis of neocortical cells began to appear after as little as 4 minutes (71,72). CA1 sector of hippocampus and the dorsolateral part of striate nucleus are particularly vulnerable. Neurons in these regions are usually irreversibly damaged after brief ischemic episodes, whereas glial and vascular cells tend to survive (73-75) In addition, the ischemic injuries may be increased by the post resuscitation disturbances in cerebral perfusion, namely no reflow or post ischemic- hyperaemia or -hypoperfusion following successful resuscitation from cardiac arrest (76-79). Phenomena involved in these cerebral perfusion disturbances, include increased blood viscosity and perivascular edema, as well as possible down-regulation of nitric oxide synthesis and expression of endothelial adhesion molecules and generation of free radicals (76-79). The rapid initiation of CPR restores minimal levels of cerebral perfusion and this might contribute to reduce brain ischemic injuries. Chest compressions, in particular, restored cortical microvascular flow to approximately 40% of the pre-arrest values (80). After ROSC, large vessel pressures and flows together with cardiac output were promptly restored but microvessel flow was restored to near pre-arrest values over 3 minutes and reversal of cerebral ischemia with normalization of cerebral cortical tissue PCO2 occurred more slowly over 7 minutes. On the other side, postischemic reperfusion closely related to events leading to neuronal death (81). An example was the evidence that even after brief forebrain ischemia, delayed neuronal death in the hippocampal CA1 subfield occured when the reperfusion exceeded 48 hours, in rats and gerbils (81-85). CA1 neurons, in fact, showed temporary recovery of their metabolic and electrophysiological activity and appeared morphologically well preserved during the first days of reperfusion until necrosis was completed 72 to 96 hours after termination of the ischemic insult (86).

The implication of all these findings is the importance to be capable to identify the therapeutic window for a prompt postischemic treatment. The early diagnosis represents the only means to achieve this goal. Under conditions of global ischemia, i.e. following cardiac arrest, neuroradiological diagnostics usually failed to demonstrate brain damage within that time period (87). Biomarkers, and specifically NSE and S-100b, as early detectors of neuronal ischemic damage, have been therefore extensively investigated in experimental settings of cerebral ischemia, following both different intervals of local ischemia as well as episodes of cardiac arrest and CPR. Conditions of cerebral cortical infarction and subsequent neurological dysfunction following an ischemic event have been proved to be associated with neuronal depletion and astroglial damage and consequent vascular redistribution of brain NSE and S-100b in animal models. Such diffusion of NSE and S-100b into the cerebral vasculature and systemic circulation from ischemic tissue ultimately resulted in measurable plasma levels and was therefore utilized as a marker for the incidence of cerebral damage after acute and chronic ischemic brain infarcts (88). These biomarkers have also been proved to be useful for investigating different therapeutic approaches and interventions during CPR and following ROSC. Biomarkers therefore provided a new option in supporting treatments reducing cell damage in postischemic cerebral tissues.

Strong correlation between levels of NSE and S-100b and neurological outcomes have been extensively confirmed. Although the increase in the serum level of

these markers following ROSC is a common evidence, lower values are usually reported in conjunction with experimental treatments leading to better functional recovery (89-90). When, in fact, those treatments led to lowering of serum biomarkers, they also accounted for a neuroprotective action against both ischemic brain injury and secondary edema events. Thus, dosage of serum NSE and S-100b reflects not only the extent of brain damage following cerebral ischemia, but may also serve as an useful assessment of new neuroprotective approaches (91).

The NSE and S100b levels have been showed to be well correlated with severity of the ischemic insult and with the histological damages. These assumptions have been clearly evidenced in a recent investigation on piglets subjected to different grade of hypoxia. Specifically, the oxygen intake was decreased to less than 7% such to suppress the EEG to <5μV for either 20 minutes, which was considered mild insult, or 40 minutes, which represented a severe insult. Piglets were then reoxygenated with 100% oxygen, and the metabolic acidosis corrected. Animals exposed to mild insult recovered quickly and demonstrated normal behavior as well as normal histology. Animals that underwent a severe insult, instead, were neurologically abnormal and demonstrated both cerebral necrosis and apoptosis. In these piglets, NSE was significantly increased after 24 hours from the ischemic event, while S-100 rose after 48 hours. The correlation among neurological outcome and histology score with NSE and S-100 concentrations were impressive at the different time points (92).

Analysis of NSE have also been shown to be both a valuable diagnostic tool in the management of the initial period following cerebral ischemia and a prognostic parameter during the postischemic course in a gerbil model of global cerebral ischemia of different duration, i.e. 5 or 15 minutes. Changes in this marker levels, in fact, presented differences with respect to magnitude and timing of occurrence. The response was especially depended on the duration of the ischemic insult. Increases of approximately 3-folds were observed after 5 minutes of ischemia, and of more than 20-folds, when ischemia was prolonged to 15 minutes. Moreover, NSE serum levels were significantly increased by 24 hours following the 5 minute ischemia, while only by 4 hours when cerebral ischemia was of 15 minutes. These elevated NSE serum levels corresponded quantitatively to the severity of cerebral ischemia, and more interestingly were detectable even before irreversible neuronal injury appeared. After 5-minute of ischemia, neuronal damage, limited to the hippocampus, became evident only in subfield CA1 by 48-hour reperfusion. After 15-minute of ischemia, instead, severe changes in neuronal cell morphology, extended to the major parts of cortical layers, appeared by 8 hours of reperfusion (87). These data further evidenced the importance of using biomarkers as early detectors of neuronal injury and as a tool to individuate the therapeutic window, such to intervene before the irreversibility of the damage appears. NSE synthesis has been shown, in fact, to be dramatically reduced in ischemically affected but viable neurons until 18 hours after ischemia (93). On the other side, persistent inhibition of protein synthesis preceded irreversible damage in neurons destined to die. The findings of both a transient postischemic reduction and recovery of NSE immunoreactivity in ischemia-resistant neocortical and hippocampal neurons and of a persistent disappearance of immunolabeling in highly vulnerable CA1 cells have been shown. Both diminution of cytoplasmic NSE visualized by immunohistochemistry and elevation of NSE serum levels measured were demonstrable before necrosis became evident in either the cerebral cortex or the hippocampus, respectively (87, 93) Au pair than NSE, Serum S-100b protein levels have also been extensively correlated to the histopathological injuries that followed periods of circulatory arrest in animal models (94).

Animal models have brought also the advantage to allowing for long term investigations of changes of biomarker levels during different time points following reperfusion after an episode of cerebral ischemia or even to investigate condition of permanent cerebral ischemia. This was in conjunction with immunohistologic techniques, which have provided further information on the complex events following a cerebral ischemic insult. It has been therefore observed that serum NSE increased significantly beginning at 2 hour following the ischemic event and peak levels were achieved within 6 to 12 hours with a persistence for the initial 2 days. A loss of NSE immunofluorescence from within neurons to the extracellular space has also been reported in the infarcted areas of rat brains. The prognostic value of this biomarker, assessed during different timing of observation, was evident in its association with the presence of important neurological deficits for more than 15 days (88).

In association with routine plasma assessment of biomarkers of cerebral injury, direct cerebrospinal fluid (CSF) dosage has also proved potential capability of providing quantitative information about the extent of neurological injury in animal models of transient brain ischemia. In particular, the combined serial serum and CSF measures in animals have been essential to understand the kinetics of such biomarkers. Initial investigations showed that after an episode of cerebral ischemia, the increase and decrease in CSF S-100b and NSE levels occurred during the second and the fourth day and that generally CSF NSE concentration was slightly higher than the S-100 value (95). A different study in a rodent model of cerebral ischemia, associable to a situation of cardiac arrest, showed positive correlation between the duration of ischemia and the level of NSE release in CSF and ultimately the severity of neuronal lesions. More specifically, the levels of NSE were measured in CSF following global brain ischemia of different durations, namely 10, 20, or 30 minutes. After 30 minutes of total ischemia, NSE levels increased of nine folds within the first few hours of reperfusion prior to slowly decreasing. Significantly high levels were in fact detected for more than 8 days. Those were accompanied by transient behavioral and neurological abnormalities during the initial 24 hour period of reperfusion. Histological observations confirmed neuronal loss and various degree of neuronal damage in several forebrain regions, including hippocampus, striatum, and thalamus. When the duration of

the ischemic event was reduced to 10 or 20 minutes, CSF NSE increases and neuronal damages were of lesser magnitude (96).

In a complex rat model of cerebral ischemia, the variable size of cerebral infarcts was well correlated to raises in NSE concentrations assessed by repeated samples of cerebrospinal fluid. The NSE concentration increased to the maximal values after 24-72 hours, after which it decreased, returning to almost normal levels after 6 days. More interesting was the significant correlation between the relative size of the cerebral infarct and the NSE concentration. The time course of all NSE concentration changes was similar among the all animals, the maximum level, however, occurred later for larger infarct in comparison to smaller ones, namely 48-72 hours *vs* 24 hours. Neuronal damage therefore continued to develop for several days after the insult and analysis of NSE concentrations in CSF appeared to be useful to study temporal effects on the development of the infarct and its final size on living animals (97).

The apparently heterogenic responses in biomarkers levels observed in some reports have to be individualized and related to different variables, such as animal model employed, duration and severity of the ischemic event, and also extra-cerebral sources (98). However, we have to recognize that other times circulating markers of central nervous system injury did not well correlate with the actual degree of brain damage. Sometimes, in fact, alterations might be limited to the astroglial cells without interesting the neurons (99). In a canine model of normothermic circulatory arrest, serial CSF and serum NSE and S-100b levels have been assessed during an 18 hour interval following reperfusion. NSE concentration in CSF increased significantly up to 15 folds after reperfusion, whereas it did not increased significantly in serum. The CSF S100b concentration increased more dramatically, up to 50 folds, reaching a plateau at the 8th hour after reperfusion. S100b, however, remained below the detection threshold in the serum (100). Biomarker analyses and interpretation may be therefore difficult and in some instances may lack to reflect the real severity of the injury. In a recent investigation in a porcine model of cardiac arrest and CPR, in which the intervals of ischemia adopted, closely reproduced the clinical scenario, with a mean duration of 8 minutes of untreated cardiac arrest and 5 minutes of CPR, serum samples for the determination of S-100 protein were drawn following ROSC. A two- to three-fold increase in S-100b serum levels was observed immediately following resuscitation, raising form 0.5 μg/L to 1.5-2 μg/L after a 10 minute interval. The biomarker levels, however, rapidly returned to pre-arrest values within 1 hour. This lack of protracted rising, although in contrast to reports from other investigations, have been explained by the possibility that the event caused only a temporary blood-brain barrier disruption, which represents an essential condition to allowing for a continuous leakage of S-100b from the damaged neurons into the peripheral blood (101, 102). In some instance, finally, there is a need for investigating more than a single marker during assessment of cerebral injury. In a recent study performed in rabbits subjected to ischemia and reperfusion, all animals presented significant increases of the serum concentration of the S-100b immediately after reperfusion. Serum NSE, however, was not increased, but rather decreased. Light microscopy and electron microscopy, revealed perivascular astrocytic swelling but only minor neuronal cell injury. Increased immunohistochemical staining of S-100b was also revealed in the astrocytic processes with immediate connection to the perivascular space and around the perivascular edema. Finally, when monoclonal mouse anti-human NSE antibody was used for the localization of NSE in the brain indicated, well preserved neurons were shown (99).

6. IMPORTANCE OF CLINICAL SIGNS IN DEFINING PROGNOSIS AFTER CA

From clinical examination, physicians use to evaluate the recovery of consciousness immediately after CA or in the subsequent days as the most important sign for prognosis. The neurological exam focused also on the reflexes of the cranial nerves, especially corneal and pupillary, and on the assessment of Glasgow Coma Scale (GCS).

In summary, the lack of brainstem reflexes, the GCS score and the presence/absence of epilepsy/myoclonic state actually represent the key points to help clinical decision making. It is important to keep in mind that sedative and analgesic effects may be misleading for a correct assessment. Lastly, it is important to keep in mind that the cerebral cortex is less resistant to H-I damage than the brainstem, and this explains the large number of patients who do not have the just mentioned unfavorable signs but still have poor outcomes. The presence of a deficiency in brainstem reflexes suggests that the cortex could be severely damaged. On the other side, preserved brainstem reflexes do not guarantee intact cortical responses. Because there is no clinical method for assessing cerebral cortical damage in the unconscious patient, reliance on clinical criteria alone requires prolonged observation for evidence of recovery. Hence, there is a need for a more direct assessment of cortical integrity and function.

7. THE ROLE OF NEUROFUNCIONAL EXAMINATION

The determination by neuro-functional and imaging examinations may guide immediate post-arrest therapeutic strategies. Various attempts, including electroencephalography (EEG), somatosensory evoked potentials (SSEPs), computed tomography (CT) and magnetic resonance imaging (MRI), have been used to assess brain damage in comatose patients soon after CA (103-107). Serial electrophysiological exams are superior to computed tomography (CT) scan, which shows the extent of hypoxic brain damage only after an unacceptable delay (108) and usually cannot be compared with a precedent one. Additionally, early CT signs such as brain swelling or lack of visualization of basal ganglia do not inevitably result in poor neurological outcome (109).

Magnetic resonance imaging (MRI) and magnetic resonance spectroscopy might provide more specific results (30, 108), but are generally of not available and comfortable use in critical care patients.

SSEPs actually are the most useful parameter in defining negative prognosis, and bilateral absence of cortical response is worldwide accepted as specific sign for poor neurological prognosis (39, 103, 110).

8. THE ROLE OF BIOCHEMICAL MARKERS

Biochemical markers of brain damage (most commonly NSE and S-100b) have been evaluated in serum and CSF samples of survivors to CA (111-116), despite it is possible to find them also in urine and other corporeal fluids. They have given an interesting prospective in defining prognosis after CA and other conditions that cause brain damage, perhaps with limited value if analyzed alone. In particular, NSE and S-100b proteins have been investigated as predictor of poor recovery. When deciding which prognostic marker to adopt in clinical practice, another important feature to consider is the ease with which sampling can be obtained. This has favored the serum markers over those in attainable only in the CSF. Serum analysis could be made serially, providing additional information on the course variation of markers levels (ΔNSE, ΔS-100b). Blood sample is also less time-consuming while lumbar puncture is more invasive, is not devoid of complications and is not considered a primary prognostic tool in a critical patient. Lastly, examination of CSF may be hazardous if there is a possibility of an intracranial mass.

NSE and S-100b are further discussed, but also other markers have been evaluated in CA patients without reaching the meaning of both the precedent. As example, serum IL-8 correlates with reperfusion injury in animal studies (117) peaking at 12h in patient that died or become brain death in the first week after ROSC and showing level higher than those of survivor over the first week (118). IL-8 levels are not an independent predictor of long-term poor neurological outcome (114). Creatinine kinase-BB isoenzyme (CK-BB) becomes elevated in association with brain injury as the enzyme is released from the destroyed neuronal cells into the extracellular fluid (39, 119-123). CK-BB in CSF peaks around 48-72h after CA (123). In the study by Zandbergen *et al* (39), a cut-off limit for CSF CK-BB of 204 U/l had higher prognostic accuracy than NSE and S-100b serum concentration. Also brain glutamic oxalic transaminase, lactate dehydrogenase and lactate in CSF (124) have shown to reflect permanent brain damage, showing association with ischemia. Other markers that have been investigated are: N-CAM (122), sE-selectin and sP-selectin (125).

9. NEUROPROTEINS: NSE AND S-100b

NSE and/or S-100b have been investigated as marker of brain damage not only in patients resuscitated (and not) from CA, but also in different other conditions with the presuppose of neuronal injury, such as peri-natal asphyxia (126), carotid thrombo-endoarterectomy (127, 128) Traumatic Brain Injury (129-131), stroke and meningoencephalitis (132, 133), but also after cardiac surgery (134-140), in surgically treated patients with spondylotic cervical myelopathy (141) and in patients with lung cancer (142). In addition, the presence of S-100b protein has been demonstrated in certain tumors, such as glioma, melanoma, schwannoma and highly differentiated neuroblastomas (143, 144).

NSE is the neuronal form of the dimeric intracytoplasmic lycolytic enzyme enolase that works as catalizator in glucose metabolism. It has a molecular weight of 78KDa and a biological half-life of 24h, occurs as γγ-enolase in neurons and as αγ-enolase in neuroendocrine cells and in small cells lung cancer (145), but NSE also exists in platelets, erythrocytes and several other organs (146-149).

S-100 is a dimer intracellular calcium-binding protein with a molecular weight of 21KDa, metabolized or eliminated by the kidney with a biological half-life of approximately 2h (150). S-100 protein has been implicated in neuronal differentiation and proliferation (151). There are 19 type of S-100 protein and at least 4 of the possible subtypes are known to be represented in human tissue: S-100A1 (striated muscles, heart, and kidneys), S-100A1B (astroglial cells), S-100B (astroglial and Schwann cells), and S-100BB (astroglial cells). S-100A1 and S-100B are formerly known as S-100a and S-100b respectively, being represented as homodimer or heterodimer (151-153). Astroglial cells are the most common cells in the brain and they form a 3D network that constitutes a supporting framework for neurons (152, 153). These cells are known to be as sensitive as neurons to hypoxic stress, and a marker for astroglial damage may indirectly reflect neuronal injury (39, 154, 155). However, small amounts of S-100b could be released from tissue outside the brain (S-100b seems to be present, at least in a limited amount, in adipocytes, chondrocytes and other tissue as well (144, 151-153), and it cannot be ruled out that some early effects in other tissues can be a confounding factor in S-100b dosage. S-100 may also be released from the heart or other injured tissue, indicating that S-100 has a heterogeneous cerebral and extracerebral release pattern (140). S-100 has a lower molecular weight than NSE and is highly soluble, and therefore more likely to cross the cell membrane (156).

Cerebral hypoxia causes death of neuronal cells as well as damage to the blood brain barrier, resulting in an elevation of the serum levels of this and other markers. The serum levels often correlated with the extent of brain damage and in some studies with the prognosis of the patients. On the other hand there is also evidence for a constant turnover of NSE in blood, making changes specifically associated with brain damage in serum levels difficult to evaluate (112, 157). For example, hemolysis caused by invasive procedures might produce a false rise in NSE (158). Moreover, it is well known that platelets are markedly activated and hemolysis may occur during early reperfusion after CA (159). Therefore, even though at least a proportion of NSE is released from neurons, the

Table 1. Studies reporting on serum NSE as prognostic indicator following cardiac arrest induced brain injury

Study	Study size (n)	Poor Outcome	Cut-off level	Sampling time (h)	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)
Rech *et al.* (2006) [170]	43	GOS 1-2 at 6m	60ng/ml	12-36	100	35	100	29
Zandbergen *et al.* (2006)[169]	231	CPC 4-5 at 1m	33µg/l	24-72	100			
Meynaar *et al.* (2003)[116]	110	No RC	25µg/l	24-48	100	59	100	10
Martens *et al.* (1998)[156]	63	No RC	20µg/l	24	89	51	86	65
Schoerkhuber *et al.* (1999)[166]	56*	CPC 3-5 at 6m	16.4 µg/l	12-72	100	8 - 70		
Pfeifer *et al.* (2005)[168]	97	GOS 1-2 at 6m	65ng/ml	72	96	50	97	
Rosen *et al.* (2001)[162]	66	GOS 1-3 at 12m	12.6 µg/l	72			100	58
Fogel *et al.* (1997)[112]	43	Barthel Index >80**	33ng/l	72	100	80	100	78
Zingler *et al.* (2003)[167]	27	No RC	43µg/l	48	100	90.9		
Tianinen *et al.* (2003)[115]	68	CPC 3-5 at 6m	25 µg/l*** 8.8 µg/l****	48 48	96 100	25 76		
Reisenger *et al.* (2007)[172]	177*	Not RC at 6m	80ng/ml	48-96	100	63	100	84

For each study the table reports the population size and the definition of poor neurologic outcome adopted. The cut-off level indicated refers to a specific sampling time after resuscitation; specificity and sensibility are also reported. PPV = positive predictive value; NPV = negative predictive value;. GOS = Glasgow outcome scale; CPC = cerebral performance category; RC = regain of consciousness; m = months. * Patient with CPC score of 5 (death) were excluded from data analysis. **A scale for rating activities of daily living to survived at the first 3 months after cardiac arrest. *** Group treated with mild therapeutic hypothermia. **** Group treated without mild therapeutic hypothermia.

determination of NSE, particularly during early reperfusion after CA, may not be absolutely brain specific. This is in line with the fact that NSE has shown promising results at later stages after CA (112, 157, 160, 161). S-100b, as marker for glial cell damage, has its highest sensitive level during the first 24h after the anoxic insult. It usually declines over the next 48h (162). In contrast to NSE, release from blood cells doesn't seem happens with S-100, and the presence of in the serum indicates cellular brain injury and damage to the blood-brain barrier (151-153, 163, 164).

Numerous investigations have already examined the value of serum NSE for the prediction of an unfavorable neurological outcome after CPR (112, 115, 116, 156, 157, 162, 165-170). In the literature is reported a cut-off value of NSE that ranges from 25 (116) to 120ng/ml (165). In Table 1 are presented values of specificity, sensibility, PPV and NPV of studies that focused on prognosis. These rates are related to a cut-off value. We also report the population size and the neurological endpoint established as poor prognosis. However, these studies are hampered by small population sizes (112, 115, 156, 157, 162, 165-168, 170) or selection bias (116, 169) and yielded conflicting results regarding the cutoff value of NSE above which no patient in the respective series ever regained consciousness.

As for NSE, although several clinical studies have shown that increased serum concentration of protein S-100b predicts ischemic brain damage, also S-100b shows different cut-off levels purposed in the literature, varying from 0.19µg/l (162) and 1.50ng/ml (168), depending also by the different time of sampling and by the dosage methodic.

Cause of its very short half-life, an early determination at admission could be interesting in assessing of comatose patients. The S-100b protein increases transiently, with the highest values seen during the first 24h after CA. Pfeifer *et al* (168) demonstrated a rise in 95% of all survivors during the first 48h after ROSC in S-100b levels, independent of the further clinical course. They interpreted the result as necrosis of neurons and glia cells in cases of complete cerebral ischemia. The neuroprotein declined after 48h in patients with recovery of consciousness, while a further increase have been seen in those with poor prognosis.

In the same manner as for NSE, we analyzed the literature about prognostic value of S-110b in victims of CA and reported the results in Table 2.

10. CONTROVERSY ON CLINICAL AND EXPERIMENTAL DATA

NSE and S-100b serum concentration as cut-off level for prognostic discrimination in the most of cases of the various prospective studies is established only in a retrospective way, searching for the lower value that give a specificity of 100% for poor neurological outcome. Then, the authors carried out different rates of sensibility, positive and negative predictive value (PPV; NPV), that depend overall on the population size, that is often very small. To reach safe values of cut-off for a poor recovery the authors assess too high level of both neuro-markers. However, proceeding in this fashion a high number of patients with poor neurological outcome remained undetected, because their neuroprotein serum concentrations are below the limit. There are still no adequate explanations for the unrevealed high values of NSE and/or S-100b in patients with poor prognosis. Probably the blood-brain barrier remains undamaged in some cases (at least in a first phase) despite severe and ongoing brain damage (112), giving reason for the lacking increase of biomarkers. We also have to consider that survivors to CA die not only cause of cerebral damage. Then the molecular weight could not be the reason, as suggested by Pfeifer *et al* (168), because NSE,

Table 2. Studies reporting on serum S-100b as prognostic indicator following cardiac arrest induced brain injury

Study	Study size (n)	Poor Outcome	Cut-off level	Sampling time (h)	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)
Mussack *et al.*(2002)[114]	20	GOS 1-3 12m	0.76ng/ml	12	100	54	100	33
Zandbergen *et al.* (2006)[169]	230	CPC 4-5 1m	0.7µg/l	24-72	98			
Hachimi-Idrissi *et al.* (2002)[173]	58	No RC	0.7µg/l	Admission-24	85-88.2	66.6-100	84-86	78-100
Martens *et al.* (1998)[156]	63	No RC	0.7µg/l	24	96	55	95	63
Pfeifer *et al.* (2005)[168]	97	GOS 1-2 6m	1.50ng/ml	72			96	
Rosen *et al.* (2001)[162]	66	GOS 1-3 12m	0.19µg/l	72			100	56
Bottiger *et al.* (2001)[111]	66	CPC 4-5 14days	1.10µg/l	48	100		75	100
Zingler *et al.* (2003)[167]	27	No RC	0.5µg/l	72	100	75		
Tianinen et 11 (2003)[115]	68	CPC 3-5 at 6 m	0.23µg/l* 0.12µg/l **	48 48	96 100	25 88		

For each study the table reports the population size and the definition of poor neurologic outcome adopted. The cut-off level indicated refers to a specific sampling time after resuscitation; specificity and sensibility are also reported. PPV = positive predictive value; NPV = negative predictive value;. GOS = Glasgow outcome scale; CPC = cerebral performance category; RC = regain of consciousness; m = months. * Group treated with mild therapeutic hypothermia. ** Group treated without mild therapeutic hypothermia.

with the higher molecular weight, was above the limit in 10 patients that had a S-100 level below the cut-off in their study. It is reasonable to think at different patterns in developing blood-brain barrier damage.

In the analysis of the studies reported in the table 1 and 2 on prognostic value of the biomarkers many other problems rise. First, the different time of sampling and the differences among the study population (that is often very small in size) could be confounded factors that makes not homogeneous the data and moreover a large variability in specificity and sensitivity could be detected in this studies. Second, a recent study by Stern *et al* (171) shows differences in seven type of assays to determine NSE values, so that cut-off values may not be reliable without validation of the laboratory method used. Other two studies assessed that different laboratory assays for determination of serum NSE may yield deviating results (142, 172). The study of Bodi *et al* (142) in healthy persons, benign pulmonary disease and advanced lung cancer patients, suggested the more sensitivity of the monoclonal IRMA method (93%) versus the polyclonal RIA one (83%). Thus, the absolute cut-off levels of NSE in different studies may not be comparable, suggesting that strict cut-off values such as the 33µg/l found in the study of Zandbergen *et al.* (169) and also included in the recent American guidelines (40) might not be applied in clinical practice. According to this opinion in the study by Reisinger *et al.* (172) if adopted the repeatedly reported cut-off value for NSE (33µg/L) (39, 40, 112, 169), 9 of 177 analyzed patients would have been falsely classified as poor neurological outcome, whereas they showed no more than a moderate cerebral disability (see below Cerebral Performance Category score of 1-2). For this reason the authors indicated a more elevated cut-off of NSE (80µg/L) in predicting patients who don't regain consciousness, as the lowest with a 100% specificity (and reported 63% of sensitivity) for the prediction of CPC score of 4 (persistent coma). Of importance, a third point of analytic disaggregation is represented by the definition of bad neurological outcome adopted in the studies. The neurological outcome is currently evaluated according to the Glasgow-Pittsburgh Cerebral Performance Category (CPC, 1-5) of the Utstein recommendation (8, 32), defined as follows: CPC-1, conscious and alert with normal neurological function or only slight cerebral disability; CPC-2, conscious and alert with moderate cerebral disability; CPC-3, conscious with severe cerebral disability precluding independent existence; CPC-4, comatose or in a persistent vegetative state; and CPC-5, brain dead. Some studies follow the precedent issue, using a CPC score of 3 as bad neurological recovery (115, 166), while others consider it as good outcome, together with CPC 1-2, focusing on the recovery of consciousness (111, 116, 156, 167, 169, 174). Other studies used the classification in performance categories according to Glasgow Outcome Scale (GOS), defined as follows: GOS 1, death; GOS 2, persistent vegetative state; GOS 3, severe disability (unable to live independently, but capable of following commands); GOS 4, moderate disability (able to live independently, but unable to return to work); GOS 5, mild or no disability (able to return to work) (175). Two studies classified a GOS score of 3 as poor outcome (114, 162), while on the other hand, Pfeifer *et al.* and Rech *et al.* considered it as good recovery (168, 170). Moreover, in the study by Fogel *et al.*, the Barthel Index, another scale for the assessment of brain disability was adopted in defining outcome (112). One interpretation of these different approaches is given by Zandbergen *et al.* They suggest that victims of CA who were conscious but severely disabled after 1 month should not be included in the "poor outcome" group because further improvement may occur later. In their cohort, one-third of such severely injured patients lived independently 1 year after CA (169). Another interpretation is given by authors that exclude the deaths from analysis cause it could be due not only to cerebral damage (166, 173). As example, sepsis, MODs or precipitating cardiac conditions can determine a secondary decline in the cerebral conditions, that are not only due to CA brain injury (174). Perhaps, in a recent study (175) no significant differences was founded in peak NSE concentration between the group of patients with CPC score of 1-2 and the group of CPC 3, with also almost identical time course of NSE. The question remains opened and maybe the most realistic method is the 1-year related outcome, although time consuming and more difficult to evaluate.

After demonstration by Sunde *et al* (176) that the determination of prognosis after CA is more difficult after introduction of MTH, a fourth problem is elicited. In the study of Zandbergen and colleagues (169) included in the recommendation of the AAN, most patients were not treated with MTH (40). In a sub-study of the Hypothermia after Cardiac Arrest trial, the prognostic value of NSE was markedly decreased in patients treated with MTH, rendering it almost useless in clinical practice because of the lower sensitivity (115). Moreover two recent study by Tiainen *et al.* (115, 177) suggested that the use of therapeutic hypothermia doesn't affect the prognostic value of evoked potentials to predict poor outcomes after CA, while reducing the value of NSE and S-100. A much higher cut-off value of NSE at 48h in MTH and non-MTH patients (with a specificity ≥96% for 25.0 and 8.8µg/l, respectively) was detected and the authors suggest also that MTH seems to reduce the prognostic accuracy of S-100b, despite it is considered the faster kinetic in increase of this marker than NSE (115). The low sensitivity of NSE in these studies might have been caused in part by small sample size. In fact, in the study by Oksanen *et al.* (178) a cut-off value of 33µg/l at 48h indicated with 100% specificity an unfavorable outcome in patients treated with MTH. Another study by Reisinger *et al.* (173) seems do not confirm the results of Tiainen *et al*, with the diagnostic accuracy of NSE that is similar for hypothermic and non hypothermic patients. However, further studies are now warranted in order to create an unique consensus on this issue.

These different approaches in methods lead to bias and makes more difficult the validation of the different findings. Once a cut-off level will be validated, other problems should be kept in mind if the clinicians decide to apply it. There are in fact different conditions that can false the interpretation of a revealed value. Overall NSE concentrations should always be viewed with caution for some reasons. First of these, as just suggested, hemolysis in the serum sample may lead to a potentially fatal misclassification of patient's prognosis. An association between haemolysis rate and an increase in NSE serum levels was first described by Pahlmann *et al.* (145, 179). Consequently, an index of haemolysis should be determined before deciding if perform NSE measurement (180). Accordingly extreme caution should be applied to NSE values determined while intra- or extra-corporeal pumping systems (Intra-Aortic Balloon Pumping, Continuous Veno-Venous Hemodialysis, ventricular assist devices, cardiopulmonary bypass) are in operation cause they may procure mechanical destruction of blood cells (181). The use of temporary circulatory support devices are now commonly widespread in cardiogenic shock patients after suffering from Acute Myocardial Infarction (AMI) (182-184). Haemolysis has been discovered as cause of incretion in serum level of NSE, but not S-100b protein (181), despite in a recent study by Pfeifer *et al.* (185), Left Ventricular Assist Device (LAVD) was associated with a significant increase not only of NSE, but also of S-100b, suggesting that an adjunctive mechanism could be involved in LVAD-induced NSE elevation. The authors interpreted the results at least as due at cerebral micro-embolic events, which could be detected by MRI in 15-22% of patients being clinically asymptomatic after coronary angiography (186, 187). Similar thromboembolic events in cerebral vessels could be induced by LVAD. Another advanced explanation could be the release of both proteins from the ischemic myocardium. Historically, only S-100a is founded in the myocardium (188), while S-100b seems to be absent or identifiably only in conducting system (153, 189). Otherwise, Tsoporis *et al.* identified immuno-histochemically S-100b in the peri-infarct region of human heart and S-100b mRNA was detected in the animal heart after coronary artery ligation (190, 191). Possible mechanisms for increase of NSE and S-100b in myocardial ischemia still remain unclear and must be investigated. Lastly S-100b can be find in the aortic vessel wall, so that a mechanical LVAD-induced alteration of the aorta may represent another hypothesis (185).

In the exceptional patients with a primary intracranial origin of their CA, the relative contributions of the intracranial mass lesion and the diffuse anoxic damage to the rise in serum NSE and S-100 concentrations may be difficult to separate. Although extremely rare, the coincidental occurrence of NSE-producing masses such as small-cell lung carcinoma, neuroblastoma, or carcinoid tumours should be kept in mind when using this parameter for prognostic evaluation in victims of CA. However, in these cases the time course of serial NSE values should be show a persistent elevated level rather than the bell-shaped pattern of patients with a poor neurological outcome after CPR.

11. USEFUL OF COMBINATION OF DIFFERENT VARIABLES

Most of the studies on the prognostic markers in CA have used single variables. Although the AAN prognostic guidelines provided strong support for the use of elevated NSE (>33 µg/l) at day 1 and day 3 to prognosticate poor outcome (40, 169), there is skepticism for the use in clinical practice especially after introduction of MTH. S-100b and CK-BB as markers of brain damage seem to provide less confidence in the prediction of poor outcome. Clinicians use in daily practice only a combination of more than one of the common prognostic indicators to guide the timing of therapeutic intervention and clinical strategies.

Several studies (103, 120, 169, 192-194) have looked at combining the biochemical values with other parameters to increase the reliability of predicting outcome. One example of union of different indicators is the study by Bassetti and colleagues (103) that assessed the value of combining clinical examination, EEG, SSEPs, and two serum biochemical markers (ionized calcium and NSE). The combination of all the variables improved the predictions to 82%. No false pessimistic prediction was observed using the combination.

In a relatively large study of 110 patients admitted to Intensive Care Unit after resuscitation (116) is interesting that in 19% of the comatose patients with SSEPs

present, NSE was >25.0μg/l: no one of them regained consciousness, suggesting that using serum NSE at 24h and 48h after C-CPR in patients with presence of SSEPs waveform, sensitivity of the prediction of poor prognosis can be increased. This in turn might lead to fewer patients with no chance of awareness being treated.

Also Zingler *et al.* (167) concluded their study suggesting that the combination of clinical evaluation with SSEPs and neurobiochemical dosing is a promising way to increase the reliability in predicting neurological outcome for CA victims.

12. CONCLUSIONS

Despite initial successful resuscitation the majority of patients ultimately die in hospital of what has been recently named as post-cardiac arrest syndrome. Severe neurological damage is a key element. Early prognostication is unreliable and may be misleading particularly when post-resuscitation strategies that mitigate the effects of the ischemia-reperfusion phenomenon are implemented. Biochemical markers, such as NSE and S-100b may contribute to offer the clinician a platform on which to base decision making on life support. All observations summarized in this review pinpoint the concept of a multi-marker approach in order to better understand the severity of the cerebral ischemic events and better prognosticate the possible outcome. The decision to continue, limit or stop intensive care is a major problem with deep ethical implications which should be postponed until 72 hours from ROSC. Only then prognostication should be attempted relying on a multimodal approach that comprises clinical examination, neuro-functional evaluation and biochemical markers. The utility of neuroimaging assessment is still a matter of controversy.

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