# *N*-methyl-D-aspartate–Receptor Antibodies, S100B Protein, and Neuron-Specific Enolase Before and After Cardiac Surgery: Association with Ischemic Brain Injury and Erythropoetin Prophylaxis

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## ABSTRACT

**Objective:** To evaluate biomarkers of acute ischemic brain injury after surgical revascularization of the heart with the use of the heart-lung machine (ie, cardiopulmonary bypass [CPB]).

**Methods:** Twenty consecutive patients were divided into 2 groups: the first 10 patients received a potential neuroprotective human recombinant erythropoietin and the remaining 10 comprised the control group. Neurological complications were monitored by measuring serum concentrations of *N*-methyl-D-aspartate (NMDA)–receptor antibodies (NR2Ab), S100B protein, and neuron-specific enolase (NSE) before and during the first 5 days after surgery, comparing the neurological outcome with the results of MRI examinations.

**Results:** The results from the erythropoietin-treated group and the control group were similar, with a significant difference shown for the postoperative NSE. Comparison of serum concentrations of the biomarkers of 16 patients without brain ischemia and 4 patients with acute ischemia displayed significant differences for only postoperative NR2Ab, regardless if the patient received erythropoietin therapy.

**Conclusions:** The analysis of NR2Ab serum concentrations might be useful for monitoring neuroprotective stroke treatment; however, further studies are required to investigate its role in acute ischemic brain injury.

**Keywords:** biomarkers, cardiac surgery, ischemic brain injury

Neurological complications after cardiac surgery are a significant problem in health care. The incidence of postoperative stroke has risen to 5.2%,[1,2] with postoperative delirium occurring in 10% to 30%[3] of patients and cognitive changes reported by 83% of patients.[4]

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## Abbreviations

NSE, neuron-specific enolase; CPB, cardiopulmonary bypass; BBB, blood-brain barrier; NMDA, *N*-methyl-D-aspartate; NR2Ab, NMDA-receptor antibodies; TIA, transient ischemic attack; ICH, intracerebral hemorrhage; NIHSS, National Institutes of Health Stroke Scale/Score; MRI, magnetic resonance imaging; rHuEpo recombinant human erythropoietin; Epo, erythropoietin-treated; ECG, electrocardiogram; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation; DWI, diffusion-weighted imaging; FLAIR, fluid-attenuated inversion recovery; ROCs, receiver operating curves; ISCH, ischemia

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During the past few years, several biomarkers have been proposed to predict, diagnose, and monitor brain injury.[5] Ischemic stroke is associated with a variety of pathophysiological changes that lead to glial and neuronal damage in the brain.[6] Neuron-specific enolase (NSE) is a form of the dimeric enzyme that is composed of 2 gamma subunits and occurs primarily in neurons. NSE converts 2-phosphoglycerate into phosphoenolpyruvate and is a marker of ischemic brain injury associated with traumatic brain injury, brain damage after stroke, and anoxic encephalopathy after cardiac arrest.[7] Serum S100B is a calcium-regulating protein found primarily in astroglial cells, which may comprise as much as 60% of the central nervous system. Astroglia, together with endothelial cells, function as a protective barrier between the cerebral microvasculature and the neurons. The biological function of S100B is unknown. Seizures, stroke, head trauma, inflammation, and surgical revascularization of the heart with the use of the heart-lung machine (cardiopulmonary bypass [CPB]) have all been shown to cause neurological dysfunction, to increase the permeability of the blood-brain barrier (BBB) and the release of S100B into the bloodstream. Serum concentrations of S100B rise gradually after acute ischemic stroke, reaching a peak 3 days after onset of symptoms.[8]

*N*-methyl-D-aspartate (NMDA)–receptor peptides and their antibodies have also been proposed as biomarkers of cerebral ischemia and stroke. NMDA receptors, which bind the glutamate neurotransmitter, are a heterogeneous group of tetrameric transmembrane proteins on neuronal cells throughout the brain and contain 2 NR1 and 2 NR2 subunits. Fragmentation of NR2 into NR2A and NR2B peptides is thought to occur with cerebral ischemia or neurotoxicity. Subunit (ie, NR2) NMDA receptors are degraded and proteolytic fragments released into the blood after neuronal death or ischemia. Antibody response is generated to the NR2A/2B subunit fragments (ie, NMDA receptor antibodies [NR2Ab]) and mediated by the immune system after ischemic events.

Only a few clinical studies have examined the role of NMDA NR2Ab as markers of stroke. A pilot study to examine the neurotoxic biomarkers in serum found that subjects with transient ischemic attack (TIA) and ischemic stroke had significantly elevated levels of NMDA NR2Ab compared with the control group.[9] In this study and in the larger follow-up trial, patients with intracerebral hemorrhage (ICH) demonstrated no significant elevation of NMDA NR2Ab, suggesting that a negative NR2Ab result did not exclude ICH**.** The patients with TIA and ischemic stroke demonstrated markedly elevated levels of NR2Ab, correlating with the National Institutes of Health Stroke Scale/Score (NIHSS) score and the amount of brain damage displayed by magnetic resonance imaging (MRI) and neurocognitive assessment. NR2Ab could not be used to differentiate ischemic stroke from TIA.[10] In a prospective, multicenter clinical trial, NR2Ab concentrations helped predict adverse neurological complications in high-risk patients undergoing coronary surgery.[11] A recent study[12] suggested that NR2Ab concentrations reflect a history of multiple strokes and may serve as a predictive factor for stroke.

Two recent reports[13-14] described tissue-protective nonhematological effects of recombinant human erythropoietin (rHuEpo) that limit or prevent ischemia-induced tissue damage. The protective effects of rHuEpo on central and peripheral neurons, cardiomyocytes, hepatocytes, vascular endothelial cells, the pancreas, and the uterus have been reported. Several mechanisms of rHuEpo neuroprotection have been suggested, including decreasing glutamate toxicity, induction of neuronal antiapoptotic factors, reduction of inflammation, decreasing nitric oxide–mediated injury, and direct antioxidant effects.

The goals of the present study were to determine whether molecular brain biomarkers could be used to determine whether ischemic brain injury was less common in CPB patients treated with rHuEpo compared to untreated controls and whether molecular biomarkers are consistent with the MRI findings.

## Materials and Methods

We prospectively evaluated 20 consecutive patients undergoing cardiac surgery on CPB. Approval was obtained by the National Medical Ethics Committee of the Republic of Slovenia; written informed consent was obtained from all the patients. Patients younger than 18 years of age with known malignant hypertension, cancer, hematological disorder, or renal or hepatic failure, as well as those who were receiving rHuEpo therapy or had allergies to medications, were excluded from the study. The patients were assigned to the erythropoietin-treated (Epo) or the control group. The 10 patients in the Epo group received 3 consecutive doses (24,000 IU) of epoetin alfa (EPREX; Janssen-Cilag Pty Limited, North Ryde, Australia) administered intravenously. The first dose was given 1 day before the operation, the second dose on the day of operation, and the third dose 1 day after the operation.

Within 2 days prior to the operation, patients underwent testing that included medical history, an electrocardiogram (ECG), an MRI scan, and physical and mental status examination by a neurologist. Blood was collected before anesthesia on the day of surgery for subsequent biomarker analysis. Blood was again collected and MRI examinations performed within 5 days after surgery (median, 4 days; range, 2 to 5). The surgical procedure was similar for all patients, involving intravenous and/or inhaled anesthesia and protocols regarding hypothermia, hemodilution, cardioplegia, and monitoring of relevant clinical parameters (arterial pressure, cardiac arrest, duration of extracorporeal circulation, etc). A delirium assessment was performed 24 and 48 hours after surgery.[16]

Blood samples were centrifuged within 30 minutes and serum was stored at –20°C until assayed in duplicate in a single batch within 6 months. Concentrations of S100B protein and NSE were measured by an automated chemiluminescent immunoassay (reagents and analyzer: LIAISON , DiaSorin S. p. A., Saluggia, Italy). NR2Ab concentrations were determined by enzyme-linked

Table 1. Comparison of Serum Preoperative and Postoperative Concentrations of Biomarkers of the Erythropoietin-Treated (Epo) Group and Control Group (Mann-Whitney Test)

| Parameter[a] (µg/L) | Epo | Control | *P* Value |
|---|---|---|---|
| Prepoperative S100B | 0.090 | 0.085 | 0.362 |
| Q1–Q3 | 0.065–0.108 | 0.065–0.095 | |
| Postoperative S100B | 0.290 | 0.310 | 0.773 |
| Q1–Q3 | 0.200–0.405 | 0.215–0.345 | |
| Pre NSE | 8.00 | 8.75 | 0.934 |
| Q1–Q3 | 7.025–9.63 | 6.25–9.60 | |
| **Postoperative NSE** | **16.20** | **11.00** | **0.026** |
| Q1–Q3 | 10.70–19.23 | 9.15–14.00 | |
| Preoperative NR2Ab | 1.99 | 2.34 | 0.364 |
| Q1–Q3 | 1.53–2.73 | 1.74–4.32 | |
| Postoperative NR2Ab | 1.36 | 1.67 | 0.563 |
| Q1–Q3 | 1.24–2.40 | 1.30–2.74 | |

[a] *median*

immunosorbent assay (ELISA) (reagent Gold Dot-1, CIS-Biotech Inc, Decatur, GA; instrument: Personal LAB, Adaltis, Inc, Montreal, Canada).

The lower limits of detection were 0.02 µg per L (S-100B), 0.04 µg per L (NSE) and 0.5 AU (NR2Ab). The reference value was set at 0.2 for S100B, 18.3 for NSE and 2.0 µg/L (1.4 AU) for NR2Ab, the upper limits of normal, according to the 95 percentile of the "healthy population" reference range provided by the manufacturer of the assay. The assay imprecision according to the manufacturer was:

*CV Intra-assay*
NSE ≤2.3% (CV estimated for 4 different concentrations, from 18 to 100 µg/L)
S100B ≤6.4% (CV estimated by manufacturer for 6 different concentrations, from 0.11 to 18.40 µg/L)
NR2Ab ≤5.6% (CV estimated for 3 different concentrations, from 1.4 to 17.0 AU).

*CV Inter-assay*
NSE ≤5.3% (CV estimated for 4 different concentrations, from 18 to 100 µg/L)
S100B ≤10.7% (CV estimated for 6 different concentrations from, 0.11 to 18.4 µg/L)
NR2Ab ≤9.2% (CV estimated for 3 different concentrations, from 1.4 to 17.0 AU).

MRI was performed using standardized protocols on a 3T instrument (MAGNETOM Trio, a Tim System 3T; Siemens AG, Munich, Germany).[14] Scans were obtained in the same order with a T1-weighed 3-plane localizer, diffusion-weighted imaging (DWI) sequence, and fluid-attenuated inversion recovery (FLAIR) sequence. The images were presented to an investigator who was unaware of the clinical assessments.

For statistical analysis MedCalc software, version 11.4.2.0 (MedCalc, Mariakerke, Belgium) was used. The Mann-Whitney independent test was applied to the data and $P < .05$ was considered significant.

## Results

The erythropoietin-treated and control groups were similar with regard to standard preoperative history, physical, and biochemical assessments,[15] and the two groups also had comparable serum NR2Ab, S100B (before and after the surgical procedure) and NSE (before the surgical procedure) results. A significant difference was noted in postoperative NSE ($P = .03$) in the two groups; however, all levels were below the upper reference limit of 18.3 µg per L (**Table 1**).

All 20 patients survived the surgical procedure; none had shown neurological dysfunction before the operation. The MRI scans performed 24 hours before the surgery confirmed chronic multiple small ischemic lesions (2-5 mm) in all patients. Only 1 patient had a large ischemic region in the left middle artery circulation. However, we observed that

Table 2. Comparison of Serum and Postoperative Concentrations of Biomarkers of Patients With and Without Brain Ischemia (NOISCH) Regardless of Erythropoietin Therapy (Mann-Whitney Test)

| Parameter[a] (µg/L) | NOISCH | ISCH | *P* Value |
|---|---|---|---|
| Preoperative S100B | 0.090 | 0.085 | 0.547 |
| Q1–Q3 | 0.063–0.100 | 0.065–0.095 | |
| Postoperative S100B | 0.290 | 0.310 | 0.803 |
| Q1–Q3 | 0.200–0.388 | 0.230–0.335 | |
| Preoperative NSE | 8.00 | 8.75 | 0.764 |
| Q1–Q3 | 6.48–9.88 | 7.30–9.55 | |
| Postoperative NSE | 15.00 | 12.85 | 0.424 |
| Q1–Q3 | 10.03–18.48 | 10.80–14.00 | |
| Preoperative NR2Ab | 1.99 | 4.32 | 0.057 |
| Q1–Q3 | 1.52–2.36 | 2.20–6.37 | |
| Postoperative NR2Ab | 1.36 | 2.74 | 0.016 |
| Q1–Q3 | 1.16–2.19 | 2.18–2.88 | |

[a] *median*

Table 3. Diagnostic Accuracy in Optimal Cut-Off Values of NR2Ab Serum Preoperative and Postoperative Concentrations (> 2.38 µg/L and > 2.51 µg/L) of Patients With and Without Brain Ischemia

| Parameter | Sensitivity | 95% CI | Specificity | 95% CI | + LR | – LR | + PV | – PV |
|---|---|---|---|---|---|---|---|---|
| Preoperative NR2Ab | 75.00 | 19.4–99.4 | 80.00 | 51.9–95.7 | 3.75 | 0.31 | 48.4 | 92.8 |
| Postoperative NR2Ab | 75.00 | 19.4–99.4 | 93.33 | 68.1–99.8 | 11.25 | 0.27 | 73.8 | 93.7 |

*CI–confidence interval; LR–likelihood ratio; PV–predictive value.*

4 of 10 patients from the control group had postoperative ischemic brain lesions; 2 of those lesions were larger than 5 mm (ie, cerebrovascular insult). The other 2 patients in the control groud had small postoperative ischemic lesions of approximately 2 mm in diameter. MRI scans revealed no acute postoperative ischemic brain lesions in patients treated with human recombinant erythropoietin and approximately 20% lower pre- and postoperative serum concentrations of NR2Ab compared with the control group (**Table 1**).

Serum concentrations of ischemia biomarkers of the 16 patients without brain ischemia were compared to the 4 patients with acute postoperative ischemia regardless of erythropoietin therapy, and displayed significant differences only in postoperative NR2Ab ($P$ = .02). Both pre- and postoperative concentrations of NR2Ab were more than 2-fold higher in patients with ischemia (**Table 2**).

## Discussion

Current measures of neurological complications after cardiac operations are not optimal, but biomarkers have recently gained attention.

Cerebral complications are important after cardiac surgery because CPB is the leading cause of disability and morbidity. The etiology of neurological complications from heart surgery is multifactorial, and includes intraoperative micro- and macroemboli, abnormal

**Figure 1**

Receiver operating curves of N-methyl-D-aspartate receptor antibodies (NR2Ab) preoperative and postoperative serum concentrations of 4 patients with and 16 patients without brain ischemia.

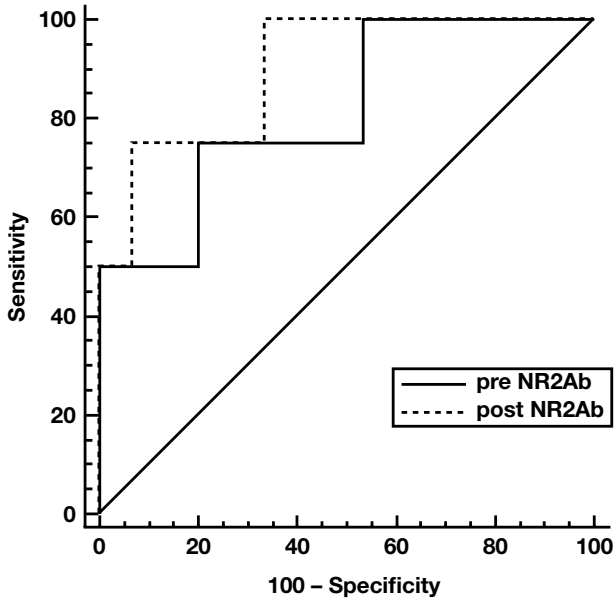


Table 4. Comparison of Receiver Operating Curves (ROC) of NR2Ab Serum Preoperative and Postoperative Concentrations of Patients With and Without Brain Ischemia Including Pairwise Comparison

| Parameter | AUC | SE | 95% CI | z | *P* Value |
|---|---|---|---|---|---|
| Preoperative NR2Ab | 0.817 | 0.136 | 0.575–0.954 | | |
| Postoperative NR2Ab | 0.900 | 0.089 | 0.675–0.989 | | |
| Preopertive NR2AB vs. Post NR2Ab | | | | 0.529 | 0.597 |

*AUC–area under curve; SE–standard error; CI–confidence interval; z–z statistic.*

cerebral perfusion, reperfusion injury, and inflammatory and neurohumoral responses. Currently, prediction and diagnosis of postoperative ischemia are based ol clinical, neuroimaging, and electrophysiological assessments. Routine application of specific biomarkers with predictive and diagnostic capabilities, in specific clinical situations, could represent a low-cost alternative that could improve management of the cardio- and cerebrovascular status in patients.[9-10] The release of neuronal and glial tissue–derived proteins before and after acute stroke correlates with the neuroradiological and neurobehavioral consequences of ischemic brain lesions and may be useful in predicting short- and long-term risk. However, the kinetics of the release of these proteins depends on the type of stroke; they do not always reflect the degree of ischemic damage. On the contrary, they often reflect complex neuronal-glial interactions as alpathological consequence of ischemic brain lesions.

We observed postoperative fresh ischemic lesions in 4 of the 10 patients who did not receive rHuEpo therapy. Two of them 4 patients had lesions larger than 5 mm and experienced delirium (ie, cerebrovascular insult). The pre-NR2Ab levels of both patients were higher than 6.0 µg/L (ie, 3-fold higher than the reference value of 2.0 µg/L). Postoperative NR2Ab levels were proportionally lower in both groups of participants, despite the fact that acute ischemic lesions were noted in the Epo group. Comparison of serum concentrations of biomarkers of 16 patients without brain ischemia and 4 patients with acute ischemia, regardless of erythropoietin therapy, displayed a significant difference in postoperative NR2Ab

($P$ = .02). Concentrations of pre- and postoperative NR2Ab were approximately 2-fold higher, on average, in ischemic patients (**Table 2**). The sensitivity at the pre- and postoperative cutoff concentrations of serum NR2Ab (>2.38 µg/L and >2.51 µg/L, respectively) in the 4 patients with, and 16 patients without, brain ischemia was relatively low compared with the specificity of postoperative NR2Ab (93.3%). The positive predictive value for ischemic stroke is relatively low (≤73.8%) compared with the negative predictive value (≥92.8%) for both NR2Ab values (**Table 3**). Comparison of receiver operating curves (ROCs) of NR2Ab preoperative and postoperative concentrations of 4 patients with and 16 patients without brain ischemia, including pairwise comparison, displayed a larger AUC for postoperative NR2Ab, but no significant difference compared with preoperative NR2Ab (**Figure 1**, **Table 4**). These findings are difficult to compare against those of previous studies that examined changes of NR2Ab, because of differences in clinical protocols and number of subjects. The sensitivity and positive predictive values obtained in our study are much lower than observed in one study of 105 patients who had experienced TIA strokes and 255 controls[9]; however, the preoperative levels of NR2Ab in high-risk patients were similar as those reported in a prospective, multicenter clinical trial to predict adverse neurological complications.[10]

Not all ischemic brain injuries from ischemia are detectable by MRI. The use of biomarkers is helpful because it may reveal injuries that eare not detected by imaging. The results of our study suggest the possibility that rHuEpo administered perioperatively may protect against ischemic brain damage. Despite a 40% difference in the incidence of stroke between the treated and control groups, postoperative NR2Ab and S100B levels were not significantly different. NSE concentrations were, paradoxically, significantly higher in the rHuEpo group with no acute clinical or radiographic signs of ischemic brain injury. Serum concentrations of the biomarkers of 16 patients without brain ischemia and 4 patients with acute ischemia, regardless of erythropoietin therapy were not significaty different. Although NR2Ab may be a marker of cerebral ischemia, S100B and NSE are considered markers of blood-brain barrier (BBB) dysfunction. Ischemic stroke is usually accompanied by BBB dysfunction; however, BBB dysfunction is not necessarily indicative of cerebral ischemia.

The key limitation of our study was the small study group. Biomarkers should be measured more frequently after cardiac surgery to obtain peak concentrations, and the association with long-term clinical outcome should be assessed. The appearance of NR2Ab is slow, and this limits the utility of measuring serum autoantibodies immediately after the onset of stroke.

In conclusion, we compared the preoperative and postoperative blood levels of NSE, S100B, and NR2Ab, as well as MRI results from erythropoietin-treated and control groups of patients undergoing surgical revascularization of the heart with CPB to diagnose cerebral ischemia and ischemic stroke. Our results regarding the diagnostic values of pre- and postoperative NR2Ab levels and brain protection with rHuEpo proved beneficial for medical intervention for the patients known to be at increased risk pre- and postoperatively. Analysis of serum concentrations of glial and neuronal tissue–derived proteins might also be the best strategy to enable the monitoring and evaluation of neuroprotective stroke treatment. Given the significant risk of stroke associated with CPB, future studies involving larger multisite trials are warranted, as is evaluation of alternative molecular biomarkers (for example, NR2 peptide fragments, which are likely formed immediately after an ischemic event) to reliably identify ischemic brain injury in this population.[17-19] LM

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