

Combined Estimation of Plasma Cell-free DNA Level and Neuron Specific Enolase Activity as Outcome Predictors of Post-resuscitation Patients

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ABSTRACT

Objectives: To evaluate the prognostic yield of estimation of plasma cell-free DNA and neuron specific enolase (NSE) activities in post-resuscitation patients concerning survival and neurologic outcome in comparison to Cerebral Performance Category (CPC) score. **Patients and Methods:** The study included 80 patients developed out-of-hospital cardiac arrest. All patients received mild therapeutic hypothermia irrespective of the initial rhythm. Blood samples were drawn at study inclusion for estimation of plasma cell-free DNA and serum NSE activity. Mortality rate was determined 1-week and 1-month after admission. Neurologic outcomes were evaluated using CPC score collectively as CPC score of 1-2 indicated favorable neurological outcome. **Results:** 1-week mortality rate was 20% and throughout a mean ICU stay of 18.4 ± 10.1 ; range: 3-42 days, another 17 patients died for late mortality rate of 21.3% and a total mortality rate of 46.3%. At time of discharge 31 patients (38.8%) were CPC1-2 (Favorable outcome), while 12 patients (14.9%) were CPC-3 (Unfavorable outcome). Mean plasma DNA and serum NSE activities were significantly higher in CPC3 patients compared to CPC1-2 patients. Survivors had significantly lower at admission plasma cell-free DNA and non-significantly lower serum NSE compared to non-survivors. Survivors had favorable outcome had significantly lower at admission plasma cell-free DNA and serum NSE compared to those had unfavorable neurologic outcome. There was positive significant correlation between plasma cell-free DNA and serum NSE activities and mortality and unfavorable outcome rates. However, the correlation was more significant with plasma DNA than with serum NSE. ROC curve analysis revealed that elevated levels of both parameters could significantly predict the unfavorable neurologic outcome, while high plasma cell-free DNA could significantly predict high mortality rate. **Conclusion:** At admission plasma levels of cell-free DNA and serum NSE act synergistically for prediction of survival and neurologic outcome of post-resuscitation patients.

Keywords: Post-resuscitation, Cell-free plasma DNA, Neuron specific enolase, Mortality, Neurological outcome

INTRODUCTION

Optimal survival following sudden cardiac arrest requires heart and brain resuscitation. In patients who achieve cardiac resuscitation, brain recovery from anoxic injury is variable. Neurological sequelae ranges from complete recovery to coma with brain death. Thus, ideally outcome assessment would incorporate functional and neurologic status⁽¹⁾.

Overall survival rate from out-of-hospital cardiac arrest has not increased in parallel with the improvements in cardiopulmonary resuscitation (CPR)⁽²⁾. The hospital discharge rate is 15% in a meta-analysis that included a total population of over 26,000 patients⁽³⁾. Additionally, the majority of patients who achieve return of spontaneous circulation after successful CPR have a high risk to death in the post-arrest period. As another problem, is the neurological outcome of the survivors who could escape the mortality risk during the post-arrest period⁽⁴⁾.

Functional and neurologic status following cardiac arrest is a more meaningful clinical outcome than simply hospital survival when trying to judge the effectiveness of resuscitation care⁽⁵⁾. Functional neurologic status consists of multiple domains including activities of daily living, cognitive function such as memory and abstract thoughts, and emotional health. Ideally then functional and neurologic status would derive from standard, validated, and repeated measures that involve direct subject communication and/or examination. In many circumstances

however, the ability to undertake that type of evaluation is not feasible because of limited resources or the practical logistics of subject contact and participation. The Cerebral Performance Category (CPC) score overcomes these challenges because assessment does not require direct subject contact, does not require assessment at specified time points and because it corresponds to quality of life and functional status derived^(6,7).

Circulating DNA in plasma is altered both qualitatively and quantitatively in various clinical conditions, including pregnancy, graft rejection trauma, cancer, stroke, myocardial infarction, sepsis, acute pancreatitis and abdominal pain. The exact mechanism of DNA occurrence in blood, however, is not fully understood. Also, knowledge about the elimination of cell-free DNA from blood is inadequate, but available data suggest that more than one mechanism is involved in its clearance⁽⁸⁻¹⁰⁾. Cell-free DNA can originate from necrotic cells or apoptotic processes, and active release of DNA fragments from living cells has also been described^(11,12).

Immediate post-resuscitation period has some similarities to the sepsis syndrome and septic shock in terms of the inflammatory cascade activation and microcirculatory hypoperfusion. As increased plasma concentrations of cell-free DNA and nucleosomes, in which fragmented DNA is packed during apoptosis, have been found in patients with sepsis and septic shock, and the plasma DNA concentration was found to be an

independent predictor for ICU mortality in these patients^(13, 14, 15).

Thus, the current study aimed to evaluate the prognostic yield of estimation of plasma cell-free DNA and neuron specific enolase (NSE) activities in post-resuscitation patients concerning survival and neurologic outcome in comparison to Cerebral Performance Category (CPC) score.

PATIENTS & METHODS

The current study was conducted at Departments of Neurology, Cardiology and Clinical Pathology, Benha University Hospital in conjunction with Medical Biochemistry Department, Faculty of Medicine through the period since June 2011 till Oct 2012. After approval of the study protocol by the Local Ethical Committee and obtaining written fully informed near patients' relative consent, 80 patients had cardiac arrest were enrolled in the study. Exclusion criteria included failure of resuscitation by emergency health provider, time lapsed since collapse till the start of CPR longer than 15 minutes, no return of spontaneous circulation be achieved within 60 minutes after collapse and history of chronic renal failure, stroke or acute coronary syndrome within the 30 days preceding collapse. Also, trauma patients, pregnant women and patients fulfilling inclusion criteria but died within 24 hours after resuscitation were excluded from the study.

All patients received mild therapeutic hypothermia irrespective of the initial rhythm. Therapeutic hypothermia was initiated after

admission with an intravenous infusion of cold saline (4°C, 1000 to 1500 ml bolus) followed by surface cooling with commercially available non-invasive devices (ArcticSun2000® Medivance, Louisville, Colorado, USA). The target temperature was maintained for 24 hours. All patients received intravenous sedation and analgesia using a combination of midazolam (0.125 mg/Kg/h) and fentanyl (0.002 mg/kg/h) in addition to muscle relaxation using repetitive administration of pancuronium (0.1 mg/kg) in order to prevent shivering.

Blood Sampling: Blood samples were drawn at study inclusion for estimation and were divided into two parts:

1. The first part was put in clean dry tube and allowed to clot and then serum was separated in clean dry Eppendorff tube to be stored at -80°C till assayed for serum NSE activity using an enzyme immunoassay (Elecsys 2010, Roche Diagnostics GmbH, Mannheim, Germany)⁽¹⁶⁾.
2. The second part was collected in heparinized tubes. Plasma was separated as soon as possible by centrifugation at 1,500 g for 15 minutes and transferred to acid-handled plastic tubes, which were stored at -80°C till assayed for plasma cell-free DNA level.

Quantification of plasma cell-free DNA:

DNA extraction and quantification of plasma cell-free DNA were performed as described by *Saukkonen et al.*⁽¹⁷⁾. Briefly, plasma samples were centrifuged at 16,000 g for 10 minutes before DNA extraction

to remove any residual cells⁽¹⁸⁾. DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the "blood and body fluid protocol". Plasma cell-free DNA was measured by real-time quantitative PCR assay for the β -globin gene using the ABI PRISM 7000 sequence detection system (Applied Biosystems). The sequences were as follows:

Forward primer 5'-GCA CCT GAC TCC TGA GGA GAA-3'

Reverse primer 5'-CAC CAA CTT CAT CCA CGT TCA-3'

PCR cycling conditions were two minutes at +50°C, 10 minutes at +95°C, and 46 cycles of 20 seconds at +95°C and one minute at +60°C. Plasma DNA was measured in duplicate samples. A 10-fold serial dilution of human genomic DNA (Roche Diagnostics GmbH, Mannheim, Germany) was used as a standard curve in the PCR assay. Results are expressed as genome equivalents (GE)/ml; 1 GE equals 6.6 picograms of DNA. Plasma cell-free DNA concentration of 4,000 GE/ml was considered as an upper limit of normal range⁽¹⁹⁾.

Clinical evaluation and outcomes:

The basal characteristics of patients and outcomes were evaluated. Mortality rate was determined one week after admission (shock-related mortality) and one-month after admission (late dead from neurological dysfunction including brain death or cardiovascular problem including myocardial infarction). Neurologic outcomes were evaluated using the Cerebral Performance Category (CPC) score collectively as CPC score of 1-2 indicated favorable

neurological outcome, whereas a CPC score of 3-5 indicated unfavorable outcome. In detail; CPC1: normal function, CPC 2: minor disability, CPC 3: severe disability, CPC 4: coma and CPC 5: death⁽²⁰⁾.

STATISTICAL ANALYSIS:

Obtained data were presented as mean \pm SD, ranges, numbers and ratios. Results were analyzed using Wilcoxon; ranked test for unrelated data (Z-test) and Chi-square test (χ^2 test). Possible relationships were investigated using Pearson linear regression. Sensitivity & specificity of estimated parameters as predictors for outcome were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared versus the null hypothesis that AUC=0.05. Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. P value <0.05 was considered statistically significant.

RESULTS

The study included 80 patients developed out-of-hospital cardiac arrest. There were 59 males (73.8%) and 21 females (26.2%) with mean age of 63.8 \pm 5.1; 49-78 years. All patients had multiple co-morbidities in varying combinations; however, the presence of underlying cardiac disorder presents the cause of cardiac arrest in 38 patients (47.5%), 18 patients (22.5%) had respiratory failure, 15 patients (18.8%) had hypovolemia and 9 patients (11.3%) had metabolic disorders.

As regards the initial cardiac arrest rhythm; 70 patients (87.5%) had

either pulseless ventricular tachycardia or ventricular fibrillation, 8 patients (10%) had asystole, and only two patients (2.5%) had pulseless electrical activity. For resuscitation, more than one resuscitation modality was tried for each patient; however, all received hypothermia as a basic line of management in conjunction with defibrillatory shock wave in 73 patients and 52 patients required coronary reperfusion therapy via PCA for acute coronary syndrome. Mean time elapsed till return of spontaneous circulation (ROSC) since arrival to emergency department was 21.2 ± 3.6 ; range: 12-25 minutes. However, the majority of patients (68.8%) required 20-25 minutes till ROSC, 22.5% of patients achieved ROSC within 15-20 minutes and only 8.7% of patients achieved ROSC within 12-15 minutes.

Twenty patients died within the first week after resuscitation for a shock-related mortality rate of 25%. Mean ICU stay was 18.4 ± 10.1 ; range: 3-42 days, throughout ICU stay 17 patients died for late mortality rate of 21.3% and a total mortality rate of 46.3%. At time of discharge 31 patients (38.8%) were CPC1-2 (Favorable outcome); 13 patients were CPC-1, while 18 patients were CPC2 and 12 patients (14.9%) were CPC-3 (Unfavorable outcome), (Fig. 1).

At admission mean levels of plasma DNA and serum NSE activity showed progressive steady increase with increased CPC score. Mean plasma DNA levels estimated in CPC3 patients were significantly ($p < 0.05$) higher compared to both CPC1 and CPC2 patients with non-significantly ($p > 0.05$) higher levels in CPC2 patients compared to CPC1

patients. Similarly, mean serum levels of NSE activity estimated in CPC3 patients were significantly ($p < 0.05$) higher compared to CPC1 patients and non-significantly ($p < 0.05$) compared to CPC2 patients with non-significantly ($p > 0.05$) higher levels in CPC2 patients compared to CPC1 patients, (Table 2).

Categorizing patients according to survival outcome; survivors had significantly ($p < 0.05$) lower at admission plasma cell-free DNA and non-significantly ($p > 0.05$) lower serum NSE activity compared to non-survivors, (Fig. 2). Categorizing patients according to neurologic outcome; survivors had favorable outcome significantly ($p < 0.05$) lower at admission plasma cell-free DNA and serum NSE activity compared to those had unfavorable neurologic outcome, (Table 2, Fig. 3).

There was positive significant correlation between plasma cell-free DNA and serum NSE activities and mortality and unfavorable outcome rates. However, the correlation was more significant with plasma DNA than with serum NSE. On the other hand, high levels of both parameters showed highly significant correlation with unfavorable neurologic outcome rate (Table 3).

Evaluation of predictivity of markers' estimation at time of admission for outcome, ROC curve analysis revealed that elevated levels of both parameters could significantly predict the unfavorable neurologic outcome (Fig. 4), while high plasma cell-free DNA could significantly predict high mortality rate among post-arrest patients, (Table 4, Fig. 5).

Table (1): Enrollment data of studied patients

Data			Findings	
Age (years)	Strata	>40-50	2 (2.5%)	
		>50-60	16 (20%)	
		>60-70	60 (75%)	
		>70	2 (2.5%)	
	Total	63.8±5.1 (49-78)		
Gender	Males		59 (73.8%)	
	Females		21 (26.2%)	
Associated co-morbidities	Obesity		50 (62.5%)	
	Diabetes		26 (32.5%)	
	Hypertension		45 (56.3%)	
	CAD		49 (61.3%)	
	CHF		28 (35%)	
	COPD/emphysema		21 (26.3%)	
Cause of arrest	Underlying cardiac disorder		38 (47.5%)	
	Respiratory failure		18 (22.5%)	
	Metabolic disorders		9 (11.3%)	
	Hypovolemia		15 (18.7%)	
Initial cardiac arrest rhythm	Ventricular fibrillation		70 (87.5%)	
	Asystol		8 (10%)	
	Pulseless electrical activity		2 (2.5%)	
Resuscitation procedure	Therapeutic hypothermia		80 (100%)	
	Defibrillatory shock		73 (91.3%)	
	Coronary reperfusion therapy		52 (65%)	
Time till ROSC	Strata	<15 min	Number	7 (8.7%)
			Mean ± SD	13.6±3.9
		15-20 min	Number	18 (22.5%)
			Mean ± SD	17.6±7.8
		>20 min	Number	55 (68.8%)
			Mean ± SD	23.4±1.5
Total		21.2±3.6 (12-25)		

Data are presented as mean ± SD & number; ranges & percentages are in parenthesis; CAD: Coronary artery disease; CHF: Chronic heart failure; COPD: Chronic obstructive pulmonary disease; ROSC: return of spontaneous circulation

Table (2): Mean (\pm SD) level of estimated parameters in studied post-resuscitation patients categorized according to outcome

			Plasma Cell-free DNA	Serum NSE
Survival outcome	Survivors	CPC1 (n=13)	2246 \pm 738 (1290-3450)	27.1 \pm 8 (20-32)
		CPC2 (n=18)	2529 \pm 730 (1000-3700)	31.7 \pm 7.8 (19-47)
		CPC3 (n=12)	5972 \pm 2098 (3000-8720) ^{AB}	40.3 \pm 14.5 (25-54) ^A
		Total (n=43)	3400 \pm 2937 (1000-8720)	32.7 \pm 8.9 (19-54)
	Non-survivors (CPC4-5; n=37)		4910 \pm 2976 (750-9700) ^C	39.3 \pm 19 (15-86)
Neurologic outcome	Favorable (CPC1-2; n=31)		2410 \pm 735 (1000-3700)	29.8 \pm 6.7 (19-47)
	Unfavorable (CPC-3; n=12)		5972 \pm 2098 (3000-8720) ^D	40.3 \pm 14.5 (25-54) ^D

A: significant versus CPC1 patients; B: significant versus CPC2 patients; C: significant versus total survivors; D: significant versus patients had favorable outcome

Table (3): Correlation coefficient “r” between outcome of post-resuscitation patients and estimated parameters

		Plasma Cell-free DNA	Serum NSE
Mortality rate	r	0.289	0.224
	p	=0.009	=0.046
Unfavorable neurologic outcome rate	r	0.793	0.536
	p	<0.001	<0.001

Table (4): ROC curve analysis for the predictivity of estimated parameters and outcome of post-resuscitation patients

		AUC	Std error	Sig.	95% CI	
					Lower	Upper
Mortality	Plasma Cell-free DNA	0.627	0.068	0.047	0.493	0.761
	Serum NSE	0.659	0.068	>0.05	0.437	0.702
Unfavorable neurologic outcome	Plasma Cell-free DNA	0.956	0.032	<0.001	0.893	1.018
	Serum NSE	0.805	0.080	=0.002	0.649	0.962

AUC: area under curve; Std error: standard error; Sig.: significance versus the null hypothesis that AUC=0.5; CI: confidence interval

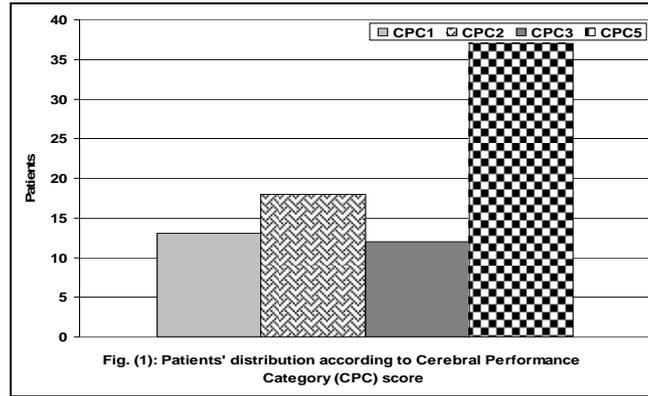


Fig. (1): Patients' distribution according to Cerebral Performance Category (CPC) score

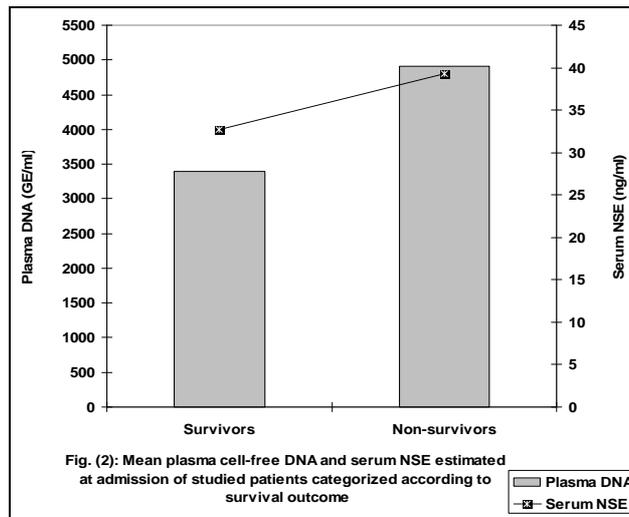


Fig. (2): Mean plasma cell-free DNA and serum NSE estimated at admission of studied patients categorized according to survival outcome

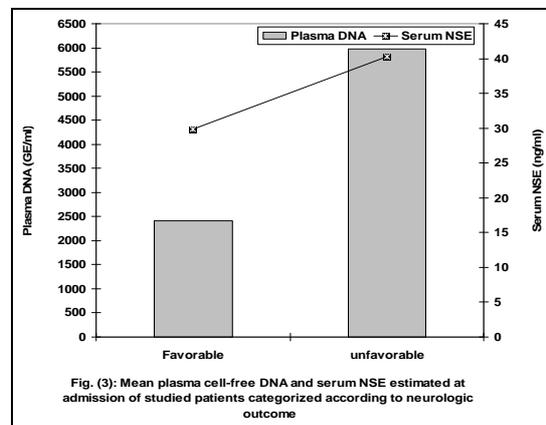


Fig. (3): Mean plasma cell-free DNA and serum NSE estimated at admission of studied patients categorized according to neurologic outcome

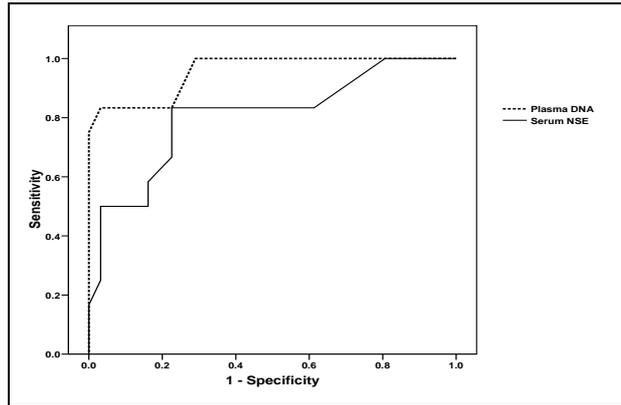


Fig. (4): ROC analysis for both parameters as predictors for neurological outcome

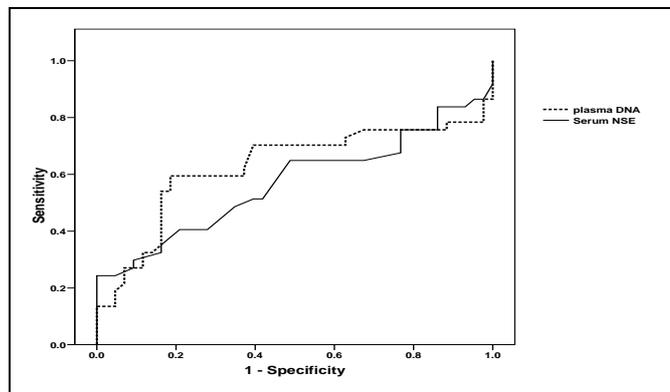


Fig. (5): ROC analysis for both parameters as predictors for vitality outcome

DISCUSSION

The predictability for outcome of post-resuscitation is critical for both patients and health care providers, so multiple studies tried to evaluate clinical parameters and laboratory markers for prediction of both survival and neurological outcome of these patients (21-24). The current study showed a significantly higher at admission levels of plasma cell-free DNA and serum NSE in non-

survivors compared to survivors and in survivors who had favorable neurologic outcome compared to those had unfavorable outcome. However, serum NSE activities showed less prognostic yield for mortality compared to plasma DNA and ROC curve analysis showed high specificity of both parameters for neurological outcome, but plasma level of DNA showed high sensitivity for survival outcome.

The obtained data supported the previous report concerning the applicability of cell-free DNA plasma level estimation as outcome predictor for critical patients cared at ICU. **Saukkonen et al.**⁽²⁵⁾ investigated the concentration of plasma DNA and its association with organ dysfunction and hospital mortality in ICU patients and found the maximum plasma DNA concentration measured during the first 96-hours of intensive care is associated with higher degree of organ dysfunction and disease severity, and the maximum DNA concentration is independently associated with hospital mortality. **Okkonen et al.**⁽²⁶⁾ studied prospectively 580 mechanically ventilated critically ill patients and found that plasma DNA levels were significantly higher in non-survivors than survivors and its level at baseline was an independent predictor of 90-day mortality.

Also, the obtained results supported the applicability of plasma DNA estimation as predictor for prognosis of acute attacks as shown by **Rainer et al.**⁽²⁷⁾ who studied patients with acute abdominal pain and found that median plasma DNA concentrations were three-fold higher in patients with systemic inflammatory response syndrome, five-fold higher in patients who died within 28 days, and eight-fold higher in patients admitted to ICU and concluded that plasma DNA may have a role in patients with acute abdominal pain as a marker for inflammation and cancer, and a predictor of ICU admission/mortality. **Arnalich et al.**⁽²⁸⁾ studied 130 consecutive patients who underwent laparotomy for suspicious of acute mesenteric ischemia (AMI)

and found that DNA concentration at admission was significantly higher in patients with AMI and in AMI patients who died compared to those with different diagnosis and concluded that plasma DNA levels may be a useful biomarker in predicting the outcome of patients with AMI.

Concerning the prognostic yield of cell-free plasma DNA level estimation in ischemic neurological affection; **Rainer et al.**⁽²⁹⁾ reported significantly higher median plasma DNA concentrations taken within 3 hours of symptom onset in patients had ischemic stroke, intracerebral hemorrhage, and transient ischemic attacks who died compared with those who survived at discharge and correlated with the volume of cerebral hematoma with 100% sensitivity and 74.4% specificity for predicting hospital mortality after stroke.

As regards prognosis of post-resuscitation patients, the reported data go in hand with those of **Arnalich et al.**⁽³⁰⁾ who found plasma DNA concentrations at admission of out-of-hospital cardiac arrest patients were higher in non-survivors at 24 hours than in survivors and were also higher in patients who died in the hospital than in survivors to discharge and concluded that plasma DNA levels may be a useful biomarker in predicting outcome after out-of-hospital cardiac arrest. **Huang et al.**⁽³¹⁾ found plasma cell-free DNA level estimated within 2-hours after cardiac arrest was higher in the non-survival group than the survival-to-discharge group and concluded that the plasma cell-free DNA level increases during the early post-cardiac

arrest phase and can be an early prognostic factor for out-of-hospital cardiac arrest patients.

As regards NSE, it was found to have has specificity for neurological outcome, that finding could be attributed to the fact that NSE, a gamma isomer of enolase, is located in neurons and neuroectodermal cells so it confers specificity for the damage of nerve cells irrespective of the effect of such damage on survival. Several studies agree that the high NSE serum activity carries the highest predictive value for neurological outcome after resuscitation.

Thus, considering the non-specific sensitivity of high levels of serum NSE for survival prediction, necessitated the combined estimation of NSE and another predictor for survival, so the current study provided the outcome of combined estimation of serum NSE and plasma DNA for coverage of the probable outcome. In hand with combined biomarker estimation; **Topjian et al.** ⁽³²⁾ found serum NSE activities are associated with neurologic outcome, whereas serum S-100B levels are associated with survival.

It could be concluded that at admission plasma levels of cell-free DNA and serum NSE act synergistically for prediction of survival and neurologic outcome of post-resuscitation patients.

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قياس مستويات الحمض النووي في بلازما الدم والإينولاز الخاص بالخلايا العصبية بالمصل كمؤشرات لنتائج ما بعد الإنعاش لمرضى السكتة القلبية

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الأهداف: تقييم القيمة التوقعية لقياس مستويات الحمض النووي الديوكسي الريبوز الحر في بلازما الدم ونشاط الإينولاز الخاص بالخلايا العصبية بالمصل في مرضى مرحلة ما بعد الإنعاش بشأن البقاء حياً وللنتائج العصبية مقارنة بمقياس الأداء الدماغي.

المرضى والطرق: شملت الدراسة ٨٠ مصاباً بالسكتة القلبية خارج المستشفى، تلقوا جميعاً إفاقة مع خفض طفيف لدرجة حرارة الجسم. وأخذت عينات من الدم لقياس مستوى الحمض النووي الديوكسي الريبوز الحر في بلازما الدم، ونشاط الإينولاز الخاص بالخلايا العصبية بمصل الدم. تم تحديد معدل وفيات خلال الأسبوع الأول والشهر الأول وتم تقييم النتائج العصبية باستخدام مقياس الأداء الدماغي.

النتائج: كان معدل وفيات في الأسبوع الأول عشرون مريضاً من ٨٠ مريضاً (٢٥٪)، وطوال فترة الإقامة بوحدة العناية المركزة والتي استمرت حوالي ١٨ يوماً توفي ١٧ مريضاً آخرًا بمعدل وفيات متأخر ٢١.٣٪ ومعدل وفيات إجمالي ٤٦.٣٪. عند خروج المرضى من وحدة العناية المركزة كان ٣١ مريضاً (٣٨.٨٪) في حالة جيدة (نتيجة إيجابية)، في حين ١٢ مريضاً (١٥٪) كانوا في حالة سيئة (نتيجة سلبية). كان مستوى الحمض النووي الحر في البلازما ونشاط الإينولاز الخاص بالخلايا العصبية بالمصل أعلى بشكل ملموس في مرضى النتيجة السلبية مقارنة بمرضى النتيجة الإيجابية. وكان مستواهما أقل بكثير عند الناجيين مقارنة مع غير الناجيين. ووجدت علاقة ارتباط ذو دلالة احصائية بين مستويات الحمض النووي الحر في البلازما ونشاطات الإينولاز الخاص بالخلايا العصبية بالمصل ومعدلات الوفيات ونتائج سلبية. ووجد بالتحليل الاحصائي أن المستويات المرتفعة للحمض النووي الحر في البلازما تمكن من التنبؤ بشكل كبير بارتفاع معدل الوفيات.

الاستنتاج: قياس مستوى الحمض النووي الحر في البلازما ونشاط الإينولاز الخاص بالخلايا العصبية بالمصل يفيد للتنبؤ بالنجاة والنتائج العصبية في مرحلة ما بعد إنعاش مرضى السكتة القلبية.