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ORIGINAL WORK

Serum Caspase-3 Levels and Early Mortality of Patients with Malignant Middle Cerebral Artery Infarction

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Abstract

Purpose: Circulating caspase-3 levels at 24 h of ischemic stroke were found to be associated with poorer functional neurological outcome in a previous study. The aim of this study was to determine whether there is an association between serum caspase-3 levels and early mortality in patients with malignant middle cerebral artery infarction (MMCAI).

Methods: We included patients with MMCAI defined as computer tomography showing ischemic changes in more than 50% of the middle cerebral artery territory and Glasgow Coma Scale ≤ 8. Serum caspase-3 levels at days 1, 4, and 8 of MMCAI were determined.

Results: Non-surviving MMCAI ($n = 34$) showed higher serum caspase-3 levels at days 1 ($p < 0.001$), 4 ($p = 0.001$), and 8 ($p = 0.01$) than surviving patients ($n = 34$). We found that the area under the curve of serum caspase-3 levels for prediction of mortality at 30 days was 88% (95% CI = 78–95%; $p < 0.001$). Multiple logistic regression showed that serum caspase-3 levels were associated with 30-day mortality (OR = 51.25; 95% CI = 8.30–316.31; $p < 0.001$).

Conclusions: The novel and more important findings of our study were that high serum caspase-3 levels were associated with mortality in MMCAI patients.

Keywords: Caspase-3, Cerebral infarction, Patients, Mortality

Introduction

Ischemic stroke is an important cause of disabilities, deaths, and consumption of health resources [1]. Cell death by apoptosis appears in cerebral ischemia [2–9], and it occurs through extrinsic pathway (or death receptor pathway) and intrinsic pathway (or mitochondrial pathway) [2–9] (Fig. 1). The activation of tumor necrosis factor receptor superfamily, which is a surface death receptor, in type I cells by its ligand tumor necrosis factor superfamily produces a death signal that initiates apoptosis by extrinsic pathway, leading to cleave pro-caspase-8 in active caspase-8, and that produces caspase-3 activation. Different agents such as interleukin (IL)-1, IL-6, and oxygen free radicals begin apoptosis by intrinsic pathway in type II cells by the liberation of cytochrome-c from the mitochondria into cytoplasm, producing caspase-3 activation. Both apoptotic pathways (intrinsic and extrinsic pathways) produce caspase-3 activation, which is the main executor of apoptosis, leading to cell death [2–9].

Some studies analyzing post-mortem brain samples from patients who died due to cerebral infarction found high expressions of caspase-3 [10–13]. Other studies found higher caspase-3 platelet expression [14] and

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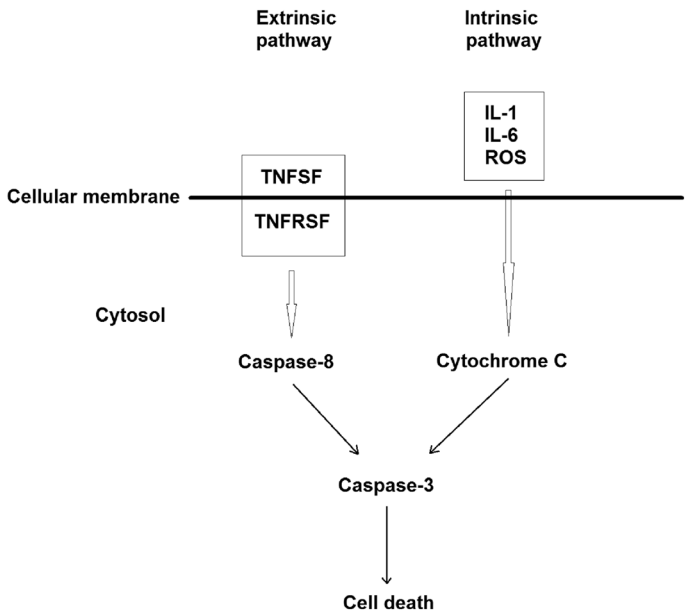

Fig. 1 Apoptosis pathways

higher plasma caspase-3 levels [15, 16] in patients with cerebral infarction compared to control subjects. In addition, one study found that plasma caspase-3 levels at 24 h of ischemic stroke were associated with poorer functional neurological outcome [15]. The aim of this study was to determine whether there is an association between serum caspase-3 levels and early mortality in patients with malignant middle cerebral artery infarction (MMCAI).

Methods

Design and Subjects

This was an observational and prospective study. The period of time of patient recruitment was 2009–2012. This multicentre study was carried out after the approval of Institutional Review Boards of all participating hospitals and with the written informed consent from legal guardians of patients. This study was performed in six Spanish Intensive Care Units: H. Universitario Dr. Negrín (Las Palmas de Gran Canaria), H. Clínico Universitario de Valencia, H. General de La Palma, H. Insular de Las Palmas de Gran Canaria, H. Universitario Nuestra Señora de Candelaria, and H. Universitario de Canarias (Tenerife).

We diagnosed ischemic stroke based on clinical and computed tomography findings [1]. The radiologist interpreting the imaging was blinded to clinical situation and serum caspase 3 levels. We included patients with MMCAI that was defined as computed tomography findings of acute massive middle cerebral artery infarction (MCA) (which consisted of large parenchymal hypodensity at least of 50% of the MCA territory and midline shift), and acute neurological deterioration consisting of a decrease in the level of consciousness compared with the baseline clinical status on admission to Glasgow Coma Scale (GCS) [17] ≤ 8.

Exclusion criteria were inflammatory or malignant disease, pregnancy, age less than 18 years, intracerebral hemorrhage or subarachnoid hemorrhage, or comfort measures only on admission or at any point during their hospitalization.

We had previously determined serum levels of other biomarkers as malondialdehyde [18], soluble CD154 [19], and caspase-cleaved cytokeratin-18 [20] in some of those patients. In this study, the aim was to determine whether there is an association between serum caspase-3 levels and mortality.

Recorded Variables

The following variables were recorded: diabetes mellitus, age, arterial hypertension, sex, chronic obstructive pulmonary disease (COPD), chronic renal failure, heart failure, Acute Physiology and Chronic Health Evaluation II (APACHE II) score [21], GCS, body temperature, sodium, glycemia, bilirubin, creatinine, lactic acid, partial pressure of arterial oxygen (PaO_2), fraction of inspired oxygen (FIO_2), leukocytes, platelets, hemoglobin, international normalized ratio (INR), fibrinogen, activated partial thromboplastin time (aPTT), infarct volume, midline shift, hemorrhagic transformation, and

decompressive craniectomy. The 30-day mortality was the end-point of study.

Blood Sample Collection and Serum Caspase-3 Concentration Determination

Blood samples were obtained on days 1, 4, and 8 of MMCAI and were placed in tubes with separator gel allowing its coagulation at room temperature for 10 min. Afterward, blood samples were centrifugated for 15 min at 1000 g. Finally, we deposited the serum in Eppendorf tubes froze them at −80 °C until the determination of caspase-3 concentration.

The Laboratory Department of the Hospital Universitario de Canarias from La Laguna (Tenerife, Spain) carried out all caspase-3 concentration determinations by means of an enzyme-linked immunosorbent assay (ELISA) with the kit Human Caspase-3 Elisa BlueGene Biotech® (Shanghai, China). This assay had an intra-assay coefficient of variation, inter-assay coefficient of variation, and detection limit of 5.6%, 7.9%, and 0.1 ng/mL, respectively.

Statistical Methods

Medians (and interquartile ranges) were used to report continuous variables, and frequencies (and percentages) to report categorical variables. Wilcoxon–Mann–Whitney test was used to compare continuous variables between patient groups, and Chi-square test to compare categorical variables. Multiple logistic regression was used to determine the association between serum caspase-3 levels and 30-day mortality controlling for lactic acid, GCS, and platelet count. Receiver operating characteristic curve was used to determine the prediction capacity of mortality at 30 days by serum caspase-3 levels. Kaplan–Meier curves of 30-day mortality with patients that had higher and lower serum caspase-3 levels than 0.17 ng/mL were performed; this cutoff value was selected based on the optimal prognostic value according to Youden J index. We analyzed the correlation between continuous variables using Spearman's rank coefficient. We considered statistically significant all p values lower than 0.05. LogXact 4.1 (Cytel Co., Cambridge, MA), SPSS 17.0 (SPSS Inc., Chicago, IL, USA), and NCSS 2000 (Kaysville, Utah) were used to perform the statistical analyses.

Results

As shown in Table 1, we did not find statistically significant differences between non-surviving ($n=34$) and surviving ($n=34$) patients in age, sex, diabetes mellitus, arterial hypertension, COPD, chronic renal failure, temperature, sodium, glycemia, creatinine, bilirubin, PaO_2, PaO_2/FIO_2 ratio, leukocytes, INR, hemoglobin, fibrinogen, aPTT, APACHE II score, volumen infarction, midline shift, hemorrhagic transformation, and decompressive craniectomy. We found that non-surviving MMCAI compared to surviving patients showed lower platelet count and GCS, and higher serum levels of lactic acid and caspase-3. The pathophysiological mechanisms leading to death were brain death in 18 patients and cardiac arrest in 16 patients.

Serum caspase-3 levels at days 1 ($p<0.001$), 4 ($p=0.001$), and 8 ($p=0.01$) of MMCAI were significantly higher in the non-surviving than in the surviving patient group (Fig. 2).

The area under the curve of serum caspase-3 levels for prediction of mortality at 30 days was 88% (95% CI = 78–95%; $p<0.001$) (Fig. 3). Survival analysis showed that patients with serum caspase-3 levels higher than 0.17 ng/mL had higher risk of 30-day mortality compared to patients with lower levels (hazard ratio = 14.9; 95% CI = 7.61–29.23; $p<0.001$) (Fig. 4). Multiple logistic regression showed that serum caspase-3 levels were associated with 30-day mortality (OR = 51.25; 95% CI = 8.30–316.31; $p<0.001$) controlling for lactic acid, GCS, and platelet count (Table 2). We have not found a statistically significant association between serum caspase-3 levels and infarct volume (rho = −0.19; $p=0.34$), or between GCS and infarct volume (rho = −0.29; $p=0.13$).

Discussion

The novel findings of our study were that non-surviving MMCAI patients showed higher serum caspase-3 levels during the first week than the surviving patients, and that high serum caspase-3 levels were associated with mortality.

In one previous study including patients with an acute ischemic stroke who had a transcranial Doppler demonstrating arterial occlusion and receiving t-PA within 3 h from symptom onset, it was found that plasma caspase-3 levels at 24 h were associated with poorer functional neurological outcome [15]. Thus, the finding of our study in respect to that serum caspase-3 levels on day 1 of the MMCAI diagnosis are associated with mortality is a new finding. We believe that the association in our study is due to the fact that we included only patients with MMCAI and GCS ≤ 8; in the study by Rosell et al. [15] this severity criterion was not included.

We did not calculate the sample size initially due that there were not studies previously reporting blood caspase-3 levels in MMCAI patients. The non-surviving patient group was relatively small, and this has been able to contribute on that we have not found an association between serum caspase-3 levels and infarct volume, or between GCS and infarct volume. However, our non-probabilistic sample size was large enough to find the

Table 1 Clinical and biochemical characteristics of patients on day 1 of the MMCAI diagnosis according to 30-day survival

	Survivors ($n=34$)	Non-survivors ($n=34$)	*P* value
Age (years)—median (p 25–75)	59 (47–68)	63 (53–70)	0.36
Gender female—*n* (%)	14 (41.2)	13 (38.2)	0.99
Diabetes mellitus—*n* (%)	4 (11.8)	9 (26.5)	0.22
Arterial hypertension—*n* (%)	19 (55.9)	16 (47.1)	0.63
COPD—*n* (%)	1 (2.9)	1 (2.9)	0.99
Chronic renal failure—*n* (%)	2 (5.9)	2 (5.9)	0.99
Heart failure—n (%)	1 (2.9)	1 (2.9)	0.99
APACHE II score—median (p 25–75)	20 (16–25)	22 (19–27)	0.06
GCS score—median (p 25–75)	7 (6–8)	6 (3–7)	0.01
Temperature (°C)—median (p 25–75)	36.4 (36.0–37.0)	36.9 (36.0–37.3)	0.15
Sodium (mEq/L)—median (p 25–75)	139 (136–145)	140 (139–145)	0.38
Glycemia (g/dL)—median (p 25–75)	127 (100–170)	136 (118–162)	0.40
Creatinine (mg/dl)—median (p 25–75)	0.80 (0.60–1.13)	1.00 (0.70–1.25)	0.19
Bilirubin (mg/dl)—median (p 25–75)	0.60 (0.40–0.83)	0.60 (0.33–1.10)	0.95
Lactic acid (mmol/L)—median (p 25–75)	1.20 (0.90–1.70)	1.55 (1.00–2.70)	0.05
PaO_2 (mmHg)—median (p 25–75)	156 (105–293)	115 (94–267)	0.26
PaO_2/FIO_2 ratio—median (p 25–75)	300 (198–369)	254 (192–325)	0.24
Leukocytes-median*$10^3/mm^3$ (p 25–75)	12.4 (9.6–16.9)	13.9 (9.7–20.1)	0.32
Hemoglobin (g/dL)—median (p 25–75)	12.1 (11.4–14.0)	12.5 (11.0–14.8)	0.81
Platelets—median*$10^3/mm^3$ (p 25–75)	202 (171–265)	175 (136–216)	0.02
INR—median (p 25–75)	1.06 (1.00–1.20)	1.20 (1.01–1.31)	0.07
aPTT (s)—median (p 25–75)	28 (25–30)	27 (26–32)	0.91
Fibrinogen (mg/dl)—median (p 25–75)	443 (416–489)	419 (337–631)	0.90
Volumen infarction (ml)—median (p 25–75)	173 (100–231)	180 (60–277)	0.64
Midline shift (mm)—median (p 25–75)	6.0 (2.5–11.5)	9.0 (3.5–15.0)	0.43
Thrombolysis—*n* (%)	11 (32.4)	10 (29.4)	0.99
Hemorrhagic transformation—*n* (%)	7 (20.6)	6 (17.6)	0.99
Decompressive craniectomy—*n* (%)	9 (26.5)	7 (20.6)	0.78
Caspase-3 (ng/mL)—median (p 25–75)	0.14 (0.11–0.18)	0.24 (0.20–0.31)	< 0.001

APACHE II Acute Physiology and Chronic Health Evaluation, aPTT activated partial thromboplastin time, *COPD* Chronic Obstructive Pulmonary Disease, FIO_2 pressure of arterial oxygen/fraction inspired oxygen, *GCS* Glasgow Coma Scale, *INR* international normalized ratio, PaO_2 pressure of arterial oxygen/fraction inspired oxygen, p 25–75 = percentile 25th–75th

association between high serum caspase-3 levels on day of MMCAI diagnosis and mortality.

We included only four variables in the multiple logistic regression analysis since the number of events (deaths) was 34 and we like to avoid an overfitting effect in the regression model [22]. In addition, we included those variables that showed higher statistically significant differences in the comparison between surviving and non-surviving patients (caspase-3, lactic acid, GCS, and platelet count).

National Institutes of Health Stroke Scale (NIHSS) [23] was not used to assess stroke severity since all our patients showed a GCS ≤ 8 and therefore was very difficult to evaluate the NIHSS items. Thus, we used GCS to assess stroke severity. We found that non-surviving MMCAI patients showed lower GCS than surviving patients, and that finding was also found in other previous studies [24, 25]. The explanation for this finding could be in relation with an association between GCS and infarct volume; however, in our study that association was not found in a statistically significant way, possibly because the sample size was not large enough to demonstrate it.

Different criteria could be used to stablish MMCAI diagnosis. We used similar computed tomography findings criteria that other studies used [26–28]; however, other studies also included basal cisterns compression in the MMCAI definition [26–28]. In addition, other studies included patients with acute neurological deterioration consisting of a decrease in the level of consciousness to somnolence or stupor compared with the baseline clinical status on admission [26–28], and in our study were included patients with GCS ≤ 8.

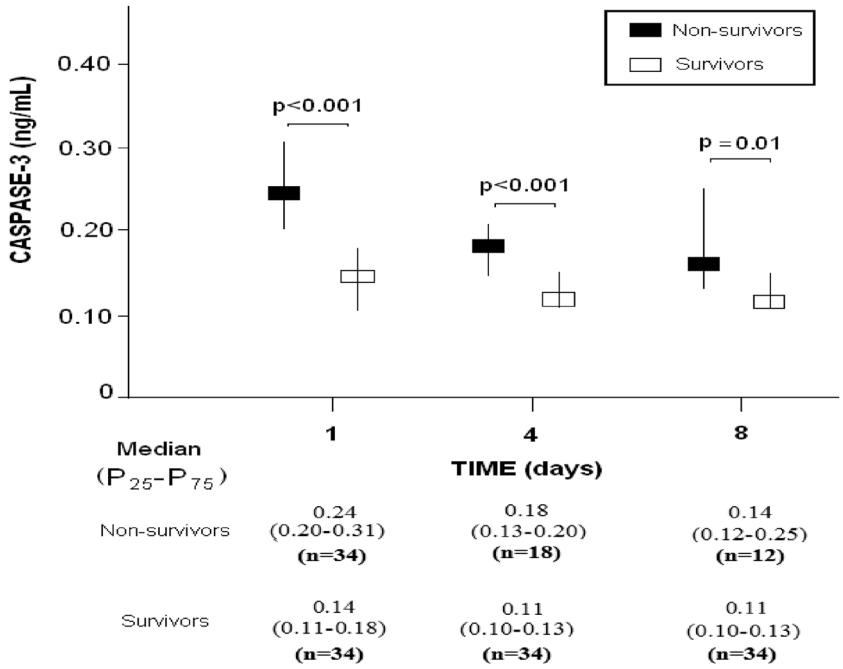

Fig. 2 Serum caspase-3 levels at days 1, 4, and 8 of MMCAI in 30-day surviving and non-surviving patients

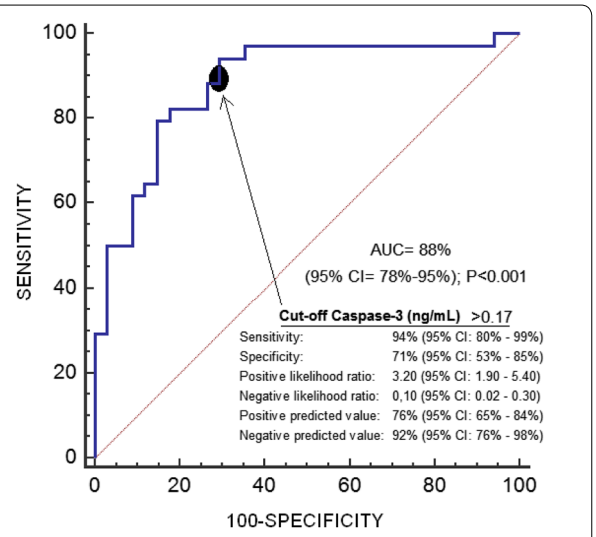

Fig. 3 Receiver operation characteristic analysis using serum caspase-3 levels to prediction of mortality at 30 days

In animal models with ischemic stroke, it has been found that the administration of different antiapoptotic agents (memantine, ulinastatin, acetylpuerarin) has been associated with lower brain caspase-3 activity, less neuronal apoptosis, and lower neurological impairment [29–31]. Thus, the administration of antiapoptotic agents could be a promising therapy for patients with ischemic stroke.

We cannot conclude that death in patients with MMCAI is directly related to high serum caspase-3 levels although we can conclude that there is an association between serum caspase-3 levels and mortality, and we think that this association could be due to an association between serum caspase-3 and brain cell death due to apoptosis. In some studies of brain samples of patients who died of cerebral infarction was found that caspase-3 and apoptotic cells (assessed by terminal transferase-mediated deoxyuridine triphosphate-digoxigenin nick end-labeling) were predominantly detected in neurones within zones of cerebral infarction [10–13]. However, we have not recollected brain samples to determine caspase-3 and apoptosis in ischemic and non-ischemic brain zones, and we have not found an association between serum caspase-3 levels and infarct volume (possibly due to the small sample of non-surviving patient group). We recognize other limitations in our study, including that we have not registered the patients excluded from the study and the causes for exclusion. Neither have we explored differences in caspase-3 concentrations between serum and plasma, and between MMCAI patients and healthy subjects. In addition, we did not have patients taking antiapoptotic agents to explore its effect in serum caspase-3 levels and survival rate. Besides, an association has been found between different genetic polymorphisms

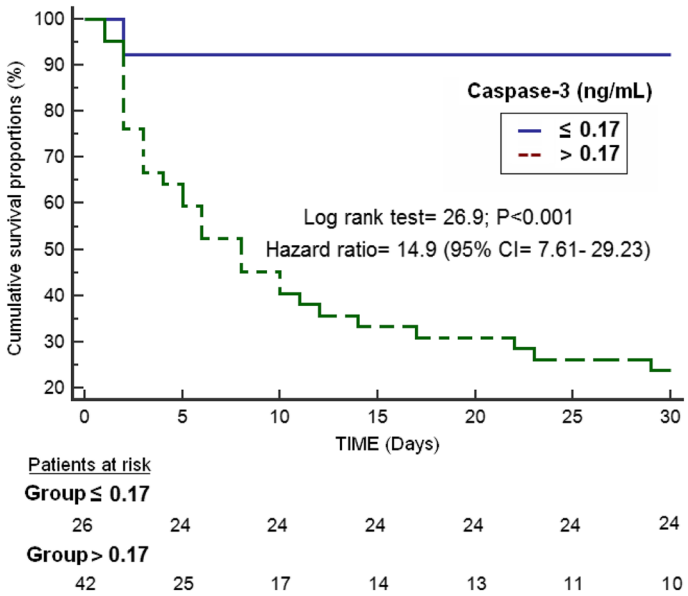

Fig. 4 Survival curves at 30 days with serum caspase-3 levels higher or lower than 0.17 ng/mL

Table 2 Multiple logistic regression analysis to predict 30-day mortality

Variable	Odds ratio	95% confidence interval	*P*
Serum caspase-3 > 0.17 ng/mL	51.25	8.30–316.31	< 0.001
Lactic acid (mmol/L)	1.17	0.54–2.28	0.78
Platelet count (each 1000/mm^3)	0.98	0.97–0.99	0.03
Glasgow Coma Scale (points)	0.80	0.54–1.12	0.28

of caspase-3, caspase-3 messenger ribonucleic acid levels, and the incidence of different cancers [32–34]; however, we have not studied genetic polymorphisms of caspase-3.

We think that our study may have some strengths. First, our novel findings (non-surviving showed higher serum caspase-3 levels during the first week than surviving patients and that there is an association between high serum caspase-3 levels and mortality) are in consonance with those of other studies. Our findings are in consonance with those of previous studies reporting high expression of caspase-3 in brain samples of post-mortem patients that died due to cerebral infarction [10–13] and higher plasma caspase-3 levels at 24 h of ischemic stroke in patients with poorer functional neurological outcome [15]. In addition, our findings are in consonance with those of a previous study by our team that showed an association between high serum caspase-3 levels and mortality of patients with traumatic brain injury [35]. We think that another strength of our study is that we have found higher serum caspase-3 levels at day 1, and also at days 4 and 8 in non-surviving than in surviving patients. We recognize that the findings of our study will not change the clinical practice at this time. However, despite the limitations of our study, we think that the novel findings of our study and the findings in ischemic stroke animal models about the benefits of antiapoptotic agents administration could open the interest to research about the role of serum caspase-3 levels for the prediction of survival and the potential use of antiapoptotic agents to reduce the risk of death of those patients.

Conclusions

The novel and more important findings of our study were that high serum caspase-3 levels were associated with mortality in MMCAI patients.

List of Abbreviations

APACHE: Acute Physiology and Chronic Health Evaluation; aPTT: Activated partial thromboplastin time; COPD: Chronic obstructive pulmonary disease; FIO_2: Fraction inspired of oxygen; GCS: Glasgow Coma Scale; INR: International normalized ratio; PaO_2: Pressure of arterial oxygen.

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Author Contributions
LL conceived, designed and coordinated the study, participated in acquisition and interpretation of data, and drafted the manuscript. MMM, RS, LR, MA, JSV, JJC, and VGM participated in acquisition of data. APC and AFGR participated in blood determination levels. AJ participated in the interpretation of data. All authors revised the manuscript critically for important intellectual content, made the final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Conflicts of interest
The authors declare that they have no conflict of interests.

Ethical Approval/Informed Consent
This study was carried after the approval of Institutional Review Board of all participating hospitals and with the written informed consent from legal guardians of patients.

Publisher's Note

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