Baseline severity and soluble vascular cell adhesion molecule 1 (sVCAM-1) as biomarker predictors of short-term mortality in acute ischemic stroke

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Abstract

The aim was to investigate the association between plasma levels of cellular adhesion molecules (CAMs) and risk factors, subtypes, severity and short-term mortality of acute ischemic stroke (IS), and to identify a panel of biomarkers to predict short-term mortality after IS. The prospective study evaluated 132 IS patients within 24 h of their hospital admission. The baseline IS severity was assessed using the National Institutes Health Stroke Scale (NIHSS) and categorized as mild (NIHSS<5), moderate (NIHSS 5-14) and severe (NIHSS≥15). After three-month follow-up, the disability was assessed using the modified Rankin Scale (mRS); moreover, the patients were classified as survivors and non-survivors. Baseline inflammatory and anti-inflammatory cytokines and soluble CAMs were evaluated. Twenty-nine (21.9%) IS patients were non-survivors and showed higher NIHSS and soluble vascular cellular adhesion molecule 1 (sVCAM-1) than the survivors. The sVCAM-1 levels positively correlated with age, homocysteine, severity, and disability. The model #3 combining sVCAM-1 and NIHSS showed better results to predict short-term mortality with an area under the curve receiving operating characteristics (AUC/ROC) of 0.8841 [95% confidence interval (CI): 0.795-0.941] than the models with sVCAM-1 and NIHSS alone, with positive predictive value of 68.0%, negative predictive value of 91.3%, and accuracy of 86.5%. In conclusion, the combined model with baseline severity of IS and sVCAM-1 levels can early predict the prognosis of IS patients who may benefit with therapeutic measures of personalized therapy that taken into account these biomarkers. Moreover, this result suggests that VCAM-1 might be a potential target for the therapeutic strategies in IS.

Introduction

Stroke is the world's second-leading cause of death and the third-leading cause of death and disability combined, and ischemic stroke (IS) is the most common type of stroke (Feigin et al., 2021). Clinical ratings, imaging and laboratory testing have all been studied as potential biomarkers for predicting short-term prognosis and mortality in patients with acute IS (Lehmann et al., 2021; Lehmann et al., 2022; Marta-Enguita et al., 2021; Reiche et al., 2019; Lehmann et al., 2015).

The National Institutes of Health Stroke Scale (NIHSS) is used to measure the severity of neurologic deficits and track the clinical evolution of the patient with IS (Brott et al., 1989). NIHSS evaluates 15 items in a neurological examination: eye movements, visual fields, motor and sensory deficits, ataxia, speech, cognition, and inattention. Patients are scored from zero (no deficit) to 42 (highest deficit) at the conclusion (Brott et al., 1989). Previous studies have indicated that NIHSS scores associated or not with other biomarkers may be useful to predict functional prognosis and mortality after IS (Chen et al., 2021; Lehmann et al., 2021; Kusvuran Ozkan et al., 2013; Saber; Saver, 2020; Zhao et al., 2018).

Blood biomarkers independent of other clinical predictors can assist in the identification of a useful laboratory predictor in prognostic scores (Richard et al., 2015). Inflammation plays a crucial role in at all stages of the ischemic cascade, from the cessation of blood flow to the late regeneration processes associated with the restoration of ischemic tissues (Pauluk et al., 2020). In the specific case of

atherothrombotic stroke, the nature of the biomarkers is expected to be inflammatory or lipid-related because of their involvement in atherosclerosis (Puig et al., 2020)

With the transendothelial migration of leukocytes in the blood brain barrier (BBB), the expression of cell adhesion molecules (CAMs), inflammatory, and anti-inflammatory molecules play a significant role in pathophysiology and prognosis of ischemia damage in acute IS (Lehmann et al., 2022; Richard et al., 2015; Supanc et al., 2011). One of the most essential CAMs is vascular cell adhesion molecule 1 (VCAM-1). VCAM-1 is a glycoprotein found on the surface of cells of the endothelium and is also available in a soluble form (sVCAM-1), which is the result of the ectodomain of VCAM-1 being released. VCAM-1 is involved in the recruitment of inflammatory cells and consequently the development of atherosclerotic plaques. Its levels are upregulated by interleukin (IL)-1β, tumor necrosis factor (TNF)-a and reactive oxygen species (ROS) (Ghaisas et al., 1997; Singh et al., 2005; Lin et al., 2015; Cook-Mills et al., 2011). Previous studies have reported that sVCAM-1 is a predictor of cardiovascular events, such as heart attacks and IS (De Lemos et al., 2000; Mulvihill et al., 2001; Hayek et al., 2021). Studies have shown that apart from E-selectin and P-selectin, the soluble intercellular adhesion molecule 1 (sICAM-1) and sVCAM-1 concentrations are increased in IS patients (Frijns and Kappelle, 2002), although the results are contradictory. Elevated concentrations of sVCAM-1 were observed in three (Fassbender et al., 1995; Bitsch et al., 1998; Blann et al., 1999) of four studies (Frijns et al., 1997; Fassbender et al., 1995; Bitsch et al., 1998; Blann et al., 1999).

The clinical significance of proving the role of CAMs in the prognosis of IS may be their utility as diagnostic and prognostic biomarker as well as therapeutic targets for patients with IS (Lehmann et al., 2022; Patel et al., 2020; Ramiro et al., 2018; Richard et al., 2015; Supanc et al., 2011). IS biomarkers of high specificity and sensitivity should be able to diagnose and differentiate between intracerebral hemorrhage (ICH) and acute IS, to predict prognosis after IS and to facilitate patient stratification for therapeutic intervention (Hasan et al., 2012).

In ischemia and hypoxia, ICAM-1 and VCAM-1 have been extensively studied. ICAM-1 levels were significantly increased in the brain three hours after IS, peaked between six and twelve hours, and remained elevated for five days (Wang and Feuerstein, 1995). Whether VCAM-1 levels are elevated after IS are still debatable (Ma et al., 2013). While Blann and coworkers (1999) found high expression of VCAM-1 by astrocytes and endothelial cells in the ischemic area from the IS patients, Vemuganti and coworkers (2004) showed that VCAM-1 mRNA expression was never changed in the ipsilateral cortex between three and 72 hours after reperfusion.

Therefore, to clarify this issue, we investigated the association between plasma levels of CAMs and risk factors, subtypes, severity and short-term mortality of acute IS, and a panel of biomarkers to predict the short-term mortality after IS.

Material And Methods

Subjects

This study included 132 eligible participants of both sexes with acute neurological signs or symptoms attributed to IS, confirmed by brain computed tomography and clinical examination. The patients with IS were recruited at the emergency room of the University Hospital, State University of Londrina in Londrina, Paraná, from January 2017 to January 2018.

The IS subtypes were classified according to the Trial of Org in 10172 Acute Stroke Treatment (TOAST) criteria (Adams et al., 1993); the baseline IS severity was determined using NIHSS and categorized as mild (NIHSS < 5), moderate (NIHSS 5–14), or severe (NIHSS ≥15) (Brott et al., 1989). All the patients were treated according to national standards for the IS treatment (Oliveira-Filho et al., 2012; Brasil, 2013; Martins et al., 2012). After three months, patients or their families were contacted via telephone (Wang et al., 2014), and the Modified Rankin Scale (mRS) was used to assess the degree of disability of the IS patients. Moreover, after three-month follow-up, the patients were classified as survivors and non-survivors (Park et al., 2015)

The exclusion criteria for IS patients were hemorrhagic stroke, transient ischemic attack (TIA), history of myocardial infarction, malignancies, chronic infections such as human immunodeficiency virus type 1, hepatitis virus B and hepatitis virus C infections, the presence of immune-inflammatory disorders, inflammatory bowel disease, chronic kidney disease, and liver failure, fever within the last seven days prior to the onset of IS symptoms, surgery or trauma within the last 30 days, angiography within the last seven days, and use of steroid or non-steroidal anti-inflammatory and immunosuppressive drugs.

A clinical evaluation and a standard questionnaire were used to obtain baseline demographic, lifestyle, and medical data. Anthropometric measurements included weight and height as reported by patients or their families. Body mass index (BMI) was determined using weight (kg) divided by height (m) squared. The ethnicity (Caucasian and non-Caucasian) were self-reported. Baseline systolic and diastolic blood pressures (SBP and DBP, respectively) were taken twice and the mean of these two values was utilized in the analysis; systemic arterial hypertension (SAH) was defined as SBP 140 mmHg or DBP 90 mmHg following the acute phase of IS. Antihypertensive medication use was also considered to diagnose SAH (James et al., 2014). Type 2 diabetes mellitus (T2DM) was defined as a fasting blood glucose level of 126 mg/dL, a non-fasting serum glucose level of 200 mg/dL, or the use of oral hypoglycemic drugs (Draznin et al., 2022). Dyslipidemia was defined as the presence of one or more of the abnormal serum lipid concentrations: total cholesterol > 200 mg/dL, low-density lipoprotein (LDL) cholesterol > 130 mg/dL, high-density lipoprotein (HDL) cholesterol < 40 mg/dL, and triglycerides > 150 mg/dL (National Cholesterol Education Program, 2002).

The protocol was authorized by the State University of Londrina's Institutional Research Ethic Committee in Paraná, Brazil (CAAE 61361416.9.0000.5231). All of the patients gave their informed consent. When the IS patients were unable to communicate, their legal guardians signed a consent form.

Laboratory biomarkers

Peripheral blood samples were collected in a vacuum collection tube without anticoagulant and with ethylenediaminetetraacetic acid (EDTA) and sodium fluoride anticoagulants within 24 hours of patient admission. After centrifugation for 10 minutes at 2500 rpm, serum and plasma samples were collected, aliquoted and kept at -80°C until use.

The plasma levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, and TNF-a, as well as the plasma levels of sICAM-1, sVCAM-1, soluble platelet endothelial cell adhesion molecule 1 (sPECAM-1), soluble E-selectin (sE-selectin), and soluble P-selectin (sP-selectin) were determined with the immunofluorimetric method with multiplex microsphere immunoassay (Novex Life Technologies, Frederick, USA). The plasma levels of transforming growth factor (TGF)-β were determined with the immunofluorimetric method with singleplex microsphere immunoassay (Novex Life Technologies, Frederick, USA). These determinations were performed in the Luminex platform on the MAGPIX® equipment (Luminex Corp., TX, USA). Plasma levels of homocysteine were determined using chemiluminescence microparticle immunoassay (CMIA, Architect™ Abbott Laboratory, Abbott Park, IL, USA). The plasma levels of glucose and lipid profile were determined using an automated biochemical analyzer (Dimension™ Dada Behring, Deerfield, IL, USA). method. All the laboratory analyses were performed according to the manufacturer's instructions and reference values.

Statistical analysis

Analyses of contingency tables checked the associations between categorical variables and diagnostic groups. In parametric tests, the Kolmogorov–Smirnov test assesses normality of distribution. The Mann-Whitney test was used for non-parametric data. Logarithmic (Ln) transformation of continuous data was used in the analysis when the variables were not normally distributed or when there was heterogeneity of variance as assessed with the Levene test, and transformation in Z-score standardized and allowed comparison of disparate distribution scores (Maes; Carvalho, 2018). For the analysis of cytokine results, two indexes, with z scores, were proposed: inflammatory: z(IL)-1+ zIL-2+ zIL-6+, zIL-12p70, zIL-17A, zIFN-γ, zTNF-α; anti-inflammatory: zIL-4, zIL-10, zTGF-β. The values of NIHSS and sVCAM-1 were also transformed as z scores, and a composite score reflecting the NIHSS + sVCAM-1 was computed.

We assessed the differences in continuous variables between groups using an analysis of the Mann Whitney or Kruskal-Wallis test. Categorical variables were expressed as an absolute number (n) and a percentage (%), while continuous variables were expressed as a median and 25.0%-75.0% interquartile range (IQR). The correlations between CAMs and clinical and inflammatory variables were assessed using Spearman correlation coefficients. Multivariate general linear model (GLM) analysis assessed the effects of explanatory variables on dependent variables (sPECAM-1, sVCAM-1, sICAM-1, sE-Selectin, sP-Selectin) with sociodemographic, clinical, and inflammatory data with independent variables. Tests for between-subject effects assess the univariate effects of significant predictor variables on the dependent variables. The partial eta squared (R^2) statistic was used to determine the magnitude of the effect of various variables in the models. Box's M statistic was used to test for the homogeneity of covariance matrices.

The association between mortality after three-months follow-up and CAMs with significant association in univariate analysis was evaluated using the hierarchy method of binary logistic regression analysis. After defining the association between NIHSS and CAMs significantly obtained, Cox Regression with hierarchy methods was applied to evaluate the survival rate. The predictors of mortality were assessed using the area under the receiver operating characteristic (AUC/ROC) curve as our measure of discrimination, with Kernel smoothing for optimizing the Youden-Index method. The AUC/ROC curve reflects the models' ability to discriminate between survivors and non-survivors. The positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity using combined models were determined. The odds ratio (OR) and 95% confidence interval (CI) were also determined. The non-parametric bootstrap method was used to simulate real-world situations as well as validate the tests. From the data obtained from the present study, we randomly generated 500 bootstrap samples of the same size, obtained by resampling and replacement of each sample, for the validation test. Statistical analyses were performed using Environment R (R Development Core Team 2020). The tests were two-tailed and $p<0.05$ indicated statistically significant results.

Results

Sociodemographic and clinical data

Table 1 shows the baseline demographic and clinical characteristics of IS patients who were measured according to three-month outcome mortality. The analysis enrolled a total of 132 eligible participants. The median age (IQR) was 66.8 years (56.0-75.5). There were 62 (47.0%) males and 70 (53.0%) females. In terms of ethnicity, 88 (66.7%) self-declared as Caucasian, 42 (31.8%) non-Caucasian and 2 (1.5%) did not present this record. There were 93 (70.4%) patients with SAH, 38 (28.8%) with T2DM, 58 (43.9%) with dyslipidemia, and 26 (19.7%) current smokers. The median BMI was 27.1 kg/m^2(24.1-30.1). According to TOAST criteria, 38 (29.2%) patients had large-artery atherosclerosis stroke (LAAS), 32 (24.6%) had cardioembolic stroke (CEI), 33 (25.4%) had lacunar stroke (LAC), 7 (5.4%) had other determined etiology (ODE), and 20 (15.4%) had undetermined etiology (UDE). After the three-month follow-up, the median disability (mRS) was 3.0 (IQR:1.0-6-6.0), and 29 (21.9%) of the 132 patients did not survive.

In the univariate analysis, the non-survivors were older ($p=0.001$) than the survivors. There was no significant difference between the subgroups in terms of sex, ethnicity, BMI, current smoking, SAH, T2DM, dyslipidemia, and TOAST subtype ($p>0.05$). On admission, the NIHSS score ranged from 0 to 24, and survivors had a lower NIHSS score, with a median (IQR) of 6.0 (3.0–12.0), compared to non-survivors, who had a median of 19.0 (12.0–22.5) ($p <0.001$). Regarding the disability after three-month follow-up, the non-survivors showed higher scores than the survivors (median of 6.0 *versus* 2.0, $p<0.001$). Regarding the CAMs, sVCAM-1 levels were found to be significantly associated with short-term mortality, with non-survivors having greater levels than survivors ($p=0.001$). Other CAMs, including sPECAM-1, sICAM-1, sE-selectin, and sP-selectin, were not associated with short-term mortality ($p>0.05$). Moreover, the inflammatory anti-inflammatory indexes proposed in the present study were not associated with this outcome (Table 1).

Effects of variables on cell adhesion molecules

Table 2 shows the results of the GLM multivariate analysis with sICAM-1, sVCAM-1, sPECAM-1, sP-Selectin, and sE-Selectin as dependent variables and sociodemographic, comorbidity, and inflammatory and anti-inflammatory biomarkers as explanatory variables. All CAMs (z score transformed) presented equality of variances by Levene's error test ($p>0.05$) and the GLM showed equality of variances by test of covariance matrices ($p=0.160$). The partial eta squared (R^2) statistic determined the magnitude of the effect of various variables in the model. When sICAM-1, sVCAM-1, sPECAM, sP-selectin, and sE-selectin were combined as dependent variables, the explanatory variables age, current smoking and anti-inflammatory index biomarkers exerted significant and strong effect of 24.4%, 25.1%, and 24.0%, respectively, in the variation of these CAMs. The effects of T2DM, dyslipidemia, homocysteine, and inflammatory biomarkers were mild. When these CAMs were evaluated individually, age and homocysteine had a mild effect on the sVCAM-1 levels (14.2% and 10.0%, respectively) and age, smoking, dyslipidemia, inflammatory and anti-inflammatory biomarkers all exerted a moderate effect on sPECAM-1 levels.

Cell adhesion molecules according to TOAST

Serum levels of sPECAM-1, sICAM-1, sP-Selectin, and sE-Selectin did not differ according to the TOAST subtype ($p>0.05$) (data not shown). On the other hand, sVCAM-1 levels were associated with TOAST ($p=0.016$). Patients with LAAS subtype showed higher sVCAM-1 compared to the patients with LAC ($p=0.011$) and ODE ($p=0.009$) (Figure 1). When the CAMs were analyzed in a dichotomized group, LAAS *versus* non-LAAS, the LAAS subtype was associated with higher levels of sVCAM-1 ($p=0.022$) than the non-LAAS. However, after adjusting by age, this association did not remain significant ($p> 0.05$).

Cell adhesion molecules and mortality

The levels of sVCAM-1 were positively correlated with age ($r=0.418$, $p<0.001$), homocysteine ($r=0.276$, $p=0.004$), NIHSS ($r=0.284$, $p= 0.011$), and disability measured with mRS ($r=0.280$, $p=0.007$) (Figure 2). In order to evaluate the sVCAM-1 as a predictor of three-month mortality, logistic regression analysis with the hierarchical method and confirmed stepwise method was performed (Table 3). In the first model, merely NIHSS was analyzed and showed an OR of 5.284 (95% CI: 2.616-10.671, $p<0.001$) and explained 44.8% of outcome, with correctly classified 87.5% of cases ($x^2=35.915$, $p<0.001$). As at #1 model, the objective of #2 model was to evaluate the effect of only sVCAM-1 and the results showed an OR of 2.206 (95% CI: 1.363-3.572, $p=0.001$) and explained 16.3% of outcome, with correctly classified 78.0% of cases ($x^2=12.401$, $p<0.001$). In the #3 model, we observed that sVCAM-1 showed significant effect in the first model (Block: $x^2=10.189$ $p=0.001$, Model: $x^2=46.104$, $p<0.001$). Together, sVCAM-1 and NIHSS explained 54.9% of the outcomes and correctly classified 86.5% of cases. In the #4 model, we added age and homocysteine variables in two blocks to evaluate the importance of these variables in this regression model. However, both showed no contribution to the prediction of three-month mortality ($x^2=0.366$, $p=0.545$ and $x^2=0.737$, $p=0.391$, respectively).

In the Cox regression analysis, hierarchical regression models confirmed the association of NIHSS and sVCAM-1 with mortality after three-month follow-up. In the #1 and #2 models, higher NIHSS (OR: 3.138, 95% CI: 2.165-4.548, p<0001) and higher serum sVCAM-1 levels (OR: 1.683, 95% CI: 1.277-2.216 (p<0.001) were shown to be associated with mortality. In the #3 regression model, there was a significant effect of sVCAM-1 with the model just with NIHSS (x^2=7.49, p=0.005); together, these variables showed a concordance of 0.860. When age was added to the model, no effect was obtained (x^2=0.005, p=0.941) (Table 4).

Further, the non-parametric bootstrapping method was used to simulate real-world situations as well as to validate these results. NIHSS and sVCAM-1 did not have a normal distribution; therefore, we assessed the robust cut-off of short-term mortality using the method of Kernel smoothing for optimizing the Youden-Index and the 95% CI was calculated with 500 bootstrapping. The optimal cut-off values for predicting the three-month mortality for NIHSS and sVCAM-1 were 12.298 and 389.60 ng/L, respectively. The combination of both variables showed significant differences in the AUC/ROC, as well as PPV and PNV for mortality outcome. The NIHSS and sVCAM-1 single models performed not as well [AUC of 0.723 (95% CI: 0.581- 0.821) for NIHSS and AUC of 0.844 (95% CI: 0.732- 0.91) for sVCAM-1] than the combined model with NIHSS and sVCAM-1 (AUC of 0.8841, 95% CI: 0.795-0.941). NIHSS was showed as a potential biomarker to predict short-term mortality in IS patients, with a PPV and NPV of 0.4651 (46.5%) and 0.9047 (90.5%), respectively. In addition, sVCAM-1 showed potential biomarker to predict short-term mortality in these patients, with PPV and NPV of 0.487 (48.7%) and 0.876 (87.6%), respectively. Moreover, when both biomarkers were analyzed in a combined model, the PPV and NPV were 0.68 (68.0%) and 0.9135 (91.3%), respectively (Table 5).

Discussion

The main finding of the present study is that both biomarkers (NIHSS and sVCAM-1) obtained within 24 h of a patient's hospital admission and diagnosed with IS performed well in predicting short-term mortality. When used together, these two biomarkers correctly classified 86.5% of the IS patients regarding their short-term mortality. This study highlights the role of NIHSS and sVCAM-1 in the prediction of mortality following the IS.

The application of the NIHSS is well-established in the literature and in IS therapy guidelines. It's even used as a criterion for deciding on reperfusion therapy and monitoring the patient's progress (Powers, 2020). Previous research indicates that NIHSS scores, whether or not they are related to other indicators, may be effective in predicting functional prognosis and death following IS (Chen et al., 2021; Kusvuran Ozkan et al., 2013; Saber; Saver, 2020; Zhao et al., 2018; Lehmann et al. 2022). According to Lehman et al. (2021), high NIHSS scores at admission strongly predict short-term disability as well as short-term mortality after IS.

Given the close pathophysiological relationship between neuroinflammatory mediators and processes related to poor outcome in IS, the role of molecules that are part of different inflammatory pathways,

such as acute phase proteins, pro-inflammatory and anti-inflammatory cytokines, and endothelial dysfunction as prognostic biomarkers of IS may be expected. CAMs play a key role in leukocyte infiltration into active endothelia and their expression increases after ischemic injury. CAMs mediate the molecular interactions between endothelium and leukocytes, such as rolling, adherence, and transendothelial migration of leukocytes that are required for these cells to pass through the BBB and reach the ischemic brain (Krieglstein; Granger, 2001; Ramiro et al., 2018; Yang et al., 2019). The immunoglobulin gene superfamily (ICAM-1 and 2, VCAM-1, and PECAM-1), selectins (E-, P-, and L-selectin), and integrins are types of CAMs. These molecules have been investigated as biomarkers and treatment targets for IS (Krieglstein; Granger, 2001; Ramiro et al., 2018; Yang et al., 2019).

VCAM-1 is a protein expressed on the surface of endothelial cells. Its expression is minimal on unstimulated endothelium but is upregulated by a number of pro-inflammatory cytokines, such as IL-1β and TNF-a. The adherence of lymphocytes and monocytes in inflamed vascular beds is mediated by the VCAM-1 (Krieglstein; Granger, 2001; Ramiro et al., 2018; Yang et al., 2019).

Previous research indicates that factors such as age and smoking influence VCAM-1 levels. Cavusoglu et al. (2004) found levels of sVCAM-1 in smokers are substantially higher than in nonsmokers. Smoking possibly causes an increase in sVCAM-1, which could be another mechanism for cigarette smoking's negative effects on the atherosclerotic process and its consequences.

Regarding the positive effect of age on the sVCAM-1, our result is in agreement with previous studies. Purschwitz et al. (2001) and Miles et al. (1997) reported an age-dependent increase in sVCAM-1 and sICAM-1 in human subjects. Age-related accumulations of ROS and reactive nitrogen species (RNS) are followed by a decrease in total thiol content, resulting in a net increase in oxidative stress. Consequently, vascular endothelial cells generate activation/dysfunction with elevated levels of sE-selectin, sP-selectin, sVCAM-1, and sICAM, which contributes to an increase in the incidence of vascular disorders with aging (Zou et al. 2004). These authors demonstrated that older animals had the highest levels of CAMs, indicating a higher vulnerability to the inflammatory stimuli with age and highlighting the crucial role that inflammation plays in age-related changes of CAMs (Zou et al., 2004). On the other hand, Morisaki et al. (1997) showed that sVCAM-1, but not sICAM-1, was positively correlated with age; moreover, a negative correlation between sE-selectin, sICAM-1, and sVCAM-1 levels and age was suggested (Nash et al., 1996).

Other biomarker that exerted a role in the CAMs levels in the present study was homocysteine. Homocysteine has also been studied as a factor that stimulates the expression of VCAM-1 and affects the progression of atherosclerotic lesions. Silverman et al. (2002) demonstrated increased adhesion of monocytes to VCAM-1-dependent endothelial cells after treatment of aortic endothelial cells with homocysteine but without explaining the underlying mechanism. Caluccio et al. (2007) demonstrated that homocysteine affects the expression of CAMs, mainly VCAM-1, by increasing their gene expression. Our findings are in agreement with these studies in which age, homocysteine, and smoking had an effect on CAMs levels, revealed by the correlation between age and homocysteine with sVCAM-1 levels.

The immunocytochemical study of brain tissue from patients who died after IS revealed intense expression of VCAM-1 by endothelial cells and infarct astrocytes (Ramiro et al., 2018; Zaremba; Losy, 2002). When VCAM-1 levels were compared between IS patients and controls, several authors found that the IS group had higher levels (Bitsch et al., 1998; Blann, 1999; Licata et al., 2009; Tuttolomondo et al., 2009). The increase in sVCAM-1 was also observed by Blann et al. (1999) when compared healthy controls and individuals with carotid atherosclerosis, and by Fassbender et al. (1995) when compared healthy controls and individuals with vascular risk factors. This change could be due to VCAM-1 upregulation in the acute phase of IS, which does not occur in individuals who do not have ischemia. VCAM-1 levels remained elevated even after three month-follow-up according to Blann et al. (1999). These findings suggest the possibility that VCAM-1 may be expressed in both the acute and chronic phases of ischemic injury and that it may play a role in both infarction and tissue repair. Other study also showed that patients with IS had a persistent increase of sVCAM (Fassbender et al., 1995).

According to Bitsch et al (1998), soluble CAMs levels exhibit substantial variation in the kinetics after the IS. While sICAM-1 levels peaked within 24 hours, sVCAM-1 levels peaked after five days, and sE-selectin levels declined after five days. These authors also showed that there was no correlation of soluble CAMs levels with infarct volume or clinical disability. However, their negative result could be explained by the small number of IS patients (n=26) included in the study and the large interindividual variability of the CAMs levels.

Other studies also failed to find association between CAMs and IS. Compared with control subjects, sP-selectin and sE-selectin were significantly elevated in acute stage of IS, but also in symptomatic carotid stenosis; however, sICAM-1 and sVCAM-1 were not increased in these patients (Frijns et al.,1997). Supanc et al. (2011) found no significant difference between the levels of sVCAM-1 and sICAM-1 when IS patients and controls were compared, with marked biological interindividual variability in all groups. Only the levels of sVCAM-1 were significantly higher in patients with the CEI subtype than in controls. Moreover, these authors found no significant correlation between the levels of sVCAM-1 and sICAM-1 and IS severity and disability.

On the other hand, increased plasma levels of sICAM-1, sVCAM-1 and sE-selectin were associated with IS, independent of age, sex and other conventional risk factors for IS (Simundic et al., 2004). Moreover, sICAM-1 and sVCAM-1 levels were significantly higher in IS patients who non-survived compared to those who survived; however, after adjusting for various confounding factors, only sICAM-1 levels were independently associated with early death (Rallidis et al., 2009).

Regarding the association of CAMs with IS prognosis, our results are in agreement with the MITICO study, which showed that patients with higher sVCAM-1 levels at admission had a higher probability of IS recurrence than those with lower sVCAM-1 levels (Castillo et al., 2009). In another investigation (Richard et al., 2015), high level of sVCAM-1 in the second to third week after IS was associated with a worse prognosis after three months. Corroborating our findings, the Cox regression analysis of the present study, hierarchical regression models confirmed the association of NIHSS and sVCAM-1 with mortality

after three-month follow-up, and patients with higher sVCAM-1 and NIHSS scores died earlier than the others of the present cohort.

Licata et al. (2009) and Tuttololondo et al. (2009) found no difference between IS subtypes in the TOAST classification despite evidence of higher sVCAM-1 levels in IS patients compared to controls. In our study, when the CAMs were analyzed in a dichotomized group, LAAS *versus* non-LAAS, the LAAS subtype was associated with higher levels of sVCAM-1 when compared to the non-LAAS. However, after adjusting by age, this association did not remain significant. As a result, age is a more critical determinant for sVCAM-1 levels in our study than the IS subtype.

After cerebral ischemia, cerebral endothelium is capable of expressing high levels of CAMs and recruiting large numbers of leukocytes and platelets. Initially, the predominant leukocytes are neutrophils followed by a more sustained increase in mononuclear cells and T lymphocyte cells. This recruitment is a highly coordinated and well-regulated two-step process that involves different CAMs expressed on vascular endothelium and circulating cells. In the first step, occurs low affinity binding manifested as rolling mediated by P-selectin upregulation as early as 15 minutes following an ischemic and E-selectin upregulation within 2 hours of ischemia. In the second step of recruitment, occurs high affinity interaction mediated by ICAM-1 that results in firm adhesion of leukocytes in cerebral microvessel. Lymphocytes and other mononuclear cells also express the α4 integrin named very late antigen-4 (VLA-4), which adheres the cell to the endothelium through its interaction with VCAM-1 (Yilmaz; Granger, 2008). Based on what is known about the expression of CAMs after IS, modulation of the VCAM-1/VLA-4 interaction may be a good strategy for reducing the post-ischemic inflammatory response than inhibition of other CAMs (Becker et al., 2001)

The importance of VCAM-1 was also demonstrated by a recent review based on results of protein-protein interaction network, enrichment, and annotation analyses. This study indicates that the activated immune response, as demonstrated by increased levels of cytokines (IL-6, IL-10, TNF-α), MACs (VCAM-1, E-selectin) and the positive acute phase protein (C-reactive protein) but lowered anti-inflammatory cytokine (TGF-β1) and negative acute phase protein (albumin), together with the activated hemostasis, thrombosis and coagulation pathways, as demonstrated by increased levels of Factor VIII, von Willebrand, and fibrinogen but reduced levels of protein C, Protein S, antithrombin, and albumin, are interrelated phenomena associated with short-term mortality after IS (Maes et al., 2021).

The main ligand of VCAM-1, the VLA-4, has been studied in the treatment of IS. Preclinical and monoclonal-clinical studies on VLA-4 that reduce leukocyte infiltration, reduce infarct volume, and improve stroke, though not in all animal models (Becker et al., 2001; Llovera et al., 2015). Researchers investigated the use of natalizumab, a monoclonal antibody that targets the α4-β1 integrin of VLA-4 in acute IS in the ACTION (Elkins et al., 2017) and ACTION II (Elkind et al., 2020) trials. Although no reduction was observed in focal infarct growth from day 1 to day 5 day 1 and day 5, as the primary study endpoint, a functional outcome improvement sustained over 90 days was observed particularly in subgroups of patients with smaller infarcts in ACTION trial (Elkins et al., 2017). Regarding the ACTION II

trial, natalizumab administered ≤ 24 hours after IS did not improve patient outcomes (Elkind et al., 2020).

The discrepancies in results between studies of sVCAM-1 and other CAMs in IS may be explained by the differences in patient inclusion and exclusion criteria, control group definitions, time between IS ictus and sample collection, or laboratory methods. Previous studies (Bitsch et al., 1998; Frijns et al., 1997) determined the CAMs levels with enzyme immunoassay (ELISA), a less sensitive method compared to immunofluorimetric assay that was used in the present study. Our study excluded all patients with evidence of acute infections on admission, chronic infections, in addition to autoimmune and auto-inflammatory diseases, and the use of corticosteroids and immunosuppressive medications. As a result, the possibility of the increase in sVCAM-1 being caused by other systemic inflammation and not by acute ischemic brain injury was reduced.

The present study has some limitations and strengths. First, the rigid inclusion and exclusion criteria limited the number of samples; however, they allowed an analysis with few confounding factors. Second, the samples were collected in the first 24 hours after the admission on the emergency room of the university hospital, the most acute phase of the event. The obtention of samples during the follow-up period could show the kinetics of the CAMs levels after cerebral ischemia and their correlation with the outcome. On the other hand, the evaluation of the potential role of CAMs in IS patients from the Brazilian population is scares and necessary and our study proposed the evaluation of predictive models with a panel of combined biomarkers. Given the heterogeneity and dynamic nature of the cellular and molecular changes that occur after IS, a single biomarker will not accurately predict all adverse outcomes of the ischemic event. The most indicated will be the combination of different biomarkers of the different pathways involved in the pathophysiology of IS, in order to achieve more significant results.

Taken together, our results underscore the important role of CAMs in the pathophysiology of IS and highlight the potential use of NIHSS and sVCAM-1 as possible biomarkers of short-term mortality in IS patients. While systematic reviews of the role of blood biomarkers in the diagnosis of IS indicate that these tests cannot be recommended in clinical practice, it is highly desirable that new studies be conducted so that a faster diagnosis of IS may be possible, even before the patient arrives at the hospital, using biological markers of cerebral ischemia or inflammation (Gagliardi et al., 2005). Research on the role of the CAMs in the pathophysiology of IS might yield useful biomarkers and treatment targets. Therefore, interventions that impede lymphocyte trafficking into the brain and lymphocyte activation may be potential therapeutic approaches for reducing brain injury after IS. Thus, medications designed to minimize endothelial activation and specifically VCAM-1 may be a viable strategy for preventing poor outcome after IS.

Declarations

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Conflict of interest

There is no conflict of interest to declare. None of the authors are involved in the publication process or have a financial or other beneficial interest in the products or concepts mentioned in the submitted manuscript.

Compliance with ethical standards

The protocol was approved by the Institutional Research Ethics Committees of University of Londrina, Paraná, Brazil (CAAE 61361416.9.0000.5231) and all of the individuals invited were informed in detail about the research and gave written Informed Consent.

Human rights

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institution and/or National Research Committee and with the World Medical Association 1964 Helsinki Declaration.

Standards for reporting

The manuscript was prepared taken into account the recommendations of the guidelines hosted by the Strengthening the Reporting of Observational studies in Epidemiology (STROBE). STROBE is used for observational studies (cohort, case-control, or cross-sectional designs) according to the STROBE statement (www.strobe-statement.org)

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

The studied participants were informed about the present research, and a written consent form was taken from all of them before their enrollment. Moreover, all the authors and co-authors participated and contributed sufficiently in the research, and all of them concur with the submission. The manuscript has been approved by the responsible authorities where the work was carried out. The authors also concur that, if accepted, the manuscript shall not be published elsewhere in the same form in either the same or any other language, without the consent of the Editor-in Chief of Metabolic Brain Disease.

Availability of data and material

The data and materials are available.

Authors' contributions

1) conception and design of the study: EMVR, ANCS; 2) acquisition of data: MCMA, DFA, ALFCL, ERT, MRN, LBF, TF, 3) statistical analysis: DFA; 4) analysis and interpretation of data: MCMA, DFA, EMVR; 5) drafting of the manuscript, tables and figures: MCMA, DFA, EMVR 6) manuscript review: EMVR.

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Tables

Table 1 Characteristics of patients with acute ischemic stroke according to short-term mortality attended at the University Hospital, Southern Brazil

Characteristics	Survivors (n=103)	Non-Survivors (n=29)	df	p value
Age (years)	66.0 (55.0-75.0)	73.0 (67.0-81.0)	1/132	0.001
Sex				
Male/Female	46 (44.7) /57 (55.3)	16 (55.2) /13 (44.8)	1/132	0.316
Ethnicity				
Caucasian/Non-Caucasian	67 (65.7) /35 (34.3)	21 (75.0) /7 (25.0)	1/130	0.351
Body mass index (kg/m^2)	26.8 (24.4-30.1)	26.1 (22.8-30.4)	1/108	0.597
Current smoking	19 (20.7)	7 (25.0)	1/120	0.625
SAH	73 (73.0)	20 (71.4)	1/128	0.953
T2DM	33 (33.0)	5 (17.9)	1/128	0.121
Dyslipidemia	46 (46.0)	12 (42.9)	1/128	0.768
Antihypertensive use	73 (73.0)	18 (64.3)	1/128	0.369
Antidiabetic drug use	20 (20.2)	5 (17.9)	1/127	0.783
Lipid-lowering use	35 (35.0)	10 (37.7)	1/128	0.944
Antiaggregating use	33 (33.0)	12 (42.9)	1/128	0.334
NIHSS	6.0 (3.0-12.0)	19.0 (12.0-22.5)	1/127	<0.001
mRS after three-month follow-up	2.0 (1.0-4.0)	6.0 (6.0-6.0)	1/127	<0.001
IS subtypes (TOAST)			4/130	0.472
LAAS	29 (28.4)	9 (32.1)		
Cardioembolic	23 (22.5)	9 (32.1)		
Lacunar	28 (27.5)	5 (17.9)		
Other determined etiology	6 (5.9)	1 (3.6)		
Undetermined etiology	16 (15.7)	4 (14.3)		
Cell adhesion molecules				
sPECAM-1 (ng/mL)	14.16 (11.48-17.02)	14.00 (11.69-15.99)	1/109	0.746
sICAM-1 (ng/mL)	44.69 (24.47-147.07)	29.54 (24.31-596.06)	1/109	0.303
sVCAM-1 (ng/mL)	325.32 (275.25-381.96)	421.69 (97.89-708.06)	1/109	0.001

sE-Selectin (ng/mL)	49.27 (36.87-70.05)	60.87 (36.36-70.80)	1/109	0.614
sP-Selectin (ng/mL)	48.65 (36.61-67.34)	50.14 (41.49-70.21)	1/109	0.629
zInflammatory index	-0.89 (-4.06-4.01)	0.73 (-0.02-5.22)	1/106	0.091
zAnti-inflammatory index	0.22 (-2.0-1.17)	0.61 (-0.65-2.55)	1/104	0.352

The continuous variables were expressed as median and 25%-75% interquartile range (IQR); the categorical variables were expressed as number (n) and percentage (%). df: degrees of freedom; IS: acute ischemic stroke;T2DM: type 2 diabetes mellitus; NIHSS: National Institutes of Health Stroke Scale; mRS: modified Rankin Scale; TOAST: Trial of Org in 10172 Acute Stroke Treatment; LAAS: Large artery atherosclerosis stroke; sPECAM-1: soluble platelet endothelial cell adhesion molecule-1; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1.

zInflammatory: interleukin z(IL)-1+ zIL-2+ zIL-6+, zIL-12p70, zIL-17A, zIFN-γ, zTNF-α.

zAnti-inflammatory: interleukin zIL-4, zIL-10, zTGF-β.

Table 2 Results of multivariate general linear model (GLM) analysis with cell adhesion molecules as dependent variables and demographic and clinical characteristics as explanatory variables

Dependent variable	Explanatory variables	df	F	p value	Eta partial square
sICAM-1	Age	5/76	4.587	0.001	0.244
sVCAM-1 sPECAM sP-Selectin	Sex	5/76	1.132	0.352	-
	BMI	5/76	0.535	0.749	-
sE-Selectin	Current Smoking	5/76	4.785	0.001	0.251
	Hypertension	5/76	0.416	0.836	-
	T2DM	5/76	2.104	0.088	0.126
	Dyslipidemia	5/76	3.088	0.014	0.179
	Homocysteine	5/76	2.372	0.048	0.143
	Inflammatory	5/76	1.979	0.096	0.119
	Anti-inflammatory	5/76	4.483	0.001	0.240
sICAM-1	Anti-inflammatory (-)	1/89	3.722	0.057	0.047
sVCAM-1	Age (+)	1/89	12.396	0.001	0.142
	Homocysteine (+)	1/89	9.089	0.004	0.108
sPECAM-1	Age (+)	1/89	7.879	0.006	0.095
	Smoking (+)	1/89	9.353	0.003	0.108
	T2DM (+)	1/89	6.186	0.0150	0.076
	Dyslipidemia (-)	1/89	13.313	<0.001	0.151
	Inflammatory (+)	1/89	6.930	0.010	0.085
	Anti-Inflammatory (-)	1/89	9.127	0.003	0.108
sP-Selectin	Smoking (+)	1/89	5.052	0.028	0.063
sE-Selectin	T2DM (+)	1/89	7.017	0.010	0.086

df: Degrees of freedom; BMI: Body mass index; T2DM:type 2 diabetes mellitus; sPECAM-1: soluble platelet endothelial cell adhesion molecule-1; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1; Inflammatory: interleukin (IL)-1β, IL-2, IL-6, IL-12p70, IL-17A, Tumor necrosis factor (TNF)-α, Interferon (IFN)-γ; Anti-inflammatory: IL-4, IL-10, transforming growth factor beta (TGF)-β. The use of anti-hypertensive, hypoglycemic and lipid-lowering drugs did not alter the results (data not shown). All dependent variables presented equality of variances (p>0.05) (Levene test).

Table 3 Models of logistic regression analysis with mortality three-months follow up outcome as dependent variable in patients with acute ischemic stroke

Model	Significant explanatory variables	B	Wald	df	*P* value	OR	95% CI	R^2 Nagel-kerke
#1	zNIHSS	1.665	21.541	1	<0.001	5.284	2.616-10.671	0.448
#2	zsVCAM-1	0.791	10.358	1	0.001	2.206	1.363-3.572	0.163
#3	zNIHSS	1.718	18.959	1	<0.001	5.573	2.572-12.075	0.549
	zsVCAM-1	0.875	8.499	1	0.004	2.355	1.324-4.189	
#4	zNIHSS	1.720	17.362	1	<0.001	5.583	2.486-12.537	0.550
	zsVCAM-1	0.859	6.665	1	0.010	2.361	1.230-4.532	
	zAge	0.250	0.350	1	0.554	1.284	0.333-1.551	
	zHcy	-0.330	0.707	1	0.400	0.719	0.333-1.551	

All variables were analyzed in z score (normalized). sVCAM-1: soluble vascular cell adhesion molecule 1;

OR: Odds Ratio; CI: confidence interval; NIHSS: National Institutes of Health Stroke Scale; Hcy: homocysteine;

Model #1: x^2=35.915, df 1, p<0,001, sensitivity=96.3%; specificity=56.5; 87.5% of cases correctly classified;

Model #2: x^2=35.915, df 1, p<0,001, sensitivity=96.3%; specificity=16.0; 78.0% of cases correctly classified;

Model #3: x^2=46.104, df 2, p<0,001, sensitivity=96.4%; specificity=56.5; 87.5% of cases correctly classified;

Model #4: x^2=47.205, df 4, p<0,001, sensitivity=95.1%; specificity=56.5; 86.0% of cases correctly classified.

Table 4 Cox Regression analysis with mortality three-months follow up outcome as dependent variable in patients with acute ischemic stroke

Regression	Significant explanatory variables	B	Wald	*P* value	OR	95% CI	Concordance
#1	zNIHSS	1.143	36.460	<0.001	3.138	2.165-4.548	0.808
#2	zsVCAM-1	1.6826	13.700	<0.001	1.683	1.277-2.216	0.702
#3	zNIHSS	1.216	32.896	<0.001	3.376	2.230 -5.112	0.860
	zsVCAM-1	0.484	9.079	0.002	1.624	1.185-2.225	
#4	zNIHSS	1.209	27.94	<0.001	3.357	2.144-5.254	0.861
	zsVCAM-1	0.477	6.930	0.008	1.615	1.132-2.303	
	zAge	0.022	0.005	0.941	1.020	0.572-1.826	

All variables were analyzed in z score (normalized); sVCAM-1: soluble vascular cell adhesion molecule 1;

OR: Odds Ratio; CI: confidence interval; NIHSS: National Institutes of Health Stroke Scale

Table 5 Analysis of cutoff for short-term mortality using Kernel smoothing test for optimizing Youden-Index in patients with acute ischemic stroke

	Non-survivor/ Survivor	**Cutoff**	**AUC**	**Sensitivity**	**Especificity**	**TP**	**FN**	**FP**	**TN**
sVCAM-1 (ng/mL)	25/84	389.60	0.7233	0.6785	0.7619	19	9	20	64
NIHSS	28/99	12.298	0.844	0.7143	0.7677	20	8	23	76
zNIHSS+ zsVCAM-1	24/82	0.7948	0.8841	0.7083	0.9024	17	7	8	74

All variables were analyzed in z score (normalized).

NIHSS: National Institutes of Health Stroke Scale; sVCAM-1: soluble vascular cell adhesion molecule-1.

AUC: area under curve – Youden Kernel smoothing test

Figures

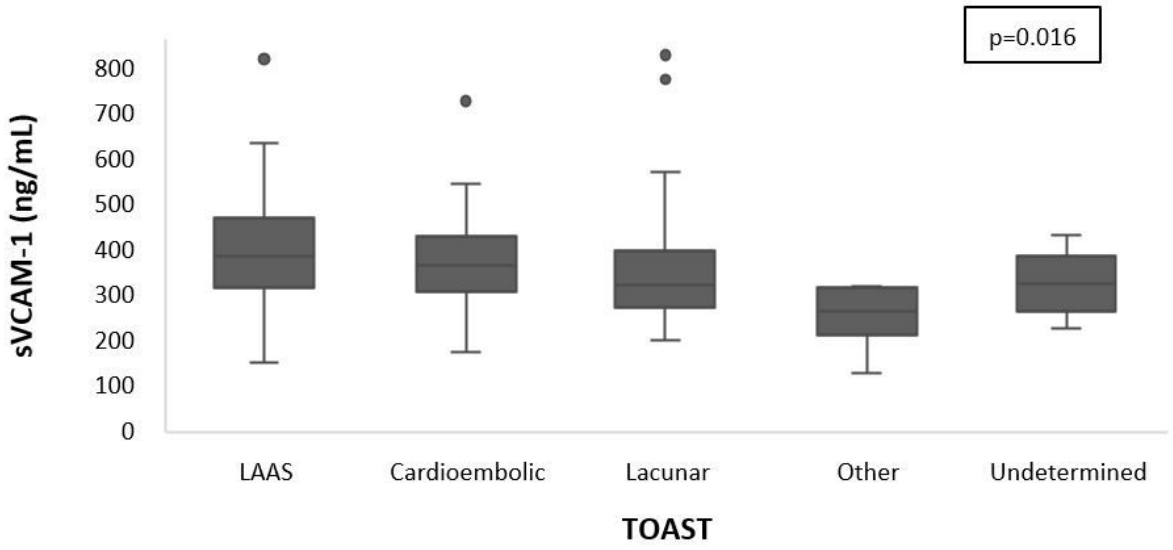

Figure 1

sVCAM-1 serum levels according to the ischemic stroke TOAST subtypes. Box-plots depict the median and interquartile range (IQR) of each group. Error bars represent limits intervals. Statistically significant differences were observed between the LAAS and Lacunar (p=0.011) and Other (p=0.009).

sVCAM-1: soluble vascular cell adhesion molecule-1; TOAST: Trial of Org in 10172 Acute Stroke Treatment; LAAS: Large artery atherosclerosis stroke; Other: other determined etiology; Undetermined: undetermined etiology

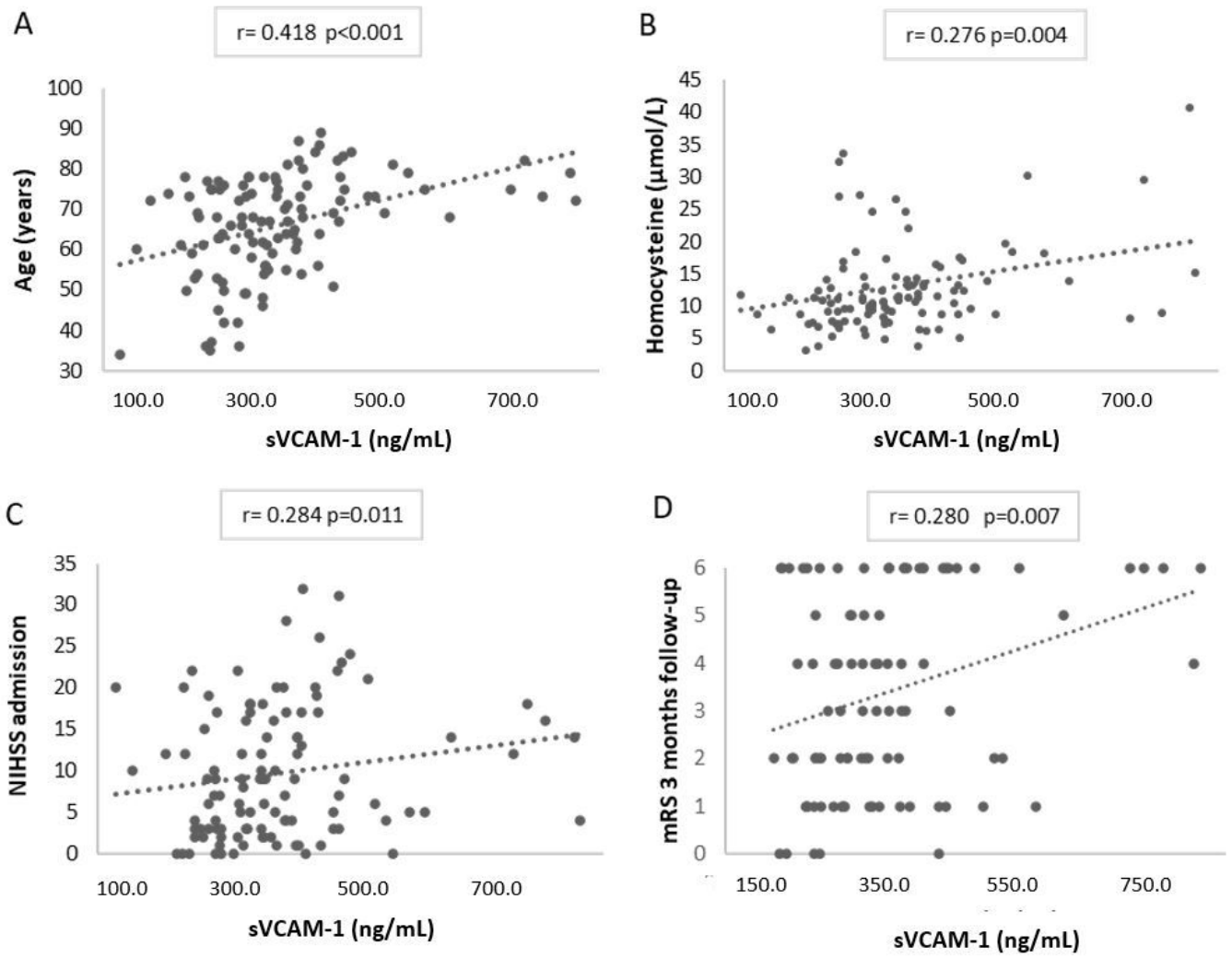

Figure 2

Correlation between sVCAM-1 with age (A), homocysteine (B), NIHSS at admission (C) and disability (mRS) three-month follow up.

sVCAM-1: soluble vascular cell adhesion molecule-1; NIHSS: National Institutes of Health Stroke Scale; mRS: modified Rankin Scale.

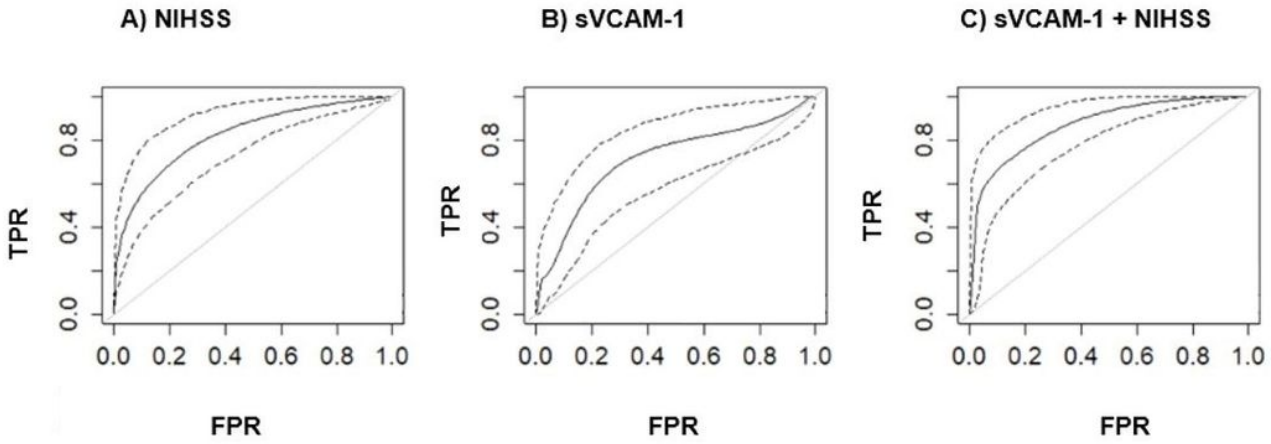

Figure 3

Receiver operating characteristic (ROC) curve to distinguish patients with acute ischemic stroke who survived from those who non-survived. A) using the National Institutes of Health Scale Score (NIHSS) with area under curve (AUC) of 0.844; B) using serum levels of soluble vascular cell adhesion molecule 1 (sVCAM-1) with AUC of 0.7233; and C) using both sVCAM-1 and NIHSS with AUC of 0.8841. FPR: False Positive Rate (1- Specificity); TPR: True Positive Rate (1-Sensitivity).