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ORIGINAL ARTICLE

Immune-inflammatory, oxidative stress and biochemical biomarkers predict short-term acute ischemic stroke death

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Abstract

The aim of the study was to define new immune-inflammatory, oxidative stress and biochemical biomarkers, which predict mortality within a period of 3 months after acute ischemic stroke (AIS). We recruited 176 healthy volunteers and 145 AIS patients, categorized as AIS survivors and non-survivors, and measured interleukin (IL)-6, high sensitivity C-reactive protein (hsCRP), ferritin, iron, total serum protein (TSP), erythrocyte sedimentation rate (ESR), white blood cells (WBC), 25 hydroxyvitamin D [25(OH)D], lipid hydroperoxides (CL-LOOH), insulin, glucose and high-density lipoprotein (HDL)-cholesterol. In patients, these biomarkers were measured within 24 h after AIS onset. We also computed two composite scores reflecting inflammatory indices, namely INFLAM index1 (sum of z scores of hsCRP+IL-6 + ferritin+ESR + WBC) and INFLAM index2 (z INFLAM index1 – z 25(OH)D – z iron + z TSP). Three months after AIS, non-survivors ($n = 54$) showed higher baseline levels of IL-6, hsCRP, ferritin and glucose and lower levels of HDL-cholesterol and 25(OH)D than survivors ($n = 91$). Non-survivors showed higher baseline ESR and lowered TSP than controls, while survivors occupied an intermediate position. Death after AIS was best predicted by increased IL-6, glucose, ferritin and CL-LOOH and lowered 25(OH)D levels. The area under the receiver operating curves computed on the INFLAM index1 and 2 scores were 0.851 and 0.870, respectively. In conclusion, activation of peripheral immune-inflammatory, oxidative and biochemical pathways is critically associated with mortality after AIS. Our results may contribute to identify new biomarker sets, which may predict post-stroke death, as well as suggest that IL-6 trans-signaling coupled with redox imbalances may be possible new targets in the prevention of short-term outcome AIS death.

Keywords Ischemic stroke · Mortality · Inflammation · IL-6 · Oxidative stress · Biomarkers

Introduction

Stroke is a major worldwide health problem and the majority of global stroke burden is in low- and middle-income countries (Benjamin et al. 2017). While age-standardized rates of stroke mortality have decreased worldwide in the past two decades, the absolute number of people who have a stroke every year, live with the consequences of stroke, and die from their stroke is increasing (Feigin et al. 2013, 2016). In 2013, there were globally almost 25.7 million stroke survivors with 71% with

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acute ischemic stroke (AIS), 6.5 million deaths from stroke (51% died from AIS), 113 million disability-adjusted life-year (DALYs) due to stroke (58% due to AIS), and 10.3 million people having a first stroke (67% AIS) (Feigin et al. 2013). Around one third of stroke victims die and another one third being permanently disabled and requiring permanent residential care. Among patients with cardiovascular disorder (CVD), stroke is the second most important contributor to hospital care costs explaining the high socioeconomic costs of this event (Benjamin et al. 2017).

Stroke results from the cumulative, long-term effects of irreversible factors, including age, sex, and ethnicity, as well as modifiable risk factors, including hypertension, heart diseases, diabetes mellitus (DM), body mass index (BMI), hyperlipidemia, smoking, excess alcohol consumption, and sedentary life style (Sacco 1997; Chen et al. 2014; Benjamin et al. 2017). The risk of having a stroke increases with age and doubles each decade after the age of 55 years (Sacco 1997; Benjamin et al. 2017), while men show a higher incidence of stroke than women (Sacco 1997; Benjamin et al. 2017). The risk of stroke is increased in individuals with hypertension, especially in those with systolic blood pressure (SPB) ≥ 140 mmHg (Sacco 1997; Chen et al. 2014; Choudhury et al. 2015; Benjamin et al. 2017). Increased BMI or body weight are associated with stroke risk factors including hypertension thereby increasing risk of stroke (Kurth et al. 2002; Choudhury et al. 2015; Benjamin et al. 2017). Biochemical biomarkers associated with BMI and DM also increase stroke risk, including increased insulin resistance (as assessed with the homeostasis model assessment of insulin resistance based on blood glucose and insulin levels), hyperinsulinemia and glucose intolerance (Denti et al. 2003; Choudhury et al. 2015; Benjamin et al. 2017; Ago et al. 2018).

Increased levels of low-density lipoprotein (LDL)-cholesterol are associated with AIS, while high-density lipoprotein (HDL)-cholesterol may have protective effects (Luo et al. 2014; Demarin et al. 2010). Current smoking may increase the risk to develop stroke (Chen et al. 2014; Choudhury et al. 2015; Benjamin et al. 2017).

Interruption of cerebral blood flow by embolism or a thrombus causes AIS leading to necrotic and glutamate-related excitotoxic cell death in the ischemic core (Tobin et al. 2014; Rodrigo et al. 2013). Neuroinflammatory (microglia activation) and oxidative processes following AIS are critically involved in the primary insult leading to apoptosis, necrosis or authophagy and consequent brain cell death in the penumbra (Rodrigo et al. 2013; Tobin et al. 2014; Becker and Buckwalter 2016). Reperfusion is accompanied by secondary neurotoxic responses induced by a second burst in reactive oxygen species (ROS) and neuroinflammatory responses including increased production of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α (Tobin et al. 2014; Rodrigo et al. 2013). Moreover, peripheral activation of immune-inflammatory pathways occurring after stroke plays a critical role in neurological outcome (Chapman et al. 2009). Immune and oxidative mediators generated in the brain may propagate into the systemic blood (spill-over) causing a peripheral immune-inflammatory response (Anrather and Iadecola 2016). Increased ROS and altered endothelial cell functions disrupt the blood brain barrier (BBB) contributing to infiltration of blood-derived neutrophils, M1 macrophages and activated T cells thereby aggravating neuroinflammation and neurotoxicity (Tobin et al. 2014; Anrather and Iadecola 2016; Shirley et al. 2014; Rodrigo et al. 2013; Wang et al. 2007). Moreover, experimental stroke causes a peripheral immune-inflammatory response, which peaks four hours after stroke and precedes the peak in neuroinflammation 20 h later. This response is characterized by elevated levels of plasma IL-6 and C-reactive protein (CRP) (Chapman et al., 2007), the latter being associated with a worse clinical outcome (Matsuo et al. 2016). Likewise, stroke is accompanied by peripheral signs of lipid peroxidation including increased lipid peroxides and lowered levels of antioxidant defenses, such as vitamin D (Rodrigo et al. 2013; Poole et al. 2006).

One third of stroke individuals will die within some months from stroke or its complications (Feigin et al. 2017). Mortality after stroke is one of the leading causes of death worldwide (Feigin et al. 2017, 2013; Strong et al. 2007). Short-term (1 week - 3 months) post-stroke death is predicted by fever, age, plasma glucose levels, previous stroke, severity of neurological deficits and size of lesion (Koton et al. 2010; Hénon et al. 1995; Das et al. 2012). Nevertheless, there are no data whether peripheral signs of activated immune-inflammatory pathways within 24 h after stroke onset may predict stroke-associated mortality three months later. The oxidative stress may also be involved in the pathophsyiology of atrial fibrillation (AF), an important factor that greatly increases the risk of AIS, through the activation of NF-kB, with consequent changes in the inflammatory mechanisms. AF was associated with increased expression of ischemia and stress-related genes and with decreased coronary artery flow (Gasparova et al. 2017). Moreover, in patients with IF, increased IL-6 and CRP levels predict cardiovascular events and death (Aulin et al. 2015). Moreover, oxidative stress and atrial fibrillation All-cause mortality due to for example CVD, pneumonia, cancer and chronic kidney disease is predicted by a number of immune and metabolic biomarkers including IL-6, CRP, albumin and glucose levels, white blood cell (WBC) counts and oxidative stress biomarkers of lipid peroxidation (Bruunsgaard et al. 2003; Lepper et al. 2012; Goodson et al. 2005; Singh-Manoux et al. 2017; Ridker 2008; Proctor et al. 2015; Huerta et al. 2005).

Hence, the aim of the present study is to examine whether the immune-inflammatory, oxidative stress and biochemical biomarkers are associated with short-term AIS death. The

design is based on the evaluation, within 24 h after stroke onset, of the IL-6, high sensitivity CRP (hsCRP), WBC count, erythrocyte sedimentation rate (ESR), ferritin, lipid hydroperoxides, vitamin D, iron, total serum protein (TSP), HDL-cholesterol, plasma insulin and glucose levels, adjusting for extraneous and confounding variables, including age, sex, smoking, BMI, DM and hypertension.

Subjects and methods

Study subjects

The protocol was approved by the Institutional Research Ethic Committee of the State University of Londrina, Paraná State, Brazil (CAAE 0250.0.268.000–11) and a written consent form was obtained from all of the individuals. A total of 145 AIS patients diagnosed with focal neurological signs or symptoms thought to be of vascular origin that persisted for >24 h, confirmed by brain computed tomography (CT) and clinic examination in baseline conditions were consecutively recruited during January 2013–2015 from the Emergency Room of the University Hospital of State University of Londrina, Paraná, Brazil. The stroke subtypes were classified according to the TOAST criteria (Adams et al. 1993). The functional impairment was evaluated using the modified Rankin Scale (mRS) (Bonita and Beaglehole 1988) applied within the first 24 h of admission (mRS baseline) and the values were used to categorize the patients as mild functional impairment (mRS <3) and moderate/severe functional impairment (mRS ≥3). The mRS was also applied after three-month follow-up through clinical examination or using telephone interviews with the patients or their relatives (Wang et al. 2014) and the values were used to categorize the patients as AIS survivors (mRS <6) and AIS death (mRS = 6) (Park et al. 2015). Firstly, our study is a case – control study with respect to the biomarker differences between controls and patients. However, it is a prospective study with regard to the prediction of mRS within 3 months after acute stroke because baseline biomarkers, which were assessed within 24 h after AIS, are used as predictors of the mRS three months later. All the patients were treated using a standardized protocol adapted from international guidelines for managing AIS (Brazil 2013).

As controls, 176 healthy individuals from general population of Londrina were enrolled in the same period, with similar demographic and anthropometric characteristics and no history of stroke/ myocardial infarction. Exclusion criteria for AIS patients and controls were the report of recent history of fever within last seven days prior to onset of stroke symptoms, acute inflammatory diseases, haemorrhagic stroke, acute transient ischemic attack (AIT); chronic infectious, such as human immunodeficiency virus type 1 (HIV-1), hepatitis B virus and hepatitis C virus infections, autoimmune diseases, renal or liver failure, history of myocardial infarction, surgery within last 30 days, angiography within last seven days, trauma within last 30 days, malignancies, steroid or non-steroidal anti-inflammatory therapies and immunosuppressive drugs use.

Demographic, epidemiological, anthropometric and clinical data

Demographic, epidemiological, anthropometric, and clinical data, as well as the AIS risk factors and the use of any therapeutic drugs before the inclusion in this study were obtained using a standard questionnaire on study admission. The anthropometric measures were verified by body weight and height reported by the individuals, when it was possible, or by the patient's family. BMI was calculated as weight (kg) divided by height (m) squared. The ethnicity was self-reported as Caucasian and non-Caucasian (Asiatic, Black, and Afro-Brazilian) (Brazil 2011). Baseline systolic blood pressure (SBP) and diastolic blood pressure (DBP) evaluations were obtained on study admission of the individuals (AIS patients and controls) using digital apparatus properly calibrated, and the mean of these measurements was used in the analysis. The use of antihypertensive medication was an indication of hypertension (James et al. 2014); DM was defined as a fasting serum glucose ≥126 mg/dL, a non-fasting serum glucose ≥200 mg/dL and/or use of hypoglycemic medication (American Diabetes Association 2014); dyslipidemia was defined by the presence of one or more than one of the abnormal serum lipid concentration: total cholesterol ≥200 mg/dL, LDL-cholesterol ≥130 mg/dL; HDL-cholesterol <40 mg/dL, and triglycerides >150 mg/dL (National Cholesterol Education Program (NCEP) Expert Panel On Detection, Evaluation 2002).

Immune-inflammatory, biochemical and oxidative stress biomarkers

Peripheral blood samples were obtained under non-fasting state, with and without EDTA as anticoagulant. From the AIS patients, the samples were obtained on hospital admission; from controls, the samples were obtained on study inclusion. Plasma and serum samples were immediately separated by centrifugation (2500 rpm for 15 min) and stored in aliquots at −80 °C until analyzes. As immune-inflammatory biomarkers, plasma levels of IL-6 were evaluated using a sandwich enzyme-linked immunosorbent assay (ELISA, eBioscience, San Diego, California, USA); WBC counts and ESR were determined using hematological autoanalyzers; serum levels of CRP determined with high sensitivity method (hsCRP), and ferritin were determined using chemiluminescence microparticle assay (CMIA, Architect, Abbott Laboratory, Abbott Park, IL, USA).

As biochemical biomarkers, the study included glucose, HDL-cholesterol, TSP, and iron levels that were evaluated using a biochemical auto-analyzer (Dimension Dade AR Dade Behring, Deerfield, IL, USA); insulin and hydroxyvitamin D [25(OH)D] levels were determined using chemiluminescent microparticle immunoassay (CMIA) (Architect, Abbott Laboratory, Abbott Park, IL, USA). As oxidative stress biomarkers, lipid hydroperoxides were evaluated by tert-butyl hydroperoxide-initiated chemiluminescence (CL-LOOH), as previously described (Gonzales-Flecha et al. 1991) and the results were expressed in counts per minute (cpm). We used new immune-inflammatory indices by using z unit weighted composite scores computed on immune-inflammatory markers (hsCRP, IL-6, ferritin, ESR, WBC), negative acute phase reactants (iron and TSP) and the anti-inflammatory 25(OH)D. The INFLAM index 1 was computed as the sum of the z transformations of all 5 immune-inflammatory markers: z transformation of hsCRP (z hsCRP) + z IL-6 + z ferritin + z ESR + z WBC count (expressed in z scores). The INFLAM index 2 was computed based on the sum of the 5 immune-inflammatory markers (after z transformation) minus the negative acute phase reactants and 25(OH) D, namely z INFLAM index 1 – z 25(OH) D - z iron - z TSP. As such, both INFLAM index1 and 2 reflect the severity of the immune-inflammatory response.

Statistical analysis

We used analyses of variance (ANOVA) to check between-group differences in scale variables and analyses of contingency tables (X^2-tests) to check associations between nominal variables. Results of multiple comparisons were p-corrected for false discovery rate (Benjamini and Hochberg 1995).

We employed multivariate general linear model (GLM) analyses to delineate the effects of survival versus non-survival versus controls (the primary explanatory), and with immune, oxidative and metabolic biomarkers as dependent variables, while adjusting for extraneous variables including age, sex, diabetes, BMI, hypertension, smoking and use of medications. When the multivariate GLM analyses were significant, we employed tests for between-subject effects to assess the univariate effects of the explanatory variables. Model-generated estimated marginal means obtained by these GLM analyses were computed and protected, pairwise post-hoc analyses were used to assess the differences in estimated marginal means between the diagnostic groups. We used binary logistic regression analysis to delineate the most important biomarkers that are associated with post-stroke death. Receiver operating characteristic (ROC) curves were computed using the biomarkers predicting post-stroke death. The area under the ROC curve (AUC ROC), and sensitivity and specificity of the biomarkers for post-stroke death were computed.

Logarithmic (Ln) transformations of continuous data were used in the analyses when the variables were not normally distributed or when there was heterogeneity of variance (as assessed with the Levene test). We also have computed two z unit weighted composite scores reflecting activation of peripheral immune-inflammatory pathways. The first (INFLAM index1) reflects the cumulative effects of CRP, IL-6, WBC count, ferritin and ESR and is computed as: INFLAM index1 = z transformation of hsCRP (zCRP) + zIL-6 + zFerritin + zWBC + zESR. Consequently we have computed the sum of zTSP + zVitamin D + zIron (three biomarkers that decrease during immune-inflammatory responses) and computed the second index as: INFLAM index2 = zINFLAM index1 – (zTSP + zVitamin D + zIron). All statistical analyses were performed using IBM SPSS windows version 22. Tests were 2-tailed and an alpha level of 0.05 indicated statistically significant results.

Results

Descriptive statistics

Table 1 shows the demographic data of the health controls and patients divided into those who survived and those who died within the first three months after stroke. P-correction showed that the results of the tests presented in Table 1 remained significant after correction for false discovery rate. Nevertheless, the results are not adjusted for the various background variables and thus less reliable than the results displayed in Tables 2, 3 and 4, which show data that were adjusted for the relevant background variables. Nevertheless, Table 1 shows the following: AIS patients non-survivors were significantly older than AIS patients who survived and healthy controls ($P < 0.001$). There were significantly more males among patients with AIS ($P < 0.001$), but no significant differences between survivors and non-survivors. There were no significant differences in BMI between the three study groups and patients with AIS showed a higher systolic (SBP) and diastolic blood pressure (DBP) as compared to controls ($P < 0.001$). The mRS score at admission (baseline mRS) was significantly higher in AIS patients who died versus survivors ($P < 0.001$). Baseline HDL-cholesterol and insulin levels were significantly different between AIS patients and controls, while glucose levels were significantly different among the three study groups and increased from controls to survivors and non-survivors ($P < 0.001$). There were significantly more smokers among stroke patients as compared with controls ($P = 0.001$), as well as CL-LOOH levels were increased in stroke victims as compared with controls ($P < 0.001$). There were significantly differences in INFLAM index 1 and INFLAM index 2 among the three groups with both values increasing from controls to stroke survivors to non-survivors (controls < AIS survivors < AIS non-survivors, $P < 0.001$). There were no significant

Table 1 Demographic and biomarkers data in ischemic stroke patients and healthy controls. Patients were divided into stroke survivors versus non-survivors (three months after stroke onset)

Characteristics	Controls [A] ($n = 176$)	Survivors [B] ($n = 91$)	Non-survivors [C] ($n = 54$)	F/X^2	df	P value
Age (years)	63.2 (11.3) [C]	65.9 (14.0) [C]	72.3 (2.0) [A,B]	12.62	2/318	<0.001
Sex						
Male	49 (27.84%) [B,C]	58 (63.73%) [A]	27 (50.00%) [A]	33.60	2	<0.001
Female	127 (72.16%)	33 (36.27%)	27 (50.00%)			
Ethnicity						
Caucasian	129	73	43	2.82	2	0.244
Non-Caucasian	47	16	11			
BMI (kg/m^2)	26.79 (4.28)	26.31 (5.48)	26.49 (5.40)	0.29	2/285	0.749
Hypertension	77 (45.56%) [B,C]	75 (82.42%) [A]	49 (92.45%) [A]	57.1	2	<0.001
SBP (mmHg)	122.5 (14.9) [B,C]	146.4 (26.1) [A]	151.9 (32.2) [A]	50.04	2/288	<0.001
DBP (mmHg)	79.1 (12.1) [B,C]	87.1 (20.6) [A]	91.0 (17.6) [A]	13.62	2/288	<0.001
Diabetes Mellitus	36 (20.45%) [B,C]	36 (39.56%) [A]	23 (42.59%) [A]	15.77	2	<0.001
Dyslipidemia	68 (40.23%)	39 (57.14%)	24 (44.44%)	0.48	2	0.788
Smoking	12 (6.82%) [B,C]	19 (20.88%) [A]	12 (22.22%) [A]	14.82	2	0.001
Previous Stroke	–	34 (37.39%)	24 (44.44%)	0.96	1	0.328
mRS	–	3.41 (1.24) [C]	4.48 (1.06) [B]	27.62	1/141	<0.001
CL-LOOH (cpm)	16,556 (11484) [B,C]	25,672 (18515) [A]	31,077 (34926) [A]	12.89	2/284	<0.001
INFLAM index1	−1.66 (2.11) [B,C]	+1.05 (2.28) [A,C]	+3.13 (2.60) [A,B]	82.47	2/224	<0.001
INFLAM index2	−2.77 (2.46) [B,C]	+1.62 (3.27) [A,C]	+4.67 (3.84) [A,B]	107.49	2/212	<0.001
Insulin (IU/L)	10.7 (7.3) [B,C]	27.7 (40.4) [A]	19.6 (18.7) [A]	15.62	2/297	<0.001
Glucose (mg/L)	98.1 (29.2) [B,C]	133.4 (53.6) [A,C]	168.0 (79.2) [A,B]	47.72	2/308	<0.001
HDL (mg/dL)	54.9 (15.3) [B,C]	43.7 (16.8) [A]	40.1 (12.9) [A]	27.29	2/315	<0.001

*F/X2: results of analyses of variance (F-values) and analyses of contingency tables (X^2 tests) performed on the three study groups (controls, survivors and non-survivors); the continuous variables were expressed as mean (±SD) and categorical variables as number (n) and percentage (%); [A,B,C]: Results of protected post-hoc analyses showing the pair-wise comparisons between controls, survivors and non-survivors; [A,B,C]: results of pairwise protected post-hoc analyses (all $p < 0.05$); BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; mRS: modified ranking scale; CL-LOOH: lipid hydroperoxide-initiated chemiluminescense; HDL: high density lipoprotein; INFLAM index1: computed as z transformation of CRP (zCRP) + z interleukin-6 + z ferritin + z sedimentation rate + z number of leukocytes (expressed in z scores); INFLAM index2: INFLAM index1 – z vitamin D - z iron - z total serum protein (expressed in z scores); HDL: high-density lipoprotein cholesterol

differences between survivors versus non-survivors with respect to previous stroke ($P = 0.328$). The prevalence of DM and hypertension, but not dyslipidemia, was significantly higher in stroke patients than in controls ($P < 0.001$ and $P = 0.788$, respectively). With regard to vitamin D, all the three groups were different each other; the patients with stroke (survivors and non-survivors) showed lower levels of 25(OH)D than controls; moreover, non-survivors showed lower levels of 25(OH)D than those survivors ($P < 0.001$).

Inspection of the inter-correlation matrices showed that IL-6 and hsCRP are correlated with ferritin (r = 0.136, $p = 0.017$, $n = 309$ and r = 0.231, $p < 0.001$, $n = 311$, respectively, $P < 0.001$) and iron (r = −0.370, $n = 326$ and r = −0.450, $n = 335$, respectively, all $P < 0.001$). IL-6 and hsCRP are inversely correlated with HDL-cholesterol (r = −0.246, $n = 331$ and r = −0.309, $n = 340$, respectively, all $P < 0.001$). IL-6 and hsCRP are positively correlated with glucose levels (r = 0.289, $n = 323$ and r = 0.417, $n = 333$, respectively, both $P < 0.001$). IL-6 and hsCRP are also correlated with TSP (r = −0.269, n = 326 and r = −0.255, n = 326, respectively, both $P < 0.001$) and 25(OH)D (r = −0.202, n = 323 and r = −0.295, $n = 332$, respectively, both $P < 0.001$). INFLAM index1 is significantly correlated with 25(OH)D (r = −335, $p < 0.001$, $n = 243$), TSP (r = −274, p < 0.001, $n = 246$), iron (r = −0.492, $P < 0.001$, $n = 252$), glucose (r = 483, $P < 0.001$, $n = 249$) and HDL-cholesterol (r = −0.339, $P < 0.001$, n = 252).

Immune/biochemical biomarkers of stroke survivors and deaths post-stroke versus controls

Table 2 shows the results of multivariate GLM analyses with INFLAM index1 and index2, CL-LOOH, HDL-cholesterol, glucose and insulin as dependent variables and diagnosis (the three study groups as shown in Table 1) as primary explanatory variable, while adjusting for sex, smoking, age and BMI. Tests for between-subject effects showed significant effects of diagnosis on all biomarkers. Post-hoc analyses performed on the model-generated estimated marginal mean values showed significant differences among the three study groups in INFLAM index1

Table 2 Results of multivariate GLM analysis with inflammatory indices, glucose, insulin, high density lipoprotein (HDL)-cholesterol (HDL) and lipid hydroperoxides (CL-LOOH) as dependent variables

Test	Dependent variables	Explanatory variables	F	df	*P* value
Multivariate	INFLAM index1 INFLAM index2 HDL, glucose, insulin, CL-LOOH	Diagnosis	16.95	12/312	<0.001
		Sex	3.79	6/156	0.001
		Smoking	1.16	6/156	0.333
		Age	2.89	6/156	0.011
		BMI	4.87	6/156	<0.001
Between subject effects	INFLAM index1	Diagnosis	45.40	2/161	<0.001
	INFLAM index2	Diagnosis	68.89	2/161	<0.001
		Sex	6.52	1/161	0.012
		Age	4.70	1/161	0.032
		BMI	4.75	1/161	0.031
	HDL-cholesterol	Diagnosis	9.68	2/161	<0.001
		Sex	11.51	1/161	0.001
		Age	6.56	1/161	0.011
	Glucose	Diagnosis	31.15	2/161	<0.001
	Insulin	Diagnosis	14.21	2/161	<0.001
		BMI	17.72	2/161	<0.001
	CL-LOOH	Diagnosis	7.52	2/161	0.001

* In this multivariate GLM analysis we entered the six biomarkers as dependent variables. Diagnosis (controls, survivors and non-survivors) was the primary explanatory variable, while we controlled for the effects of extraneous variables (sex, smoking, age and BMI). The between-subject effects show the significant associations between each of the dependent variables and the explanatory variables (only the significant associations are shown)

Model-generated estimated marginal means (±SE and expressed as z-values as obtained by the GLM analysis presented in Table 2) in healthy controls and stroke victims divided into survivors and non-survivors three months after stroke onset

Variables	Controls [A]	Survivors [B]	Non-survivors [C]
INFLAM index1	−1.57 (0.35) [B,C]	+1.25 (0.37) [A,C]	+2.61 (0.49) [A,B]
INFLAM index2	−2.70 (0.45) [B,C]	+1.90 (0.48) [A,C]	+3.87 (0.63) [A,B]
z Glucose	−0.39 (0.13) [B,C]	+0.35 (0.14) [A,C]	+0.98 (0.18)[A,B]
z Insulin	−0.27 (0.14) [B]	+0.59 (0.15) [A,C]	+0.04 (0.20) [B]
z HDL-cholesterol	−0.03 (0.14) [B,C]	−0.38 (0.15) [A,C]	−0.88 (0.19) [A,B]
z CL-LOOH	−0.16 (0.14) [B,C]	+0.44 (0.15) [A]	+0.36 (0.20) [A]

INFLAM index1: computed as z transformation of CRP (zCRP, C-reactive protein) + z interleukin-6 + z ferritin + z sedimentation rate + z number of leukocytes; INFLAM index2: INFLAM index1 – z vitamin D - z iron - z total serum protein; BMI: body mass index;

[A,B,C] : results of pairwise protected post-hoc analyses (all $p < 0.05$)

and INFLAM index2, glucose and HDL-cholesterol with INFLAM index1 and INFLAM index2 increasing from controls to AIS survivors to AIS non-survivors (controls < AIS survivors < AIS non-survivors). Insulin levels were significantly higher in stroke survivors than in controls and non-survivors. CL-LOOH levels were significantly higher in stroke patients than in controls. Figure 1 shows the mean values of the INFLAM index 1 and INFLAM index2 in the three study groups.

Immune-inflammatory biomarkers of death after stroke

Figure 2 shows the mean values of the z transformed values of the 11 biomarkers in the three study groups. Table 3 shows the results of multivariate GLM analysis with WBC count, IL-6, hsCRP, ferritin, ESR, 25(OH)D, TSP and iron as dependent variables and diagnosis (three study groups as in Table 2) as primary explanatory variable while adjusting for sex, smoking, age and BMI. Tests for between-subject effects showed a significant impact of diagnosis on all immune-inflammatory variables. Model-generated estimated marginal mean values and protected post-hoc analyses show that IL-6, hsCRP and 25(OH)D were significantly different between all three study samples. Thus, IL-6 and hsCRP increased, while 25(OH)D decreased from controls to stroke survivors to stroke non-survivors (controls < AIS survivors < AIS non-survivors). ESR was significantly higher, while TSP was significantly lower in post-stroke non-survivors versus controls. Serum ferritin levels were significantly higher in non-survivors versus controls and stroke survivors. Serum iron

Table 3 Results of multivariate GLM analysis with 8 immune-inflammatory biomarkers as dependent variables

Test	Dependent variables	Explanatory variables	F	df	*P* value
Multivariate	Leukocytes, IL-6, hsCRP, Ferritin, ESR, 25(OH)D, TSP, Iron	Diagnosis	9.80	16/354	<0.001
		Sex	3.20	8/177	0.002
		Smoking	2.31	8/177	0.022
		Age	2.11	8/177	0.037
		BMI	1.45	8/177	0.180
Univariate	IL-6	Diagnosis	27.92	2/263	<0.001
		Age (+)	13.68	1/263	<0.001
	hsCRP	Diagnosis	55.43	2/275	<0.001
		BMI (+)	5.49	1/275	0.020
	ESR	Diagnosis	4.88	2/216	0.008
		BMI (+)	5.69	1/216	0.018
	Ferritin	Diagnosis	8.74	2/254	<0.001
		Sex (M > F)	4.52	2/254	0.034
	Iron	Diagnosis	24.99	2/275	<0.001
	Leukocytes	Diagnosis	37.29	2/232	<0.001
	25(OH)D	Diagnosis	17.52	2/273	<0.001
	TSP	Diagnosis	4.89	2/228	0.008

Model-generated estimated marginal means (±SE and expressed as z-values as obtained by the GLM analysis presented in Table 3) in healthy controls and stroke victims divided into survivors and non-survivors three months after stroke onset

Variables	Controls [A]	Survivors [B]	Non-survivors [C]
IL-6	−0.38 (0.10) [B,C]	+0.27 (0.11) [A,C]	+0.74 (0.15) [A,B]
CRP	−0.51 (0.09) [B,C]	+0.38 (0.10) [A,C]	+0.83 (0.13) [A,B]
ESR	−0.12 (0.12) [C]	+0.14 (0.13)	+0.45 (0.17) [A]
Ferritin	−0.28 (0.10) [C]	−0.13 (0.11) [C]	+0.38 (0.14) [A,B]
Iron	+0.44 (0.10) [B,C]	−0.42 (0.12) [A]	−0.33 (0.16) [A]
Leukocytes	−0.56 (0.11) [B,C]	+0.34 (0.11) [A]	+0.64 (0.14) [A]
25(OH)D	+0.42 (0.11) [B,C]	−0.16 (0.12) [A,C]	−0.54 (0.17) [B,C]
TSP	+0.16 (0.10) [C]	−0.07 (0.11)	−0.36 (0.15) [A]

All data are shown in z scores obtained by z transformations; IL: interleukin; hsCRP: high sensitive C reactive protein; ESR: erythrocyte sedimentation rate; 25(OH)D: 25-hydroxyvitamin D, TSP: total serum protein; [A,B,C]: results of pairwise protected post-hoc analyses (all $p < 0.05$)

was lower while WBC count was higher in stroke patients than controls.

Biomarkers of death after stroke (non-survivors versus survivors and controls)

Table 4 displays the results of a multivariate GLM analysis with the immune-inflammatory and biochemical variables as shown in Table 2 as dependent variables and diagnosis (two study groups, namely non-survivors versus survivors + controls) as primary explanatory variable, while controlling for sex, smoking, age and BMI. Tests for between-subject effects showed a significant impact of diagnosis on INFLAM index1 and INFLAM index2, glucose and HDL-cholesterol. Model-generated estimated marginal mean values showed that INFLAM index1, INFLAM index2 and glucose were significantly higher, while HDL-cholesterol was significantly lower in stroke non-survivors than in survivors. Figure 3 shows the mean of the z transformed values of both INFLAM index 1 and INFLAM index2, HDL-cholesterol, insulin, glucose and CL-LOOH in both patient groups. The differences in INFLAM index1 ($F = 8.60$, $df = 1/75$, $P = 0.004$), INFLAM index2 ($F = 5.30$, $df = 1/75$, $P = 0.024$), glucose ($F = 8.64$, $df = 1/75$, $P = 0.004$) and HDL-cholesterol ($F = 4.84$, $df = 1/75$, $P = 0.031$) were significant.

Effects of background variables

The multivariate GLM analysis in Table 2 showed significant effects of sex, age and BMI on the dependent variables. GLM

Table 4 Results of multivariate GLM analysis with inflammation indices, high-density lipoprotein (HDL)-cholesterol, insulin, glucose and lipid hydroperoxides (CL-LOOH) as dependent variables and diagnosis (post-stroke death versus all other subjects) as primary explanatory variable, while adjusting for confounding variables

Type test	Dependent variables	Explanatory variables	F	df	*P* value
Multivariate	INFLAM index1 and index2, HDL-cholesterol, Insulin, Glucose, CL-LOOH	Diagnosis	12.25	6/157	<0.001
		Sex	4.31	6/157	<0.001
		Smoking	1.24	6/157	0.290
		Age	3.05	6/157	0.008
		BMI	3.05	6/157	0.008
Between subject effects	INFLAM index1	Diagnosis	32.04	1/162	<0.001
	INFLAM index2	Diagnosis	40.33	1/162	<0.001
	HDL-cholesterol	Diagnosis	19.41	1/162	<0.001
	Insulin	Diagnosis	0.03	1/162	0.868
	Glucose	Diagnosis	32.60	1/162	<0.001
	Cl-LOOH	Diagnosis	1.91	1/162	0.169

Model-generated estimated marginal means (±SE and expressed as z-values as obtained by the GLM analysis presented in Table 4) in stroke victims divided into survivors and non-survivors three months after stroke onset

Variables	Controls +Survivors	Non-survivors
INFLAM index1	−0.28 (0.34)	+2.77 (0.55)
INFLAM index2	+0.60 (0.47)	+4.14 (0.77)
z HDL-cholesterol	−0.15 (0.11)	−0.95 (0.19)
z Glucose	−0.06 (0.12)	+1.02 (0.19)

INFLAM index1: computed as z transformation of high sensitivity C reactive protein (z hsCRP) + z interleukin-6 + z ferritin + z erythrocyte sedimentation rate + z white blood cell count

INFLAM index2: INFLAM index1 – [z 25-hydroxyvitamin D (z 25(OH)D) - z iron - z total serum protein]; HDL: high-density lipoprotein

analysis showed significant effects of sex on INFLAM index2 (mean ± SE: in females: 1.63 ± 0.45 and in males: 0.42 ± 0.45) and HDL-cholesterol levels (mean ± SE: in females: −0.18 ± 0.14 and in males: −0.68 ± 0.14). Parameter estimates showed that age is associated with INFLAM index2 (positively) and HDL-cholesterol (inversely), while BMI is associated with INFLAM index2 and insulin (both positively).

We have also examined the possible effects of hypertension ($n = 118$ subjects), diabetes ($n = 50$ subjects) and dyslipidemia ($n = 63$) on the results. There was a significant effect of hypertension on the above-mentioned dependent variables ($F = 4.38$, $df = 6/155$, $P < 0.001$), while tests for between-subject effects showed a significant impact on HDL-cholesterol ($F = 9.95$, $df = 1/160$, $P = 0.002$; lower in those with hypertension) and glucose ($F = 10.06$, $df = 1/160$, $P = 0.002$; higher in those with hypertension). There was a significant effect of DM ($F = 14.05$, $df = 6/155$, $P < 0.001$), with tests for between-subject effects showing an impact on CL-LOOH ($F = 5.29$, $df = 1/160$, $P = 0.023$; higher in DM) and glucose ($F = 70.20$, $df = 1/160$, $P < 0.001$; higher in diabetes). There was a significant effect of dyslipidemia ($F = 2.20$, $df = 6/155$, $P = 0.045$), which significantly impacted CL-LOOH ($F = 4.69$, $df = 1/160$, $P = 0.032$) and glucose ($F = 5.71$, $df = 1/160$, $P = 0.0189$ (both higher in dyslipidemia). Most importantly, the entry of these three diagnoses in the GLM regression shown in Table 2 did not change the results presented in Table 2 or the pairwise post-hoc tests showing differences between the three categories (also Table 2).

Consequently, we have examined possible effects of the drug state of the participants. Sixty-one subjects were taking antihypertensive drugs there were no significant effects of these drugs on the biomarkers shown in Table 2 ($F = 1.58$, $df = 6/146$, $df = 0.158$). Use of hypolipemiant drugs (taken by 46 subjects) showed no significant effect on the biomarkers ($F = 1.44$, $df = 6/146$, $P = 0.205$). In the multivariate analysis, treatment with hypoglycemic drugs ($n = 38$) showed a significant effect ($F = 7.89$, $df = 6/146$, $P < 0.001$), while tests for between-subject effects showed a significant effect on glucose ($F = 37.86$, $df = 1/151$, $P < 0.001$). AIS patients who were treated with hypoglycemic drugs had higher glucose values than those without (0.98 ± 0.14 versus 0.05 ± 0.11, expressed in z scores). This indicates that patients with higher glucose levels were treated with hypoglycemic drugs, rather than causal effects of hypoglycemic drugs. Most importantly, the significant differences in the biomarkers (see Table 2) remained unchanged after covarying for use of hypoglycemic, hypolipemiant and antihypertensive drugs.

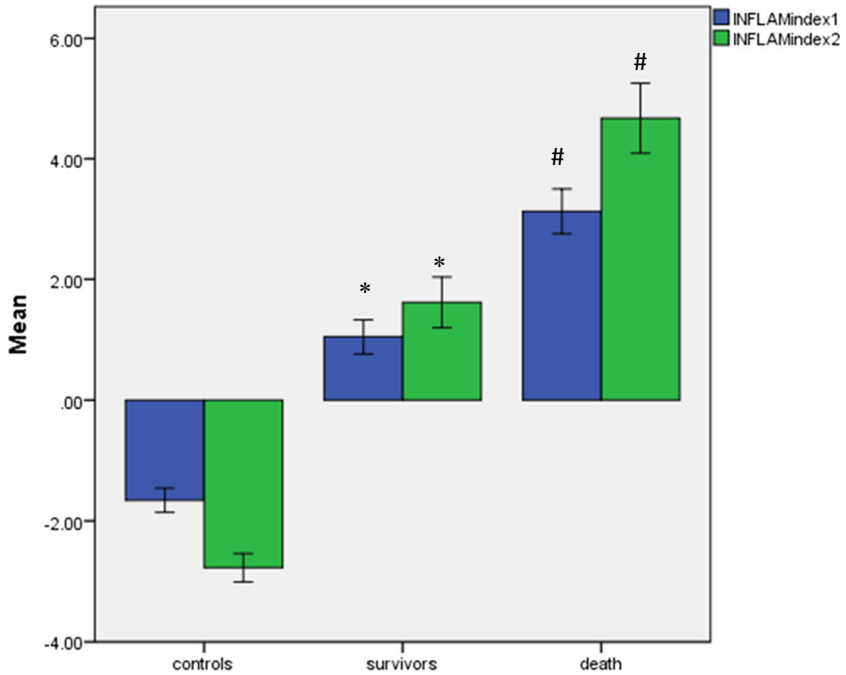

Fig. 1 The mean values of the INFLAM index 1 and INFLAM index2 among the three study groups: healthy controls and those survivors and non-survivors patients after three-month follow-up of the acute ischemic stroke. Post-hoc analyses performed on the model-generated estimated marginal mean values showed significant differences among the three study groups, with INFLAM index1 and INFLAM index2 increasing from controls to stroke survivors to stroke non-survivors. INFLAM index 1 was computed as z transformation of inflammatory variables, such as high sensitivity C reactive protein (z hsCRP) + interleukin 6 (z IL-6) + z ferritin + erythrocyte sedimentation rate (z ESR) + white blood cell count (z WBC), expressed in z scores. INFLAM index 2 was computed as INFLAM index 1 – z 25-hydroxyvitamin D [z 25(OH) D] - z iron - z total serum protein z (TSP), expressed in z scores. SE: standard error. The results of pairwise protected post-hoc analyses. *survivors versus controls: $P < 0.001$; #non-survivors versus controls and versus survivors: $P < 0.001$

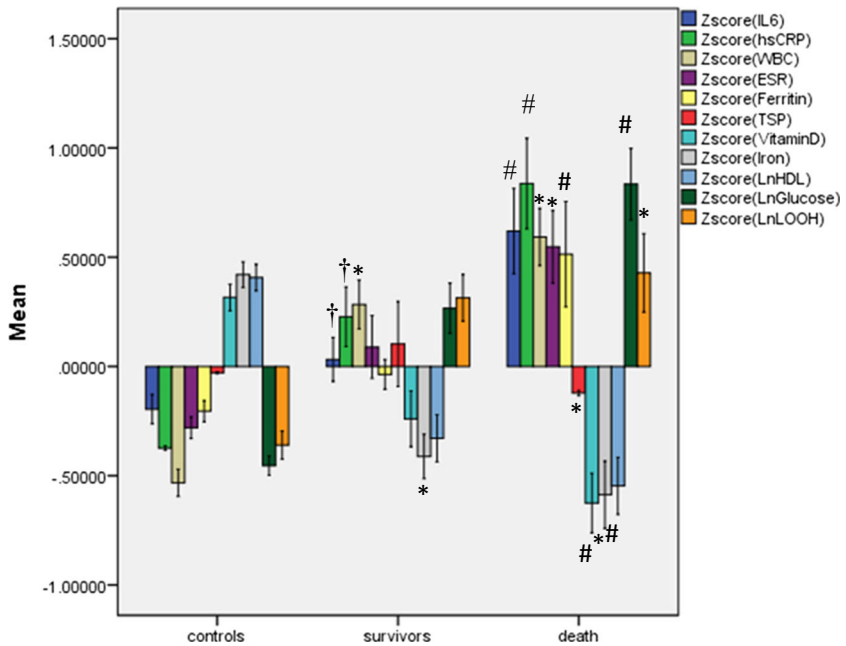

Fig. 2 The z transformed values of the 11 biomarkers in the three study groups: healthy controls and those survivors, and non-survivors patients after three-month follow-up of acute ischemic stroke. IL-6: Interleukin 6; hsCRP: high sensitivity C reactive protein; WBC: White blood cell count; ESR: erythrocyte sedimentation rate; TSP: total serum protein; LnHDL: Logarithmic transformation of HDL-cholesterol values; LnGlucose: Logarithmic transformation of glucose; LnLOOH: Logarithmic transformation of CL-LOOH values; SE: standard error. The results of pairwise protected post-hoc analyses. *: non-survivors versus controls ($P < 0.05$); # non-survivors versus survivors and versus controls ($P < 0.05$); †: survivors versus controls and versus non-survivors ($P < 0.05$)

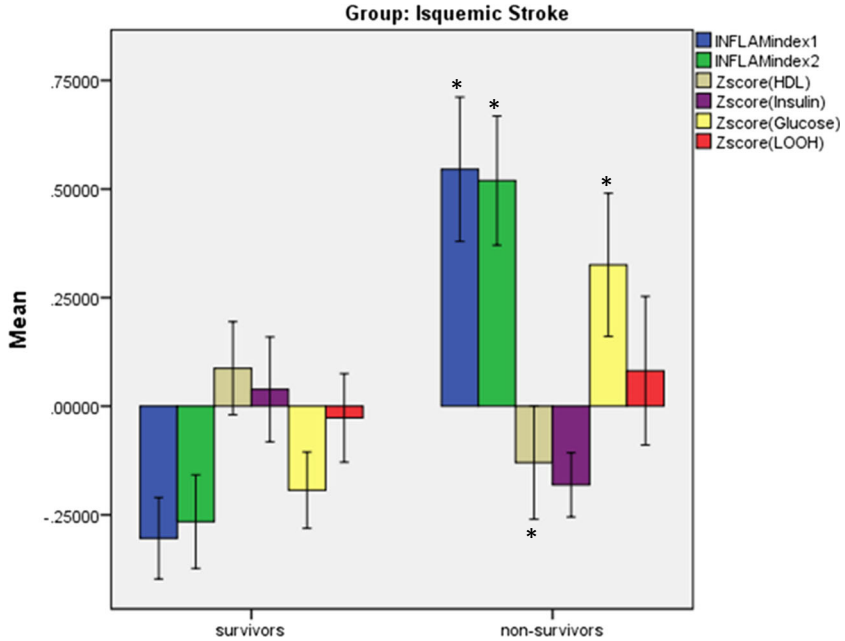

Fig. 3 The z transformed values of both INFLAM index 1 and INFLAM index2, HDL-cholesterol, insulin, glucose and CL-LOOH in both acute ischemic stroke patient groups: survivors and non-survivors after three-month follow-up. INFLAM index 1 was computed as z transformation of inflammatory variables, such as high sensitivity C reactive protein (z hsCRP) + interleukin 6 (z IL-6) + z ferritin + erythrocyte sedimentation rate (z ESR) + white blood cell count (z WBC), expressed in z scores. INFLAM index 2 was computed as INFLAM index 1 – z 25-hydroxyvitamin D [z 25(OH) D] - z iron - z total serum protein z (TSP), expressed in z scores. SE: standard error. The differences in INFLAM index1 ($p = 0.004$), INFLAM index2 ($p = 0.024$), glucose ($P = 0.004$) and HDL-cholesterol ($P = 0.031$) were significant. The results of pairwise protected post-hoc analyses. *: non-survivors versus survivors ($P < 0.05$)

Best prediction of death after stroke

In order to delineate the best predictors for death after AIS we have performed binary regression analyses with death following AIS as dependent variable (and no death as reference group) and using the significant biomarkers as delineated in Tables 2 and 3 as explanatory variables, namely hsCRP, IL-6, INFLAM1 index, HDL-cholesterol, glucose, ferritin and vitamin D and in addition CL-LOOH. Table 5 (regression #1) shows that glucose, ferritin, IL-6, CL-LOOH and 25(OH)D significantly predicted stroke death ($X^2 = 74.52$, df = 5, $P < 0.001$; Nagelkerke = 0.461; correctly classified subjects = 87.8% with 42.1% of sensitivity and 96.5% of specificity). The best predictor was IL-6 followed by glucose. We performed a second logistic regression analysis performed on the patients only and with mRS basal and INFLAM index 1 as explanatory variables. Table 5, regression #2 shows that INFLAM index1 coupled with baseline mRS score significantly predicted death after AIS ($X^2 = 35.26$, df = 2, $P < 0.001$; Nagelkerke = 0.361; correctly classified subjects = 79.6% with 76.6% of sensitivity and 81.8% of specificity.

AUC/ROC analysis

In order to examine whether some of the variables may be used in the clinical practice to predict death after AIS we have performed ROC curve analyses with the most relevant biomarkers as discriminatory variables (Table 6). INFLAM index1 and INFLAM index2 showed the best diagnostic performance with an AUC equaling 0.851 and 0.870, respectively. INFLAM index1 showed that 71.7% of the death cases were correctly classified with specificity of 85.3%. hsCRP (AUC = 0.776) and IL-6 (AUC = 0.776) showed satisfactory results with a 48.6% of sensitivity and of 89.3% specificity for hsCRP >13.5 mg/L and a 51.4% of sensitivity and 84.6% of specificity for IL-6 > 20.4 pg/mL. We have rerun these analyses in the patient study groups examining the discrimination of non-survivors versus survivors. For example, INFLAM index2 yielded a significant separation with an AUC ROC = 0.728 [standard error (SE) = 0.051] and a sensitivity = 55.7% and specificity = 75.0%.

Discussion

The main finding of this study is that death within three-month follow-up after AIS was significantly associated with immune-inflammatory, oxidative and biochemical biomarkers, which are measured within 24 h after hospital admission. Thus, increased levels of IL-6, hsCRP, ferritin and glucose and lower levels of HDL-cholesterol, insulin and 25(OH)D were significantly associated with death after AIS. Moreover, high ESR and low TSP were observed in AIS victims who had died as compared with controls, while AIS survivors occupied an intermediate position.

Table 5 Results of binary regression analyses with post stroke death as dependent variable

Test	Explanatory variables	Wald	df	*P* value	OR	95% CI
Regression #1	Glucose	10.25	1	0.001	2.05	1.32–3.17
	Ferritin	3.89	1	0.049	1.60	1.00–2.57
	IL-6	18.71	1	<0.001	2.97	1.81–4.86
	CL-LOOH	4.89	1	0.027	1.73	1.06–2.80
	25(OH)D	4.62	1	0.032	0.63	0.41–0.96
Regression 2#	INFLAM index1	6.10	1	0.013	1.22	1.04–1.43
	mRS basal	11.38	1	0.001	2.49	1.46–4.23

OR: odds ratio; CI: confidence interval with upper and lower limits; IL: interleukin; 25(OH)D: 25-hydroxyvitamin D; CL-LOOH: lipid hydroperoxides; INFLAM index1: computed as z transformation of high sensitivity C reactive protein (z hsCRP) + z interleukin-6 + z ferritin + z erythrocyte sedimentation rate + z white blood cell count; mRS: modified ranking scale; Regression #1: performed in patients and controls; Regression #2: performed in stroke victims

Increased peripheral levels of IL-6, which are associated with increased hsCRP, higher ESR and WBC counts, indicate that IL-6-associated immune-inflammatory responses play a critical role in death after AIS. These results extend those of previous reports showing that increased peripheral levels of IL-6 and hsCRP predict increased mortality in individuals with serious medical conditions (Goodson et al. 2005; Singh-Manoux et al. 2017; Ridker 2008; Proctor et al. 2015; Gröschel et al. 2007; Böger et al. 2005; Aulin et al. 2015). It may be hypothesized that increased IL-6 trans-signaling rather than classical IL-6 signaling is critically involved in mortality after AIS, because the former is pro-inflammatory and the latter is protective (Maes et al. 2014). Firstly, increased IL-6 trans-signaling and enhanced ROS production may reciprocally induce each other, causing greater oxidative damage to membrane lipids, proteins, DNA and mitochondria (Maes et al. 2014). Secondly, increased IL-6 may cause a T helper (Th)-17 shift with increased IL-17 production thereby facilitating autoimmune responses and formation of neoantigens directed against oxidatively modified lipid structures (Hirano 2010; Jones et al. 2010; Maes et al. 2014). Thirdly, IL-6 trans-signaling may block the development of T regulatory (Treg) cells thereby changing the balance between autoreactive effector versus protective Treg cells (Dominitzki et al. 2007; Hirano 2010). Fourthly, increased overexpression of IL-6 in plasma and the brain enhances neurodegenerative processes by aggravating neuroinflammation, favouring recruitment of T cells to the brain, lowering hippocampal neurogenesis and neuroplasticity and enhancing the activity of the tryptophan catabolite (TRYCAT) pathway resulting in enhanced detrimental effects trough N-methyl-D-aspartate (NMDA) receptors (Maes et al. 2014; Erta et al. 2012; Anderson et al. 2013). Nevertheless, to fully appreciate the effects of IL-6 trans-signaling we would need additional measurements of the soluble IL-6 receptor (sIL-6R), which may bind sIL-6 to form a sIL-6R/IL-6 complex that propagates IL-6 trans-signaling, and soluble gp130 molecule, which attenuates IL-6 trans-signaling (Maes et al. 2014).

The current study found that increased ferritin is a biomarker of AIS and short-term AIS death. Increased plasma levels of ferritin indicate the presence of an immune-inflammatory or acute phase response (Maes et al. 1996; Millerot et al. 2005; Kell and Pretorius 2014; Wang et al. 2010). Interestingly, elevated levels of plasma ferritin measured within 24 to 48 h after stroke may predict a worse prognosis after AIS (Dávalos et al. 2000; Erdemoglu and Ozbakir 2002), while increased ferritin levels are a risk factor for consequent AIS in postmenopausal women (van der A et al. 2005). In a rodent model, induced brain ischemia is accompanied by increased levels of plasma ferritin in rats subjected to severe insults (Millerot et al. 2005). In our study, plasma iron levels were not associated with post-stroke death but were significantly lower in AIS patients than in controls. Generally, the activation of immune-inflammatory pathways is accompanied by reduced serum levels of iron and other erythron markers (Maes et al. 1996). The associations between AIS and serum iron (lowered) and ferritin (increased) may be explained by effects of IL-6 trans-signaling. Thus, IL-6 enhances hepatic ferritin production and lowers plasma iron through effects of IL-6 on hepcidin, the main regulator of plasma iron levels (Maes et al. 1996; Kobune et al. 1994; Nakagawa et al. 2014). Moreover, our findings show significant inverse associations between increased IL-6 (and the consequent elevations in hsCRP) and increased ferritin and lowered iron levels. Reduced plasma iron and elevated ferritin have protective functions during immune-inflammatory responses mainly by preventing iron overload, which may have direct effects on ferroptosis (Morris et al. 2018), the development of thrombosis (Day et al. 2003) and enhanced hydroxyl radical production through the Fenton reaction (Miller et al. 2016). Moreover, ferritin stores cellular iron thereby attenuating the production of hydroxyl radicals and, therefore, increased ferritin protects against the consequences of oxidative stress (Wang et al. 2010; Zielińska-Dawidziak 2015). Nevertheless, ferritin may also display detrimental effects including nuclear factor-κB activation, induction of apoptosis and increased ROS

Table 6 Area under curve of the receiver operating curve of inflammatory and metabolic biomarkers as discriminatory variables to predict death after acute ischemic stroke

Variables	AUC ROC	SE	*P* value	95% CI	Cut-off	Sens (%)	Spec (%)
INFLAM index1	0.851	0.030	<0.001	0.792–0.910	>1.93	65.9	85.3
INFLAM index2	0.870	0.030	<0.001	0.812–0.928	>2.91	71.7	87.1
IL-6 (pg/mL)	0.775	0.037	<0.001	0.703–0.848	>20.40	51.4	84.6
hsCRP (mg/L))	0.776	0.040	<0.001	0.698–0.853	>13.50	48.6	89.3
WBC (cells/mm^3)	0.756	0.042	<0.001	0.675–0.838	>10.570	50.0	90.0
ESR (mm)	0.709	0.045	<0.001	0.621–0.790	>22.50	45.5	81.8
Glucose (mg/dL)	0.767	0.042	<0.001	0.685–0.849	>142.5	48.6	88.6
25(OH)D (ng/mL)	0.717	0.043	<0.001	0.633–0.802	<18.90	48.6	85.2

AUC/ROC: area under curve of receiver operating curve; SE: standard error; CI: confidence interval; Sens: sensitivity; Spec: specificity; IL: interleukin; hsCRP: high sensitivity C reactive protein; WBC: white blood cell; ESR: erythrocyte sedimentation rate; 25(OH)D: 25-hydroxyvitamin D, TSP: total serum protein; INFLAM index1: computed as z transformation of hsCRP + z IL-6 + z ferritin + z ESR + z WBC; INFLAM index2: INFLAM index1 – z 25(OH)D - z iron - z TSP

production through its iron-storing ability (Lombardi et al. 2016; Knovich et al. 2009; Bresgen et al. 2010).

Other major finding of this study is that increased levels of lipid hydroperoxides (CL-LOOH), an oxidative stress biomarker, are associated with short-term AIS death. Previously, it was shown that AIS is accompanied by increased levels of CL-LOOH and malondialdehyde (MDA) (Rodrigo et al. 2013) and that increased MDA levels predict mortality in institutionalized elderly, while antioxidant defenses, such as superoxide dismutase and vitamin E are associated with survival (Huerta et al. 2005). As explained in the Introduction section of the present study, increased ROS production and damage to lipids and proteins, DNA and mitochondria is a key component of neuronal damage in the penumbra, while ROS-induced BBB disruption may lead to increased passage of activated T cells, effector cells and M1 macrophages. Moreover, ROS supports the formation of cerebral edema, the leading cause of death after AIS. Vasogenic cerebral edema follows breakdown of the BBB and affects white matter, while cytotoxic edema is a primary mechanism of cerebral edema during ischemia by generating hydroxyl radical formation and formation of peroxynitrite (Rodrigo et al. 2013).

Our results also extend those reported by previous studies showing that AIS is accompanied by lowered serum vitamin D (Poole et al. 2006; Kim et al. 2017), and that lowered vitamin D increases risk towards AIS (Sun et al. 2012; Kienreich et al. 2013; Gupta et al. 2014; Manouchehri et al. 2017; Alfieri et al. 2017). Vitamin D has strong anti-inflammatory, antioxidant and neuroprotective effects (Kienreich et al. 2013; Calton et al. 2015) and may suppress expression and/or production of pro-inflammatory mediators and cytokines, including IL-6 (Calton et al. 2015; Berridge 2017). Vitamin D enhances superoxide dismutase activity, improves glutathione system activities, controls the expression of Nrf2, maintains mitochondrial respiration and protects against lipid peroxidation (Mokhatari et al. 2017; Berridge 2017). Moreover, vitamin D has neuroprotective properties, for example by enhancing neurotrophin release and intracellular calcium homeostasis, and reduces neuronal injury and inflammation following traumatic brain injury (Lawrence and Sharma 2016; Wrzosek et al. 2013). Therefore, the inverse association between vitamin D and the inflammation indexes (INFLAM index1 and INFLAM index2) found in the current study may suggest that lowered vitamin D levels in AIS victims aggravate the ongoing immune-inflammatory and oxidative responses and attenuate neuroprotection thereby contributing to detrimental processes leading to death.

Finally, we also observed that some biochemical variables were significantly associated with death after AIS, namely glucose/insulin and HDL-cholesterol. Our results extend previous findings that increased blood glucose levels and AIS risk are significantly associated (Wang et al. 2017; Natuva et al. 2016) and may predict risk of early death in heart failure patients and in patients hospitalized for pneumonia (Lepper et al. 2012). Previous reports showed that aberrations in glucose metabolism may predict stroke (Choudhury et al. 2015) and that glucose intolerance highly significantly increases risk of brain infarction in nondiabetic individuals (Denti et al. 2003). In animal models of ischemia, hyperglycemia is associated with increased neuronal damage (Ginsberg et al. 1980; Pulsinelli et al. 1982). Following intracerebral hemorrhage, hyperglycemia exacerbates brain edema and causes perihematomal cell death (Song et al. 2003). Blood glucose may cross the BBB and may promote free radical generation, impair radical scavenging and glutathione cycle functions, induce aberrant osmolarity in brain cells, and promote protein glycation with formation of advanced glycation end-products (Tomlinson and Gardiner 2008). Regarding the stress hyperglicemia and AIS outcome, some studies have shown conflicting results. A previous systematic review and meta-analysis study showed that acute hyperglycemia predicted increased risk of in-hospital mortality after AIS in nondiabetic patients and increased risk of poor functional recovery in nondiabetic stroke survivors (Capes et al. 2001). Other study also showed that prolonged stress hyperglycemia in AIS patients increases the risk of short-

term mortality, especially in non-diabetic patients (Kes et al. 2007). However, a prospective study showed that stress hyperglycemia does not appear to be directly associated with the outcome of AIS (Tziomalos et al. 2017); however, given that patients evaluated in the last mentioned study that showed stress hyperglycemia had higher prevalence of cardiovascular risk factors than patients with normoglycemia and that these authors did not evaluate glucose tolerance of the AIS patients, the role of stress hyperglicemia does not be excluded from the pathophysiology of AIS outcome.

Mounting evidence suggest that HDL-cholesterol may protect against stroke (Choudhury et al. 2015). Decreased HDL-cholesterol is associated with AIS especially in individuals less than 70 years old (Luo et al. 2014). Individuals with low HDL-cholesterol show a trend towards higher stroke risk (Demarin et al. 2010) and increased risk towards cardiovascular events (Acharjee et al. 2013; Demarin et al. 2010). Interestingly, in our study there was a significant inverse association between HDL-cholesterol levels and inflammation indexes, IL-6 and hsCRP levels, suggesting that attenuated HDL-cholesterol-associated antioxidant defenses participate in the immune-inflammatory pathophysiology of death after stroke. Furthermore, the present study showed that TSP was significantly lower in stroke non-survivors versus healthy controls, while survivors showed an intermediate position. There was a significant inverse association between TSP and the z unit weighted inflammation index as well as IL-6 and hsCRP. An acute phase response is indeed accompanied by lowered levels of TSP and albumin, indicating that lowered TSP is an indicant of the ongoing inflammatory process (Van Hunsel et al. 1996).

In our study, significant associations were established between AIS and male sex, hypertension, DM and smoking, but not dyslipidemia. Nevertheless, we could not observe that any of these AIS predictors was associated with death after stroke. Most importantly, the associations between death after stroke and the abovementioned biomarkers remained significant after adjusting for these established predictors of AIS. Nevertheless, non-survivors showed a higher age than survivors, while increasing age also contributes to INFLAM index2 and HDL-cholesterol. This indicates that aberrations in immune-inflammatory and oxidative pathways fueled by changes in HDL-cholesterol and glucose levels are more important in predicting death than the established predictors of stroke, while age may have a minor contribution to the inflammatory response. Furthermore, in the present study we found that the effects of the biomarkers predicting death were not affected by use of hypoglycemic, hypolipemiant and antihypertensive drugs. This shows that in the clinical practice the predictive value of the biomarkers is not substantially affected by these different confounding and extraneous variables. In this respect, we computed z unit weighted composite scores based on immune-inflammatory biomarkers, which yielded a good prediction of death three months after stroke with an area under the ROC curve of 0.870.

The results of the present study should be interpreted with respect to its strengths and limitations. Using more immune-inflammatory biomarkers, including other cytokines and receptor levels such as IL-1β and soluble IL-1 receptor antagonist (sIL-1RA), TNFα and its sTNF-receptors, sIL-6R, IL-4 and IL-10, may result in a better prediction of death after stroke. In addition, the measurement of adipokines, such as leptin, resistin, apelin and visfatin, which have been proposed as potential biomarkers of occurrence of AIS as weel as cardiovascular morbidity, mortality and therapeutic target (Opatrilova et al. 2018) may be included in further studies. Regarding the oxidative stress biomarkers, the evaluation of xanthine oxidase, superoxide dismutase, catalase, PON1 and glutathione peroxidase, and lipid and protein oxidation products, including MDA, 4-hydroxynonenal, protein carbonyls or advanced protein oxidation products (AOPP), may also help to enhance the diagnostic performance of our composite scores. Finally, the model herein proposed deserves validation in an independent study sample. Strengths are the prospective design of our study and the multivariate approach to delineate cumulative effects of the immune-inflammatory and metabolic biomarkers to predict short-term AIS death.

Importantly, our results show that activated immune-inflammatory pathways, oxidative stress and lowered levels of vitamin D and HDL-cholesterol are possible new drug targets in the prevention of death after AIS. New possible treatments are targeting immune-inflammatory pathways by IL-6 trans-signaling blockade (tocilizumab) and maybe blockade of TNF-α signaling (etanercept) and IL-1β signaling. Moreover, compounds with combined anti-inflammatory, antioxidant and neuroprotective properties may have some benefit including allopurinol, minocycline and statins (Maes et al. 2012; de Melo et al. 2017). These treatments can be combined with antioxidant supplements, which target the same pathways including vitamin D, curcumin, coenzyme Q10, resveratrol and zinc (de Melo et al. 2017).

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Authors' contributions Study concept and design: EMVR and ANCS; acquisition of data: JRG, DFA, TF, MFL, MCMA; analysis and interpretation of data: EMVR, ANCS, MM; drafting of the manuscript: EMVR and MM; statistical analysis: MM; study supervision: EMVR, ERDA.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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