

Wound Healing and Inflammation/Infection

Immuno-inflammatory activation in acute cardio-embolic strokes in comparison with other subtypes of ischaemic stroke

Giuseppe Licata*; Antonino Tuttolomondo*; Domenico Di Raimondo; Salvatore Corrao; Riccardo Di Sciacca; Antonio Pinto

Dipartimento Biomedico di Medicina Interna e Specialistica, Università degli Studi di Palermo

Summary

Few studies have examined the relationship between inflammatory biomarker blood levels, cardioembolic stroke subtype and neurological deficit. So the aim of our study is to evaluate plasma levels of immuno-inflammatory variables in patients with cardio-embolic acute ischaemic stroke compared to other diagnostic subtypes and to evaluate the relationship between immuno-inflammatory variables, acute neurological deficit and brain infarct volume. One hundred twenty patients with acute ischaemic stroke and 123 controls without a diagnosis of acute ischaemic stroke were evaluated. The type of acute ischaemic stroke was classified according to the TOAST classification. We evaluated plasma levels of IL-1 β , TNF- α , IL-6 and IL-10, E-selectin, P-selectin, sICAM-1, sVCAM-1, vWF, TPA and PAI-1. Patients

with ischaemic stroke classified as cardio-embolic (CEI) showed, compared to other subtypes, significantly higher median plasma levels of TNF- α , IL-6 and IL-1 β . Furthermore stroke patients classified as lacunar showed, compared to other subtypes, significantly lower median plasma levels of TNF- α , IL-6 and IL-1 β . Multiple linear regression showed a significant association between the Scandinavian Stroke Scale (SSS) score at admission and diagnostic subtype, infarct volume of cardio-embolic strokes and some inflammatory variables. Our findings confirm that cardio-embolic strokes have a worse clinical presentation and produce larger and more disabling strokes than other ischaemic stroke subtypes reporting a possible explanation of higher immuno-inflammatory activation of the acute phase.

Keywords

Cerebral infarct, cerebrovascular accident, cerebrovascular disease, inflammation, risk factors, stroke, cytokines

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Introduction

The prevalence of cardio-embolic stroke is increasing as the population of patients with atrial fibrillation (AF) is growing markedly with the increasing number of the elderly (1). In general, the prognosis of cardio-embolic stroke tends to be poor as a result of obstruction of the major brain arteries by large fibrin-rich thrombi to produce large brain infarcts (2). Cerebral ischaemia initiates a complex cascade of events at genomic, molecular, and cellular levels, and inflammation is important in this cascade, both in the central nervous system (CNS) and in the periphery (3, 4). Furthermore, recent research points to the role of chemokines, normally key factors in inflammation, in thrombogenesis (5), probably also explaining hyper-responsiveness of platelets in ischaemic stroke (6). Recently our group reported that lacunar strokes compared to non-lacunar ones exhibited significantly lower plasma levels of tumour necrosis factor

(TNF)- α and interleukin (IL)1- β , P-selectin and intercellular adhesion molecule 1 (ICAM-1) 24–72 hours (h) and 7–10 days after stroke onset (7). One previous study examined inflammatory pathways in lacunar study (8), whereas no study, to our knowledge, evaluated inflammatory pathways in acute cardio-embolic strokes. Furthermore, few studies have examined the relationship between blood levels of inflammatory biomarkers and stroke outcome (9, 10), but data on a relation between inflammation parameters and diagnostic subtype remain scarce.

So the aim of our study is to evaluate cytokines, selectins and adhesion molecule plasma levels in patients with acute ischaemic stroke in relation to diagnostic subtype and to evaluate the relationship between clinical, laboratory and immuno-inflammatory variables and acute neurological deficit. We chose to evaluate these biomarkers because acute ischaemic stroke has been associated with serum elevations of a series of immuno-inflammatory variables such as TNF- α , IL-6, IL1- β , selectins and

Correspondence to:
Dr. Antonino Tuttolomondo
Dipartimento Biomedico di Medicina Interna e Specialistica
Università degli Studi di Palermo
Pizza delle Cliniche n.2, 90127 Palermo, Italy
E-mail: brunotutto@unipa.it

*These Authors contributed equally to the article.

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adhesion molecules (11–14) and of markers of impaired haemostasis and thrombosis (15–18). Thus, it appears that inflammation, mediated by both molecular components, including cytokines, selectins and adhesion molecules and cellular components, such as leukocytes and microglia may be important in the mechanisms of cerebral injury and repair. So it may be useful to evaluate immuno-inflammatory and thrombotic-fibrinolytic variables in acute ischaemic stroke and to evaluate their relation with acute neurological deficit and stroke volume.

Materials and methods

Patient population

We enrolled all consecutive patients with a diagnosis of acute ischaemic stroke admitted to the Internal Medicine and Cardio-Angiology Department at the University of Palermo (Italy) between November 2002 and January 2006. Controls were patients admitted, in the same period, to our department for any cause other than acute cardiovascular and cerebro-vascular events or exclusion criteria (see exclusion criteria section).

Stroke was defined by focal neurological signs or symptoms thought to be of vascular origin that persisted for >24 h confirmed by brain computed tomography (CT) and/or magnetic resonance imaging (MRI) scan in baseline conditions and brain CT with contrast medium after 48–72 h (19).

In order to also match patients with acute ischaemic stroke and controls for cardiovascular risk and previous cardiovascular morbidity, controls were included if they had vascular risk factors or a history of myocardial infarction or cerebrovascular disease or peripheral vascular disease, but they were excluded if they had either current or recent (up to six months) cerebro-vascular disease or one of the exclusion criteria (see exclusion criteria section).

Cardiovascular risk factors were evaluated for both subjects and controls on the basis of the following criteria:

- Hypercholesterolemia was defined as the presence of total cholesterol blood levels ≥ 200 mg/dl. Hypertension was defined as present if subjects had been previously diagnosed according to the World Health Organization/ International Society of Hypertension guidelines and were routinely receiving antihypertensive therapy. Patients were defined as type 2 diabetics if they had known diabetes treated by diet, oral hypoglycaemic drugs or insulin before stroke.
- Previous coronary artery disease was determined on the basis of a history of physician-diagnosed angina, myocardial infarction, or any previous revascularisation procedure assessed by a questionnaire
- Previous cerebrovascular disease (transitory ischaemic attack [TIA]/ischaemic stroke) was assessed by history, specific neurologic examination executed by specialists, and hospital or radiological (brain CT or MRI scan) records of definite previous stroke.
- Subjects were classified as having previous peripheral artery disease (PAD) when they had a history of arterial brachial index (ABI) < 0.9 and/or of claudication intermittens or of critical limb ischaemia or when they had undergone a peripheral arterial bypass surgery or amputation.

Since several drugs commonly used by subjects suffering from hypertension, diabetes, and hypercholesterolemia display anti-inflammatory properties, we took into account at the time of recruitment if patients enrolled are being treated with these drugs.

The study protocol was approved by the local ethics committee, and all participants (or the nearest relative when patients were unable) gave written informed consent.

Every subject with ischaemic stroke was matched for age (± 3 years), sex, and cardiovascular risk factor prevalence with one control subject.

Patients with inflammatory or infectious diseases, autoimmune and rheumatic diseases, cancer, haematological diseases and severe renal or liver failure, as well as those who were under treatment with anti-inflammatory drugs, were excluded. We also excluded patients with fever and recent venous thromboembolism.

The type of acute ischaemic stroke was classified according to the TOAST classification (20): 1) Large Artery Atherosclerosis (LAAS); 2) Cardio-Embolic Infarct (CEI); 3) Lacunar infarct (LAC); 4) stroke of Other Determined Etiology (ODE); 5) stroke of Undetermined Etiology (UDE).

Clinical and instrumental evaluation

All the ischaemic stroke patients underwent: medical history with recording of potential stroke risk factors, blood and coagulation tests, 12-lead electrocardiogram (ECG), 24-h electrocardiography monitoring, transthoracic echocardiography, carotid ultrasound, brain CT scan at admission (in some cases repeated at 4–7 days).

Blood samples

Blood samples were obtained in the non-fasting state. After 10 minutes of rest in the supine position, vital signs were recorded and blood samples were collected from the antecubital vein.

EDTA-anticoagulated peripheral blood was drawn from each patient within 12 h of symptom onset. Serum and plasma were immediately separated by centrifugation and stored in aliquots at -80°C until analysis.

Biochemical evaluation of immuno-inflammatory variables

We evaluated plasma levels of IL-1 β , TNF- α , IL-6 and IL-10, E-selectin, P-selectin, sICAM-1 and sVCAM-1 as markers of immuno-inflammatory activation, von Willebrand factor (VWF) plasma levels as a marker of endothelial dysfunction and platelet activity, tissue plasminogen activator (TPA) antigen and plasminogen activator inhibitor (PAI)-1 plasma levels as thrombotic/fibrinolytic markers.

IL-1 β , TNF- α , IL-6 and IL-10 and VWF antigen were measured using a sandwich ELISA (Human IL-1 β , TNF- α , IL-6 and IL-10 Quantikine, R&D Systems; VWF ELISA kit, Instrumentation Laboratory, Milano, Italy); VCAM-1, ICAM-1, E-selectin, P-selectin, PAI-1 and TPA-antigen were measured by commercial bioimmunoassay (Human sICAM-1, sVCAM-1, sE-selectin and sP-selectin Parameter, Quantikine, R&D Systems; Gentaur AssayMax Human Plasminogen Activator Inhibitor-1 (PAI-1) ELISA Kit, Gentaur AssayMax Tissue Plasminogen Activator (TPA) ELISA Kit).

The minimum detectable concentrations for the diagnostic tests are: TNF- α : 1.6 pg/ml; IL1- β : <1 pg/ml; IL-6: <0.70 pg/ml; IL-10: >3.9 pg/ml; ICAM-1: <0.35 ng/ml; VCAM-1: 0.6 ng/ml; E-selectin: <0.1 ng/ml; P-selectin: <0.5 ng/ml; VWF: 1.0%; TPA: 0.3 pg/ml; PAI-1: <50 pg/ml.

Intraassay and interassay coefficients of variation were: TNF- α : 4.2% and 4.6%; IL1- β : 3.3% and 4.2%; IL-6: 1.6% and 3.3%; IL-10: 4.3% and 7.5%; ICAM-1: 4.8% and 6.1%; VCAM-1: 3.5% and 7.7%; E-selectin: 4.8% and 5.7%; P-selectin: 4.9% and 8.8%; VWF: 5% and 10%; TPA: 4.8% and 5%; PAI-1: 5.7% and 8.3%.

Table 1: Demographic, clinical and immunoinflammatory variables and anti-thrombotic and cardiovascular medications for stroke patients and controls.

Variable	Stroke patients (n = 120)	Controls (n = 123)	P-value
Age (years)	72 (64–82.5)	69 (65–83)	0.749
Glucose blood levels (mg/dl)	145.5 (99–233)	119 (87.5–143.5)	0.001
Cholesterol blood levels (mg/dl)	232 (199–260)	201 (178–225)	<0.001
Triglycerides (mg/dl)	187.5 (140.75–211.75)	154 (98–200)	0.007
White body cells (WBC) (per mm ³)	8000 (6700–10000)	6400 (5500–8800)	<0.001
Neutrophils (%)	5780 (4071–8000)	3843 (336–6320)	<0.001
CRP (mg/dl)	3.5 (1.2–4.6)	1.3 (0.8–1.5)	<0.001
Sex (M/F)	68/52	55/68	0.062
Diabetes (n/%)	50 (41.7)	59 (48%)	0.363
Hypertension (n/%)	74 (61.7%)	77 (62.6%)	0.101
Previous stroke (n/%)	39 (32.5)	37 (30.08)	0.75
CAD (n/%)	25 (20.8)	24 (19.5)	0.46
PAD (n/%)	14 (11.6)	17 (13.8)	0.23
IL-1 β (pg/ml)	9 (4–11)	3 (2–5)	<0.001
IL-6 (pg/ml)	11 (6–30)	9 (2.9–18)	<0.001
TNF- α (pg/ml)	31.5 (10.25–41)	3.7 (1.1–4.3)	<0.001
E-selectin (ng/ml)	2.25 (2.0–4.0)	2 (1–2)	<0.001
P-selectin (ng/ml)	4 (2–6.3)	3.1 (2.1–4)	0.004
VICAM (ng/ml)	20 (15.1–23)	14 (13–17)	<0.001
ICAM (ng/ml)	20.8 (16.2–24)	15.9 (12–18.1)	<0.001
IL-10 (pg/ml)	3.75 (2–9)	4 (2–10)	0.433
vWF (ng/ml)	10 (6–10)	4 (3–8)	0.0001
PAI-1 (pg/ml)	142 (109.5–175)	21 (12–26)	<0.001
TPA (pg/ml)	24 (15.75–36)	74 (59–98)	<0.001
<i>Premorbid antithrombotics</i>			
Antiplatelet drugs (n/%)	36 (30)	35 (29.1)	0.56
Anticoagulants (n/%)	38 (31.6)	56 (45.5)	0.23
<i>Premorbid cardiovascular drugs</i>			
Statins (n/%)	41 (34.16%)	44 (35.7%)	0.23
ACE inhibitors (n/%)	47 (39.1%)	49 (39.8%)	0.23
Angiotensin-receptor blockers, (ARBs) (n/%)	27 (22.5)	29 (23.5)	0.25
<i>Antidiabetic drugs (n/%)</i>			
– Biguanids (n/%)	18 (15)	21 (17.5)	0.53
– Sulphonilureas (n/%)	12 (10)	14 (11.6)	0.68
– Thiazolidinediones (n/%)	4 (3.3)	5 (4.06)	0.41
Insulin (n/%)	16 (13.3)	19 (15.44)	0.60

Demographic and anamnestic variables are expressed as n° (percentage). Laboratory variables are expressed as median and interquartile (lower and upper quartile). WBC: white body cell count; CRP: C-reactive protein; CAD: coronary artery disease; CHF: congestive heart failure; PAD: peripheral artery disease; TNF- α : tumour necrosis factor α ; IL-1 β : interleukin-1 β ; IL-6: interleukin-6; IL-10: interleukin 10; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; vWF: von Willebrand factor; TPA: tissue plasminogen activator; PAI-1: plasminogen activator.

Ischaemic brain lesion volume

Brain CT was routinely performed on patient admission and repeated (in some cases) at 4–7 days (mean 4.5 days) after stroke onset.

Each scan was performed with 120 kV, 160 mA, 5 mm thickness and 2 seconds acquisition time. We used a time-consuming but accurate technique for calculating the volume of ischaemic brain lesion according to the Cavalieri principle (20). With this method we avoided imprecise estimations of the lesion volume due to the different three-dimensional form of the lesion itself (pyramidal or ellipsoid). In detail, for each CT slice in which the ischaemic lesion appeared, we calculated the surface of the lesion (mm²). In the same slice we multiplied the lesion surface by 5, a number expressing the CT axial sections thickness of 5 mm each, to obtain the volume (3 mm) on the given CT slide. Subsequently, for each patient we summed the volumes of the individual slices where the lesion appeared.

Acute neurological deficit evaluation

Neurological deficit score on admission was evaluated by Scandinavian Stroke Scale (SSS). SSS assesses neurological deficit through an evaluation of consciousness level, eye movement, strength in arms, hands, and legs, orientation, language, facial weakness and gait, giving rise to a score ranging from 58 (absence of deficit) to 0 (death)

Statistical analysis

Data are reported as median (lower Quartile-> upper Quartile). Comparisons between groups were performed by Mann-Whitney U test. The Kruskal-Wallis test was performed as non-parametric analysis of variance for multiple comparisons, and the Conover-Inman procedure was used as post-hoc test to make all possible pair-wise comparisons between groups. The Wilcoxon signed ranks test statistic was performed to compare controls with baseline data. Pearson Chi-Square was computed for cat-

Table 2: Demographic, clinical variables and antithrombotic and cardiovascular medication by ischaemic stroke TOAST subtype.

Variable	LAAS	Lacunar	CEI	ODE	P-value
Number	50 (42%)	46 (38%)	20 (17%)	4 (3.3%)	
Age (years)	75 (66–86)	66.5 (60–76)	70 (64–81)	66.8	0.007
Sex (M/F)	40/10	21/25	18/2	3/1	<0.0001
Diabetes (n%)	12 (24%)	31 (67%)	7 (35%)	-	<0.0001
Hypertension (n%)	29 (58%)	27 (59%)	14 (70%)	-	=0.623
Hypercholesterolaemia (n%)	28 (56%)	15 (33%)	5 (25%)	-	=0.034
Previous TIA (n%)	24 (48%)	22 (48%)	11 (55%)	-	=0.9206
Previous stroke (n%)	28 (56%)	15 (33%)	10 (50%)	2 (50)	=0.0651
Glucose blood levels (mg/dl)	117 (96–226)	167.5 (101–244)	109 (87–199)	96 (77–142)	<0.0001
WBC (cell/ μ l)	9010 (8000–10500)	7850 (6500–9200)	10250 (8700–12000)	9500 (8300–11000)	0.033
CRP (mg/dl)	3.2 (1.3–4.0)	3.1 (1.4–4.1)	3.4 (1.5–4.3)	3 (1.5–3.9)	0.35
CAD (n%)	17 (33)	14 (30.4)	5 (25)	0	=0.024
CHF (n%)	6 (12)	11 (23.9)	4 (20)	0	=0.05
SSS score (median)	36 (29–39)	49.5 (47–50.7)	29 (11–34)	33 (31–10)	<0.0001
Ischaemic brain volume lesion (mm ³)	11894 (\pm 2100)	9894 (\pm 1200)	12273 (\pm 2400)	10273 (\pm 1800)	=0.024
<i>Premorbid antithrombotics</i>					
Antiplatelet drugs (n%)	21 (42)	9 (19.5)	5 (25)	1 (25)	<0.0001
Anticoagulants (n%)	5 (10)	18 (39.1)	11 (55)	4	<0.0001
<i>Premorbid cardiovascular drugs</i>					
Statins (n%)	17 (34.8)	16 (34.7)	7 (35)	-	0.38
ACE inhibitors (n%)	20 (40)	18 (39.1)	9 (45)	1	0.071
Angiotensin-receptor blockers, (ARBs) (n%)	11 (22)	11 (23.9)	5 (25)	1	0.54
<i>Antidiabetic drugs</i>					
– Biguanids (n%)	4 (8)	10 (21.71)	4 (20)	-	0.034
– Sulphonilureas (n%)	3 (6)	9 (19.56)	1 (5)	-	0.021
– Thiazolidinediones (n%)	1 (2)	2 (4.34)	-	-	0.38
Insulin (n%)	4 (8)	10 (21.71)	2 (10)	-	<0.05

Demographic and anamnestic data are expressed as n° (percentage). Laboratory variables are expressed as median and interquartile (lower and upper quartile). TOAST: Trial of Org 10172 in Acute Stroke Treatment WBC: white body cell count; CRP: C-reactive protein; CAD: coronary artery disease; CHF: congestive heart failure; previous TIA: previous transitory ischaemic attack; SSS score: Scandinavian Stroke Scale score.

egorical variables. A two-tailed p-value <0.5 was considered statistically significant.

Multiple linear regression was performed to investigate associations between some relevant variables and SSS score as a dependent variable.

According to sample size calculation a sample size of 110 patient-control pairs had 80% power at the 5% significance level to detect a 10% difference in selected biomarker plasma levels between control subjects and patients and between each subtype of stroke.

Results

We enrolled 156 subjects with acute ischaemic stroke and 123 matched controls (without ischaemic stroke). We excluded 36 subjects for one or more exclusion criteria (6 for rheumatologic disease, 6 for infectious diseases; 10 for previous treatment with anti-inflammatory drugs; 8 for fever and 6 for cancer). The final sample consisted of 120 subjects with acute ischaemic stroke. Demographic, clinical, laboratory and immuno-inflammatory variables of patients with acute ischaemic stroke and controls are shown in Table 1.

Among stroke patients, according to TOAST Classification, 50 were classified as LAAS, 46 as lacunar, 20 as CEI and 4 as ODE.

Stroke patients compared to controls showed significantly higher median plasma levels of IL1- β , IL-6, TNF- α , E-selectin P-selectin V-CAM-1 and ICAM-1 (see Table 1).

Demographic, laboratory and clinical variables of stroke patients in relation to TOAST subtype are listed in Table 2. The median time of blood sampling was 36 h (8–48 h). Among subjects with acute ischaemic stroke classified as LAAS, 40 were male and 10 female, 12 (24%) were diabetic, 29 (58%) had hypertension, 28 (56%) had hypercholesterolaemia, 24 (48%) had a previous TIA and 28 (56%) a previous stroke, 17 (33%) had a previous diagnosis of CAD and six (12%) a previous diagnosis of congestive heart failure (CHF) (see Table 2). Among subjects with acute ischaemic stroke classified as cardio-embolic, 18

were male and two female, seven (35%) were diabetic, 14 (70%) had hypertension, five (25%) had hypercholesterolaemia, 11 (55%) had a previous TIA and 10 (50%) a previous stroke, 5 (25%) had a previous diagnosis of CAD and 4 (20%) a previous diagnosis of CHF (see Table 2).

Among subjects with lacunar stroke, 21 were male and 25 female, 31 (67%) were diabetic, 27 (59%) had hypertension, 15 (33%) had hypercholesterolaemia, 22 (48%) had a previous TIA and 15 (33%) a previous stroke, 14 (30.4) had a previous diagnosis of CAD and 11(23.9) a previous diagnosis of CHF (see Table 2).

In Table 2 we also reported any difference in use of drugs such as statins, sulphonilurea metformin, thiazolidinediones, ACE-inhibitors, angiotensin receptor blockers (ARBs) and other cardiovascular drugs among different stroke subtypes.

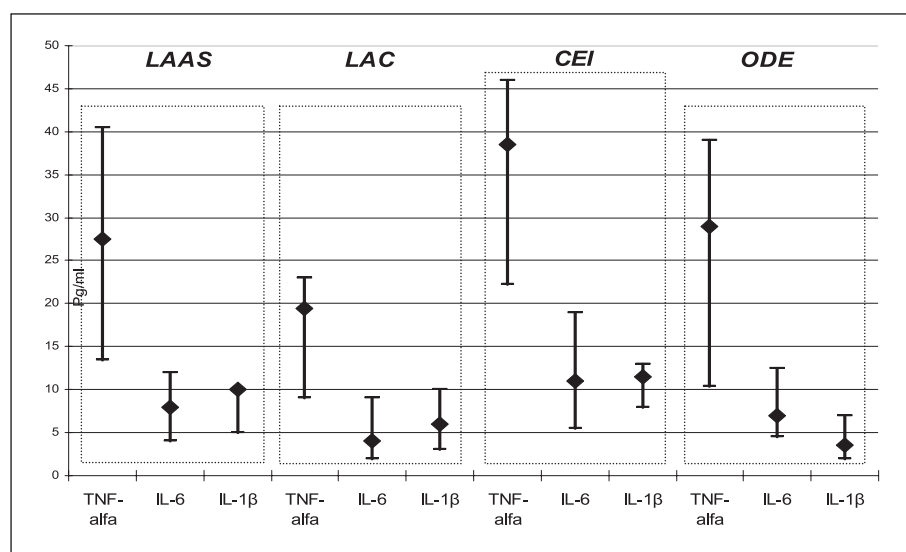
The mean ischaemic lesion volume on brain CT was 1077.7 mm³. The brain lesion mean volume was larger in patients with cardio-embolic stroke infarction (mean 12273 mm³) than in those with atherosclerotic lesions (mean 11894 mm³) and lacunar infarct (mean 9894 mm³).

Interestingly, patients with cardio-embolic subtype showed a significantly lower median SSS score [29 (11–34)] compared to other subtypes, whereas stroke patients classified as lacunar showed a significantly higher median SSS score [49.5 (47–50.7)] (see Table 1).

Patients with ischaemic stroke classified as cardio-embolic (CEI), compared to other subtypes, showed significantly higher median plasma levels of TNF- α [38.5 (22.2–46) pg/ml; p<0.0001], IL-6 [11 (5.5–19) pg/ml; p=0.0029], IL1- β [11.5 (8–13) pg/ml; p<0.0001] (see Fig. 1), whereas plasma levels of VWF in patients with cardio-embolic subtype were higher compared to other subtypes but were only near statistical significance [10 (5–12) ng/ml (p= 0.0053)].

Furthermore, stroke patients classified as lacunar showed, compared to other subtypes, significantly lower median plasma levels of TNF- α [19.4 (9–23) pg/ml; p<0.0001]; IL-6 [(4 (2–9) pg/ml ; p=0.0029)], IL1- β [6 (10–3) pg/ml p<0.0001] (see Fig. 1), whereas plasma levels of VWF [6 (3–8) ng/ml (0.0057)] in

Figure 1: TNF- α , IL-6 and IL1- β plasma levels in relation to TOAST subtype of ischaemic stroke. Data are expressed as median and interquartile (lower and upper quartile). TOAST = Trial of Org 10172 in Acute Stroke Treatment; TNF- α = tumour necrosis factor alpha; IL-6 = interleukin 6; IL-1 β = interleukin 1 beta.



	Large artery atherosclerosis strokes (n = 50)	Cardioembolic strokes (n = 20)	Lacunar strokes (n = 46)	P-value
Neuroimaging findings				
Cortical	31 (62%)	16 (80%)	8 (17.3%)	<0.0001
Subcortical	19 (38%)	4 (20%)	28 (60.8%)	<0.0001
- Internal capsula	10 (52.6%)	4 (100%)	10 (35.7%)	<0.005
- Thalamus	4 (21%)	0	7 (25%)	<0.0001
- Basal ganglia	5 (26.3%)	0	5 (21.7%)	<0.0001
- Pons	0	0	2 (7.1%)	<0.0001
- Corona radiata	0	0	4 (14.2%)	<0.0001
No lesion CT- or MRI-detectable	0	0	10 (21%)	<0.0001

TOAST: Trial of Org 10172 in Acute Stroke Treatment.

Table 3: Infarct site at neuroimaging findings (brain CT or MRI) in relation of TOAST diagnostic subtype.

patients with lacunar subtype were lower in comparison with other subtypes, but were only near statistical significance.

Patients with LAAS subtype of acute ischaemic stroke compared with subjects with cardio-embolic subtype had a lower mean brain infarct volume (11894 mm³ vs. 12273 mm³) and significantly lower median plasma values of TNF- α [27.5 (13.4–40.5) pg/ml vs. 38.5 (22.2–46) pg/ml], IL-6 [8 (4–12) pg/ml vs. 11 (5.5–19) pg/ml], IL1- β (9 (5–10.5) pg/ml vs. 11.5 (8–13) pg/ml] (see Fig. 1). No significant difference was observed between each TOAST subtype with regard of ICAM-1, VCAM-1, E-selectin, P-selectin, VWF, PAI-1 and TPA plasma levels.

Nevertheless comparing only the seven patients among LAAS and CEI strokes, that were comparable on the basis of matched mean brain infarct volume (11350 mm³ vs. 11455 mm³), patients with cardio-embolic stroke showed similar median plasma levels of TNF- α [26.2 (11.4–37.5) pg/ml vs. 26.7 (11.9–38.1) pg/ml], IL-6 [7 (3–11) pg/ml vs. 7.5 (5.5–13) pg/ml], IL1- β [10 (5–11.5) pg/ml vs. 11.5 (8–13) pg/ml] and similar mean SSS score (34 vs. 33).

Infarct sites at brain CT in relation to TOAST diagnostic subtype are shown in Table 3. Interestingly, among patients with lacunar stroke no significant difference in median plasma levels

of TNF- α , IL-6 and IL1- β was observed between subjects with and without a lacunar detectable lesion on neuroimaging (19.8 pg/ml, 9 pg/ml and 6 pg/ml vs. 18.9, pg/ml, 10 pg/ml, 5.5 pg/ml, respectively; $p = 0.87$).

Multiple linear regression performed to investigate associations between some relevant variables and SSS score as a dependent variable showed a significant association between SSS score at admission and diagnostic subtype: lacunar ($b=3.206$; $p=0.0338$) or [cardio-embolic ($b=-7.819$; $p = 0.0006$)], infarct volume of cardio-embolic strokes and some inflammatory variable [TNF- α ($b = -0.013$; $p < 0.0001$) or IL-6 ($b = -0.074$; $p < 0.0001$)] see Table 4.

Discussion

Our findings show that among patients with acute ischaemic stroke, patients with cardio-embolic subtype showed significantly higher plasma levels of TNF- α , IL-6 and IL1- β , whereas lacunar subtype showed significantly lower plasma levels of these cytokines.

Our study further supports the hypothesis that inflammation may have an important role in the pathogenesis of ischaemic strokes, but our findings also suggest a peculiar immuno-inflammatory pattern in each subtype of acute ischaemic stroke.

The first cells responding to brain ischaemia are glial cells, particularly microglia, with transcription of early pro-inflammatory cytokines such as IL1- β and TNF- α able to activate additional inflammatory pathways leading to induction of nitric oxide, adhesion molecules and IL-6 (9, 10).

Patients with cardio-embolic strokes, in comparison with other subtypes, showed a higher degree of acute neurological deficit at admission evaluated by SSS scale and a higher degree of immuno-inflammatory activation of the acute phase. On the other hand in patients with lacunar stroke, compared to other subtypes, we observed a lower degree of acute neurological deficit at admission evaluated by SSS scale and a lower degree of immuno-inflammatory activation of the acute phase. Nevertheless patients with LAAS stroke subtype showed higher median plasma levels of some immuno-inflammatory markers (TNF- α , IL-6 and IL1- β) compared to lacunar subtype, but lower median plasma levels of these biomarkers compared to the CEI subtype.

Table 4: Multiple linear regression about association between immunoinflammatory and clinical variables and SSS score.

IL-6	$b1 = -0.074$	$p < 0.0001$
TNF-α	$b2 = -0.013$	$p < 0.0001$
Age	$b3 = -0.121$	$p = 0.0596$
SBP	$b4 = 0.078$	$p = 0.0603$
Previous stroke	$b6 = -2.342$	$p = 0.0974$
TOAST subtype (lacunar)	$b7 = 3.206$	$p = 0.0338$
TOAST subtype (cardioembolic)	$b8 = -7.819$	$p = 0.0006$
Infarct volume of Cardioembolic Stroke	$b8 = -6.519$	$p = 0.0218$

TNF- α : tumour necrosis factor α ; IL-6: interleukin-6; SBP: systolic blood pressure; SSS: Scandinavian Stroke Scale; TOAST: Trial of Org 10172 in Acute Stroke Treatment.

Our finding concerning higher plasma levels of immuno-inflammatory markers in patients with cardio-embolic stroke underlines the role of inflammation in the pathogenesis of cerebral cardio-embolism. Evidence is growing that inflammation may be associated with AF (22) and it may underlie pathogenesis of the arrhythmia (21, 22). Inflammation leads to the adhesion, recruitment and trans-endothelial migration of leucocytes into the intima, which is mediated by adhesion molecules on the endothelial cell membrane; leading to the initial capture and rolling of leucocytes along the endothelium (23).

Patients with cardio-embolic stroke showed a significantly lower median SSS score compared to patients with other subtypes of stroke, whereas patients with lacunar stroke showed a significantly higher SSS score compared to other clinical subtypes of stroke. Moreover, on regression analysis stroke subtype (lacunar or cardio-embolic), infarct volume of cardio-embolic stroke and plasma levels of IL-6 and TNF- α were the variables most strictly associated with acute neurological deficit evaluated by SSS score, with cardio-embolic subtype and higher plasma levels of these cytokines associated with a poor clinical presentation (lower SSS score) and lacunar subtype and lower plasma levels of cytokines associated with a better clinical presentation (higher SSS score).

Infarct size could represent a possible factor influencing immuno-inflammatory marker plasma levels, although this issue is not always confirmed (24–26), but it is conceivable that other factors could represent an additional factor that influence immuno-inflammatory activation after acute ischaemic stroke. Indeed ischaemic brain injury secondary to arterial occlusion is characterized by acute local inflammation, which involves accumulation of poly-morphonuclear neutrophils (PMN). Factors that influence the recruitment of PMN on infarct site could modify local cytokine production. Identification of factors involved in the selective recruitment and accumulation of inflammatory cells into ischaemic brain tissue is likely to expand our understanding of the mechanisms that result in transient or permanent neurological deficits, but the mechanisms behind the entry of leukocytes to sites of ischaemia are not well understood.

Nevertheless in 25% to 40% of patients with ischaemic stroke, neurological symptoms progress during the initial hours (27, 28). Although several clinical, radiological, and biochemical factors have been associated with early neurological deterioration, most of them have a low predictor value (29, 30).

Our study showed a linear relationship between infarct volume and outcome only in patients with cardio-embolic stroke, whereas this relationship was not observed in patients with lacunar stroke in which we also did not observe any difference in immuno-inflammatory marker plasma levels in relation to the presence of detectable CT lesion. On regression analysis we also showed a significant relationship between some inflammatory markers (TNF- α , IL-6) and demographic variables (age), and diagnostic subtype (lacunar or cardio-embolic) and SSS score. Age is a widely described outcome predictor, but particularly interesting is the apparent link between diagnostic subtype, immuno-inflammatory activation of the acute phase and outcome. In our study cardio-embolic strokes showed the highest degree of immuno-inflammatory activation of the acute phase and the lowest SSS score as an expression of a higher neurological deficit of the acute phase.

This poor initial clinical presentation in terms of neurological deficit of the acute phase of our patients with cardio-embolic strokes coincides with Sherman's opinion (31) that strokes related to AF are more often large and disabling compared to other causes of brain infarction.

Moreover, Lin et al. (32) reported that ischaemic stroke associated with AF was nearly twice as likely to be fatal as non-AF stroke, whereas Jorgensen et al. (33) reported that functional deficits were more likely to be severe among survivors. This could probably be due to the larger median infarct volume of car-

What is known about this topic?

- Cerebral ischaemia initiates a complex cascade of events at genomic, molecular, and cellular levels, and inflammation is important in this cascade, both in the central nervous system (CNS) and in the periphery.
- Recent research points to the role of chemokines, normally key factors in inflammation, in thrombogenesis, probably also explaining hyper-responsiveness of platelets in ischaemic stroke.
- Recently our group reported that lacunar strokes compared to non-lacunar ones exhibited significantly lower plasma levels of TNF- α and IL-1 β , P-selectin and ICAM-1 24–72 hours and 7–10 days after stroke onset.
- Few studies have examined the relationship between blood levels of inflammatory biomarkers and stroke outcome, but data on a relation between inflammation parameters and diagnostic subtype remain scarce.

What does this paper add?

- Our findings show that among patients with acute ischaemic stroke, patients with cardio-embolic subtype showed significantly higher plasma levels of TNF- α , IL-6 and IL-1 β , whereas lacunar subtype showed significantly lower plasma levels of these cytokines.
- Our finding concerning higher plasma levels of immuno-inflammatory markers in patients with cardio-embolic stroke underlines the role of inflammation in the pathogenesis of cerebral cardio-embolism.
- In our study we showed a linear relationship between infarct volume and outcome only in patients with cardio-embolic stroke, whereas this relation was not observed in patients with lacunar stroke in which we also did not observe any difference in immuno-inflammatory marker plasma levels in relation to the presence of detectable CT lesion.
- Infarct size could represent a possible factor influencing immuno-inflammatory marker plasma levels, although this issue is not always investigated and confirmed, but it is conceivable that other factors could represent an additional factor that influence immuno-inflammatory activation after acute ischemic stroke. Indeed, ischaemic brain injury secondary to arterial occlusion is characterised by acute local inflammation, which involves accumulation of polymorphonuclear neutrophils (PMN).

dio-embolic strokes as suggested by our findings of higher mean infarct volume and higher median TNF- α , IL-6 and IL1- β in subjects with cardio-embolic stroke and of similar median plasma levels of these biomarkers and mean SSS score in patients of LAAS and CEI subtypes matched for similar infarct size.

Possible limitations of the study are the fact that we have not evaluated neurobiochemical markers such as S100B (n:S100 calcium binding protein B), NSE (neuron-specific enolase) or glial fibrillary acidic protein (GFAP). S100B, however, is expressed not only in brain tissue but also in a variety of other cell types, under both physiological and pathological conditions. Moreover, S-100B expression in the latter conditions was far below the activity measured after acute central nervous system disorders and the brain specificity of S-100B release was questioned by a number of investigators, and specificity of NSE and GFAP in ischaemic stroke is not fully demonstrated.

Single-point measurement could be another limitation of the study since repeated measures may be necessary to define the variance as well as the predictive nature of a biomarker, but in support for our findings, several studies previously reported the predictive value of single-point measurement of inflammatory biomarkers in AF (34) and other clinical conditions such as CHF (35) acute myocardial infarction (36). Moreover, in further support of our single-point evaluation, Lynch et al. (37) recently reported that two markers of inflammation and one marker of thrombosis are highly correlated with stroke at six and 24 h and that the extended time evolution of TNF- α response in humans, peaking at 2–3 days sustains our choice to evaluate biomarker plasma levels within 72 h of symptom onset.

Regarding our patients with lacunar stroke, about 20% of our patients (see Table 4) did not have visible infarction on CT or MRI, which is similar to the results of previous studies (38, 39). Our patients with lacunar stroke with no lesion detectable on brain CT or MRI do not show any significant difference with re-

gard to cytokine plasma levels of the acute phase compared to patients with lacunar lesion (CT- or MRI-detectable). So beyond a possible direct relationship between infarct size and cytokine levels, infarct site could also have an additional role in immuno-inflammatory activation of the acute phase. Interestingly, in our patients most of the lacunar strokes had a sub-cortical location, whereas most of the cardio-embolic strokes had a cortical location (see Table 3). In particular in our patients with lacunar stroke and a sub-cortical location, most were located in basal ganglia and thalamus and only a minority in corona radiata. Sub-cortical infarcts have been classified into two pathogenically different types: striatocapsular infarcts and terminal supply area infarcts (38, 39).

On this basis, could the higher sub-cortical distribution of lacunar strokes with high percentage of striato-capsular infarcts and the higher cortical distribution of cardio-embolic and LAAS strokes explain our findings concerning the low immuno-inflammatory profile of acute lacunar strokes and the high immuno-inflammatory profile of LAAS and particularly of cardio-embolic strokes? Is it plausible to hypothesize that cortical and sub-cortical sites could have a different resident immuno-inflammatory background?

To our knowledge no study has addressed this issue, but several recent studies suggested that neurons and astrocytes generate those cytokines after ischaemia (3). Moreover, two major cellular components that migrated into the infarcted area to eliminate tissue debris were macrophages and granulocytes (40). Nevertheless the precise derivation of microglia in the ischaemic regions, whether they arrive from the circulation or whether there are some differences with regard to microglial cell distribution between cortical and subcortical sites are important open questions that could, in our opinion, explain the different immuno-inflammatory pathway of the acute phase of lacunar strokes and that could provide ample basis for future studies.

References

- Kimura K, Kazui S, Minematsu K, et al. for the Japan Multicenter Stroke Investigator's Collaboration. Analysis of 16,922 patients with acute ischaemic stroke and transient ischaemic attack in Japan. A hospital-based prospective registration study. *Cerebrovasc Dis* 2004; 18: 47–56.
- Furie KL, Homma S, Kistler JP. Cardiac diseases. In: *Stroke: Pathophysiology, Diagnosis, and Management*. 4th ed. Churchill Livingstone, New York, 2004: pp. 747–759.
- Ekdahl CT, Kokaia Z, Lindvall O. Brain inflammation and adult neurogenesis: The dual role of microglia. *Neuroscience* 2008; 158: 1021–1029.
- Welsh P, Lowe GD, Chalmers J, et al. Associations of proinflammatory cytokines with the risk of recurrent stroke. *Stroke* 2008; 39: 2226–2230.
- Lambert MP, Sachais BS, Kowalska MA. Chemokines and thrombogenicity. *Thromb Haemost* 2007; 97: 722–729.
- Fateh-Moghadam S, Htun P, Tomandl B, et al. Hyperresponsiveness of platelets in ischaemic stroke. *Thromb Haemost* 2007; 97: 974–978.
- Licata G, Tuttolomondo A, Corrao S, et al. Immuno-inflammatory activation during the acute phase of lacunar and non-lacunar ischaemic stroke: association with time of onset and diabetic state. *Int J Immunopathol Pharmacol* 2006; 19: 639–646.
- Castellanos M, Castillo J, Garcia M, et al. Inflammation-mediated damage in progressing lacunar infarctions. A potential therapeutic target. *Stroke* 2002; 33: 982–987.
- Vila N, Castillo J, Davalos A, et al. Proinflammatory cytokines and early neurological worsening in ischaemic stroke. *Stroke* 2000; 31: 2325–2329.
- Sotgiu S, Zanda B, Marchetti B, et al. Inflammatory biomarkers in blood of patients with acute brain ischaemia. *Eur J Neurol* 2006; 13: 505–513.
- Kim JS, Yoon SS, Kim YH, et al. Serial measurement of interleukin-6, transforming growth factor-beta, and S-100 protein in patients with acute stroke. *Stroke* 1996; 27: 1553–1557.
- Beamer NB, Coull BM, Clark WM, et al. Interleukin-6 and interleukin-1 receptor antagonist in acute stroke. *Ann Neurol* 1995; 37: 800–805.
- Perini F, Morra M, Alecci M, et al. Temporal profile of serum anti-inflammatory and pro-inflammatory interleukins in acute ischaemic stroke patients. *Neurol Sci* 2001; 22: 289–299.
- Vila N, Castillo J, Davalos A, et al. Proinflammatory cytokines and early neurological worsening in ischaemic stroke. *Stroke* 2000; 31: 2325–2329.
- Fon EA, Mackey A, Cote R, et al. Hemostatic markers in acute transient ischaemic attacks. *Stroke* 1994; 25: 282–286.
- Takano K, Yamaguchi T, Uchida K. Markers of a hypercoagulable state following acute ischaemic stroke. *Stroke* 1992; 23: 194–198.
- Sato M, Suzuki A, Nagata K, et al. Increased von Willebrand factor in acute stroke patients with atrial fibrillation. *J Stroke Cerebrovasc Dis* 2006; 15: 1–7.
- Clatterbuck RE, Sipsos EP. The efficient calculation of neurosurgically relevant volumes from computed tomographic scans using Cavalieri's direct estimator. *Neurosurgery* 1997; 40: 339–342.
- Hatano S. Experience from a multicenter Stroke register; a preliminary report. *Bull World Health Organ* 1976; 54: 541e53.
- Adams HP, Bendixen BH, Kappelle J, et al. and the TOAST Investigators. Classification of subtype of acute ischaemic stroke. *Stroke* 1993; e24:358.
- Chung MK, Martin DO, Sprecher D, et al. C-reactive protein elevation in patients with atrial arrhythmias: inflammatory mechanisms and persistence of atrial fibrillation. *Circulation* 2001; 104: 2886–2891.
- Frustaci A, Chimenti C, Bellocci F, et al. Histological substrate of atrial biopsies in patients with lone atrial fibrillation. *Circulation* 1997; 96: 1180–1184.

24. Aukrust P, Halvorsen B, Yndestad A, et al. Chemokines and cardiovascular risk. *Arterioscler Thromb Vasc Biol* 2008; 1909-1919.
25. Nakase T, Yamazaki T, Ogura N, et al. The impact of inflammation on the pathogenesis and prognosis of ischaemic stroke. *J Neurol Sci* 2008; 271: 104-109.
26. Smith CJ, Emsley HC, Gavin CM, et al. Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome. *BMC Neurology* 2004; 4: 2-9.
27. Rincon F, Dhamoon M, Moon Y, et al. Stroke location and association with fatal cardiac outcomes. Northern Manhattan Study (NOMAS). *Stroke* 2008; 39: 2425-2431.
28. Barber M, Wright F, Stott DJ, et al. Predictors of early neurological deterioration after ischaemic stroke: a case-control study. *Gerontology* 2004; 50: 102-109.
29. Serena J, Rodríguez-Yáñez M, Castellanos M. Deterioration in acute ischaemic stroke as the target for neuroprotection. *Cerebrovasc Dis* 2006; 21 (Suppl 2): 80-88.
30. Lubart E, Leibovitz A, Baumohl Y, et al. Progressing stroke with neurological deterioration in a group of Israeli elderly. *Arch Gerontol Geriatr* 2005; 41: 95-100.
31. Ferrarese C, Masciarucci P, Zoia C, et al. Increased cytokine release from peripheral blood cells after acute stroke. *J Cereb Blood Flow Metab* 1999, 19: 1004-1009.
32. Sherman DG. Atrial fibrillation and stroke. *J Stroke Cerebrovasc Dis* 1997; 6: 165-166.
33. Lin HJ, Wolf PA, Kelly-Hayes M, et al. Stroke severity in atrial fibrillation. The Framingham Study. *Stroke* 1996; 27: 1760-1764.
34. Jorgensen HS, Nakayama H, Reith J, et al. Acute stroke with atrial fibrillation. The Copenhagen Stroke Study. *Stroke* 1996; 27: 1765-1771.
35. Conway DS, Buggins P, Hughes E, et al. Prognostic significance of raised plasma levels of interleukin-6 and C-reactive protein in atrial fibrillation. *Am Heart J* 2004; 148: 462-466.
36. Chin BS, Blann AD, Gibbs CR, et al. Prognostic value of interleukin-6, plasma viscosity, fibrinogen, von Willebrand factor, tissue factor and vascular endothelial growth factor levels in congestive heart failure. *Eur J Clin Invest* 2003; 33: 941-948.
37. Valgimigli M, Ceconi C, Malagutti P, et al. Tumor necrosis factor-alpha receptor 1 is a major predictor of mortality and new-onset heart failure in patients with acute myocardial infarction: the Cytokine-Activation and Long-Term Prognosis in Myocardial Infarction (C-ALPHA) study. *Circulation* 2005; 111: 863-870.
38. Lynch JR, Blessing R, White WD, et al. Novel diagnostic test for acute stroke. *Stroke* 2004; 35: 57-63.
39. Lavados PM, Sacks C, Prina L, et al. Incidence, case-fatality rate, and prognosis of ischaemic stroke subtypes in a predominantly Hispanic-Mestizo population in Iquique, Chile (PISCIS project): a community based incidence study. *Lancet Neurol* 2007; 6: 140-148.
40. Lindgren A, Norrving B, Rudling O, et al. Comparison of clinical and neuroradiological findings in first-ever stroke: a population based study. *Stroke* 1994; 25: 1371.
41. Gregersen R, Lambertsen K, Finsen B. Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. *J Cereb Blood Flow Metab* 2000; 20: 53-65.