



## Fetuin-A and CD40 L plasma levels in acute ischemic stroke: Differences in relation to TOAST subtype and correlation with clinical and laboratory variables

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### ABSTRACT

**Introduction:** Accumulating evidence suggests that inflammation plays an important role in the acute phase of ischemic stroke. CD40 L is a well recognized atherosclerotic inflammatory marker, whereas recent evidence suggests a pro-inflammatory role of Fetuin-A. To analyze the role of an inflammatory marker such as CD40 L and of a candidate pro-inflammatory marker such as Fetuin-A in acute stroke we evaluated their serum levels in subjects with acute ischemic stroke and their possible association with other laboratory and clinical variables.

**Materials and methods:** We enrolled 107 consecutive patients with a diagnosis of acute ischemic stroke admitted to the Internal Medicine Department at the University of Palermo between November 2006 and January 2008, and 102 hospitalized control patients without a diagnosis of acute ischemic stroke.

**Results:** Patients with acute ischemic stroke in comparison to control subjects without acute ischemic stroke had significantly higher CD40 L levels and Fetuin-A serum levels. No significant differences in plasma CD40 L or Fetuin-A levels among different TOAST groups were detected. At intragroup (intra-TOAST-subtype) correlation analysis, among subjects classified as lacunar, CD40 L plasma levels were positively correlated with LDL-cholesterol and with diabetes, whereas Fetuin-A was significantly (positively) correlated with hypertension and white blood cell count. Among subjects with LAAS subtype, CD40 L levels were positively correlated with triglyceride plasma levels and Fetuin-A, whereas Fetuin-A levels were positively correlated with LDL-cholesterol.

**Discussion:** Our findings suggest a pro-inflammatory role of Fetuin-A and CD40 L in acute stroke setting. Whether this role should be construed as direct or as a simple expression of a general inflammatory activation will be up to future studies to clarify.

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### 1. Introduction

Recent investigations have focused on the potential role of Fetuin-A or alpha2-Heremans–Schmid glycoprotein (AHSG) as a non-traditional cardiovascular risk factor [1]. Fetuin-A is a member of the cystatin superfamily of cysteine protease inhibitors [2], it is expressed in the adult liver and accumulates in the skeleton during mineralization, due to its high affinity for hydroxyapatite

[2]. There is a general consensus that Fetuin-A acts as a systemic inhibitor of ectopic calcification [3], but the clinical significance of its serum concentration is not entirely clear. In dialysis patients, low Fetuin-A levels have been found to be related to increased vascular calcification [4], to calcific uraemic arteriolopathy [5], and to cardiovascular mortality [6,7].

CD40 ligand (CD40 L), a member of the tumor necrosis family of transmembrane glycoproteins, is rapidly and transiently expressed on the surface of recently activated CD4<sup>+</sup> T cells. Interactions between CD40 L and CD40 induce B cell immunoglobulin production as well as monocyte activation and dendrite cell differentiation. Some authors have demonstrated that the multi-potent immunomodulator CD40 L and its receptor CD40, expressed on vascular endothelial cells, smooth muscle cells, mononuclear phagocytes, and platelets, promote a wide array of pro-atherogenic functions *in vitro* [8–11].

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A recent study provided evidence that human plasma Fetuin-A levels are strongly associated with the metabolic syndrome and an atherogenic lipid profile [12]. These states are characterized by subclinical inflammation and hypoadiponectinemia [13]. More recently Hennige et al. [14] provided novel evidence that the secreted liver protein Fetuin-A induces low-grade inflammation in terms of TNF and IL1 $\beta$  mRNA expression and represses adiponectin production in animals and in humans.

There is very little information on the relation between serum levels of Fetuin-A and the severity of disease in patients with atherosclerosis not associated with end stage renal disease (ESRD) or dialysis treatment.

Accumulating evidence suggests that inflammation plays an important role in the acute phase of ischemic stroke. Recently our group [15–17] showed that stroke patients, compared to controls without stroke, have significantly higher median plasma levels of some cytokines such as TNF- $\alpha$ , IL-6, IL-1 $\beta$  until 72 h after onset of symptoms. CD40 L is a well-recognized atherosclerotic inflammatory marker [8–11], whereas recent evidence suggests a pro-inflammatory role of Fetuin-A [14]. Nevertheless, no study, to our knowledge has evaluated the role of Fetuin-A in acute cardiovascular events such as acute myocardial infarction (AMI) or stroke. Few studies have evaluated the role of CD40 L in acute ischemic stroke and no study, to our knowledge, has analyzed the relationship between CD40 L and vessel calcification markers such as Fetuin-A.

To analyze the role of an inflammatory marker such as CD40 L and of a candidate pro-inflammatory marker such as Fetuin-A in acute ischemic stroke we evaluated Fetuin-A and CD40 L plasma levels both as expression of the atherosclerotic process and of inflammatory cascade in subjects with acute ischemic stroke and their possible association with other laboratory and clinical variables.

## 2. Materials and methods

### 2.1. Patient selection

We enrolled consecutive patients with a diagnosis of acute ischemic stroke admitted to the Internal Medicine Department at the University of Palermo between November 2006 and January 2008, and hospitalized control patients without a diagnosis of acute ischemic stroke. Control subjects were patients admitted, in the same period, to our Internal Medicine Department for any cause other than acute cardiovascular and cerebrovascular events.

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

In order to match patients with acute ischemic 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) cerebrovascular disease or one of the exclusion criteria (see above). We selected this control group matched for cardiovascular risk factor from a large population sample represented by all the patients of age >60 years admitted to our department from 2005 to 2008 (*total number*: 1858).

Cardiovascular risk factors were evaluated for both cases and controls on the basis of the criteria shown below. Hypercholesterolemia was defined as the presence of total cholesterol blood levels  $\geq 200$  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 revascularization procedure assessed by a questionnaire.

Previous cerebrovascular disease (TIA/ischemic stroke) was assessed by history, specific neurologic examination performed by specialists, and hospital or radiological (brain computerized tomography or brain magnetic resonance) records of definite previous stroke.

The study protocol was approved by the local ethics committee, and all participants gave written informed consent.

Every subject with ischemic stroke was matched for age ( $\pm 3$  years) and cardiovascular risk factors.

*The type of acute ischemic stroke was classified according to the TOAST classification [18]:* (1) Large-Artery AtheroSclerosis (LAAS); (2) CardioEmbolic Infarct (CEI); (3) LACunar infarct (LAC); (4) stroke of Other Determined Etiology (ODE); (5) stroke of UnDetermined Etiology (UDE).

#### 2.1.1. Large-artery atherosclerosis (LAAS)

These patients have clinical and brain imaging findings of either significant (>50%) stenosis or occlusion of a major brain artery or branch cortical artery, presumably due to atherosclerosis. Clinical findings include those of cerebral cortical impairment (aphasia, neglect, restricted motor involvement, etc.) or brain stem or cerebellar dysfunction. Cortical or cerebellar lesions and brain stem or subcortical hemispheric infarcts greater than 1.5 cm in diameter on CT or MRI are considered to be of potential large-artery atherosclerotic origin. Supportive evidence by duplex imaging or arteriography of a stenosis of greater than 50% of an appropriate intracranial or extracranial artery is needed. Diagnostic studies should exclude potential sources of cardiogenic embolism.

#### 2.1.2. CardioEmbolic infarcts (CEI)

This category includes patients with arterial occlusions presumably due to an embolus arising in the heart. Cardiac sources are divided into high-risk and medium-risk groups based on the evidence of their relative propensities for embolism. At least one cardiac source for an embolus must be identified for a possible or probable diagnosis of cardioembolic stroke. Clinical and brain imaging findings are similar to those described for large-artery atherosclerosis. Evidence of a previous TIA or stroke in more than one vascular territory or systemic embolism supports a clinical diagnosis of cardiogenic stroke. Potential large-artery atherosclerotic sources of thrombosis or embolism should be eliminated. A stroke in a patient with a medium-risk cardiac source of embolism and no other cause of stroke is classified as a possible cardioembolic stroke.

#### 2.1.3. LACunar infarct (LAC)

The patient should have one of the traditional clinical lacunar syndromes and should not have evidence of cerebral cortical dysfunction. A history of diabetes mellitus or hypertension supports the clinical diagnosis. The patient should also have a normal CT/MRI examination or a relevant brain stem or subcortical hemispheric lesion with a diameter of less than 1.5 cm demonstrated.

#### 2.1.4. Stroke of Other Determined Etiology (ODE)

This category includes patients with rare causes of stroke, such as non-atherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders. Patients in this group should have clinical and CT or MRI findings of an acute ischemic stroke, regardless of the size or location. Diagnostic studies such as blood tests or arteriography should reveal one of these unusual causes of stroke. Cardiac sources of embolism and large-artery atherosclerosis should be excluded by other studies.

#### 2.1.5. Stroke of UnDetermined Etiology (UDE)

In several instances, the cause of a stroke cannot be determined with any degree of confidence. Some patients will have no likely etiology determined despite an extensive evaluation. In others, no cause is found but the evaluation was cursory.

All the ischemic stroke patients underwent: medical history with recording of potential stroke risk factors, blood and coagulation tests, 12-lead ECG, 24-h electrocardiography monitoring, trans-thoracic echocardiography, carotid ultrasound, brain CT or MRI at admission (*repeated between the third and the seventh days of stroke onset*).

Neurological deficit score on admission was evaluated by NIH Stroke Scale (NIHSS).

Blood samples were collected within 72 h of the ischemic event. We immediately placed blood samples on ice after drawing and processed them according to the recent recommendations regarding the appropriate specimen and preparation for laboratory evaluation of soluble CD40 L. Thus, blood samples were anticoagulated in Na citrate 3.8% (1:9, v:v), centrifuged at 2000 × g for 10 min at 4 °C. Supernatants were collected and platelets and other cell types were counted (Beckman Coulter). Plasma was stored at –80 °C until analysis.

All subjects were asked to fast overnight for ≥8 h before additional blood specimen collection. Fasting glucose, triglycerides, total cholesterol, LDL-cholesterol (LDL-C), white body count (WBC), and haematocrit (HCT) were measured.

Serum Fetuin-A was determined by an enzyme linked immunosorbent assay (ELISA) (Epitope Diagnostics, San Diego, USA). Briefly, 10,000-fold diluted serum was added to microwells coated with a polyclonal anti-Fetuin-A antibody; after a 2-h-incubation, peroxidase-conjugated antibody was added to the wells. Incubation with peroxidase substrates allows color devel-

**Table 1**  
General characteristics and immuno-inflammatory variables of stroke patients and controls.

Variable	Stroke pts (n: 107)	Controls (n:102)	p
Age (years)	71 (63–80.5)	68 (63–80)	0.79
Sex (M/F)	66/41	57/55	0.022
Glucose Blood Levels (mg/dl)	148.5(97–213)	129 (81.5–163.5)	0.001
Total Cholesterol Blood Levels (mg/dl)	231(189–250)	220 (168–215)	<0.001
LDL-cholesterol (mg/dl)	138 (104–164)	129 (1047–155)	0.003
Triglyceride Blood Levels	177.5 (130.75–201.75)	141 (96–205)	0.004
White blood cells (WBC) (per mm <sup>3</sup> )	9200 (6000–13000)	7400 (6500–9800)	<0.001
CRP (mg/dl)	3.2 (2.1–3.8)	1.8 (0.8–2.1)	0.003
Neutrophils (%)	6180 (5071–9000)	4040 (3200–5800)	<0.001
Diabetes (n/%)	44 (41.12)	41 (40.19%)	0.263
Hypertension (n/%)	50 (45.72%)	46(45.09%)	0.301
Body weight (kg)	25.8	25.2	0.421
BMI	76.2	74.5	0.351
Fetuin (g/L)	0.450 (0.489–0.586)	0.125 (0.24–0.354)	0.031
CD40 L (ng/ml)	0.775/(1.19–1.83)	0.375 (0.77–1.13)	0.022
NIHSS	19.41 ± 10.06	–	–
msRankin score at discharge	–	–	–
I	20 (18.69)		
II	22 (20.56)		
III	20 (18.69)		
IV	24 (22.45)		
V	21 (19.62)		
Death (n/%)	7 (6.5)	–	<0.001
Previous TIA/stroke	35 (32.71)	32 (30.39)	0.52
CAD (n/%)	38 (35.51)	37 (36.27)	0.44
CHF (n/%)	21 (19.62)	15 (14.70)	0.065
Microalbuminuria (n/%)	41 (38.31)	18 (17.64)	<0.001
Carotid plaque (n/%)	61 (57)	33 (32.35)	<0.001
LVH (n/%)	45 (42.05)	21 (20.58)	<0.001
WMHLS (n/%)	27 (25.23)	12 (11.76)	<0.001
<i>Premorbid antithrombotics</i>			
Antiplatelet drugs (n/%)	41 (38.31)	34 (33.33)	0.74
Antivitamin K drugs (n/%)	33 (30.84)	27 (26.47)	0.54
<i>Premorbid cardiovascular drugs</i>			
Ace-inhibitors (n/%)	39 (36.44)	30 (29.41)	0.041
ARBs (n/%)	28 (26.18)	21 (20.58)	0.40
Statins (n/%)	42 (39.25)	32 (31.37)	0.35
<i>Antidiabetic drugs (n/%)</i>			
Biguanids (n/%)	12 (11.21)	14 (13.72)	0.35
Sulphonilureas (n/%)	9 (8.41)	8 (7.8)	0.67
Thiazolidinediones (n/%)	–	–	–
Insulin	23 (21.49)	19	0.28

SSS: Scandinavian Stroke Scale score; NIHSS: National Institutes of Health Stroke Scale; CAD: coronary artery disease; CRP: C reactive protein; CAD: coronary artery disease; CHF: congestive heart failure; LVH: left ventricular hypertrophy; WMHLS: white matter hyperintensity lesions; ARBs: angiotensin II receptor blockers; demographic and anamnestic data are expressed as n (percentage). Fetuin-A, CD40 L and other laboratory variables are expressed as median and interquartile (lower and upper quartile).

opment. Fetuin-A concentration was determined by interpolation with a standard curve. The analytical sensitivity of the assay was 0.05 g/L.

Serum CD40 L was determined by an enzyme linked immunosorbent assay (ELISA) (Bender MedSystems, Vienna, Austria) with a similar protocol. The analytical sensitivity of the assay was 0.005 ng/ml.

### 3. Statistical analysis

Results are expressed as median (interquartile range), with  $p \leq 0.05$  considered significant.

Statistical significance for intergroup differences with regard to CD40 L and Fetuin-A blood levels was assessed by Mann–Whitney test.

To study the intragroup (intra-TOAST-subtype) correlation between quantitative variables, Pearson or Spearman test was used.

Therefore, to calculate the number of patients to be enrolled, we defined as meaningful a significant difference in Fetuin-A and CD40 L plasma levels between subjects with acute ischemic stroke and controls without acute stroke and among each TOAST subtype, with a beta error of 20% and a power of 0.80. The estimated sample size was 100 patients.

### 4. Results

We enrolled 107 patients with acute ischemic stroke and 102 control subjects matched for age, sex, cardiovascular risk factors and previous cardiovascular morbidity.

According to the TOAST criteria, the etiology of stroke was large-artery atherosclerosis (LAAS) in 41 (38.31%) patients, cardioembolism (CEI) in 31 (28.97%) patients, lacunar stroke in 32 (29.92%) patients, while 3 (2.80%) subjects were classified as ODE.

Patients with acute ischemic stroke had significantly higher CD40 L levels and significantly higher Fetuin-A levels compared to control subjects (Table 1).

**Table 2**  
Premorbid cardiovascular risk factors, clinical characteristics and antithrombotic medication by ischemic stroke subtype.

Variable	Lacunar	LAAS	CEI	ODE	p
Number	32	41	31	3	<0.001
Age (years)	76.31 ± 7.37	72.17 ± 6.83	76.00 ± 8.79	49.00 ± 45.50	<0.001
Sex (M/F)	19/12	29/12	23/8	4/0	<0.001
Diabetes (n/%)	17 (56.25)	18 (43.90)	9 (29.03)	0	<0.001
Hypertension (n/%)	18 (56.2)	17 (41.46)	14 (45.16)	1 (33.3)	<0.001
Hypercholesterolaemia (n/%)	7 (21.87)	16 (39.02)	9 (29.03)	0	0.038
Atrial fibrillation (n/%)	3 (9.3)	5 (12.19)	24 (77.41)	0	<0.001
Previous TIA (n/%)	5 (15.6)	13 (31.07)	10 (32.2)	1 (33.3)	<0.05
Previous stroke (n/%)	7 (21.8)	8 (19.5)	7 (22.05)	2 (50)	<0.05
Glucose blood levels (mg/dl)	169.12 ± 74.06	117.0 ± 53.44	102.29 ± 7.68	92.29 ± 17.68	<0.05
Total cholesterol blood levels (mg/dl)	179.56 ± 40.07	175.02 ± 46.42	165.71 ± 30.64	169.00 ± 49.46	<0.05
LDL-cholesterol (mg/dl)	95.62 ± 38.65	103.247 ± 28.9	84.67 ± 33.99	99.20 ± 32.81	<0.05
Triglyceride blood levels (mg/dl)	170.16 ± 82.40	190.82 ± 162.12	112.35 ± 32.36	148.00 ± 83.439	0.041
White blood cells (per mm <sup>3</sup> )	10041.37 ± 4167.31	11742.82 ± 9267.20	16282.41 ± 2556.64	10785.00 ± 5437.65	<0.05
Neutrophile (%)	7384.51 ± 3769.68	7803.87 ± 4303.00	6996.13 ± 3107.86	8418.00 ± 7481.19	0.031
HCT (%)	39.20 ± 3.9	40.70 ± 4.24	41.386 ± 3.196	38.25 ± 9.54	0.044
Fetuin (g/L)	0.440 (0.428–0.565)	0.495 (0.472–0.627)	0.431 (0.422–0.675)	0.411 (0.405–0.421)	0.56
CD40 L (ng/ml)	0.650 (0.228–1.759)	0.825 (0.220–1.932)	0.850 (0.250–2.159)	0.820 (0.210–1.640)	0.059
NIHSS	18.65 ± 14.59	20.51 ± 16.06	15.64 ± 8.73	19.00 ± 12.72	0.044
msRankin score at discharge (n/%)					
I	8 (25)	7 (17.07)	4 (12.90)	1 (33.3)	
II	13 (18.75)	6 (14.63)	3 (9.6)	–	
III	6 (40.62)	9 (21.95)	5 (16.19)	1 (33.3)	
IV	3 (9.37)	10 (24.39)	10 (32.22)	1 (33.3)	
V	2 (6.25)	9 (21.95)	9 (29.03)	–	
Death (n/%)	–	2 (4.8)	5 (16.12)	–	<0.001
CAD (n/%)	13 (40.6)	18 (43.91)	7 (22.58)	0	<0.001
CHF (n/%)	4 (12.5)	5 (12.19)	11 (35.48)	1 (33.3)	<0.001
Microalbuminuria (n/%)	24 (75%)	11 (26.82)	6 (19.35)	0	<0.001
Carotid plaque (n/%)	22 (68.7)	27 (65.85)	14 (45.16)	0	<0.05
LVH (n/%)	18 (56.25)	17 (41.46)	10 (32.25)	0	<0.05
Previous brain infarct at neuroimaging	13 (40.62)	15 (36.58)	10 (32.2)	2 (50)	0.65
WMHLS (n/%)	13 (40.6)	8 (25.80)	5 (16.1)	1 (33.3)	<0.05
Premorbid antithrombotics					
Antiplatelet drugs (n/%)	11 (38.31)	20	10	–	<0.05
Antivitamin-K drugs (n/%)	4	7	21	1	<0.05
Premorbid cardiovascular drugs					
Ace-inhibitors (n/%)	18	11	10	–	<0.05
ARBs (n/%)	10	11	7	–	0.041
Statins (n/%)	15	20	7	–	0.031
Antidiabetic drugs (n/%)					
Biguanids (n/%)	10 (31.25)	7 (17.07)	4 (12.9)	–	<0.05
Sulphonilureas (n/%)	6 (18.75)	3 (7.31)	2 (6.45)	–	<0.05
Thiazolidinediones (n/%)	4 (12.5)	4 (9.7)	2 (6.45)	–	<0.025
Insulin	11 (34.37)	6 (14.63)	6 (19.35)	–	<0.05

SSS: Scandinavian Stroke Scale score; NIHSS: National Institutes of Health Stroke Scale; CAD: coronary artery disease; CHF: congestive heart failure; LVH: left ventricular hypertrophy; WMHLS: white matter hyperintensity lesions; HCT: haematocrit, CRP: C-reactive protein; demographic and anamnestic data are expressed as n (percentage). ARBs: Angiotensin-receptor blockers. Fetuin-A, CD40 L and other laboratory variables are expressed as median and interquartile (lower and upper quartile).

**Table 3**

Pearson correlation coefficients (*r*) between clinical and laboratory variables in subjects with acute ischemic stroke.

	CD40 L		Fetuin	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Diabetes	<b>0.361</b>	<b>0.021</b>	0.191	0.321
Hypertension	<b>0.331</b>	<b>0.033</b>	0.211	0.440
Total cholesterol	0.171	0.321	0.320	0.410
LDL-cholesterol	0.111	0.544	<b>0.347</b>	<b>0.021</b>
Triglyceride	0.229	0.210	0.198	0.207
HCT	<b>-0.210</b>	<b>0.061</b>	<b>-0.294</b>	<b>0.021</b>
NIHSS	0.271	0.072	0.291	0.0791
WBC	<b>0.455</b>	<b>&lt;0.05</b>	<b>0.426</b>	<b>&lt;0.05</b>
Fetuin-A	<b>0.418</b>	<b>&lt;0.05</b>	–	–

NIHSS: National Institutes of Health Stroke Scale; WBC: white blood cell count; HCT: haematocrit. Significant *r* and *p* are signed in bold.

No significant differences in plasma CD40 L and Fetuin-A levels among different TOAST groups (see Table 2) were detected.

Correlation analysis (see Table 3) among subjects with acute ischemic stroke showed a positive and significant correlation between CD40 L levels and diabetes ( $r=0.361$ ;  $p=0.021$ ), hypertension ( $r=0.331$ ;  $p=0.033$ ), WBC ( $r=0.455$ ;  $p<0.05$ ), Fetuin-A ( $r=0.418$ ;  $p<0.05$ ), whereas Fetuin-A levels were significantly and positively correlated with LDL-C ( $r=0.347$ ;  $p=0.021$ ), WBC ( $r=0.426$ ;  $p<0.05$ ) and negatively with HCT ( $r=-0.294$ ;  $p<0.021$ ).

At intra-group (TOAST subtype) correlation analysis (see Table 4), among subjects classified as lacunar group CD40 levels was positively correlated with LDL-C levels ( $r=0.410$ ;  $p=0.02$ ) and with diabetes ( $r=0.349$ ;  $p=0.015$ ), whereas Fetuin-A levels were significantly (positively) correlated with hypertension ( $r=0.405$ ;  $p=0.021$ ), and white body cell count ( $r=0.396$ ;  $p=0.031$ ).

**Table 4**

Pearson correlation coefficients (*r*) between clinical and laboratory variables for each stroke subtype.

	CD40 L		Fetuin	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
<b>Lacunar subtype</b>				
Diabetes	<b>0.349</b>	<b>0.015</b>	0.145	0.211
Hypertension	0.29	0.087	<b>0.405</b>	<b>0.021</b>
Total cholesterol	0.185	0.072	0.307	0.060
LDL-cholesterol	<b>0.410</b>	<b>0.021</b>	0.185	0.613
Triglyceride	-0.07	0.68	0.125	0.601
HCT	0.073	0.97	0.163	0.399
NIHSS	0.185	0.07	-0.015	0.936
WBC	0.185	0.34	<b>0.396</b>	<b>0.031</b>
Fetuin-A	-0.130	0.477	–	–
<b>Large-artery atherosclerosis subtype</b>				
Diabetes	0.210	0.078	0.185	0.299
Hypertension	0.184	0.11	0.167	0.10
Total cholesterol	0.123	0.53	0.315	0.079
LDL-Cholesterol	0.034	0.833	<b>0.421</b>	<b>&lt;0.05</b>
Triglyceride	<b>0.394</b>	<b>0.011</b>	0.185	0.199
HCT	<b>-0.344</b>	<b>0.032</b>	<b>-0.432</b>	<b>&lt;0.05</b>
NIHSS	0.205	0.065	0.303	0.061
WBC	<b>0.410</b>	<b>0.031</b>	<b>0.448</b>	<b>&lt;0.05</b>
Fetuin-A	<b>0.469</b>	<b>0.002</b>	–	–
<b>Cardioembolic subtype</b>				
Diabetes	-0.107	0.573	0.0185	0.231
Hypertension	0.125	0.48	<b>-0.390</b>	<b>0.030</b>
Total cholesterol	0.113	0.789	-0.242	0.085
LDL-cholesterol	0.090	0.096	0.211	0.089
Triglyceride	0.607	0.132	0.14	0.44
HCT	0.496	0.341	-0.045	0.89
NIHSS	0.060	0.45	-0.073	0.696
WBC	0.234	0.42	-0.279	0.067
Fetuin-A	-0.024	0.897	–	–

NIHSS: National Institutes of Health Stroke Scale; WBC: white blood cell count; HCT: haematocrit. Significant *r* and *p* are signed in bold.

Among subjects with Large-Artery Atherosclerosis stroke, CD40 L levels were positively correlated with triglyceride levels ( $r=0.394$ ;  $p=0.011$ ), WBC ( $r=0.410$ ,  $p=0.031$ ) and Fetuin-A plasma levels ( $r=0.469$ ;  $p<0.05$ ) and negatively correlated with haematocrit ( $r=-0.432$ ;  $p<0.05$ ), whereas Fetuin-A levels were positively correlated with LDL-cholesterol levels ( $r=0.421$ ;  $p<0.05$ ) and white body cell count ( $r=0.448$ ;  $p<0.05$ ) and negatively correlated with haematocrit ( $r=-0.432$ ;  $p<0.05$ ).

Among subjects with cardioembolic stroke Fetuin-A levels were negatively correlated with hypertension ( $r=-0.390$ ;  $p=0.030$ ).

## 5. Discussion

Our study showed higher serum levels of Fetuin-A and CD40 L in subjects with acute ischemic stroke compared to controls without acute stroke, but no significant difference with regard to these biomarker levels in relation to each TOAST subtype of ischemic stroke. Nevertheless at intra-group (intra-TOAST-subtype) analysis we observed some significant correlation between these biomarkers and some clinical and laboratory variables.

These data may support the hypothesis that fetuin-A and CD40 L may have an inflammatory role after acute ischemic stroke possibly representing a novel adjunctive pathway in the development of ischemic neuronal damage due to an immuno-inflammatory activation.

Inflammation is involved throughout the different stages of atherosclerosis and is a strong determinant of plaque disruption and thrombosis [19]. In a large study in humans, high serum fetuin-A levels were found to be positively associated with the metabolic syndrome and subclinical inflammation, suggesting that fetuin-A may be causally involved in the pathophysiology of these conditions [20]. Furthermore, a recent study [14] found that fetuin-A levels correlate positively with CRP levels, and extended the information on fetuin-A action by showing that fetuin-A promotes cytokine expression in monocytes and adipocytes, and represses the production of the insulin-sensitizing adipokine adiponectin.

It has been reported that CD40 L propagates endothelial pro-inflammatory reactions by stimulating CD40 L synthesis [21] and by enhancing production of reactive oxygen species that act to limit endothelial migration [22]. Ligation of CD40 on vascular wall cells may promote mononuclear cell recruitment, participate in the weakening of the plaque, and set the stage for thrombotic events of crucial importance in the atherothrombotic process [21]. Furthermore, ligation of CD40 on endothelial cells, smooth muscle cells, or mononuclear phagocytes triggers the expression of various pro-inflammatory mediators, such as the cytokines interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor- $\alpha$ , and interferon- $\gamma$ ; the chemokines IL-8, monocyte chemoattractant protein-1, and RANTES (regulated upon activation, normal T cell expressed and secreted); intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, matrix metalloproteinases as well as the procoagulant tissue factor [21].

Cerebral ischemia initiates a complex cascade of events at genomic, molecular, and cellular levels, and inflammation is important in this cascade, both in the CNS and in the periphery. The entire spectrum of inflammatory processes is likely to act in concert in stroke, but cytokines [22,23] are important mediators of stroke-induced immunological/inflammatory reaction which contributes to brain infarct progression as well as to the disease severity and outcome.

Our findings of higher plasma levels of Fetuin-A and CD40 L in patients with an acute ischemic cerebrovascular event appear to be novel and possible explanations are multifactorial:

First, high levels of both CD40 L and Fetuin-A may be an expression of a more extensive atherosclerotic disease.

Second, CD40 and Fetuin-A plasma levels may be due to acute immuno-inflammatory activation in terms of cytokines, adhesion molecules and selectins that have been reported to occur after acute ischemic stroke [22,23].

Third, increased plasma levels of Fetuin-A may express the pro-inflammatory role of this biomarker, possibly also explaining CD40 L and other cytokine plasma level increases in subjects with acute ischemic stroke.

Our findings of no significant difference in terms of Fetuin-A and CD40 L serum levels in relation to each TOAST subtype, are not useful to clarify the possibility of Fetuin-A as having a pathogenic role or a simple inflammatory epiphenomenon owing to the fact that we do not report any difference in Fetuin-A levels in atherothrombotic types of stroke such as those of LAAS subtype or in those subtypes of stroke that we reported in our previous studies [15–17] as characterized by higher plasma levels of markers of immuno-inflammatory activation, such as those of LAAS and CEI subtypes.

Possible explanations of our findings regarding no difference among each TOAST subtype with regard to Fetuin-A and CD40 L plasma levels may be:

First, Fetuin-A and CD40 L do not express infarct lesion volume so as not to be differently activated in different subtypes of stroke with different infarct size;

Second, we did not find any correlation with clinical score of acute neurological deficit at admission such as NIHSS. This finding could explain no difference in the two biomarkers between TOAST subtypes in relation to any difference in acute stroke severity of each subtype;

Third, Fetuin-A and CD40 L may represent only inflammatory vascular markers not directly related, nor immediate expression of the neuronal damage, but strictly linked to acute vascular events such as thrombosis or embolic occlusion both in small and large brain arteries starting the inflammatory cascade that can lead to damage and neuronal death.

On this basis the role of these two biomarkers in acute stroke setting seems to be different compared to the part played by some pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$  reported [15,16,22,23] as significantly associated with infarct size and prognosis, whereas Fetuin-A and CD40 L appear to act in this context mainly as acute vascular injury markers.

Nevertheless, it is possible to obtain a further answer to the question of the possible role of CD40 L and in particular of Fetuin-A in acute ischemic stroke by evaluating our findings of some significant correlations at intragroup analysis in each TOAST subtype between Fetuin-A and CD40 L and some clinical and laboratory variables.

In subjects with lacunar subtype of stroke we showed a significant correlation between plasma levels of Fetuin-A and hypertension and white blood cell count and this finding could underline the possible link of Fetuin-A with hypertensive small-artery disease (SAD) and sub-inflammation expressed by WBC.

In LAAS subtype we showed a significant (positive) correlation between Fetuin-A and white blood cell, LDL and CD40 L plasma levels confirming in this typical atherothrombotic stroke subtype the possible link between inflammation, large-artery atherosclerosis and Fetuin-A plasma levels whereas the finding of negative correlation between haematocrit and Fetuin-A plasma level is not easily explainable. Weikert et al. provided [24] evidence for a link between high plasma Fetuin-A levels and an increased risk of myocardial infarction and ischemic stroke (IS) suggesting a possible atherogenic and inflammatory role of this biomarker.

Our correlation analysis among the various subtypes of stroke may confirm the possible inflammatory role of Fetuin-A showing significant correlation with white blood cells on one side

and its atherogenic role showing significant correlation with LDL and triglycerides plasma levels on the other. These correlations observed in LAAS and lacunar stroke subtypes confirm the role of these two biomarkers both in atherosclerosis and microvessel disease setting. In particular they may indicate a dual role of fetuin-A as an atherogenic and pro-inflammatory marker.

This finding is also in agreement with literature reports concerning Fetuin-A and atherosclerosis, with a strict relation between Fetuin-A plasma levels and coronary and valvular calcification rate mainly in patients with ESRD [6] and a pro-inflammatory and anti-adiponectin role in diabetic subjects [14], delineating two possibly different pathways of atherogenesis linked to Fetuin plasma levels.

Only point measurement could be a limitation of the study since repeated measures and a time course analysis of fetuin-A and CD40 L may be necessary to define the variance as well as the predictive nature of a biomarker [25], but in support of our findings several studies previously reported the predictive value of only point measurement of inflammatory biomarkers in atrial fibrillation [26] and other clinical conditions such as congestive heart failure [26] and acute myocardial infarction [27].

Another possible limitation is that our control subjects are age matched ( $\pm 3$  years) but not always sex matched.

In conclusion we reported high plasma levels of both Fetuin-A and CD40 L in subjects with acute ischemic stroke and significant correlation between these two biomarkers and some clinical and laboratory variables at intragroup (TOAST subtype) analysis.

The higher plasma levels of Fetuin-A and CD40 L in subjects with acute ischemic stroke and their correlation with WBC count underline a possible pro-inflammatory role of Fetuin-A and CD40 L. Whether this role should be construed as direct or as a simple expression of a general inflammatory activation and further interrelation between these two vascular biomarkers both as expression of atherogenesis and inflammation will be up to future studies to clarify.

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