

Research Article

Role of Interleukin-6 Correlated to *C. pneumoniae* Infection as a Biomarker for Prediction of Stroke Severity in Young Patients with Acute Ischemic Stroke

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Background. Stroke in young is the leading cause of morbidity and mortality in the Indian subcontinent with a reported incidence of 15–30% of all stroke patients. The mechanisms for stroke in the young may include unconventional risk factors such as infections. Causative role of *C. pneumoniae* infection in patients with acute ischemic stroke (AIS) remains unresolved till date, although the link between *C. pneumoniae* and cerebrovascular disease has been investigated in many studies. This study examined the upregulation of IL-6 after acute cerebral ischemia and correlated the same with the *C. pneumoniae* antibody titres (IgG, IgA, and IgM). **Methods.** We studied blood samples from eighty ($n = 80$) acute stroke patients and healthy age- and sex-matched controls. Venous blood samples were drawn within one week from the onset of stroke. Detection of IgA, IgG, and IgM antibodies to *C. pneumoniae* was done with a validated microimmunofluorescence (MIF) technique from 5 mL of serum in all subjects. Interleukin-6 was estimated with sandwich ELISA method. **Results.** The IL-6 levels were elevated in patients with a mean 28.9 ± 8.6 pg/mL as compared to 4.7 ± 1.8 pg/mL in healthy age-matched controls (95% CI: 37.7 to 78.4; $P = 0.004$). On correlation of IL-6 to stroke severity, it was found that 30 patients with NIHSS between 0 and 15 had mean IL-6 of 24.6 pg/mL and 50 patients had NIHSS of 18.8 (severely affected) with a mean IL-6 of 43.8 pg/mL. On multivariate analysis after adjusting for sex, hypertension, diabetes mellitus, smoking, and alcohol, the IgA seropositivity yielded an adjusted OR for stroke (4.72; 95% CI: 1.61, 13.83; $P = 0.005$), while IgG seropositivity did not show a statistically significant result. We also observed that 81% of cases were seropositive for IgA versus 32% of controls ($P = 0.003$) followed by IgG, as 52.7% of cases were seropositive versus 17.3% of controls ($P = 0.05$). Multiple regression analysis was done with IL-6 as dependent variable to antibody with IL-6 as dependent variable to Cp-IgA, Cp-IgG, and IgM with 10.4% change in the IL-6 titres showing statistical significant result $F(4, 115) = 3.32$, $P = 0.01$. **Conclusions.** IL-6 has important role after acute ischemic stroke and is correlated with stroke severity and may correlate to acute or chronic infectious states with *C. pneumoniae*.

1. Introduction

Young stroke patients constitute 15–30% of all stroke patients in India, to that of 3.0–8.5% of all stroke patients reported from the West [1]. Stroke in young, including stroke in children and young adults (<45 years), is an important cause of morbidity throughout the world, especially in developing countries [2]. The initial data from the Indian Collaborative

Acute Stroke Study (ICASS) performed under the aegis of World Health Organization (WHO), supports a high prevalence of vascular risk factors and a relatively younger age of stroke cases in India [3–5]. Recognition of trends of stroke burden in India is essential to assist target prevention and management strategies.

Atherosclerosis is a leading cause of mortality and morbidity worldwide whereas the known conventional risk

factors including hypertension, cigarette smoking, diabetes mellitus, and hyperlipidemia do not fully explain the incidence of ischemic stroke in the young age group of 18–45 years [6, 7]. Inflammation has been postulated to play an important role in the initiation and development of atherosclerosis [8]. Recently, increasing interest has focused on the putative causal role of chronic infections such as *Helicobacter pylori* [9] and *C. pneumoniae* (Cp) in acute stroke pathophysiology. *C. pneumoniae* is a Gram-negative intracellular bacterium that commonly causes upper respiratory tract infections [10]. Proinflammatory cytokines, oxidized low-density lipoproteins, interleukin-1 (IL-1, 6) [11], tumor necrosis factor- α (TNF- α), selectins, inflammatory stimuli like interleukin-6 (IL-6), C-reactive protein (CRP), and acute-phase reactants [12] are potential mediators which help to identify and monitor the ongoing inflammatory response to injury/ischemia.

Several retrospective and cross-sectional studies have shown association between previous infections with *C. pneumoniae*, herpes simplex virus (HSV), *Cytomegalovirus* (CMV), hepatitis A (HAV) [13], and other respiratory and oral pathogens with cardiovascular and cerebrovascular diseases. A panel of inflammatory markers has shown association with coronary artery disease in some studies reported from the West [14]. Causative role of *C. pneumoniae* infection in patients with acute ischemic stroke (AIS) remains unresolved till date, although the link between *C. pneumoniae* and cerebrovascular disease has been investigated in a number of seroepidemiological studies [15]. The source of increased cytokines in stroke is another matter of controversy, as they are known to be secreted by cells of the central nervous system and leukocytes. IL-6 is an inflammatory biomarker and its increased production is associated with disturbances of homeostasis such as inflammatory diseases of vessel wall, trauma, or sepsis [16]. The objective of the present study was to assess the prevalence of inflammatory biomarker interleukin-6 (IL-6) in young patients with acute stroke and to correlate them with IgG, IgM, and IgA antibody titers of *C. pneumoniae*.

2. Methods

This study was prospective observational case control design performed at a tertiary care hospital with specialized acute stroke care. Young patients (18–45 years), diagnosed with acute ischemic stroke (AIS) within one week of index event, were recruited into the study. Complete medical history of hypertension, smoking, diabetes mellitus, hypercholesterolemia, ischemic heart disease, stroke, or TIA was recorded with neurological examination included recording of National Institute of Health for Stroke Severity (NIHSS). CT head was performed in all and if needed MRI/CTA/MRA was performed according to the clinical requirement. Controls selected for the above analysis were healthy age- and sex-matched subjects, who did not have any history of cerebrovascular, neurological, or long standing medical illness. Blood samples were drawn within one week from the onset of stroke. In each case, 5 mL of serum was obtained by

centrifugation at +4°C and stored at –70°C until analysis. Detection of IgA, IgG, and IgM antibodies to *C. pneumoniae* was done with a validated microimmunofluorescence (MIF) technique. The cutoff point for seropositivity was 1/16 for IgA and 1/64 for IgG. Inflammatory biomarker IL-6 was determined using Immulite Kit. Detection of Cp-IgA, Cp-IgG, and Cp-IgM antibodies was done with validated quantitative sandwich enzyme immunoassays according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

3. Statistical Methods

All serological results were dichotomized as positive or negative. Simultaneously, an alpha level of 0.05 was set as the level of significance. SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For continuous variables such as age, NIHSS, and calculation of IL-6, descriptive statistics was calculated and reported as means \pm SD. Normality of distribution of continuous variables was verified using the parametric tests.

4. Results

Eighty ($n = 80$) AIS patients with the mentioned inclusion criteria with eighty healthy ($n = 80$) age- and sex-matched controls were recruited for the study. The mean age in study group was 43.6 ± 6.7 years (range 25 to 45) and 43.2 ± 7.3 years in controls with diabetes mellitus 23.4%, hypercholesterolemia 35.4%, and smoking 22.3%. There were no cardiovascular risk factors in the control group. The sensitivity of the minimum detectable dose of IL-6 was 0.09 pg/mL. In all our patients, values for IL-6 were above the highest standard of 7 pg/mL and were then recorded as >7 pg/mL as positive and less as negative. The IL-6 levels were elevated in patients with a mean 28.9 ± 8.6 pg/mL as compared to 4.7 ± 1.8 pg/mL in healthy age-matched controls (95% CI: 37.7 to 78.4; $P = 0.004$). On correlation of IL-6 to stroke severity, it was found that 30 patients with NIHSS between 0 and 15 had mean IL-6 of 24.6 and 50 patients had NIHSS of 18.8 (severely affected) with a mean IL-6 of 43.8 (Figure 1).

From our previous cohort [17], out of 51 patients to that of 48 controls, Cp-IgA seropositivity was significantly increased and associated with acute ischemic stroke (unadjusted odds ratio: 3.1; 95% CI: 1.38, 6.96; $P = 0.005$), whereas Cp-IgG (unadjusted OR 0.44; 95% CI: 0.18, 1.09; $P = 0.07$) and Cp-IgM (unadjusted OR: 1.1; 95% CI: 0.36, 3.3; $P = 0.88$) did not. There was no difference in IgA or IgG positivity in different stroke subtypes. On multivariate analysis after adjusting for sex, hypertension, diabetes mellitus, smoking, and alcohol, the IgA seropositivity yielded an adjusted OR for stroke (4.72; 95% CI: 1.61, 13.83; $P = 0.005$), while IgG seropositivity did not show a statistically significant result.

In our patients, 81% of cases were seropositive for IgA versus 32% of controls ($P = 0.003$) (Figure 2). This difference was less striking for IgG, as 52.7% of cases were seropositive versus 17.3% of controls ($P = 0.05$). When considering both IgA and IgG indices, the percentage of stroke patients

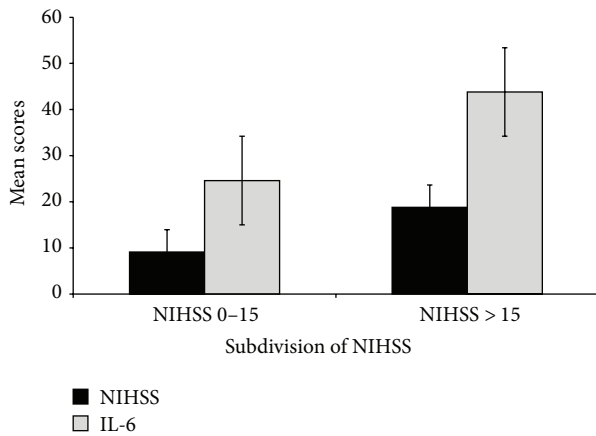


FIGURE 1: Mean IL-6 and mean NIHSS scores in moderate and severely affected patients.

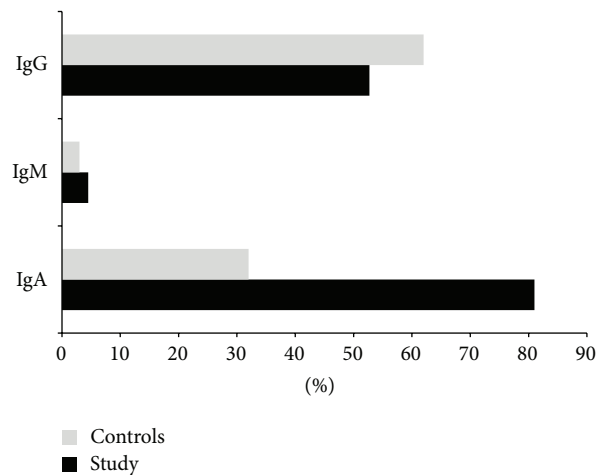


FIGURE 2: Percentage of seropositivity of Cp-IgA, IgM, and IgE in study (patients) versus controls.

with serologic evidence of *C. pneumoniae* infection was significantly higher (71%) than in controls (20%; $P < 0.01$). IgM antibodies were found to be similar and negative in patients as well in controls. This variation was not found to be statistically significant between patients and controls ($P > 0.05$). Multiple regression analysis was done with IL-6 as dependent variable to antibody with IL-6 as dependent variable to Cp-IgA, Cp-IgG, and IgM with 10.4% change in the IL-6 titres to the other variables showing statistical significant result $F(4, 115) = 3.32$, $P = 0.01$ (Figure 3).

5. Discussion

The aim of this study was to study the upregulation of IL-6 within a week post-AIS and determine the possible relationship between IL-6 and stroke severity and to Cp-IgA, IgE, and IgG antibody titers.

Interleukin-6 (IL-6) is an inflammatory biomarker, whose increased production is associated with disturbances of homeostasis, such as trauma, sepsis, or inflammatory

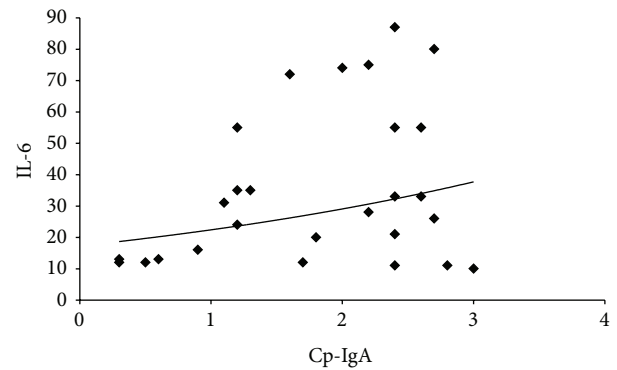


FIGURE 3: Correlation of IL-6 to Cp IgA antibodies. A strong correlation was observed ($r = 0.78$; $P = 0.01$) in patients with high IgA titres >1 have high IL-6 levels.

diseases. Waje-Andreassen et al. reported that IL-6 levels were increased in the acute phase of stroke compared with healthy controls ($P = 0.002$) and correlated with larger stroke volume ($P = 0.012$) and less favorable prognosis after 1 year, measured by ESS ($P = 0.014$) and BI ($P = 0.006$). This study was conducted on eleven stroke patients with symptoms over one year [11]. We correlated IL-6 levels with the severity of stroke and found that greater levels of IL-6 (mean 45.2 pg/mL) was highly correlated with greater NIHSS scores ($r = 0.89$; $P = 0.002$) (Figure 1) suggesting putative role of IL-6 in the cellular and molecular inflammatory cascade of cerebral ischemia.

Clinical and experimental data has shown that leukocyte recruitment is a hallmark that occurs over hours to days after cerebral ischemia. In mouse models of transient MCAO, elevated IL-1 β mRNA expression occurs within the first 15–30 min after permanent MCAO and elevated IL-1 β protein expression occurs a few hours later and remains elevated for up to 4 days [12, 14]. Flow cytometric examination of the inflammatory cell infiltration in the ischemic brain reveals that (CD3) T cells increased relatively late, 3–4 days after ischemia, whereas activated CD11b, microglia/macrophages, and neutrophils increased significantly early. The expression of IL-23 (most likely derived from activated microglia/macrophages) increases on day 1 after reperfusion injury, whereas IL-17 levels are elevated after day 3, and its induction was dependent on IL-23 [18].

IL-6 is a key regulator of the acute-phase response and elevates the temperature set point in the hypothalamus and stimulates the expression of other acute-phase proteins in the liver [19, 20]. It has been well demonstrated that IL-6 is produced locally by resident brain cells for poststroke angiogenesis in experimental models of stroke [21]. At 2nd day after mild transient brain ischemia, there was an increase in IL-6 gene transcription and protein levels in ischemic brain that was accompanied by a significant early upregulation of angiogenesis-associated gene [22]. Genetic polymorphism of IL-6 has been studied and its expression is mainly regulated at the transcriptional level [23]. The promoter of the human IL-6 gene contains several polymorphisms that influence IL-6 gene transcription in selected cell types inside the brain. In

a randomized trial for acute stroke, IL-1R (receptor), readily crossed the blood-brain barrier, was safe to use and seemed to afford some benefit, particularly for patients with cortical infarcts [24].

Acalovschi et al. [25] found that IL-6 levels in patients with ischemic stroke were dependent not only on the infarct size, but also on the haplotype of IL-6 promoter region. However, stroke is a complex multifactorial disease in which several genes and environmental factors are important role models [26]. The infarct size was not calculated from our results and hence could not be correlated with the biomarker levels.

The source of the increased IL-6 levels detected in the systemic circulation in the acute phase of ischemic stroke is unknown. As IL-6 is correlated with the stroke volume, it is possible that endothelial cells, microglia, astrocytes, and leukocytes are responsible for the cytokine production [27]. IL-6 and IL-6 mRNA have been found in the pyramidal cells of the hippocampus, and it is therefore possible that damaged neurons also contribute to the increased IL-6 levels. An intrathecal synthesis of IL-6 is supported by previous results showing peak levels of IL-6 in the CSF 2-3 days after stroke onset [28].

IL-6 effects on leukocyte adhesion, infiltration, and activation have been vastly studied [28]. Hahn et al. demonstrated by flow cytometric analysis the infiltration of T lymphocytes in mouse brain 72 h after transient middle cerebral artery occlusion [29]. However, we did not find any correlation with total number of leucocytes (TLC) and differential leucocyte counts (DLC) in peripheral blood smear, although reports suggest that there is a strong association of IL-6 with an increased TLC and DLC following cerebral ischemia [30].

Cp-IgG antibodies may be considered as markers of "chronic" infection and they could be in circulation for 3–5 years following infection. The IgA antibodies may be considered as markers of a primary, acute, or chronic infection and stay in circulation on an average for 3–5 days or longer [31, 32]. Several studies have demonstrated the presence of *C. pneumoniae* in atherosclerotic lesions in coronary arteries, aorta, and carotid arteries obtained from autopsy and end-arterectomy specimens [33]. One Indian study which has shown association of high C-reactive protein (CRP) in stroke patients lends further credence to the hypothesis that infection with *C. pneumoniae*, acute or chronic, may be associated with atherosclerosis through the mechanism of low-grade inflammation [34].

The important group of factors significantly elevating IL-6 level constitutes vascular risk factors: hypercholesterolemia and hypertension followed by obesity and cigarette smoking. In our report, patients who had previous history of cardiac disease (29.1%) and higher cholesterol levels (29.4%) had greater IL-6 levels as compared to the other risk factors which may indicate that an increased levels of IL-6 is a silent marker of coronary events and thereby a risk factor for stroke (OR, 95% CI: 37.7 to 78.4; $r = 0.87$; $P = 0.002$).

One of the major limitations of the study was that acute stroke subtype was not done and hence could not be correlated according to ASCO or TOAST score. In relation

to age and gender, no difference was detected between the groups. Such data corroborate the previous report which did not find any difference between young people and the elderly or men and women [35].

Multiple regression analysis was done with IL-6 as dependent variable to antibodies IgA, IgG, and IgM with 32.4% sensitivity in IL-6 titres to Cp-IgA ($F(4, 115) = 3.32$, $P = 0.01$). In our patients IL-6 was recorded at 1st day (35 patients), 2nd day (20 patients), and 4th day (25 patients) and it was by chance that we found out that mean IL-6 was high in patients whose measurement were taken at 4th day as compared to those taken at 1st and 2nd day which is in congruence with the reports stating that IL-6 peaks after 3rd day in cerebral ischemia [36–38].

The infection caused by *C. pneumoniae* has been described as an independent risk factor for cerebrovascular diseases; however, it is still under debate whether the microorganism would be the causal agent to the ones that believe it would merely be an innocent bystander. Despite the strong association found through several epidemiologic and experimental studies, the causality of the facts cannot be simply established. The Northern Manhattan study [35], suggested that chronic *C. pneumoniae* infection may be a risk factor for stroke in the elderly as well as the young. Also Cp-antibody levels could rise after stroke primarily because of immune response to common epitopes in the infarcted brain tissue (i.e., "molecular mimicry") or nonspecific immunological activation [39, 40]. Future prospects of the same study would be to evaluate whether *C. pneumoniae* is causative organism, to study the titres at one week, one month, and 3 months (one time sample collection), or to investigate the titres repeatedly in one patient after a week, two weeks, and one month.

The pathogenesis of cerebral brain ischemia in the young has been implicated as a result of dissection of extracranial vessels, hyperhomocysteinemia, cardiac causes, and immunological disturbances. Accumulations of erythrocytes is known to be the initial step in the pathological cascade, which is followed by blood-brain barrier (BBB) disarray finally leading to microbleeds that cause necrotic tissue damage in terms of microinfarcts. Dissection of the carotid or vertebral arteries is associated with disorders of connective tissue such as Ehlers-Danlos syndrome, Marfan's syndrome, pseudoxanthomas, and fibromuscular dysplasias. There is an association with intracranial aneurysms and possibly with alpha-1-antitrypsin deficiency. Lupus anticoagulant and anticardiolipin antibodies have activity against protein-phospholipid complexes. They are detected in 50% of patients with lupus and other autoimmune disorders such as rheumatoid arthritis and giant cell arteritis leading to another important cause of ischemia in younger population. The most common cardiac lesions are rheumatic valve disease, bacterial endocarditis, cardiomyopathies, ischemic dyskinetic segments, patent foramen ovale, and so forth [41].

It is hypothesized that stroke outcomes may be improved by antileukocyte strategies (including antiadhesion molecule strategies), which are targeted specifically to the reperfusion period [42, 43]. Despite intense investigations, it remains unclear why anti-inflammatory therapy succeeded in animal

models but not in clinical applications in management of acute stroke. Experimental studies demonstrated that antileukocyte strategies may extend the therapeutic time window of tPA reperfusion therapy in acute stroke [44].

6. Summary and Conclusion

Raised IgA antibodies as compared to IgM and IgG indicating probably recurrent acute infections may have set the stage for the acute ischemic events with other atherosclerotic risk factors in these patients [43–45]. These results are supported by the fact that inflammation and immune reaction secondary to acute infection with *C. pneumoniae* may trigger an acute stroke. We acknowledge the limitations of our study: infarct volume, CRP levels were not measured, stroke subtype and serial measurements of IL-6 were not recorded in a single patient. Third generation tetracyclines, for example, minocycline is an attractive attempt to promote neuroprotective effects after stroke, especially in a developing country where infection and cerebrovascular disease contribute significantly to health mortality and morbidity. It would be conclusive to state that IL-6 has important role after acute ischemic stroke and is correlated with stroke severity and may correlate to acute or chronic infection with *C. pneumonia*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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