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Aspirin resistance and blood biomarkers in predicting ischemic stroke recurrence: An exploratory study

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Abstract:

BACKGROUND: Recurrent strokes cause greater complications and worse outcomes by adding to the existing neurological deficit. There is the paucity of data on serum markers of inflammation as predictors of recurrent stroke. This study was planned to analyze the clinico-etiological profile of recurrent noncardioembolic ischemic stroke, estimate aspirin resistance among regular aspirin users and evaluate blood biomarkers high-sensitivity C-reactive protein (hsCRP), Tumor necrosis factor-alpha (TNF-α), Lipoprotein-associated phospholipase A_2 (Lp-PLA2) as probable predictors of stroke recurrence.

METHODS: Patients of recurrent noncardioembolic ischemic stroke fulfilling the inclusion criteria were enrolled. Detailed history, clinical examination, and investigations were obtained as per protocol. Aspirin resistance was determined by light transmission aggregometry. Serum hsCRP, TNF-α, and Lp-PLA2 levels were estimated.

RESULTS: This study included 34 males and 16 females. Majority of the patients were > 60 years ($n = 30$, 60%). Thirty (60%) cases had a repeat stroke after 1 year of primary event. Thirty-nine (78%) study participants had hypertension, while 15 (30%) had diabetes. Middle cerebral artery ($n = 40$, 80%) was the most common vascular territory. Thirty-one (62%) cases belonged to TOAST subtype 1 (large artery atherosclerosis). Seventy two percent cases were prescribed aspirin after index stroke, but only 36% were compliant. Median (range) hsCRP level was 7.5 (0.3–155) mg/L with 72% of patients having high hsCRP level (>3 mg/L). Median (range) serum PLA2 level was 11.98 (3.31–87.24) ng/ml in patients and 6.96 (0.15–61.42) ng/ml in controls ($P = 0.029$). Median (range) serum TNF-α level in patients was significantly higher than controls (68.22 [1.3–287] pg/ml versus 0.098 [0.002–36.31] pg/ml, $P < 0.001$). Aspirin resistance was found in 41.7% patients while 16.7% were semi-resistant. Mean % platelet aggregation was 34.75 ± 21.58 in patients and 64.75 ± 16.98 for controls ($P < 0.001$).

CONCLUSIONS: Majority of patients with recurrent stroke were elderly (>60 years), hypertensive, and non-compliant with aspirin. Aspirin resistance was an important factor in patients with antiplatelet compliance. Inflammatory biomarkers hsCRP, PLA2, and TNF-α were found to be significantly elevated in patients compared to controls.

Keywords:

Aspirin resistance, high-sensitivity C-reactive protein, lipoprotein-associated phospholipase A_2, recurrent ischemic stroke, tumor necrosis factor alpha

Introduction

Stroke is the second-leading cause of death worldwide causing 5.5 million

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deaths in 2016 and the second-most common cause of disability-adjusted life years (DALYs).[1] In India, the prevalence of stroke has increased by 12.2% from 1990 to 2016.[2] It was the fifth-leading cause for DALYs in 2016 and the second-leading cause of death in 2018.[3,4] The first 5-year cumulative incidence of stroke recurrence ranges from 16% to 30% in the Western world.[5,6]

Inflammation leads to atherosclerosis which in turn leads to ischemic stroke.[7-9] Various studies have evaluated pro-inflammatory biomarkers such as high-sensitivity C-reactive protein (hsCRP), Tumour necrosis factor-alpha (TNF-α), Lipoprotein-associated phospholipase A_2 (Lp-PLA2) in stroke.[9-16] However, insufficient information is available regarding its role in the prediction of recurrent stroke.

Aspirin, the cornerstone of antiplatelet therapy, reduces the risk of vascular disease by about 25% as per the Antithrombotic Trialists' collaboration. One-third to one-half of patients who experience a recurrent stroke, are already on antiplatelet medications.[17] Poor compliance, impaired absorption and metabolism, drug interactions, nonplatelet sources of thromboxane A2 production (monocytes, macrophages), other pathways of platelet activation, increased platelet turnover, genetic polymorphisms, nonatherothromboembolic pathology, and tachyphylaxis are some of the possible reasons for recurrence while on antiplatelet therapy.[18]

There is a dearth of literature on the prevalence of aspirin resistance and markers of inflammation predicting recurrence in noncardioembolic stroke. Hence, the present study was undertaken to analyze the clinico-etiological profile of recurrent non-cardioembolic ischemic stroke, estimate aspirin resistance among regular aspirin users, and evaluate blood biomarkers (hsCRP, TNF-α, Lp-PLA2) as probable predictors of stroke recurrence.

Methods

This observational, case–control hospital-based study was conducted in the Department of Neurology, Institute of Medical Sciences, Banaras Hindu University, Varanasi from January 2019 to October 2020. The study was approved by the institutional research ethics panel (No. 2019/EC/1137) and confidentiality of records was maintained.

Inclusion criteria

Patients with age ≥18 years having clinical features of recurrent stroke were included after obtaining valid informed consent. The diagnosis of patients was based on the clinical history and neuroimaging evidence (Computed Tomography and Diffusion-Weighted Imaging in magnetic resonance imaging). Age- and sex-matched healthy individuals were included as control.

Exclusion criteria

Patients with hemorrhagic stroke/venous infarct, malignancy, head trauma, severe edema, acute or chronic inflammatory disease, autoimmune disease, chronic NSAID users (>3 days/week for past 3 months), history of hemorrhagic disorder within the past 4 weeks, coagulopathy, thrombocytopenia (<90,000/μl), chronic liver or renal disease, high risk of cardio-embolism (rheumatic heart disease, coronary artery disease or cardiac arrhythmia), history of dual antiplatelets intake and not giving informed consent were excluded from the study.

Detailed history, physical and neurological examination, and investigations were done as per pre-fixed protocol. NIHSS and Modified Rankin Scale (mRS) scoring was done for quantification of stroke severity and degree of disability poststroke, respectively.[19,20] Etiological classification was done as per Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.[21] Apart from routine laboratory parameters, special biochemical tests included aspirin response test, hsCRP, TNF-α, and PLA2 levels in serum.

Method of platelet aggregation testing

Among the 50 patients, 36 were prescribed aspirin out of which only 13 patients were on regular aspirin treatment during stroke events. Platelet aggregation testing was done on those 13 cases [Figure 1]. Fresh blood was collected from antecubital veins of patients as well as controls and centrifuged at 100 x g for 20 min. Platelet-rich plasma was collected and stirred at 1200 rpm in an optical lumi-aggregometer (Chrono-log model 700-2) at 37°C for 1 min, followed by the addition of 2.5 μM adenosine diphosphate (ADP). Transmittance was then recorded. Aggregation was measured as the percentage change in the light transmission where 100% refers to transmittance through platelet-poor plasma.

Aspirin resistance is defined as mean platelet aggregation of ≥20% with 0.5 mM arachidonic acid (AA) and ≥70% with 10 μM ADP. Fulfilling of any one parameter, either mean platelet aggregation of ≥20% with 0.5 mM AA or ≥70% with 10 μM ADP, is defined as aspirin semi-resistance. Aspirin sensitivity means platelet aggregation of ≤20% with 0.5 mM AA and ≤70% with 10 μM ADP.[22] However, this definition is widely accepted for cardiovascular diseases with no such universal definition for stroke. Since we had used lower concentration of ADP (2.5 μM) in our study owing to a fresh batch of reagent producing exaggerated aggregation at the higher standard concentration (10 μM ADP) and

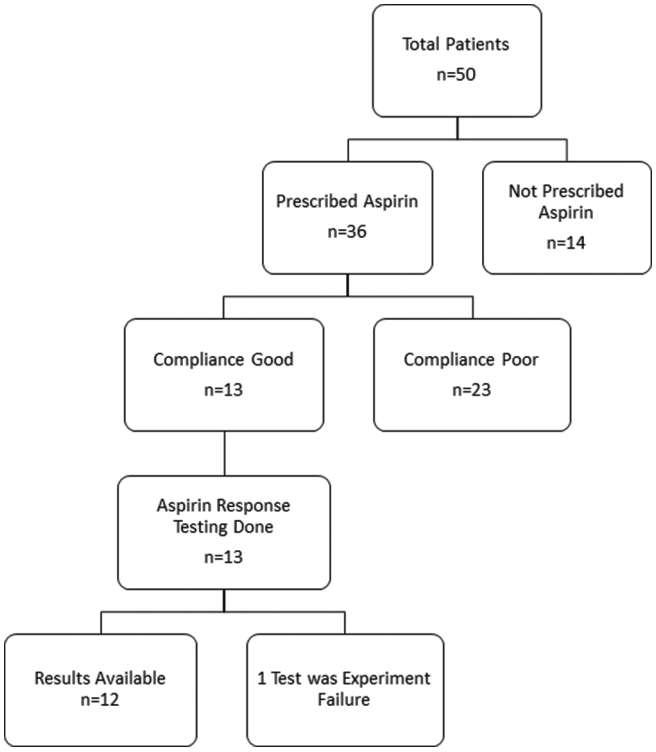

Figure 1: Flowchart of aspirin response testing

nonavailability of AA in our biochemistry laboratory, we had reduced the cut-off values for defining aspirin resistance. Greater than 50% aggregation was deemed as aspirin resistant, close to the values set by Sane *et al*. (>60%); while 25%–50% aggregation was considered semi-resistant and <25% as aspirin-sensitive in this study.[23]

Serum samples were collected within 24 hours of hospital arrival. Serum hsCRP levels were measured by the highly sensitive nephelometry method. Based on hsCRP levels, cases were classified as low risk (<1 mg/L), moderate risk (1–3 mg/L), and high risk (>3 mg/L) as per American Heart Association and Centers for Disease Control and Prevention criteria.[24]

We used GenLISA™ Human TNF-α enzyme-linked immunosorbent assay (ELISA) kit (REF: KB1145, LOT: TA0920) and Human PLA2 ELISA kit (REF: KBH4468, LOT: PLA21120) from KRISHGEN BioSystems for *in vitro* quantitative determination of TNF-α and PLA2 in serum respectively for patients as well as controls.

Results

Fifty patients with clinical and neuroradiological evidence of recurrent noncardioembolic ischemic stroke were included during our study period. There were 34 males (68%) with a male: female ratio of 2:1. Overall, the mean age of study participants was 62.92 ± 11.8 years. Majority of the patients were >60 years (N = 30, 60%). Thirty-nine (78%) patients had a second stroke while 6 (12%) cases had a third vascular event. The median (range) time interval for stroke recurrence following index stroke was 27 (0.2-276) months. Four (8%) patients had recurrence within 1 month of index stroke while 6 (12%) patients had a re-stroke between 1 and 6 months of the first stroke. Thirty (60%) cases had a repeat stroke after 1 year of the primary event [Table 1].

Thirty-nine (78%) study subjects had hypertension while 15 (30%) had diabetes. Two (4%) patients had coronary artery disease. Fifteen (30%) patients had multiple risk factors. Only 17/39 (43.6%) hypertensives and 8/15 (53.3%) diabetics were on regular treatment. Fourteen (28%) patients had dyslipidemia. Thirty-six (72%) patients had started aspirin treatment following the index stroke. Only 13 (36%) patients sincerely adhered to treatment while the remaining 23 (64%) cases had poor drug compliance [Table 1].

Fifteen (30%) patients had an NIHSS score of 5-15 while 10 (20%) cases had high score of 21–42. Twenty-one (42%) patients had an mRS score of 5 followed by 14 (28%) cases with a score of 4.

Middle cerebral artery was the commonest vascular territory involved in 40 (80%) cases followed by vertebrobasilar in 7 (14%) cases. Thirty-one (62%) cases belonged to TOAST subtype 1 (Large artery atherosclerosis) whereas 11 (22%) cases were subtype 3 (Small vessel occlusion). Remaining 8 (16%) cases were grouped under subtype 5 (Stroke of undetermined etiology). Forty (80%) patients survived despite stroke recurrence [Table 1].

Median (range) hsCRP level was 7.5 (0.3-155) mg/L. High hsCRP level (>3 mg/L) was present in 72% patients. hsCRP levels and NIHSS score showed moderately strong positive correlation (Pearson's coefficient 0.57) [Table 2]. Median hsCRP levels were found to be higher in patients with greater NIHSS scores as compared to those with lower NIHSS grades (P < 0.001) [Table 3]. However, hsCRP levels had a statistically insignificant correlation with the number of stroke episodes (Pearson's coefficient 0.09).

PLA2 ELISA and TNF-α ELISA were done for 24 patients and matched controls. Median (range) serum PLA2 levels in patients was 11.98 (3.31–87.24) ng/ml which was significantly higher compared to 6.96 (0.15–61.42) ng/ml for healthy controls (P = 0.029). PLA2 levels showed a strong positive correlation with the number of stroke episodes (Pearson's coefficient 0.864). However, correlation with NIHSS score was

Table 1: Clinico-etiological profile of recurrent ischemic stroke (noncardioembolic) cases (*n*=50)

Parameters	*n* (%)
Males	34 (68)
Age	
≤40	3 (6)
41-60	17 (34)
>60	30 (60)
Diet	
Vegetarian	21 (42)
Substance use	
Smoking	7 (14)
Alcoholism	7 (14)
Tobacco	17 (34)
Multiple (>1)	6 (12)
Stroke episode number	
2	39 (78)
3	6 (12)
4	3 (6)
5	2 (4)
Interval between 1st and 2nd stroke	
<1 month	4 (8)
1-6 months	6 (12)
6-12 months	10 (20)
>1 year	30 (60)
Risk factors	
Hypertension	39 (78)
Diabetes	15 (30)
CAD	2 (4)
Dyslipidemia	14 (28)
Multiple (>1)	15 (30)
Drug compliance for risk factors	
Hypertension	17/39 (43.6)
Diabetes	8/15 (53.3)
CAD	1/2 (50)
Aspirin treatment	
Yes	36 (72)
No	14 (28)
Aspirin compliance	
Good	13/36 (36.1)
Poor	23/36 (63.9)
NIHSS score	
0 (no stroke symptoms)	6 (12)
1-4 (minor stroke)	11 (22)
5-15 (moderate stroke)	15 (30)
16-20 (moderate to severe stroke)	8 (16)
21-42 (severe stroke)	10 (20)
mRS score	
0	2 (4)
1	6 (12)
2	3 (6)
3	4 (8)
4	14 (28)
5	21 (42)
Vascular territory	
MCA	40 (80)
ACA	1 (2)
VB	7 (14)
Multiple	2 (4)
TOAST subtype	
1: Large-artery atherosclerosis	31 (62)
2: Cardio embolism	0
3: Small vessel occlusion	11 (22)
4: Stroke of other determined aetiology	0
5: Stroke of undetermined aetiology	8 (16)
Outcome	
Survived	40 (80)
Expired	10 (20)

ACA: Anterior cerebral artery, CAD: Coronary artery disease, MCA: Middle cerebral artery, mRS: Modified Rankin Scale, NIHSS: National Institutes of Health Stroke Scale, VB: Vertebrobasilar, TOAST: Trial of Org 10172 in acute stroke treatment

weaker (Pearson's coefficient 0.196). Median (range) serum TNF-α levels in patients was 68.22 (1.3-287) pg/ml which was significant compared to 0.098 (0.002-36.31) pg/ml for matched controls ($P < 0.001$). TNF-α levels correlated strongly with the number of stroke episodes (Pearson's coefficient 0.759). However, TNF-α values correlated weakly with the NIHSS score (Pearson's coefficient 0.295).

Five (41.7%) patients had platelet aggregation of >50% with 2.5 μM ADP and were considered aspirin resistant. Two (16.7%) patients were semi-resistant while 41.7% of regular aspirin users were aspirin-sensitive [Table 2].

Overall, the mean % aggregation in patients on regular aspirin treatment was 34.75 ± 21.58 compared to 64.75 ± 16.98 for healthy controls ($P < 0.001$). On correlating platelet aggregation values with the number of stroke episodes; Pearson's coefficient was 0.696 which indicates a strong positive correlation, i. e., greater the platelet aggregation and aspirin resistance, more is the number of recurrent stroke episodes [Figure 2].

Discussion

Recurrent ischemic stroke (RIS) is a prevalent clinical entity despite advances in stroke prevention and treatment. Recurrent strokes have been associated with greater mortality and morbidity.[25,26] As there is the scarcity of data from the Indian terrain, this study was conducted to fathom deeper into the clinical profile of RIS.

We registered 50 patients with recurrent noncardioembolic ischemic stroke visiting our hospital for approximately 2 years. Male: female ratio was 2:1 in our study which indicates male preponderance similar to the study by Kocaman *et al*. wherein the majority of cases were males (55%).[27]

Table 2: Distribution of cases based on high-sensitivity C-reactive protein levels and platelet aggregation

Parameters	Number of patients, *n* (%)
hsCRP level (mg/L) (*n*=50)	
<1	3 (6)
1-3	11 (22)
>3	36 (72)
Percentage aggregation (*n*=12)	
Sensitive (<25%)	5 (41.7)
Semi-resistant (25-50%)	2 (16.7)
Resistant (>50%)	5 (41.7)

hs-CRP: High-sensitivity C-reactive protein

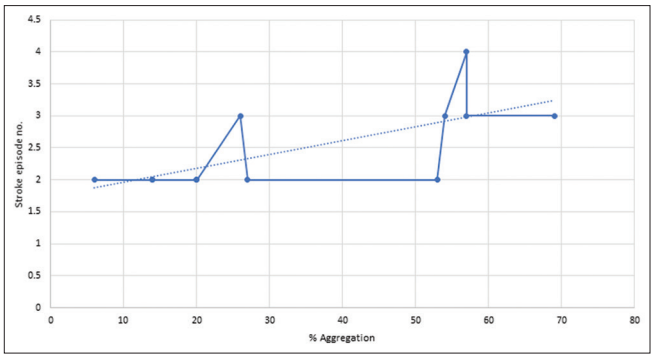

Figure 2: Scatter plot with a positive trendline indicating increase in number of stroke episodes with greater platelet aggregation/aspirin resistance

Sixty percent of patients were elderly people above 60 years. This was similar to the findings of Jung *et al*.[28] In general, age is a vital nonmodifiable risk factor for stroke and an independent predictor of recurrent stroke.[29-31]

Majority of patients (78%) had a second stroke attack. Two patients were found to have fifth stroke event which was the maximum number of episodes reported in this study. Mean time interval for recurrent stroke was 51.93 months which was similar to the study by Zhu *et al*. wherein the mean interval for stroke recurrence was 58.42 months.[32]

Risk factor assessment showed that hypertension was present in 78% of our study participants. Kocaman *et al*. reported that hypertension was the commonest risk factor seen in 85% of RIS patients similar to our study.[27] Hypertension is the most common modifiable risk factor for stroke.[33-35] The AHA/ASA 2011 guidelines and The European Stroke Organization (ESO) recommended that prevention of recurrent stroke is closely related to aggressive treatment of hypertension (Class I, Level of Evidence A).[36-38] PROGRESS study reported 28% reduction in recurrent stroke with antihypertensive drugs perindopril and indapamide.[39] Diabetes mellitus was reported in 30% of our cases, similar to RESQUE study where 24% of cases were diabetics.[33]

Only 43.6% hypertensives and 53.3% diabetics were on regular treatment while 90% of hypertensive patients in RESQUE study and 86% of hypertensives in Kocaman *et al*. study were on regular antihypertensive drugs.[27,33] Even though 72% of patients in our study had started aspirin treatment after index stroke, only 36% of patients had good compliance in contrast to western studies where the degree of aspirin compliance was far better. Kocaman *et al*. found that 67% of cases were consuming aspirin regularly while RESQUE study reported 79% of patients on antiplatelet agents.[27,33] Such poor compliance to antihypertensives, oral hypoglycaemic agents, and antiplatelets might be the responsible factor for a greater number of stroke recurrences (>2) (n = 11) in our study.

Aspirin resistance was calculated for patients on regular aspirin treatment before the current stroke episode. Aspirin resistance was present in 41.7% of patients in the present study.

The strong relationship between hs-CRP and intima-media thickness (IMT) may potentially account for the complex role of hs-CRP and IMT in the pathogenesis of cerebrovascular events.[8] LIMITS study provides evidence that hsCRP predicts risk of recurrent ischemic stroke and major vascular events among patients with recent lacunar stroke.[12] In present study also, 72% patients had high hsCRP level of >3 mg/L.

Median serum PLA2 levels in patients were higher compared to controls (P = 0.029). PLA2 levels also had a strong positive correlation with the number of stroke episodes. Tian *et al*. suggested that blood Lp-PLA2 levels could potentially be used as a predictor of recurrent vascular events in patients with the transient ischemic attack or first ischemic stroke. They also found elevated Lp-PLA2 levels in general population signifying its association with the risk of stroke.[40] Median serum TNF-α levels in patients was statistically significant compared to controls (P < 0.001). Furthermore, TNF-α levels had a strong positive correlation with the number of stroke episodes. Elevated TNF levels have been associated with higher risk of coronary artery disease and ischemic stroke.[41] Furthermore, Welsh *et al*. also reported that interleukin 6 and TNF-α were significant risk predictors of recurrent ischemic stroke.[42] However, larger studies are required to correctly assess the recurrent stroke prediction ability of PLA2 and TNF-α.

Sixty-two percent of our cases belonged to TOAST subtype 1 similar to Kocaman *et al*. who reported large-artery atherosclerosis as the most common subtype (34%).[27] Sixteen percent of cases were in category 5 similar to RESQUE study (15%).[33] All patients (100%) in our study underwent neuroimaging (computed tomography [CT]/magnetic resonance imaging [MRI]) similar to RESQUE study where 99.7% of patients had a

Table 3: Distribution of serum high-sensitivity C-reactive protein levels across various National Institutes of Health Stroke Scale grades

Total (*n*=50)	NIHSS			*P*
	0-4 (*n*=17)	5-15 (*n*=15)	16-42 (*n*=18)	
Median (range) hsCRP level (mg/L)	2.1 (0.3-13.4)	7.6 (5.39-47.7)	21 (5.4-155)	<0.001

hs-CRP: High-sensitivity C-reactive protein, NIHSS: National Institutes of Health Stroke Scale

CT scan.[33] Thus, meticulous efforts were taken to ensure the correct etiological classification of RIS.

Ours is the first study which analyzed the clinico-radiological profile of recurrent noncardioembolic ischemic stroke, assessed various inflammatory markers as possible predictors of RIS and assessed aspirin resistance in those patients who developed re-stroke despite on regular aspirin treatment. Biochemical tests were done with 1:1 matched patient and control blood samples. Neuroimaging (CT/MRI/magnetic resonance angiography brain) was done in all cases to ensure precise TOAST classification of RIS.

Small study cohort with a limited sample size, presence of confounding factors (age, sex, co-morbidities), and no serial measurements of inflammatory markers were major limitations of our study. The small sample size was chiefly due to coronavirus disease 2019 (COVID-19) pandemic which led to poor internal as well as external validity of the study findings. In addition, referral bias due to the inclusion of the patients from a single tertiary care center was also one of the limitations.

Conclusions

Majority of recurrent noncardioembolic stroke cases were aspirin noncompliant. Among the aspirin-compliant patients, a considerable proportion was detected to have aspirin resistance. Pro-inflammatory biomarker hsCRP was significantly raised in patients with higher NIHSS scores while PLA2 and TNF-α positively correlated with the number of stroke episodes. However, long-term follow-up studies with a larger sample size are necessary to better delineate the cause of stroke recurrence and strengthen the role of biomarkers in the prediction of recurrent vascular events.

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Nil.

Conflicts of interest

Prerana Dash and Varun Kumar Singh share first authorship together because of equal contribution. Rest there is no conflicts of interest among authors.

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