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**BIOCHEMICAL ASPIRIN RESISTANCE IN STROKE PATIENTS –
A CROSS-SECTIONAL SINGLE CENTRE STUDY**

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ABSTRACT

Background: Aspirin use is known to reduce the recurrence of stroke. However, the clinical response to aspirin has been mixed. The rate of stroke recurrence whilst on aspirin treatment is still unacceptably high. A plausible explanation for this may be resistance to the effects of aspirin. The causes of aspirin resistance are manifold and multi-factorial. We conducted a study to investigate the prevalence rate of biochemical aspirin resistance in a cohort of aspirin-naïve stroke patients. We also sought to determine the inherent factors that may predispose towards the development of aspirin resistance.

Method: This was a cross-sectional, observational study conducted on patients admitted to our centre with an acute stroke who were aspirin-naïve. The diagnosis of an acute stroke was confirmed by clinical history and brain imaging. Fifty consecutive patients were prospectively enrolled. Socio-demographic data were collected and baseline blood investigations were performed. Patients were tested for biochemical aspirin resistance using Multiplate® platelet analyser (Dynabyte, Munich, Germany) after 5 doses of aspirin, corresponding to a total dose of 900 mg.

Results: The median age of patients was 65.5 years and 54 % of patients were female. There were 11 smokers; of these 10 were male. Twenty-six (52 %) patients were Chinese, 21 (41 %) were Malay and 3 (6.0 %) were Indian. Aspirin resistance was present in 14 % of our patients. There was an inverse relationship between the presence of aspirin resistance and plasma HDL levels ($r = -0.394$; $p = 0.005$). There was no relationship observed between aspirin resistance and total cholesterol, triglycerides, LDL, HbA1c, ALT, ALP, urea and creatinine levels. There were no significant differences in demographic profiles or smoking status between the aspirin resistant and non-aspirin resistant groups. We did not find any link between ethnicity and aspirin resistance.

Conclusions: Our results indicate that a lower HDL level is associated with biochemical aspirin resistance. This may increase platelet aggregation and consequently increase the risk of a recurrent stroke. The clinical implications for aspirin resistance are far reaching. Any evidence that correctable factors may negatively influence the action of aspirin warrants further investigation. The prevalence rate of biochemical aspirin resistance in our study is compara-

ble to the findings in other studies performed in an Asian population. Further research is required to determine how our findings translate into clinical aspirin resistance and stroke recurrence.

Keywords: ischaemic stroke, aspirin resistance, antiplatelet therapy, Asia, developing countries, risk factors, aspirin

INTRODUCTION

It is estimated that there were 5.7 million stroke deaths worldwide in 2005. By 2015, this number is predicted to rise to 6.5 million and the majority of cases will be in developing economies (Strong et al., 2007). Correspondingly, stroke related disability was judged to be the sixth most common cause of reduced disability-adjusted life-years (DALYs), and is expected to be the fourth most common cause in 2030 (Murray and Lopez, 1997; Lopez et al., 2006). From an economic standpoint, worldwide stroke consumes 2-4 % of total health care costs (Donnan et al., 2008). All these facts and figures underline the importance of proper secondary prevention of stroke.

Secondary prevention of stroke involves adequate risk factor control (Davis and Donnan, 2012). Aspirin remains the standard first-line anti-platelet therapy in the secondary prevention of stroke in developing economies due to its low-cost. Aspirin use results in a 13 % to 25 % reduction in stroke recurrence (Algra and van Gijn, 1996). However, as many as three-quarters of patients may still suffer a recurrence of stroke whilst on aspirin treatment (Anti-thrombotic Trialists' Collaboration, 2002). This could be due to biochemical aspirin resistance that leads to a failure to inhibit platelet aggregation.

There are several reasons why aspirin resistance is thought to occur. NSAIDs competitively bind to the COX-1 substrate binding site and adversely influence the effectiveness of platelet inhibition by aspirin (Capone et al., 2005). Concomitant use of proton-pump inhibitors (PPI) may reduce the enteral absorption of aspirin (Würtz et al., 2010). Inherent patient factors may also

play a role in the development of aspirin resistance. Single nucleotide polymorphism (SNP) in platelet genes can affect the platelet inhibition of aspirin (Hankey and Eikelboom, 2006). Studies have shown that there is an increase in the prevalence of aspirin resistance in patients with diabetes (Di Chiara et al., 2007). Hypertriglyceridaemia has been demonstrated to adversely affect the platelet response to aspirin (Karepov et al., 2008). Reduced platelet responsiveness to aspirin is associated with a worse stroke severity at onset (Schwammental et al., 2008).

The aim of this study was to investigate the prevalence of biochemical aspirin resistance in acute ischaemic stroke patients who were aspirin naïve. We also investigated patient factors that might contribute towards the development of aspirin resistance. We used the Multiplate® platelet analyser to measure biochemical aspirin resistance in our sample of patients.

MATERIALS AND METHODS

This was a cross-sectional observational study conducted at UKM Medical Centre from May 2009 to November 2009. Consecutive patients who were admitted with an acute stroke during the recruitment period were screened. Aspirin naïve patients who were confirmed to have a stroke based on clinical examination and imaging findings were invited to participate. Patients who provided informed consent had blood drawn for baseline investigations, which included Full Blood Count (FBC), Renal Profile (RP), Liver Function Test (LFT), HbA1c and Fasting Serum Lipid (FSL), as part of routine blood investigation at this centre. Patients were excluded if they were

allergic to aspirin, had recent NSAID's, antiplatelet or anticoagulant therapy, and had any form of blood disorders, recent or past history of haemorrhagic strokes, liver failure and any history of gastrointestinal bleeding.

On day 1, patients were given 300 mg of aspirin as a loading dose, followed by 150 mg daily thereafter for 5 days. On day 5, 4.5 ml of venous blood was collected. The samples were stored in vials containing heparin-lithium as the anti-coagulating agent.

The study complies with the Declaration of Helsinki and was approved by the hospital ethics committee.

Measurement of aspirin resistance using Multiplate® platelet analyser

The analysis of platelet function was carried out using the Multiplate® platelet analyser (Dynabyte, Munich, Germany), a whole blood impedance aggregometer. The method for using Multiplate® has been described in greater detail elsewhere (Calatzis et al., 2004, 2005; Toth et al., 2006). In brief, the device has 5 test cells, and each test cell has 2 independent sensor units. The system works on the principle of increased electrical impedance between the two sensor wires caused by activation of platelet adhesion and aggregation. Arachidonic acid (AA) (ASPItest, 0.5 mM) was used to initiate platelet aggregation. The increased impedance caused by aggregation of platelets onto the sensor wires was recorded for 6 minutes. The data from the aggregation of platelets onto the sensor wires were converted to arbitrary units labeled as "AU". The converted data is then plotted as a graph of aggregation curve versus time. The area under the aggregation curve was labeled as AUC*min. An AUC*min value of > 440 was defined as 'biochemical aspirin resistance' (von Pape et al., 2007).

All data were analysed using SPSS 16.0 for Mac® statistical software package. Quantitative and qualitative demographic characteristics were summarized and data

were tabulated and tested for normality using *Shapiro-Wilk* test because the sample was below 100.

Categorical variables were analyzed using χ^2 test whereas continuous variables were analysed using *Mann-Whitney U* test. Associations between parameters were examined using *Spearman's Rank* correlation test.

All statistical tests were considered significant if *p*-value < 0.05. All data were expressed as mean +/- standard deviation (SD), median (95 % CI) and inter-quartile range (IQR).

RESULTS

During the recruitment period, 217 patients were admitted to UKM Medical Centre with an acute stroke. Fifty consecutive patients fulfilling all the inclusion and exclusion criteria were enrolled into the study. The median age was 65.5 years (IQR 18) and 27 (54 %) patients were female. Twenty-six (52.0 %) patients were Chinese, 21 (41.0 %) were Malay and 3 (6.0 %) were Indian. There were 11 smokers; of these 10 were male and one female. Baseline haematological and biochemical parameters are shown in Table 1.

Using the Bamford Oxford stroke classification, 20 (40 %) patients were admitted with Partial Anterior Circulation Syndrome (PACS), 18 (36 %) presented with Lacunar Syndrome, 7 (14 %) patients with Total Anterior Circulation Syndrome (TACS) and the remaining 5 (10 %) patients presented with Posterior Circulation Syndrome (Bamford et al., 1991).

Seven out of 50 patients (14 %) were found to have biochemical aspirin resistance. The median AUC was 258.0 AUC*min (IQR 143.5).

Using the Spearman's Rank Correlation test, only HDL level had a statistically significant negative correlation with AUC ($r = -0.394$; $p = 0.005$) (Figure 1). There were no significant correlations between age, haemoglobin, platelet, HbA1c, total

cholesterol, triglycerides, LDL, ALT, ALP, urea and creatinine with AUC (Table 2).

The study population was then divided into aspirin resistant and non-aspirin resistant groups based on the AUC (AUC*min \leq 440, non-aspirin resistant; AUC*min $>$ 440, aspirin resistant).

Analysis using χ^2 -test showed that there was no significant difference in ethnicity between the aspirin resistant group and the non-aspirin resistant group ($p = 0.516$). Although the results showed that 18.2 % of smokers were aspirin resistant and only 12.8 % of non-smokers were resistant to aspirin, this was not statistically significant ($p = 0.651$). It was shown that 22.2 % of females were aspirin resistant as compared to only 4.3 % in males, but this was not statistically significant ($p = 0.16$).

Table 1: Baseline characteristics of the study population

Demographics	(n=50)
Age	65.5 (18.0)
Sex	
Male	23 (46 %)
Female	27 (54 %)
Race	
Malay	21 (42 %)
Chinese	26 (52 %)
Indians	3 (6 %)
Smoking	
Yes	11 (22 %)
No	39 (78 %)
Metabolic parameters	
Haemoglobin (g/dL)	13.5 (2.8)
Platelet ($\times 10^9$ g/dL)	271.0 (115.5)
Haemoglobin A1c (%)	7.1 (2.7)
Total Cholesterol (mmol/L)	5.0 (2.1)
Triglycerides (mmol/L)	1.4 (0.7)
HDL (mmol/L)	1.1 (0.4)
LDL (mmol/L)	3.0 (1.9)
ALT (mmol/L)	17.5 (9.5)
ALP (mmol/L)	73 (34)
Urea (mmol/L)	5.5 (3.1)
Creatinine (umol/L)	70.5 (38.5)

Data is expressed as median (IQR).

HDL: high density lipoprotein, LDL: low density lipoprotein, ALT: alanine transaminase, ALP: alkaline phosphatase

Comparison between the two groups showed no significant differences for age, haemoglobin, platelet, HbA1c, total cholesterol, triglycerides, HDL, LDL, ALT, ALP, urea and creatinine (Table 3).

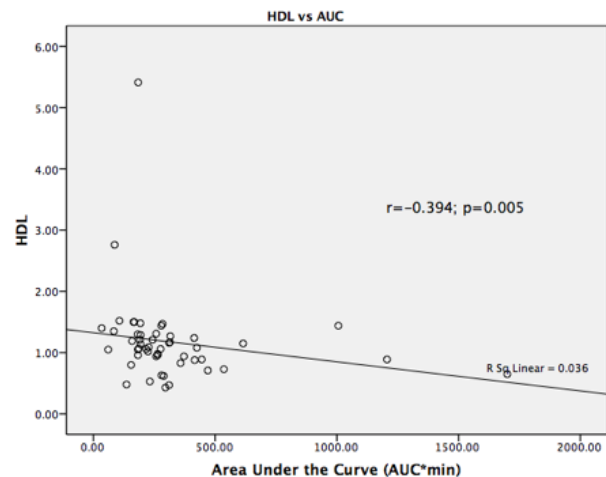


Figure 1: Correlation of HDL with AUC

Table 2: Correlation of AUC with age, biochemical, lipid and haematological profiles

Variable	Correlation coefficient (n=50)	p-value
Age (years)	0.098	0.498
Haemoglobin (g/dL)	0.120	0.407
Platelet ($\times 10^9$ g/dL)	0.041	0.775
Haemoglobin A1c (%)	0.007	0.964
Total Cholesterol (mmol/L)	-0.074	0.611
Triglycerides (mmol/L)	0.079	0.588
HDL (mmol/L)	-0.394	0.005*
LDL (mmol/L)	0.32	0.826
ALT (mmol/L)	0.274	0.054
ALP (mmol/L)	0.254	0.075
Urea (mmol/L)	0.057	0.694
Creatinine (umol/L)	-0.139	0.337

HDL: high density lipoprotein, LDL: low density lipoprotein, ALT: alanine transaminase, ALP: alkaline phosphatase; all data analysed using Spearman's Rank Correlation test AUC; Area Under the Curve

*Data is considered significant when $p < 0.05$

To further investigate the relationship between hyperglycaemia and aspirin response, patients were grouped using the HbA1c level as a surrogate marker. HbA1c \leq 6.5 % were grouped as good glycaemic control whereas HbA1c $>$ 6.5 % were labeled as poor control. χ^2 test did not show any significant statistical difference ($p = 1.00$). Aspirin resistance occurred in 14.3 % of patients with poor glycaemic control and 13.8 % of patients with good glycaemic control.

Patients receiving calcium-channel blockers, proton-pump inhibitors and HMG-CoA reductase inhibitors (statins) during the 5 days of the study period were selected and the aspirin response was compared.

Of those who were deemed to be aspirin resistant, 6 (85.7 %) patients were on statins whereas only one (14.3 %) patient was not. Analysis using χ^2 -test showed that there was no statistically significant difference in the number of patients taking statins be-

tween the aspirin resistant group and non-aspirin resistant group ($p = 1.00$).

In the aspirin resistant group, 3 (42.9 %) patients were on a proton-pump inhibitor and 4 (57.1 %) of the patients were not. Analysis using χ^2 -test showed that there was no statistically significant difference in the number of patients taking a proton-pump inhibitor between the aspirin resistant group and non-aspirin resistant group ($p = 0.345$).

In the aspirin resistant group, 3 (42.9 %) patients were on a calcium-channel blocker and 4 (57.1 %) of the patients were not. Analysis using χ^2 -test showed that there was no statistically significant difference in the number of patients taking a calcium-channel blocker between the aspirin resistant group and non-aspirin resistant group ($p = 1.00$).

Table 3: Comparison between aspirin resistant group and non-aspirin resistant group in terms of AUC, demographic and baseline parameters

Variable	Aspirin Resistant (n=7)	Non-Aspirin Resistant (n=43)	p-value
AUC (AUC*min)	615.0 (445.0)	227.0(107.0)	< 0.001
Age	72 (18)	65 (18)	0.493
Haemoglobin (g/dL)	14.3 (4.5)	13.3 (2.4)	0.989
Platelet ($\times 10^9$ g/dL)	301 (113)	260 (109)	0.275
Haemoglobin A1c (%)	6.0 (3.9)	6.1 (2.5)	0.706
Total Cholesterol (mmol/L)	5.12 (1.70)	4.96 (2.12)	0.605
Triglycerides (mmol/L)	1.28 (0.82)	1.44 (0.72)	0.511
HDL (mmol/L)	0.89 (0.44)	1.09 (0.36)	0.117
LDL (mmol/L)	3.56 (1.21)	2.88 (1.88)	0.511
ALT (mmol/L)	14.0 (25.0)	18.0 (9.3)	0.654
ALP (mmol/L)	94.0 (33.0)	72.0 (33.3)	0.281
Urea (mmol/L)	6.5 (4.2)	5.5 (2.8)	0.529
Creatinine (μ mol/L)	68.0 (52.0)	72.0 (35.5)	0.655

Data is expressed as median (IQR).

All data analysed using Mann Whitney U test.

Data is significant when p value is $<$ 0.05.

HDL: high density lipoprotein, LDL: low density lipoprotein, ALT: alanine transaminase, ALP: alkaline phosphatase

DISCUSSION

This is a single-center cross-sectional observational study of 50 acute stroke patients who were aspirin-naïve. The evidence of biochemical aspirin resistance was determined using *in vitro* impedance aggregometry. 14 % of our patients were found to have aspirin resistance. It is difficult to make a meaningful head-to-head comparison between our studies and others due to differences in the methods of testing aspirin resistance. Previous studies on aspirin resistance have reported a wide range of prevalence rate, ranging from 5 % to 45 % (Mason et al., 2005). Nonetheless, one study investigating aspirin resistance in stroke patients in an Asian population found a prevalence rate of 12 %, which is comparable to our finding (Seok et al., 2008).

There is no cut-off value of HDL level that determines pathology or cerebrovascular disease. We therefore analysed the data to determine a correlation between HDL levels and AUC. We found that the HDL levels had a significant negative correlation with AUC ($r = -0.394$; $p = 0.005$). This suggests that a lower level of HDL may increase the risk of aspirin resistance. *In vitro*, HDL inhibits thrombin, collagen, adenosine diphosphate and thrombin-induced binding of fibrinogen on platelets (non-COX-1 platelet aggregation pathway), which may explain how HDL affects platelet function (Gum et al., 2003). Watala et al. (2004), using a different method to assess platelet function also found that a lower concentration of HDL levels amongst diabetic patients was associated with reduced aspirin-induced platelet inhibition. In another study, Naqvi et al. (1999) demonstrated that a low HDL level is an independent predictor of acute platelet-dependant thrombus formation.

The majority of previous studies on aspirin resistance was conducted in countries that have a fairly homogenous population (Krasopoulos et al., 2008; Snoep et al., 2007). The population of Malaysia is ethnically heterogeneous. We had hoped to

demonstrate a link between ethnicity and aspirin resistance. Although our study showed that there is no statistically significant difference in prevalence of aspirin resistance between the three different ethnic groups, our sample is too small for this to be a meaningful result.

There have been several reports that have demonstrated possible risk factors for aspirin resistance. Some studies have shown that women and older patients are more likely to be aspirin resistant (Gum et al., 2001). Obesity, diabetes and anaemia have been described by certain authors to be factors for reduced aspirin-induced platelet inhibition (Watala et al., 2004; Tamminen et al., 2003). We found no significant differences in demographic and other biochemical parameters between the groups who were aspirin resistant and those who were not. Our results are not unique as there have been other previous studies that have also published equally conflicting findings (Friend et al., 2003; Ozben et al., 2011). It is not unusual for findings in different studies relating to risk factors for aspirin resistance to be contradictory and inconsistent. In our study, this may be due to a small sample size. It also raises the question whether the current methods to measure aspirin resistance requires further improvement.

The relationship between biochemical aspirin resistance and clinical aspirin resistance is being actively investigated. The present body of knowledge seems to suggest that there is a direct relationship between the two but there is no worldwide consensus. Presently, clinical guidelines advocate the reduction of LDL levels in reducing the rate of stroke recurrence. Our results open up the possibilities of addressing the role of modulating HDL as part of the strategy in the secondary prevention of stroke.

Our findings could also be viewed from a socio-economic standpoint. If we can assume that presence of biochemical aspirin resistance infers clinical aspirin resistance,

standard practice dictates that aspirin should be substituted with an alternative anti-platelet agent. In the developing world, where clopidogrel is usually the alternative of choice, albeit an expensive one, increasing HDL instead of prescribing clopidogrel has significant economic repercussions. Unfortunately, recent studies investigating this approach have so far been disappointing (Barter et al., 2007; Luscher et al., 2012).

One of the major confounding factors in studies relating to aspirin resistance is the matter of compliance for aspirin in patients being studied. We addressed this issue by standardizing the method of aspirin administration and measurement of platelet function. We ensured that aspirin was taken under direct supervision by a health personnel and the measurement of platelet function was performed by one personnel for all the patients to remove the effect of inter-operator variability.

The duration of aspirin therapy has been shown to influence the prevalence rate of aspirin resistance (Pulcinelli et al., 2004). We attempted to remove this variable by measuring for biochemical aspirin resistance at a standard pre-determined total dosage of aspirin for all of our patients. It is hoped that this uniformity would make our results more scientifically robust.

This study investigated the prevalence of biochemical aspirin resistance, as opposed to clinical aspirin resistance, i.e. recurrent stroke despite secondary prevention therapy. How our results translate into clinical outcome cannot be answered by our study, as it was not designed to investigate this aspect. We are planning a follow-up study to see how many of our patients develop recurrent stroke.

There are several weaknesses in our study. Due to the small sample size, the prevalence of aspirin resistance in our study can hardly be extrapolated to the general stroke population, but our finding is still food for thought. Secondly, we had to fall back on the manufacturer's recommendation for the level of biochemical aspirin re-

sistance, as we do not have the financial resources to establish the level in our own population.

CONCLUSION

The prevalence of *in vitro* aspirin resistance using the Multiplate® platelet analyser in this study was 14 %. Low HDL levels were found to be associated with higher AUC levels, indicating that HDL may be associated with the development of aspirin resistance.

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