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Circulating Micro-Particles as Potential Hemostatic Biomarkers for Cerebrovascular Ischemic Infarction

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ABSTRACT

Background: Cellular micro-particles (MPs) are submicron plasma membrane derived vesicles shed into the circulation by a variety of blood cells and vascular cells during cellular activation and apoptosis. Currently no practical, rapid and sensitive test is available for the diagnosis of acute ischemic stroke. Current knowledge from earlier studies on MPs suggests that they represent reliable biomarkers as they are cell specific and released early in the pathophysiological cascade of the disease.

Patients and Methods: This study included 20 patients with acute cerebrovascular ischemic infarction confirmed by neuroimaging as well as 20 matched healthy controls. Full evaluation of clinical, radiological and laboratory data was done. Peripheral blood endothelial, platelet, erythrocyte and monocyte micro-particles were measured by flowcytometry using their corresponding monoclonal antibodies (anti CD 62 E, anti CD 61 P, anti CD235 and anti CD 14) respectively.

Results: A significantly higher CD 235 and highly significantly elevated CD 61P and CD 14 were observed in stroke patients compared to controls where platelet derived micro-particles PMPs were the most commonly occurring (28.8%). Co-expression of CD61P and CD62E was a common feature in stroke patients (39.5%) which was found to be highly significant when compared to controls. A cutoff value for the co-expression of CD 61P and CD 62 E as a marker of thrombotic stroke was suggested to be 13.5% using ROC curve statistical method. This co-expression was higher among stroke patients with diabetes mellitus and cardiac disease while CD61P expression was significantly higher in diabetic patients when compared to non-diabetics. A significant higher expression of CD 61P and CD 235 was found in patients not receiving anticoagulation at the time of sampling when compared to controls.

Conclusions: The higher levels of CD 61P, CD 62 E, CD 14 and co-expression of CD 61P and 62E suggests that the systemic endothelial, platelet and inflammatory cell activation increases the risk for cerebrovascular morbidities especially in patients with diabetes mellitus and history of cardiac disease. MPs co-expressing CD62E and CD 61P can be used as a test for the early diagnosis of thrombotic stroke with high sensitivity and specificity. Establishing a cutoff value for co-expression of CD62E and CD61P in stroke patients can contribute to the clinical applications as using MP assay in diagnosis of thrombotic propensity, monitoring of anticoagulant therapy, and detection of risk of stroke and ischemic heart disease in high risk patients.

Key Words: *Stroke – Micro-particles – Biomarker – Flowcytometry.*

INTRODUCTION

Cellular micro-particles are small submicron plasma membrane derived vesicles which are shed into the circulation by a variety of blood cells and vascular cells during cellular activation or apoptosis **[1]**. Micro-particles are present in low concentrations in normal plasma and possess some specific cell surface proteins that indicate their cell of origin, as well as other cell surface molecules that regulate their physiological and pathologic interactions as coagulation, cell signaling and cellular interactions **[2]**.

Their procoagulant properties are mediated through their phospholipid rich surfaces as well as cell surface molecules reflecting their cell of origin: Tissue factor (TF), large multimers of vWF and P-selectin **[3]**. In the recent years circulating MPs of endothelial, platelet and leucocyte origin have been implicated in contributing to the pathogenesis of thrombosis in different thrombotic disorders **[4]**. Their newly diagnosed role in vascular accidents is an area of immense interest that promises to yield important advances into diagnosis and therapy **[5]**.

Increasing studies are exploring the role of circulating MPs in thrombotic stroke. Currently, no practical, rapid and sensitive test is available for the diagnosis of acute ischemic stroke. A number of soluble molecules have been identified that are merely associated to these cerebrovascular accidents. Current knowledge suggests that these membrane derived micro-particles may represent reliable biomarkers as they are cell specific and released early in the pathopysiological cascade of a disease. MPs can be found not only in the cerebrospinal fluid but also in tears and circulating blood in case of blood brain barrier dysfunction. They represent a new challenge in stroke diagnosis and management **[6]**.

The aim of this work was to study circulating platelet, endothelial, monocyte, and erythrocytes micro-particles in patients with ischemic stroke in comparison with healthy controls to assess their clinical application as sensitive biomarkers for diagnosis, prediction and management of the disease.

SUBJECTS AND METHODS

An informed written consent was obtained from all patients or their legally authorized relatives as well as the healthy controls prior to their enrolment. Approval of the Institutional Ethical Review Board was obtained.

This is a case control cross-sectional study which included 20 patients and 20 age matched controls. Patients enrolled were those who have had experienced focal neurological symptoms and signs lasting 24 hours or longer with a relevant ischemic lesion within the brain assessed by neuroimaging. Patients above 50 years of both genders were recruited from the stroke unit at Kasr Al Aini Hospital. Patients were excluded if they presented with stroke of other determined etiology as cardioembolic, hemorrhagic, non-atherosclerotic vasculopathy, hypercoagulable states or borderzone infarction secondary to cerebral infarction.

Patients were subjected to full medical history taking and clinical assessment with emphasis on previous thrombotic or bleeding disorders, previous angina, myocardial infarction (MI), transient ischemic attacks (TIA), previous cerebrovascular accidents, presence of cerevascular risk factors as hypertension, diabetes mellitus, smoking, hyperlipidemia and history of medications as antihypertensives and anticoagulants.

Both patients and controls were subjected to neuroimaging studies (CT brain or MRI), echocardiography and duplex, ECG, routine laboratory investigations (complete blood count, renal functions, liver functions, lipid profile and hemostatic profile) and flowcytometric measurement of circulating micro-particles.

Citrated whole blood (2ml) was collected and processed for platelet poor plasma (PPP). Micro-particles were obtained by centrifugation in two steps. The initial centrifugation to obtain platelet poor plasma was done at 3000 rpm for 25 minutes at room temperature (20ºC-24ºC). The supernatant was removed and transferred to another test tube leaving 200µl above the cell pellet. This was followed by another centrifugation of the supernatant at 5000 rpm for 5 minutes at room temperature. One ml supernatant was collected for micro-particle measurement leaving a 100µl pellet which was discarded. MPs were stained for 30 minutes in the dark at room temperature, with periodic vortexing, by mixing 100µl of PPP with 20µl of each of the anti-human fluorochrome- conjugated antibodies (fluorescein isothiocynate-labeled anti-CD 61P with phycoerythrin-labeled anti-CD 62E in one tube and allophycocyanin-labeled anti-CD 14 with phycoerythrin-labeled anti-CD 235 in a second tube). All antibodies were obtained from Beckman Coulter except CD 235-PE which was obtained from Dako, Denmark. Then 100µl of red cell lysing buffer was mixed with the sample, followed by vortexing and 10 minutes incubation in the dark. The sample was diluted with 200µl phosphate buffered saline (PBS) analyzed on a Beckman Coulter MCL-XL2 setting the stop condition for both patients and controls at 3000 events. An initial micro-particle-size gate was set at 1.0µ based on the forward scatter results.

Statistical method:

In this case-control study, sample size calculation was not possible due to insufficient literature that suggests the expected mean difference of micro-particle expression in stroke patients compared to control subjects.

Clinical and laboratory data were analyzed using SPSS software (SPSS 17.0, SPSS Inc., Chicago, Il). Continuous data was calculated

as mean and standard deviation (SD). Clinical findings were reported in frequency tables. Micro-particles phenotypic expression comparison was performed using the Student *t*-test when data was of normal distribution. For non-parametric data, Mann-Whitney U test was used for comparison of mean values. Receiver operator characteristic (ROC) curve was used for estimation of cutoff value for expression of CD61P/CD62E in stroke patients, to contribute to the future test evaluation. Data were found significant when *p*-value was less than 0.05.

RESULTS

The patient population comprised 20 cases including 17 males and 3 females with an age range of 50-73 with a mean of 63.65±6.43 and a median of 64 years.

As regards risk factors, hypertension was encountered in 18 (90%), hyperlipidemia and diabetes, each in 15 (75%), thrombophilia and family history of stroke, each in 2 (10%) and 12 (60%) of patients were smokers.

Other pathological conditions included associated cardiac disease and renal affection, each in 5 (25%) as well as respiratory disease and hepatic affection, each in 3 (15%) of patients. Presenting complaint and neurological symptoms and signs encountered in the patients' cohort are presented in Table (1). Table (2) presents the medications received by the patients at the time of diagnosis.

Various circulating micro-particles levels are presented in Table (3). Table (4) represents the findings in various patients' subgroups.

No association was encountered between any of the circulating micro-particle types on one hand and any of the presenting complaints, symptoms or signs on the other hand except for an association between erythroid micro-particles (CD235) and blurred vision. Patients who presented with blurred vision showed a level of 31.0500±29.37667 versus 9.7700±8.44552 for those who did not present with blurred vision (p=0.043).

Studying the difference between micro-particles levels in patients and controls as regards the variable risk factors, no statistically significant association was found with either hyperlipidemia, hypertension or smoking. Neither was there any statistically significant association with the various neurological symptoms and signs (headache, neuropathy, vertigo, muscle weakness or memory affection) or with receiving medication.

Receiver operating characteristic (ROC) curves were used to determine a cutoff value for CD61P/CD62E co-expression as a marker of thrombotic stroke. Suggested cutoff value is 13.5%. Other micro-particles showed an overlap between patients and controls and calculation of cutoff values were not possible.

Table (1): Complaint and neurological symptoms/signs in 20 stroke patients.

Presentation	No. (%)	Neurological symptoms/signs	No. (%)
Coma	8 (40.0)	Fainting attack	1 (5.0)
Drowsiness	3 (15.0)	Fits	3 (15.0)
Rt side hemiplegia	3 (15.0)	Headache	11 (55.0)
Lt side hemiplegia	3 (15.0)	Neuropathy	10 (50.0)
Muscle paresis	3 (15.0)	Memory affection	2 (10)
Quadriplegia	2 (10.0)	Muscle weakness	9 (45)
		Vertigo	2 (10)
		Blurred vision	8 (40)

Table (2): Type of medications received by 20 stroke patients at time of sampling.

Medication	Frequency	Percentage
Oral anticoagulant	5	25.0
Antiplatelet therapy	2	10.0
Aspirin + oral anticoagulant	4	20.0
Antihypertensive drugs	18	90.0
Insulin	11	55.0
Oral hypoglycemic	2	10.0

Table (3): Type of medications received by 20 stroke patients at time of sampling.

Cell specific micro-particle	Patients	Controls	*p*-value
Platelet (CD 61P)	28.81±22.67*	5.31±5.79	0.001
Endothelial (CD 62E)	2.02±3.16	3.58±4.81	0.233
Erythrocyte (CD 235)	22.67±22.62	7.72±3.71	0.031
Monocyte (CD 14)	15.05±19.31	2.45±3.43	0.007
Co-expression CD 61P/CD 62E	39.52±24.79	1.95±1.90	0.023*

Mean ± SD

Table (4): Circulating micro-particles level in 20 stroke patients' subgroups.

Patients' subgroups	Circulating Micro-particle Type									
	Platelet (CD 61P)		Endothelial (CD 62E)		Erythrocyte (CD 235)		Monocyte (CD 14)		CD 61P/ CD 62E	
Cardiac:										
Yes	20.82±21.54*	0.37**	1.98±1.55	0.97	17.80±15.96	0.89	15.76±21.29	0.92	66.73±26.97	0.006
No	31.47±23.12		2.03±3.58		21.54±24.68		14.79±19.40		25.91±5.04	
Diabetes:										
Yes	23.08±23.08	0.047	2.12±3.35	0.802	19.11±20.44	0.97	12.54±18.64	0.286	44.67±26.07	0.05
No	46.00±17.78		1.70±2.81		19.62±32.96		24.45±21.58		21.50±0.707	

* Mean ± SD ** *p*- value

DISCUSSION

Although micro-particles are present in low concentration in normal plasma, increased levels are generated in response to platelet activation, direct vascular endothelial damage and thrombin activity on the cell surface. Indeed their altered numbers and characteristics that are exhibited in many vascular diseases with increased thrombotic predilection (both arterial and venous) has urged for the need to achieve further advances towards their use in diagnosis and therapy in vascular accidents.

Since few reports are available on the role of MPs in thrombotic cerebral stroke, the current study has aimed to assess the clinical application of circulating MPs in the diagnosis, prediction and management of thrombotic cerebrovascular accidents through the measurement of levels of platelet (CD61P), endothelial (CD 62E), erythrocyte (CD 235) and monocyte (CD 14) microparticles by flowcytometry in a cross sectional sample of 20 patients and 20 healthy controls.

In the current study, the most common type of micro-particles was PMP (28,8%), MPs co-expressing both platelet and endothelial markers (39.5%) followed by MP of erythrocyte origin (19.2%), then those of monocyte origin (15%) and finally endothelial derived MP (2%). Comparing MP levels in stroke patients versus controls revealed significantly higher CD 235 expression and highly significant greater expression of both CD 61P and CD 14 in stroke patients compared to control but not endothelial MPs. This finding is in accordance with other studies. A study of cerebro-occlusive events, reported high PMP levels in cerebrovascular accidents in small and large vessels and multifactorial dementia with no apparent effect of antiplatelet therapy on PMP levels **[7]**. Again other studies reported increased levels of PMP in both acute and chronic phase of cerebral infarction **[8,9]** and another study reported that this significantly correlated with intima media thickness and intracranial stenosis of carotid arteries **[10]**. In contrast to this study, other studies detected elevation of endothelial derived MP levels in acute ischemic stroke **[11,12]**. Also a recent study on endothelial micro-particles reported that high levels of CD 62E was associated with cardiovascular events in patients with stroke. This study did not simultaneously assay PMP **[13]**.

In the current study, CD 61P/CD62E co-expression was a common feature in stroke patients. Only a single report on co-expression of platelet and endothelial markers was found. This "remarkable" finding, as stated by the authors, was suggested to be a result of an interaction between platelets (or platelet fragments) and endothelial cells resulting in cellular activation and generation of micro-particles of bi-lineage origin **[14]**.

In healthy control subjects of the present study erythroid MPs exhibited the highest level of expression, followed by platelet then endothelial and the least was monocyte derived MPs. This is not in agreement with other reports, in which the majority of micro-particles in healthy controls were of platelet origin, followed by endothelial, red blood cells and monocytes.

MPs level was compared according to presence or absence of other clinical data of stroke patients. MPs of erythroid origin were significantly higher in patients complaining of blurred vision. However this finding was not supported by other studies on stroke patients. In the current

study, both CD 61P and CD61P/CD 62E co-expression were significantly higher in diabetic patients when compared to non-diabetics. In partial agreement with our results, endothelial derived MPs were found to be elevated in patients with DM with no reports on co-expression of endothelial and platelet MPs **[15]**. Other studies have shown that elevated MPs in diabetic patients especially of endothelial, platelet and monocyte origin correlate with diabetic complications **[16]**. Another study related platelet and endothelial MPs levels in diabetic patients to vascular complications **[17]**.

No significant difference in MPs levels was found between hypertensive and non–hypertensive stroke patients in the present study. This is in contrast to other studies which reported the association between elevated levels of endothelial derived MPs and hypertension **[18,19]**.

The current study showed no significant difference when comparing MPs levels in patients with renal dysfunction with disease free patients. This is in disagreement with a study done on patients with chronic renal failure that reported that renal disease is accompanied by endothelial activation and their results in all their patient subgroups showed an increase mainly of platelet-derived MPs, with minor populations of endothelial and TF bearing MPs **[20]**.

A recent review on micro-particles as reliable markers of thrombosis highlighted that the difficulty in identification, standardization and quantification methods of MPs, hinders its use as a practical and clinical diagnostic tool. Further prospective studies and more evaluation of diagnostic value of MP assays in different diseases are worth doing to promote the utility of this technique in clinical practice **[21]**. Improved decision making in diagnosis and patient management is only one route by which tests affect patient health, and empirical evaluations are needed to compare the effect of test strategies on patient health. To establish whether a new diagnostic test will change health outcomes, it must be examined as part of a broader management strategy **[22]**.

In conclusion, increased circulating microparticles (CD 61P, CD 62 E, CD 14 and co-expression of CD 61P and 62E) suggests that the systemic endothelial, platelet and inflammatory cell activation increases the risk for cerebrovascular morbidities especially in patients with diabetes mellitus and history of cardiac disease. Establishing a cutoff value for co-expression of platelet and endothelial circulating MPs and significant elevation of its level in stroke patients especially those with history of DM and cardiac ischemia can contribute to the clinical application. Evaluation of its plasma level may be used in diagnosis of thrombotic propensity, monitoring of anticoagulant therapy, and detection of risk of stroke and ischemic heart disease in diabetic patients.

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