Elevated Cellular-microparticlesCoexpressing Endothelial and Platelets Markers in Cerebro-vascular Ischemic Infarction

A Possible New Challenge for Early Diagnosis

Abstract

Background: Stroke is one of the leading causes of morbidity and mortality in the world. Current diagnosis of thrombotic cerebral stroke remains delayed due to lack of a suitable mechanism for rapid, accurate, and analytically sensitive biomarker-based testing. A single set or multiple sets of identified potential blood biomarkers that could be used in an acute setting to diagnosis stroke, or even predict an initial/reoccurring stroke would be extremely valuable in improving patient outcome and quality of life.

Study Design and Methods: 20 patients above 50 years of age were recruited from the stroke unit of Kasr Al Aini hospital with focal neurological symptoms & signs lasting 24 hours or longer. The levels and the cellular origin of microparticles were determined by flowcytometric analysis. The following fluorescent monoclonal antibodies were assayed:Endothelial: CD 62 E, Platelet: CD 61p, Monocyte: CD 14 and Erythrocyte: CD 235. 20 age and sex matched controls were included.

Results: Patients withcerebrovascular ischemic infarction had highly significant higher level of microparticlescoexpressing CD 61P and CD 62E (mean 39.5 vs 1.95), expressing CD 61p (mean 28.81 vs 5.31), CD 235 (19.23 vs 7.71) and CD 14 (mean 15.0 vs 2.44) comparing to control group.

Conclusion: Rapid diagnosis of cerebrovascular infarction is essential for optimal patient treatment. Cell derived microparticlesmay represent reliable biomarkers for diagnosis, prediction of consequences, forecasting recovery and outcome and might

improve diagnostic certainty. They represent a new challenge in stroke diagnosis andmanagement.

Key words: Cell derived-microparticles, stroke, biomarkers, CD62 antigen, CD61 antigen.

Introduction:

Stroke is the third most frequent cause of mortality after ischemic heart disease and cancer, and the first cause of disability in adults worldwide (1). Most authors define stroke as CNS dysfunction of vascular origin; this broad definition includes ischemic stroke, intracerebral hemorrhage, subdural hemorrhage, epidural hemorrhage and subarachnoid hemorrhage. Cerebral ischemia leads to cerebral infarction is the most common type of stroke and is usually caused by arterial pathology, although it may rarely be caused by venous dysfunction (2,3).

The two principal diagnostic challenges in overall stroke management are difficulties in diagnostic certainty and differentiating brain ischaemic attacks from haemorrhages and stroke-like disorders. Stroke is a preventable and treatable disease, if it is recognized early, hence the adage: "Time is Brain." However, to date, rapid blood tests for stroke assessment are still investigational (4).

A biomarker is any measurable physiological characteristic or substance that marks the riskfor or manifestation of a stroke-related process. Several categories of biomarkers arestudied in stroke – physical markers, imaging markers, electrophysiological markers,histological markers, genetic markers, systemic (serum) markers and neuronal markers (5, 6).

Cellular microparticles are plasma membrane vesicles mainly composed of lipids and proteins,which are released into the circulation by blood cells and vascularcells during cellular activation or apoptosis (7). Microparticlesare heterogeneous, differing in size, as well as in phospholipidsand protein composition. In addition, microparticlesdisplaysome specific cell surface proteins that indicate their cellularorigin. Investigation into their biological activity has revealed diverse actions

in coagulation, cell signalling and cellular interactions. These actions are mediated through their phospholipid rich surfaces and the expression of cell surface molecules, which reflect their cell of origin and its state of activation (7,8).

Indeed, the numbers and characteristics of circulatingmicroparticleshave been found to be altered in many vasculardiseases associated with an increased risk of both arterialand venous thrombosis (7). The complex role of microparticles in vascular accidents is an area of immense interest that promises to yield important advances into diagnosis and therapy (9). Few reports are available on role of circulating microparticles in thrombotic cerebral strokes.Currently, no practical, rapid and sensitive test is available for the diagnosis of acute ischemic stroke (10). A number of soluble molecules have been identified that are merely associated to these cerebrovascular accidents. Current knowledge from the field of cell-derived microparticles suggests that these membrane fragments may represent reliable biomarkers as they are cell-specific and are released early in the pathophysiological cascade of a disease (10,11).

In our study, the aim wasidentification, quantitation & function appreciation of circulating microparticlesassensitive and early markers for diagnosis, predictionand prognosis of cerebrovascular ischemic infarction. Based on our results, we suggest that measurement of circulating microparticles levels specially those expressing endothelial and platelets markers may provide a new option assessing a patient's risk of cerebrovascular ischemic infarction.

Materials and Methods:

Study subjects

20 patients above 50 years of age were recruited from the stroke unit of Kasr Al Aini hospital over a period of 10months (fromOctober 2011 to July 2012)with focal

neurological symptoms & signs lasting 24 hours or longer, with a relevant lesion within the brain as assessed by neurological imaging, both males and females.All patients were subjected to full clinical evaluation, revision of their archived clinical progress reports, radiological and laboratory data.Thecriteria for exclusion of patients were cardio embolic cause of ischemic stroke, possibility of artery to artery embolism excluded by duplex, vacuities diagnosed clinically or laboratory, presence of haemorrhagic ischemia detected by neuro-imaging, pregnant females, hematological evidence of ischemic infarction as anti cardiolipinAb, hypercoagulable state & TTP, border zone infarction secondary to cerebral hyperperfusion detected by neuro imaging.20 matched age and gender hospital attendees were recruited as a control group who have no clinical evidence of thrombotic disease & the same exclusion criteria as the patients.Informedconcent was obtained from patients and control groups for routine clinical management and extra laboratory sample for research procedure. Fayoum university ethical committee approved the research proposal before the start of actual recruitment.

Sampling and preparation of platelets poor plasma

Citrated whole blood samples were drawn.Platelet-poor plasma was obtained by centrifugation in two steps. The initial step consisted of two centrifugations at 3000 rpm for 20 minutes at room temperature. After the first centrifugation the supernatant was transferred into a new tube, leaving 200 μL above the cell pellet, and was centrifuged again for five minutes at maximumspeed. After centrifugation, the supernatant was transferred into a new tube, while discarding the last 500 μL at the base of the centrifuged tube. All buffers (Phosphate buffered saline) were sterile-filtered with a 0.2 μmfilter. For standardization purposes in 8 of our patients citrated

whole blood was used in addition to compare results with PPP for isolation of microparticles.

Microparticles isolation and staining

Platelet poor plasma samples and citrated whole blood were incubated with fluorescent monoclonal antibodies for endothelial CD 62 E,plateletsCD 61 P,monocyticCD 14 and erythrocyte CD 235.Two protocols were performed, the first including CD 61 P FITC with CD 62 E PE and the second including CD 235 PE with CD 14 PC5.This was followed by acquisition of the samples and saving of scattergraphs produced by flowcytometer software including forward and side F1 and F2 scatter graph for each protocol.

Microparticle enumeration by flow cytometry

For the detection of microparticles by flow cytometry, an initial microparticle-size gate was set with the help of calibrating fluorescent 0.8 μm and 3.0 μm latex beads Forward scatter and side scatter had a logarithmic gain. The absolute count of microparticles was measured setting the stop condition for True Count beads at 3000 events. In order to separate true events from background noise and unspecific binding of antibodies to debris, we defined microparticles as particles that were less than 1.0 μm in diameter.

Data processing

Analysis of data started with gating based on forward scatter results and microparticles were identified by size. Results of expression of each marker were recorded.

Statistical analysis

Clinical and laboratory data were analysed using SPSS-14 software and test selection for mean comparison depended on data distribution and test efficiency followed.

Association between micropaticle type and level and other relevant parameters were performed by correlation analysis.

Results:

Flowcytometry assay of circulating microparticles in studied cases revealed that the most common type of microparticles present in thrombotic stroke were microparticlescoexpressing both platelet and endothelial markers (39.5%)and platelet derived micropartcles (28.8%), followed by MP with erythrocyte origin (19.2%), then those of monocytic origin (15%) and finally endothelial derived MP (2%).

Comparing MP assay data in stroke patients with controls revealed, highly significant higher levels of coexpressionCD61P and CD 62 E (table 1), significant difference in CD 235 and highly significant difference in both CD 61p and CD 14 where expression of these markers were higher in stroke patients compared to controls.The means and standered deviation of Microparticle assay results among studied casesand controls were presented in (tables 2-3) respectively.Receiver operating characteristic (ROC) curves were used in determine a cutoff value for a coexpression of CD61p and CD62 E as a marker of thrombotic stroke. Suggested cutoff value is 13.5%. Other MP assay markers showed overlap between patients and control and calculation of cutoff values were not possible (figures 1,2).

Table4 shows significant difference in Comparing MP assay coexpressing CD 62 E, CD 61p in patients with and without cardiac disease,whereacoexpression of these markers was higher in stroke patients with cardiac disease compared to stroke patients without cardiac disease.

Regarding comparison of MP assay data in patients with and without Diabetes mellitus it showed higher CD 61p expression in stroke patients with diabetes.

Similarly coexpressing of CD 62 E, CD 61p was significantly higher in DM (table 5,6).

In comparison of MP assay data in patients not receiving anticoagulant therapy at time of sampling with controls there was significant higher expression of both CD 61p and CD 235 in patients compared to controls (table 7).

Discussion:

Stroke is a devastating condition encompassing a wide range of pathophysiological entities that include thrombosis, hemorrhage, and embolism (12). Current diagnosis of stroke relies on physician clinical examination and is further supplemented with various neuroimaging techniques but remains hampered and delayed due to lack of a suitable mechanism for rapid, accurate, and analytically sensitive biomarker-based testing (2,12). Serum biomarkers could increase diagnostic certainty by helping to distinguish cerebral ischemia from common mimics such as focal seizure, complicated migraine, and psychogenic spells (13).A single set or multiple sets of blood biomarkers that could be used in an acute setting to diagnosis stroke, differentiate between stroke types, or even predict an initial/reoccurring stroke would be extremely valuable (12,14).

Cellular microparticles are plasma membrane vesicles mainly composed of lipids and proteins,which are released into the circulation by blood cells and vascularcells during cellular activation or apoptosis (15).Microparticlesare heterogeneous, differing in size, as well as in phospholipidand protein composition. In addition, microparticlesdisplaysome specific cell surface proteins that indicate their cellularorigin. Investigation into their biological activity has revealed diverse actions in coagulation, cell signalling and cellular interactions (16).

Microparticles are present in low concentrations in normal plasma. Increased levels are generated by a number of mechanisms including platelet activation, direct vascular endothelial damage, thrombin activity on the cell surface.Several techniques are currently used to study the generation and nature of circulating microparticles (9). Indeed, the numbers and characteristics of circulatingmicroparticleshad been found to be altered in many vasculardiseases associated with an increased risk of both arterialand venous thrombosis (9). The complex role of microparticles in vascular accidents is an area of immense interest that promises to yield important advances into diagnosis and therapy. Few reports are available on role of circulating microparticles is thrombotic cerebral strokes (17).

Despite presence of many laboratory markers that can be used in the early diagnosis and treatment monitoring in cardiovascular accidents and myocardial infarction that are used clinically as Troponin level and cardiac enzymes (18), yet no practical, rapid and sensitive test is available for the diagnosis of acute ischemic stroke that can be used clinically (19). A number of soluble molecules had been identified that are merely associated to these cerebrovascular accidents in the past 20 or 30 years but none of them can be used till now as a marker in cerebrovascular accidents (19,20).

In the current study Flowcytometry assay of circulating microparticles in studied cases revealed that the most common type of microparticles present in thrombotic stroke were platelet derived micropartcles (28.8%), microparticlescoexpressing both platelet and endothelial markers (39.5%), followed by MP with erythrocyte cell of origin (19.2%), then those of monocytic origin (15%) and finally endothelial derived MP (2%).

Comparing MP assay data in stroke patients versus controls revealed, a significantly higher CD 235 expression and highly significant greater expression both CD 61p and CD 14 in stroke patients compared to controls.

Coexpression of CD61p and CD62E was a common feature in stroke patients and it exhibits a highly significant elevation in stroke patients compared to control subjects. In a recent review on microparticles as reliable markers of thrombosis, higher numbers of microparticles expressing platelet and endothelial markers were described, suggesting ongoing endothelial activation. The same review 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).

Most studies of diagnostic tests evaluate only their accuracy. Although such studies describe how well tests identify patients with disease (sensitivity) or without disease (specificity), further evidence is needed to determine a test's true clinical value. Firstly, since tests are rarely used in isolation, studies are needed to evaluate the performance of testing strategies, accounting for when and how a new test is used within a diagnostic pathway, and how its findings are combined with results of other tests. Secondly, decision-making involves selecting among multiple testing strategies; thus studies that compare test strategies and estimate differences in sensitivity and specificity are more informative than those that evaluate the accuracy of one test or diagnostic strategy. Thirdly, improvements in test accuracy will not benefit patients unless they lead to changes in diagnoses and patient management, requiring evaluations of the effect of improved accuracy on decision making. Finally, improved

decision-making 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).

So far, accumulating data suggests that MP play a role in thrombosis and vascular dysfunction. Additional studies are necessary to delineate more precisely the pathways involved in microparticle regulation of vascular function. Clarification of their composition as well as the underlying mechanisms involved in their effects will help us to develop additional interventional strategies for prevention and treatment of thrombotic disorders.

To date, the measurement and detection of levels of microparticles in various conditions have not translated into therapeutic or diagnostic strategies in the management of the disease conditions in which they have been shown to be relevant. However they have helped to shift the understanding of the pathophsiological mechanisms of several diseases. The detection of chronically elevated levels of circulating microparticles provides an insight into the chronic endothelial attack, and may provide an important tool in measuring the protective effects of therapeutic interventions in an early and non-invasive manner

Active, prothromboticmicroparticles in the circulation during several disease states may contribute directly to the disease process, and constitute an important therapeutic target in their own right.

Conclusion:

Although several markers of different physiological processes were associated with a diagnosis of cerebrovascular stroke and with recurrent vascular events after stroke, no marker improved the diagnosis of stroke or prediction of recurrent events over

established clinical variables. Major challenges in the diagnosis of stroke are both the variety of conditions that mimic stroke and the heterogeneity of stroke itself.

Our study revealed that MP coexpressing CD 62E and CD 61p could be used as a test for the early diagnosis of thrombotic stroke with high sensitivity and specificity.

Establishing a cutoff value forcoexpression of CD 62E and CD 61p 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.

Recommandations :

- Furtherstudieswithlargersample size isrecommended for betterevaluation and clinicalcorrelation of MP assayrole in ischemic stroke.

- TechnicalStandardizationstudies are essential beforestart of clinical application of MP assay.

- A prospective cohortstudyisneeded for betterassessment of coexpression of CD 61p & CD 62^E as a risk factor of thrombotic accidents.

- Assessment of the effect of anticoagulant protocols on MP assayisrecommendedwithpotentialrole of thisassay in better monitoring of anticoagulant therapy, and prevention of thrombotic accidents.

Table 1 : Comparison of MP assaydatacoexpression of CD62 E and CD61p in stroke patients versus control

	Group	Mean	Std. Deviation	Significance
Coexpression of CD62 E and CD61p	Stroke patients	39.5222	24.78633	Significant
	Controls	1.9500	1.90498	

Table 2: Mean and SD of Microparticleassayresultsamongstudied case

	N	Minimum	Maximum	Mean	Std. Deviation
CD62 E	20	.00	11.90	2.0200	3.16155
CD61p	20	.00	64.00	28.8100	22.67263
CD235	20	1.00	80.00	19.2278	22.61633
CD14	20	.00	52.60	15.0474	19.31161

Table 3: Mean and SD of Microparticleassayresultsamong control cases

	N	Minimum	Maximum	Mean	Std. Deviation
CD62 E	20	.00	20	3.5800	4.80686
CD61p	20	.00	20	5.3100	5.78582
CD235	20	3.5	17	7.7150	3.70608
CD14	20	.00	11	2.4450	3.42644

Table 4 : Comparison of MP assaycoexpressing CD 62 E, CD 61p in patients with and withoutcardiacdisease

	Cardiac	Mean	Std. Deviation	Significance
Coexpression of CD62 E and CD61p	**Absent**	25.9167	5.04397	Significant
	Present	66.7333	26.97857	

Table 5 : Comparison of MP assay data in patients with and withoutDiabetesmellitus

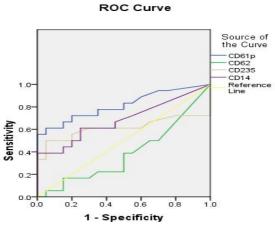|	D.M	Mean	Std. Deviation	Significance
CD62 E	Absent	1.7000	2.81957	Not significant
	Present	2.1267	3.35335	
CD61p	Absent	46.0000	17.78707	Significant
	Present	23.0800	21.59931	
CD235	Absent	19.6250	32.96810	Not significant
	Present	19.1143	20.44501	
CD14	Absent	24.4500	21.58418	Not significant
	Present	12.5400	18.64579	

Table 6: Comparison of MP assaycoexpressing CD 62 E, CD 61p in patients with and withoutDiabetesmellitus

	DM	Mean	Std. Deviation	Significance
coexpression of CD62 E and CD61p	Absent	21.5000	.70711	Significant
	Present	44.6714	26.07423	

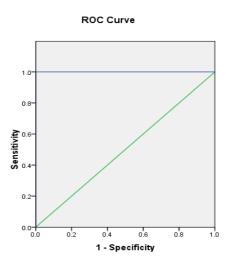Table 7: Comparison of MP assay data in patients receiving anticoagulants at time of samplingwithcontrols

	Anticoagulants	Mean	Std. Deviation	Significance
CD62 E	Patients	1.5125	2.39370	Not significant
	Controls	3.5800	4.80686	
CD61p	Patients	19.1875	22.38140	Significant
	Controls	5.3100	5.78582	
CD235	Patients	21.5000	27.07366	Significant
	Controls	7.7150	3.70608	
CD14	Patients	8.2857	14.63596	Not significant
	Controls	2.4450	3.42644	

Figure 1 : Receiver operating characteristic (ROC) curvesfor MP assay in diagnosis of thrombotic stroke

Figure 2 :Receiver operating characteristic (ROC) curves for coexpression of CD62 E and CD61p in diagnosis of thrombotic stroke.

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