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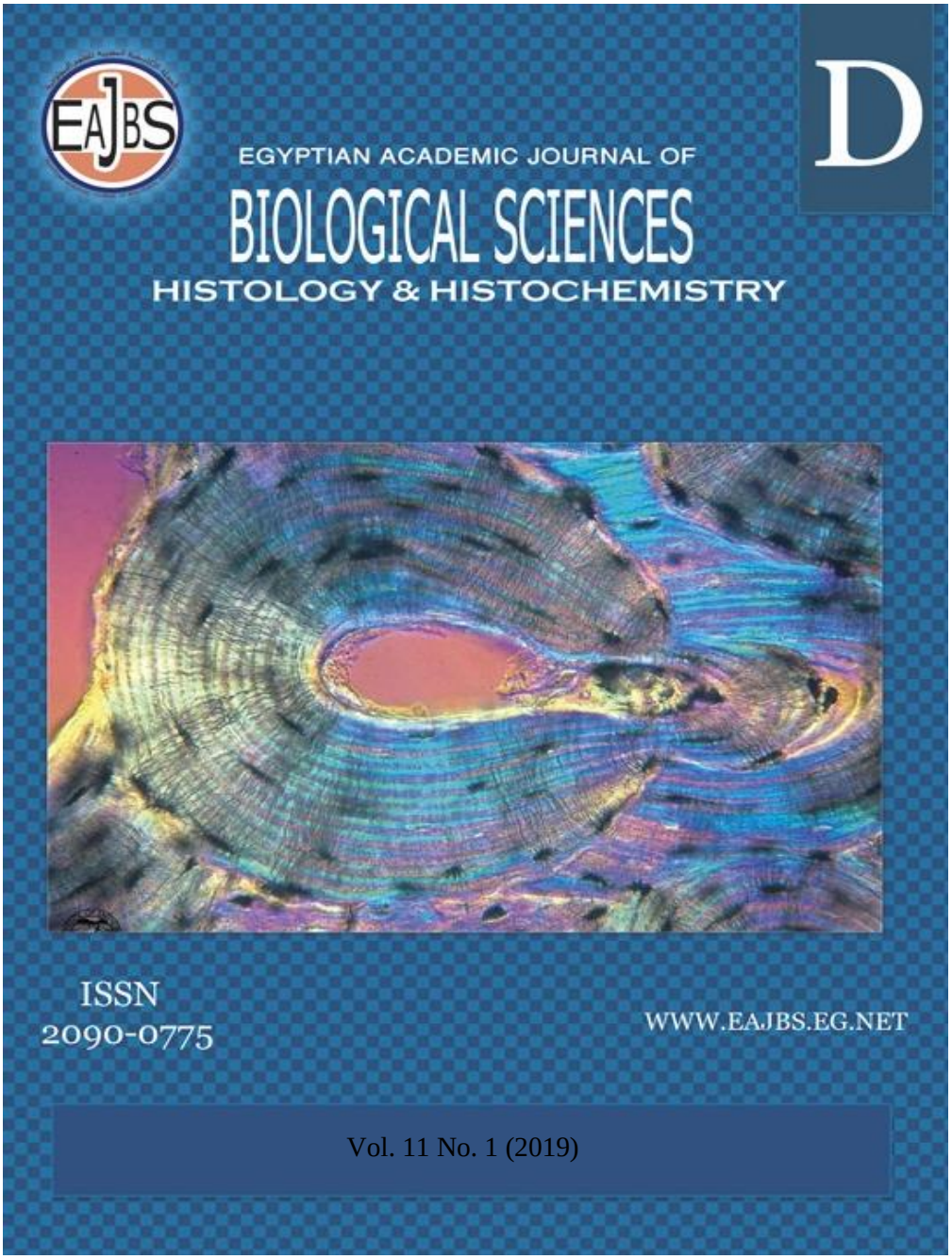

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Elevated Cellular-microparticles Expressing Platelets and Endothelial Markers in Cardio-vascular Ischemic Infarction (A Possible New Challenge for Early Diagnosis)

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ABSTRACT

Cellular microparticles are plasma membrane vesicles of <1 μm in diameter, mainly composed of lipids and proteins, which are released into the blood circulation by blood cells and vascular cells during cellular activation or apoptosis. Microparticles play an important procoagulant role in several diseases, especially in thrombotic accidents. In the present study, we assess the effect of anticoagulant treatment on microparticles in patients having thrombotic accidents for better monitoring of the anticoagulant therapy. We collected blood samples from 20 patients with myocardial infarction at the time of diagnosis and four weeks after suffering the MI. All patients were subjected to full clinical evaluation, revision of their archived clinical progress reports, radiological and laboratory data. In addition assay of circulating, microparticles for both quantitation and determination of cell of origin using flow cytometry technique was performed. The following fluorescent monoclonal antibodies were assayed: Endothelial: CD 62 E, Platelet: CD 61p. Comparing MP assay data in MI patients versus controls revealed a significantly higher Coexpression of CD61p and CD62E and highly significant expression of CD 61p in MI patients compared to controls. In comparison of MP assay data in patients at time of diagnosis as MI and after a month of surviving the MI and receiving anticoagulant treatment, there was no statistically significant difference in MP markers following intervention in all cases, from (22.39±9.41 to 21.72±7.77) for CD 62E/61, from (62.12±14.25 to 61.18±13.18) for CD 61, and from (0.040±0.043 to 0.034±0.037) for CD 62E ($p > 0.05$). A cutoff value for the expression of CD61p as a marker of thrombotic MI was suggested to be (58.70) revealed (70%) sensitivity and (85%) specificity and as regards CD 62E/61, taking (22.25) as a cut-off point revealed (60%) sensitivity and (95%) specificity using ROC curve statistical method. In conclusions, the antithrombotic properties of low dose antiplatelet or anticoagulant therapy are not strong enough to suppress shedding of the microparticles. A cut off value for expression of CD61 and coexpression CD 62E/61 in MI patients can contribute to the clinical applications as using MP assay in early diagnosis of thrombotic propensity, monitoring of anticoagulant therapy, and detection the risk of ischemic heart diseases in high-risk patients.

INTRODUCTION

Microparticles are described as a heterogeneous population of membrane-delimitated vesicles 50–1000 nm in size released from the cells in which they form and retain certain antigens of their cells of origin (Van der Pol *et al.,* 2012). It has been reported that approximately 80% of these circulating vesicles are derived from platelets (Flaumenhaft *et al.,* 2009). These particles carry surface proteins and cytoplasmic material of their parent cells, allowing their origin to be identified (Diamant *et al.,* 2004; Rautou *et al.,* 2011). The investigation into their biological activity has revealed diverse actions in coagulation, cell signaling 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 (Horstman *et al.,* 2004). There has been a resurgence of interest in circulating microparticles from endothelial cells and platelets because of their newly recognized diverse physiologic and pathologic role. They were reported to contribute to the pathogenesis of thrombosis in different diseases, including cancer-associated thrombosis and sepsis (Mackman, 2006). Cardiovascular diseases (CVDs) was the most common underlying cause of death in the world in 2013, accounting for an estimated 17.3 million, representing 31.5% of all global deaths (Benjamin *et al.,* 2017). In spite of advances in assessment and treatment of common cardiovascular risk factors, including hypertension, obesity, diabetes, hyperlipidemia, the world health organization estimates the number of death from CVDs will increase to almost 24 million by 2030 (Mathers and Loncar, 2006; Alwan, 2011). Acute myocardial infarction (MI) is associated with a 30% mortality rate; about 50% of the deaths occur prior to arrival at the hospital. An additional 5-10% of survivors die within the first year after their myocardial infarction. Approximately half of all patients with MI are rehospitalized within 1 year of their index event. Overall, the prognosis is highly variable and depends largely on the extent of the infarct, the residual left ventricular function, and whether the patient underwent revascularization (Amsterdam *et al.,* 2014). To characterize the cellular origin of MPs in peripheral blood, the most common approach is to stain MPs with fluorescently-labeled AB directed against antigens of parental cells (for example CD41, CD61 and platelet activation marker CD62 for platelets; glycophorin for erythrocytes; CD45 for lymphocytes; CD14 for monocytes; and so on) and to perform subsequent analysis by flow cytometry (Zahra *et al.,* 2011). Until the 1990s, no biological importance had been given to MP, which was considered an inert particle resulting from cell destruction or only a marker of apoptosis (Franca *et al.,* 2014). In 1996, however, Raposo *et al.* suggested that MP played an important role in the adaptive immune response. Since then, several studies have shown the importance of MP as vectors of intracellular exchange of biological information, by the use of identification, characterization and quantification of MP in several situations, such as obesity, diabetes mellitus, infarction, depression, cancer, HIV and renal failure (Franca *et al.,* 2014). The procoagulative properties of MPs are largely linked to their physical characteristics with two specific surface features thought to be responsible for this procoagulant activity. First, the externalization of anionic phospholipids (predominantly phosphatidylserine) results in a negatively charged surface; this negatively charged surface allows for interaction with cationic domains in clotting proteins, the subsequent

assembly of coagulation factors and ultimately thrombin formation (Owens and Mackman, 2011). Elevated levels of MPs are found in patients with CVD and in groups with risk factors for CVD. Subpopulations of MPs are promising biomarkers for improving risk prediction, as well as monitoring treatment (Christersson *et al.*, 2016).

MATERIALS AND METHODS

The present study is Case-control. 20 patients were included in this study with 20 age and sex-matched controls. Patients were selected according to the following criteria.

Inclusion Criteria:

Patients who had experienced chest pain typical with criteria of cardiac origin, diagnosed as myocardial infarction, assessed medically by ECG and labs, pre and post anticoagulation therapy. A full history was obtained

Exclusion Criteria:

Included patients having other cardiac causes of chest pain other than myocardial infarction as pericarditis, myocarditis, stress cardiomyopathy and aortic dissection.

Control subjects:

Matched age & gender group were recruited from the hospital attendees who have no clinical evidence of thrombotic disease & the same exclusion criteria as the patients, who consent for the investigative laboratory procedure.

Informed Consent:

Objective of the study and steps were clearly explained to every patient.

Examination:

Assay of circulating microparticles for both quantitation and determination of cell of origin using flowcytometry technique using Beckmann Coulter Flowcytometer MCL-XL2.

Blood Collection:

Platelet-poor plasma samples and citrated whole blood were then incubated with the following fluorescent monoclonal antibodies: Endothelial - CD 62 E, Platelet - CD 61 P

A Protocol Was Performed Including CD 61 P FITC with CD 62 E PE:

-100µ sample was added to 20 µ monoclonal antibodies and then vortexed.

-Tubes were then incubated for 30 minutes in the dark

-100 µ lyse reagent was added to plasma and 300 µ lyse to whole blood then vortexed.

- Tubes were incubated for 10 minutes in the dark.

- 200 µ PBS was then added.

- This was followed by the acquisition of the samples and saving of scatter graphs produced by flowcytometer software including forward and side F1 and F2 scatter graph for each protocol.

Microparticle Enumeration by Flowcytometry:

For the detection of microparticles by flowcytometry, 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. Analysis of data started with gating based on forward scatter results and microparticles were identified by size.

The Statistical Analysis:

Clinical and laboratory data were analyzed using SPSS software version 18 in Windows 7 and test selection for mean comparison depended on data distribution and test efficiency followed. Association between microparticle type and level and other relevant parameters were performed by correlation analysis. The data obtained in the study were expressed as means± SD. Sensitivity and specificity test for

testing a new test with ROC curve "Receiver Operating Characteristic". The level $P \leq 0.05$ was considered the cut-off value for significance.

RESULTS

The present study included 20 MI patients and 20 healthy individuals as controls group. All patients were subjected to full clinical evaluation, revision of their filed clinical progress, radiological and laboratory data. In these clinical settings, we aimed to assess the effect of anticoagulant treatment in patients having thrombotic accidents for better monitoring of anticoagulant therapy using the flowcytometer to identify the origin of the MP so it can be used to monitor the response to treatment. Regarding age, there was no statistically significant difference between cases and controls, $P > 0.05$ (mean ± SD: 61.1 ± 12.7 vs. 59.7 ± 10.3). Also, there was no statistically significant association between sex and study groups, $P > 0.05$ which indicated proper matches between groups.

Our results showed that the patients had a higher value than the control for CD 62E/61 and CD 61 (mean ± SD: 22.39±9.41 vs. 14.75±4.82, and 62.12±14.25 vs. 53.55±5.28) for coexpression of both markers CD62E/61 and CD 61 respectively, with statistically significant difference ($P<0.05$). Regarding CD 62E, although patients had a higher value than control (mean ± SD: 0.040±0.043 vs. 0.035±0.037) but the difference was not statistically significant, ($P > 0.05$) as shown in table (1) and figures (1, 2, 3 and 4).

Table (1): Differences in markers before intervention (at time of diagnosis) according to study groups

Variable	Cases (N=20)	Controls (N=20)	P-value
	Mean ± SD		
CD 62E/61	22.39±9.41	14.75±4.82	**0.003***
CD 61	62.12±14.25	53.55±5.28	**0.019***
CD 62E	0.040±0.043	0.035±0.037	0.666

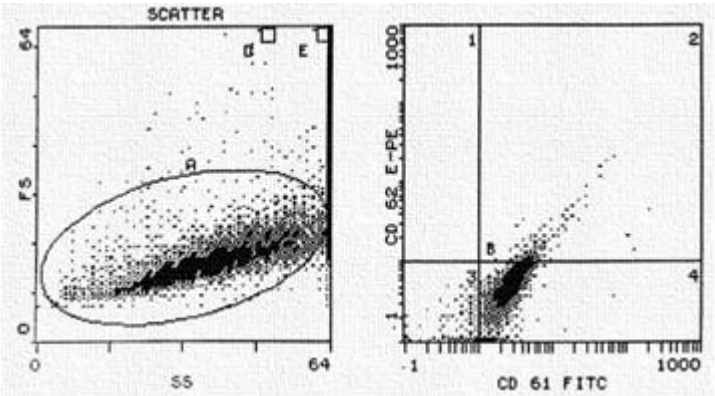

Fig. (1): A histogram shows CD61 FITC positive events represents PMPs and shows CD62E PE positive events which represent EMPs of one of the cases group.

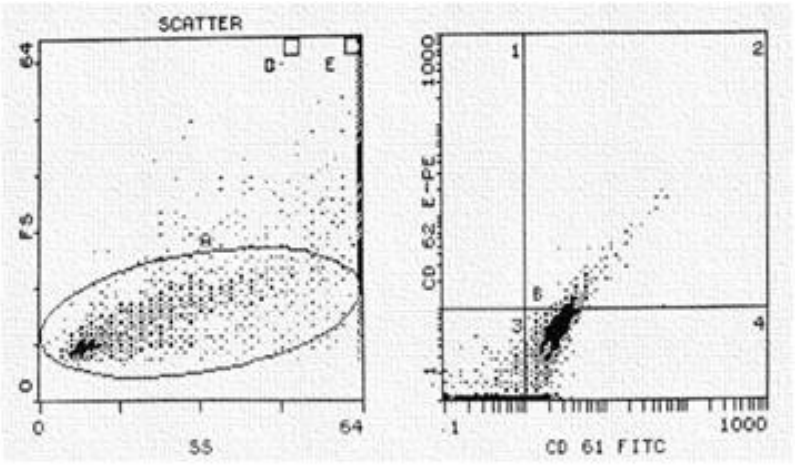

Fig. (2): A histogram shows CD61 FITC positive events represents PMPs and shows CD62E PE positive events which represent EMPs of one of the controls group.

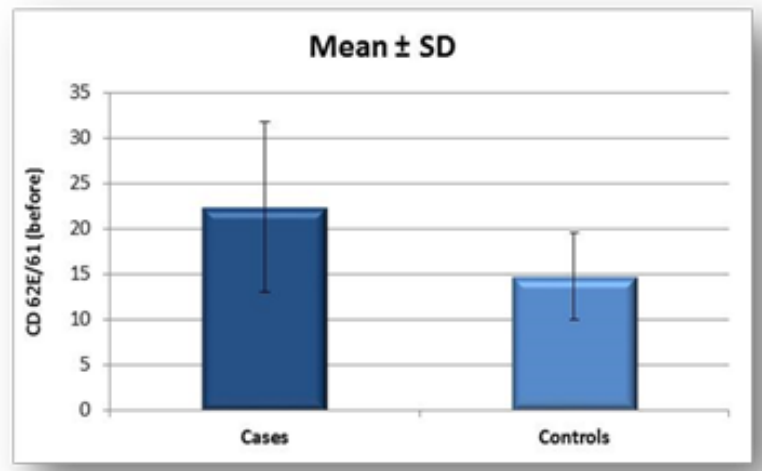

Fig. (3): Differences in CD62E/61 before intervention according to study groups

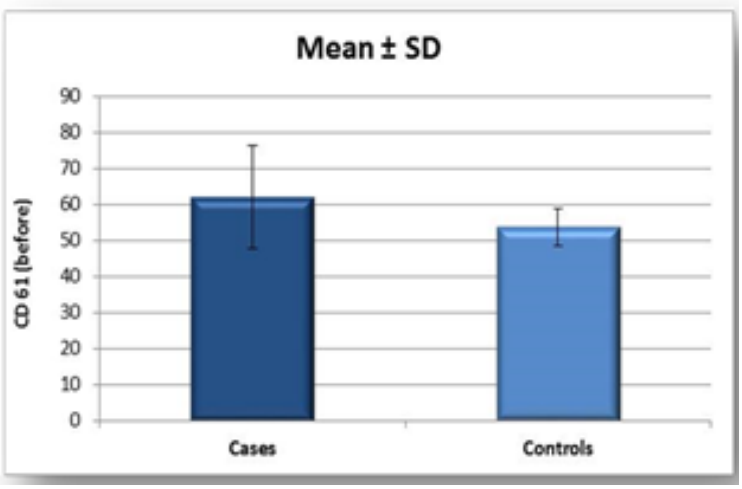

Fig. (4): Differences in CD61 before intervention according to study groups

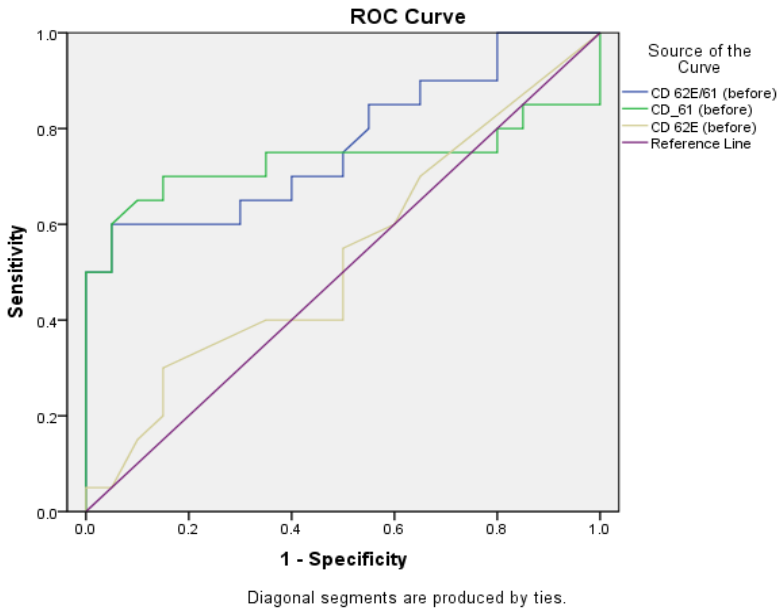

Fig. (5): ROC curve of microparticles as markers in differentiating cases from control

As regards CD 62E/61, taking 22.25 as a cut-off point revealed (60%) sensitivity and (95%) specificity. Regarding CD 61, taking 58.70 as a cut-off point revealed (70%) sensitivity and (85%) specificity. On the other hand, taking 0.025 as a cut-off point for CD 62E indicated (55%) sensitivity and (50%) specificity, figure (5). Table (2) reveals that there was no statistically significant improvement in MP markers following intervention in all cases (p >0.05).

Table (2): Differences in markers before intervention (at time of diagnosis as MI) and after a month of surviving the MI in all cases

Variable	Before	After	P-value
	Mean ± SD		
CD 62E/61	22.39±9.41	21.72±7.77	0.444
CD 61	62.12±14.25	61.18±13.18	0.830
CD 62E	0.040±0.043	0.034±0.037	0.660

DISCUSSION

Microparticles (MPs) are vesicles less than 1 µm in diameter shed from the plasma membranes of cells that are injured, activated, or undergoing apoptosis (Boulanger, 2010). Microparticles are heterogeneous, differing in size, as well as in phospholipid and protein composition. In addition, microparticles display some specific cell surface proteins that indicate their cellular origin. The investigation into their biological activity has revealed diverse actions in coagulation, cell signaling and cellular interactions (Horstman *et al.*, 2004). The complex role of microparticles in vascular accidents is an area of immense interest that promises to yield

important advances into diagnosis and therapy. Apart from providing physiological procoagulant activity, microparticles may play an important procoagulant role in several diseases especially in thrombotic accidents.

The aim of the current study was to assess the effect of anticoagulant treatment on microparticles in patients having thrombotic accidents for better monitoring of anticoagulant therapy.

The study included 20 MI patients with 20 age and sex-matched controls. All patients were experiencing typical chest pain with criteria of cardiac origin, diagnosed as myocardial infarction from the cardiac care unit of Fayoum university hospital.

In addition assay of circulating, microparticles for both quantitation and determination of cell of origin using flowcytometry technique was performed. Flow cytometry is the method mainly used for microparticle analysis, having the ability to detect its cellular origin by specific antibodies (Poncelet *et al.*, 2015).

In the current study flowcytometry assay of circulating microparticles in studied cases revealed that the most common type of microparticles present in thrombotic MI was platelet-derived microparticles (62.12%), microparticles coexpressing both platelet and endothelial markers (22.39%), and endothelial-derived MP (0.040%). Also, in healthy control subjects of the present study platelet markers (53.5%) exhibited the highest level of expression followed by microparticles coexpressing both platelet and endothelial markers (14.75%) and the least were endothelial microparticles (0.035%).This is in agreement with other reports, in which the majority of microparticles in healthy controls were of platelet origin, followed by those of endothelial origin (Tesselaar *et al.*, 2007; Vasina *et al.*, 2013).

Other authors stated that Circulating endothelial derived MP are detectable in the plasma of healthy subjects and their amount increased under pathological conditions associated with increased thrombotic risk and endothelial dysfunction (Bulut *et al.*, 2009; Nozaki *et al.*, 2009; Rautou *et al.*, 2011).

High levels of platelet-derived MP have been reported in a study of platelet microparticles in survivors of myocardial infarction (Michelsen *et al.*, 2008).

In this study, the authors concluded that the observation of an independent association between PMPs and thrombin generation may provide evidence for a procoagulant role of platelet microparticles in MI.

Stepien *et al.* 2012 reported that the increased generation of MPs from platelets and endothelial cells. It suggests that interactions between platelets and endothelial cells play an important role in the pathogenesis of myocardial ischemia, these observations support the concept that platelet activation, and formation of PMPs in particular, plays an important role in the thrombotic process.

As stated before, coexpression of both platelet and endothelial markers on MP was a common feature in the present study being expressed in 22.4% 0f cases. Only a single report of coexpression of platelet and endothelial markers was found in a study by Marijke *et al.* 2009. The authors described the finding as remarkable and stated that These CD62E-positive microparticles were not normal endothelial microparticles since they co-expressed platelet markers as well. An explanation for this double positivity was suggested to be an interaction between platelets (or platelet fragments) and endothelial cells resulting in cellular activation and generation of microparticles of bilineage origin (Marijke *et al.*, 2009).

Comparing MP assay data between results of samples taken from patients at the time of diagnosis as MI before intervention versus samples taken 4 weeks after surviving the MI

which revealed that there was no statistically significant difference in microparticles after a month of therapy and surviving the thrombotic accident. This may be due to the short duration of therapy after the occurrence of the accident. These results were in agreement with Chiva-Blanch *et al.* 2017 who found that no statistical differences in the number of circulating MPs from any cell origin were observed between samples taken at 2 or 8 weeks after the AMI.

Most studies of diagnostic tests evaluated only their accuracy. Although such studies describe how well tests identify patients with the disease (sensitivity) or without the 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 a part of broader management strategy (Ferrante di Ruffano *et al.*, 2012).

In the current study, Receiver operating characteristic (ROC) curves were used to determining a cutoff value for expression of CD61p as a marker of thrombotic MI.

The suggested cutoff values are 58.7, 22.25 for CD61 and CD 62E/61 respectively in the present study. Other MP assay markers showed an overlap between patients and control and calculation of cutoff values were not possible.

Establishing a cutoff value for coexpression of platelet and endothelial markers on circulating MP and significant elevation of this marker in MI patients especially those with history of hypertension can contribute to the clinical application as using MP assay in diagnosis of thrombotic propensity, monitoring of anticoagulant therapy, and detection of risk of myocardial infarction and coronary heart diseases.

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

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