Study of Platelet and Endothelial Microparticles in Patients with Type 2 Diabetes Mellitus

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Abstract: Microparticles (MP) are membrane nano-fragments with a diameter of 0.05-1 μm and an intact vesicular appearance. MPs are produced by various circulating cells and also by endothelial cells after their activation or apoptosis. MPs, including both the platelet microparticles (PMP) and endothelial microparticles (EMP) are considered bioactive because of their crucial role in coagulation, inflammation and activation of angiogenesis. The purpose of the current study was to characterize circulating platelet and endothelial MPs in patients with type 2 diabetes mellitus (T2DM) compared to healthy controls, in order to prove their role as predictive marker of vascular complications in these patients. To achieve this aim, detection of PMP and EMP by flow- cytometer was done using anti CD62E, anti CD62P, anti CD31and anti CD42b monoclonal antibodies. PMP was defined as CD31+/42b+ and CD62P + while EMP was defined as CD31+/42b- and CD62E+. The study involved enrollment of 30 diabetic patients divided in 2 groups , group I included 15 diabetic patients with no vascular complication ,and group II included 15 diabetic patients with known cardiovascular complication .Fifteen age and sex matched healthy volunteers were included in the study as a control group . PMP and EMP were significantly higher in diabetic patients compared to the control group with no significance difference detected between the 2 diabetic groups. CD62E/CD31+42b- ratio was ≤ 1 suggesting an apoptotic mechanism of EMP generation in diabetic individuals. The significant correlation between EMP (CD62+) and HbA1c indicated that the uncontrolled diabetic patients might be more prone to develop vascular injury and endothelial dysfunction, also the positive significant correlation detected between low density lipoprotein (LDL) and EMP(CD31+/CD42-) points to the importance of LDL in inducing arterial stiffness releasing EMP. These results can point to the importance of PMP and EMP as markers for haemostatic activation, and development of thrombotic events.

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1. Introduction:

Cellular microparticles (MPs) are defined as plasma membrane fragments that are shed by almost every cell after being subjected to a number of stress conditions, including both cellular activation and apoptosis (1), (2). Since the description of "platelet dust" by **Wolf** (3), numerous studies had worked on these sub cellular vesicles and reported their presence in centrifuged plasma. They have crucial roles in coagulation, cellular signaling, vascular injury and homeostasis (4). Attention has been drawn towards them as biomarkers for various diseases (5).

According to research conducted by **Jy et al** (6), MPs are small in size and "bear surface membrane antigens reflecting their cell of origin". This is why MPs that arise from the cellular components of blood and the endothelial lining of blood vessels are commonly named blood MPs because they expose the anionic phospholipid (PL) on the outer leaflet of their membrane. **Freyssinet et al** also went farther into other areas and noted that "apart from blood MPs, plasma contains smaller membrane vesicles (40–100 nm) termed exosomes and larger vesicles (>1.5 μm) termed apoptotic bodies that are derived from blood and vascular cells" (7).

Understanding the causes of mortality cases across all diabetic patients is paramount in combating its complications. Type I has its own set of features that may be somewhat different from Type II diabetes, not to mention the complications associated with each type, microangiopathy is considered a common finding in both types of diabetes mellitus. However, in type II diabetes, macroangiopathy is more prevalent. (8). However, the traditional risk factors such as obesity and hypertension remain the root cause of either type.

Hyperglycemia has a dominant role in development of both micro- and macroangiopathy in diabetic patients as it is the cause of an accumulation of "advanced glycation end products" (9). It has also been proven that it contributes to a heightened oxidative stress level which is responsible for early alterations of endothelial function. Considering type 2 diabetes, hyperlipidemia and hypertension are also

responsible for endothelial damage that leads to atherosclerosis progression (9).

Atherosclerosis is a common feature in diabetic patients due to activation of virtually all blood and vascular cells .As evidenced by increased expression of adhesion molecules such as CD11b/CD18 or CD11c on polymorphonuclear cells (10). Furthermore, increased tissue factor expression through the interaction between endothelial cells and monocytic cytokines induce activation of the coagulation cascade, thus it has been suggested as an indicator "biomarker of diabetic macroangiopathy" (11). The thrombotic tendency of platelets smolder in the expression of several types of adhesion molecules "P-selectin, thrombospondin, CD62, CD63, and activated glycoprotein IIb/IIIa" (12) in which they circulate in type1 diabetes manifesting the signs of vascular complications.

MPs can prompt pro-inflammatory responses in target cells and hence can promote thrombosis in diabetic patients. Accordingly, new insights in the pathogenesis and treatment of vascular complications of diabetes can be achieved (13). The aim of this study was to characterize circulating platelet, endothelial MPs in type 2 diabetic patients compared to healthy controls, in order to shed light on their role as predictive and diagnostic markers of vascular complications in these patients

2. Subjects and Methods:

The present study was conducted in Kasr El Aini Hospitals; Cairo University over a period of 6 months starting from September 2009 to March 2010 on 30 diabetic patients together with 15 healthy normal volunteers. Informed consent was obtained from all the participants prior to the study accompanied by detailed explanation of the procedure and its outcome, and this study was carried out in accordance to the guidelines approved by the Ethics Committee , Kasr Al Aini Hospitals , Cairo University.

The study involved enrolment of 30 (type 2) diabetic patients according to the WHO diagnostic criteria. They were 12 males (66.6%) and 18 females (33.3%). Their ages ranged between 28 and 72 years with mean± of 51.37±9.08 years. These patients were subdivided into 2 groups: **Group 1**: included 15 diabetic patients who were free from any vascular complications and not on any specific therapy, other than the hypoglycemic drugs. **Group 2**: included 15 diabetic patients suffering from vascular complications (cardiovascular diseases) in the form of unstable angina or myocardial infarction for CABG operation, the patients were on hypoglycemic therapy in addition to lipid lowering drugs and aspirin.

Fifteen healthy individuals matched for age and sex, with normal fasting and 2 hours post prandial (PP) glucose levels were also included as a control group. These individuals were healthy normal volunteers. They were 5 males (33.3%) and 10 females (66.7%).Their ages ranged between 20 and 50 years old with mean value ± SD equal to 30.15±8.61 years.

Detection of EMP by flow-cytometry using anti CD62E, anti CD31 and anti CD42b monoclonal antibodies and PMP using anti CD62P, anti CD31 and anti CD42b monoclonal antibodies were done to both groups.

Two ml of venous blood from patients and control subjects were withdrawn on 3.2% sodium citrate in vacutainer tubes under complete aspetic conditions. Platelet rich plasma (PRP) was prepared immediately after venipuncture by centrifuging whole blood at 160 g for 10 minutes, the PRP was separated in another tube, then samples recentrifuged at 6000 rpm for 5 minutes to obtain platelet poor plasma (PPP), after centrifugation samples were evaluated with the coulter counter for the presence of other cells, this step validated that the platelet count of $< 1 \times 10^3$ /µl and other cells were absent from the sample. The PPP was then frozen at – 40 ºc (14).

At time of analysis, frozen samples were thawed in 37 ºc water bath for 5 minutes then vortexed for assay of platelet and endothelial microparticles by flow cytometry. Repeated freezing-thawing cycles had been avoided, as this may give rise to falsely elevated levels of microparticles in the sample (15). 5 µl of PE-conjugated anti CD31 monoclonal antibody and 5 µl of FITC-conjugated anti CD42 monoclonal antibody were added to 50 µl of PPP in a tube, and in another tube 5 µl of PE-conjugated anti CD62P monoclonal antibody and 5 µl of FITC-conjugated anti CD62E monoclonal antibody were added to 50 µl of PPP in a tube. Tubes were incubated in the dark for 30 min at 4 °C and diluted with 500 ml of filtered phosphate-buffered saline (PBS). Flow-Count Fluorospheres were mixed by a vortex for 10 - 12 seconds. Fifty ul of flow - count™ fluorospheres (flow beads of standard size) were added and mixed by vortex for 5 seconds immediately before analysis by flow cytometry. As a control for analysis, 50 µl of PPP in a separate tube free from CD 42, CD62 P, CD31, and CD62E were diluted with 500 ml PBS.

Plasma samples were measured using EPICS XL-MCL Beckman coulter, a logarithmic scale was implemented for forward scatter signal, side scatter signal and each fluorescent channel. The proper protocols for each monoclonal antibody were loaded and used for its interpretation. Control sample was introduced for (each sample) in the machine and

forced in the sheath by the sample pressure "run button" to adjust auto fluorescence region.

The fractions of microparticles coated by monoclonal antibodies were determined inside the gated population of microparticle. Data acquisition was stopped after 10,000 fluorospheres were counted. The absolute count of PMPs and EMPs were automatically calculated by the following equation which was used according to **Baran et al** (16)

$$\text{Microparticles} = \frac{\text{No. of events in region containing MP}}{\text{No. of events in absolute count bead region}} \times \frac{\text{No. of beads per test}}{\text{Test volume (µl)}}$$

3.Results:

Medical history of the diabetic patients revealed that 2/15 (13.3%) of group I and 5/15(33.3%) of group II were smokers. Clinical examination revealed that 5/15 (33.3%) of group II diabetic patients were hypertensive, while no hypertension was detected among patients in group I 0/15(0%).

As regard treatment of diabetic patients, 13/15 (86.5%) of group I and 1/15 (6.7%) of group II were on oral hypoglycemic, while 2/15(13%) of group I and 14/15 (93.3%) of group II were on insulin therapy .Moreover, all patients in group II were on lipid lowering drugs and Aspirin therapy, while no one in group I was on other specific therapy.

Table (1): The statistical comparative study of FBS and HbA1c among the studied groups:

Parameter	Control (n=15)	group I (n=15)	Group II (n=15)
FBS (mg/dl)	96.20±9.41	149.20±61.10*	163.93±73.47*
HbA1c	5.12±0.33	9.41±2.06*	9.26±1.54*

* Statistically significant from control group ($p<0.05$).

On statistical analysis, serum cholesterol, and LDL levels were significantly higher in both group I and group II compared to the control group. Serum triglycerides levels were significantly higher in group II compared to the control. While HDL levels were significantly lower in both group I and group II compared to the control group. No significant difference was detected regarding these parameters among both diabetic groups.

In addition, Risk ratios (total cholesterol/HDL and LDL/HDL) were significantly elevated in both group I and group II compared to the control group, without detected comparative significance between both diabetic groups (Table2).

Table (2): the statistical comparative study of the lipid profile and high risk ratio among the studied groups:

	Control (n=15)	group I (n=15)	Group II (n=15)
Chol.(mg/dl)	167.73±15.82	236.27±40.20*	230.07±63.11*
TG (mg/dl)	122.20±35.13	158.13±66.49	203.47±101.04*
HDL(mg/dl)	48.60±6.51	29.00±6.29 *	31.80±8.13 *
LDL (mg/dl)	94.16±12.79	175.48±33.84*	165.23±55.61*
Chol./HDL	3.37±0.52	8.50±2.37 *	7.32±1.33 *
LDL/HDL	1.95-0.29	6.34±1.99 *	5.20±1.09 *

* Statistically significant from control group ($p<0.05$).

Platelet MP, measured by CD31+/CD42+, CD62P and endothelial MP, measured by CD31+/CD42- ,CD62E+ were significantly higher in diabetic patients compared to the control group. CD62P+ and CD62E+ microparticles show higher significant difference between group II and the control group but no significant difference is found between group I and the control group (Table3, Figs 1-4).

A positive correlation between CD62E and HbA1c was detected in group II ($r=0.539$, $p=0.38$) (Fig 5). In addition, there was a significant positive correlation between LDL and CD31+/CD42- in the control group ($r=0.532$, $p=0.41$) (Fig 6) and a significant positive correlation between CD62E and CD62P among diabetic patients ($r=0.538$, $p=0.002$) (Fig 7).

Table (3): The statistical comparative study of PMPs and EMPs between control and diabetic patients:

Microparticles (count// u l)		Studied groups		P- value
		Control group (n=15)	Diabetic patients (n=30)	
CD42b + / CD 31+	Mean+ SD	33.67±7.8	75.4±44.2	*
	Median (range)	33 21-46	67 17-155	
CD 62 P+	Mean+ SD	36.8±15.7	78.9±62.3	*
	Median(range)	39 13-60	61 14-250	
CD42b - / CD 31+	Mean+ SD	28.5±8.5	173.8±82.5	*
	Median (range)	31 17-44	158.5 42-330	
CD 62 E +	Mean+ SD	26±10.5	61.5±80	
	Median(range)	25 13-45	39.5 14-440	*
CD 62 /CD31+,42- (Ratio)	Mean+ SD	0.96±0.52	0.33±0.25	*
	Median (range)	0.8 0.38-2.5	0.27 0.05-1.21	

*Statistically significant from control group (< 0.05)

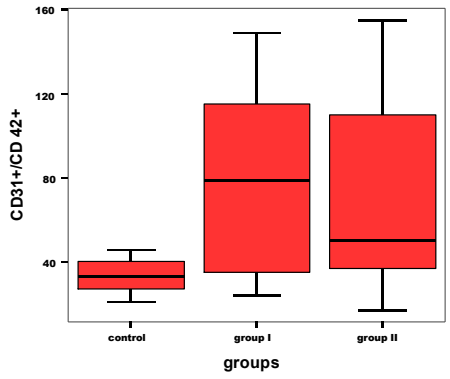

Fig (1): CD31+/CD42b+ (Count/ul) in the studied groups:

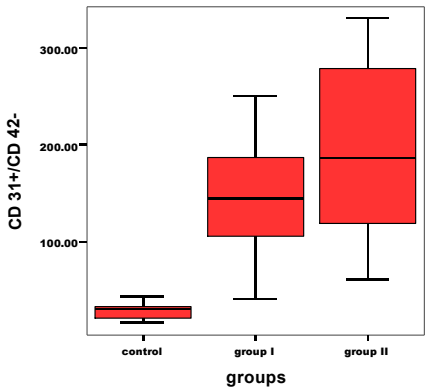

Fig (3): CD31+/CD42b- (Count/ ul) in the studied groups

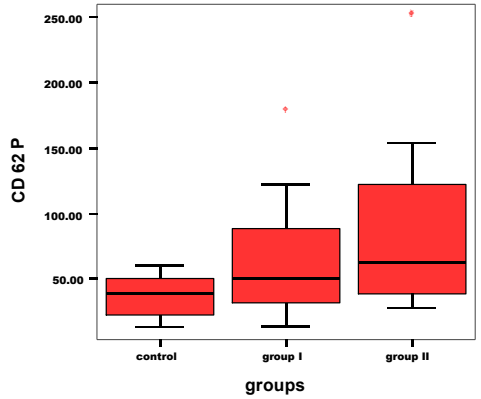

Fig (2): CD62P (Count/ul) in the studied groups

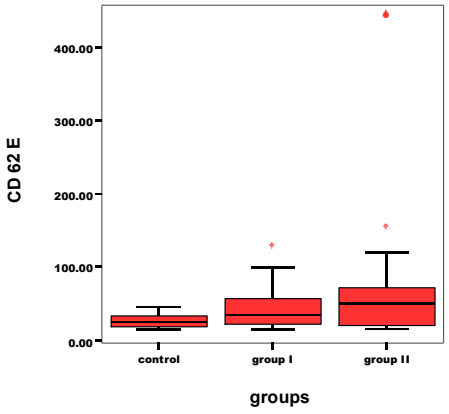

Fig (4): CD62E (Count/ul) in the studied groups

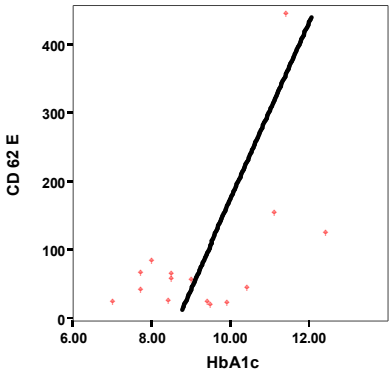

Fig (5): Correlation between CD62E and HbA1c in group II:

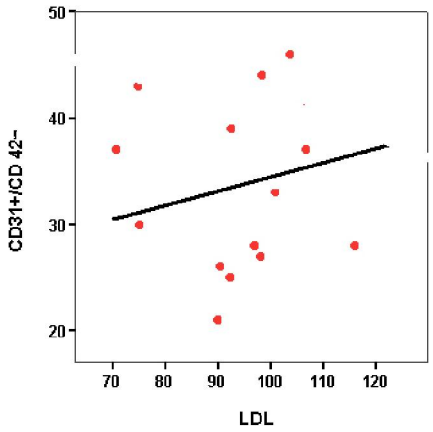

Fig (6): correlation between LDL and CD31+/CD42b- in control group:

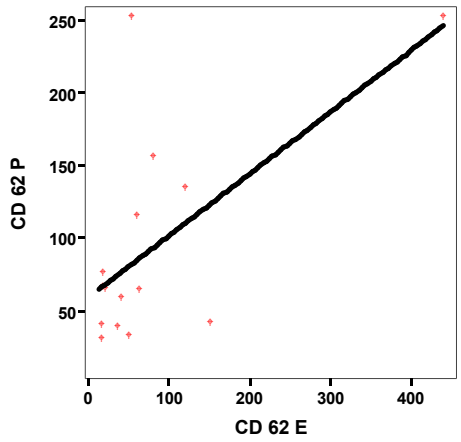

Fig (7): Correlation between CD62P and CD62E in both group I and group II:

4. Discussion:

T2DM is considered highly prevalent diseases nowadays and the urge for management of diabetic thrombophilia becomes one of the highest public health priorities nowadays (17). Alteration in Virchow's triad is the main feature of T2DM which harbor a combination of pro-inflammatory and hypercoagulable states that define the pathology of the disease (18).

Only small amounts of microparticles can be detected in healthy people but their circulating levels can be strikingly increased in many pathological conditions including diabetes, coronary artery disease, hypertension and metabolic syndrome which suggest the involvement of MPs in multiple patho-physiological events such as thrombosis, inflammatory conditions and atherogenesis (19).

Results obtained in this study revealed that Fasting blood glucose level and HbA1c were significantly higher in both diabetic groups (group I and group II) compared to the control group. Large clinical studies have demonstrated that hyperglycemia plays an important role in the pathogenesis of microvascular complications in both type I and type II diabetes (20), Also hyperglycemia is responsible for the non-enzymatic glycation of platelet membrane proteins leading to changes in protein structure and conformation. High HbA1c levels, which are defined as markers of poor glycemic control, strongly correlate with platelet expression of P-selectin and CD63 (21).

The current study detected that the levels of cholesterol, triglyceride, LDL, Chol/HDL ratio, and LDL/HDL ratio, were significantly elevated in both diabetic groups compared to the control group. As regards HDL, it was significantly higher in the control group compared to both diabetic groups. However no statistically significant difference in the levels of the lipid profile parameters was observed between the two diabetic groups.

A state of progressive vascular endothelial damage and inflammation is considered a constant feature of metabolic syndrome which is defined as cluster of many risk factors such as hypertension, dyslipidaemia, obesity, insulin resistance, hyperinsulinaemia and impaired fibrinolysis (22). The disturbed ratios between Chol/HDL and LDL/HDL especially if above 5.0 can cause deposition of fat in the microvascular circulation of the heart, brain and kidney (23).

The study of MPs was done using flow cytometric assay. PMP were defined as CD31+/CD42+ , and by the platelet activation marker CD 62P+, while EMP were defined as CD31+CD42- , and by endothelial activation marker CD 62 E+ (24).

The obtained results showed that the circulating levels of PMP (CD31+/42b+ , CD62P+) were significantly elevated in type 2 diabetic patients (both group I and II) compared to the control group (p=0.002,p= 0.009 respectively). This is in accordance with **Tan et al** (25) study which is the first to report that the presence of symptomatic atherosclerosis is associated with higher plasma levels of PMPs in patients with type 2 diabetes; this study is the only one which provides support to the study (26), who found an increase in ex vivo platelet reactivity to calcium ionophore as measured by PMP release in patients with type 2 diabetes.

Our results were different from **Sabatier et al** (9), who showed that a significant elevation of PMP occurs in type 1 diabetes, while no significant increase was observed between type 2 diabetic patients and their age-matched control subjects.

In this work, results revealed a significant increase in the circulating levels of EMP (CD31+/CD42b- and CD62E) in diabetic patients (both group I and II) compared to the control group (p<0.001, p=0.036 respectively). This is in accordance with **Meigs (27)** who identified elevated levels of 62E+ (E-selectin) EMPs in type 2 diabetic patients that trended towards significance. E-selectin is also expressed in activated endothelium and was found to be an independent predictor of DM. **Tramontano et al.** (28) demonstrated that DM is associated with increased levels of circulating EMPs. This corroborates with previous studies where levels of EMPs were associated with microalbuminuria and microvascular complications in patient with T2DM suggesting that EMPs could be a marker of the diabetes-associated endothelial dysfunction, they also found that circulating levels of CD31+ EMPs were significantly increased in patients with DM compared to non-diabetic control patients.

However, in our study, we did not find any statistically significant difference in the levels of endothelial and platelets microparticles between group I and group II diabetic patients. This is in accordance with **Tramontano et al** (28) who found no significant relation of EMP levels with the presence or severity of CAD. On the contrary the study of **Tan et al** (25), found difference between both groups, as they confirmed that the presence of symptomatic atherosclerosis is associated with higher plasma levels of MPs, and this controversy could be attributable to small numbers in each diabetic group which hampered reaching a definite conclusion.

Moreover, our study showed that although the difference between the two diabetic groups was statistically insignificant, we noticed that EMPs levels showed considerable elevation, compared to the humble elevation in the levels of PMPs between the patients group, this may point to an intimate relation and the important role of EMP in endothelial dysfunction / injury in type 2 diabetic patients.

As regards the ratio of [CD62E+ to CD31+CD42b-] EMP populations, which represents the ratio of MP produced by endothelial activation (CD62) to that produced via apoptotic stimuli (CD31+CD42b-), we found that it was significantly lower in all the diabetic patients on comparison with the control group (p<0.001).This finding clarify that the circulating levels of CD31+/CD42b- showed significant increase in their levels compared that of CD62E. However, we could not find statistically significant difference of this ratio among group I and group II diabetic patients.

Tramontano et al (28) who suggested that analysis of EMP phenotypic profile may provide clinically useful information on the status of the endothelium. In order to distinguish between cellular activation and apoptosis he utilized the ratio of CD62E+/CD31+ EMP populations, instead of using only absolute numbers. A ratio ≥10 identifies activation while ratio ≤1.0 suggests apoptosis. Our data suggest that apoptosis may be important stimulants of EMP release in the DM population.

In this study, significant positive correlation was detected between HbA1c and CD62E+ microparticle levels among diabetic patients with cardiovascular diseases(group II) (r=0.539,p=0.38) which indicates that patients with uncontrolled diabetes mellitus are prone to vascular injury and endothelial dysfunction. We also found a positive significant correlation between LDL and EMPs (CD31+/CD42b-) among the control group**.** This result is in accordance with **Nomura et al** (26) who suggested that oxidized LDL could contribute to the endothelial membrane vesiculation**.**

Pirro et al (24) showed that hypercholesterolemic patients have higher circulating CD31+/CD42– MPs than normocholesterolemic subjects , this was the first observation that hypercholesterolemia may contribute to large artery stiffness both by increasing the release of microparticles that are mainly of endothelial origin and by reducing the number of circulating endothelial progenitors.

A study done by **Tsimerman et al** (29) showed that the highest levels of platelet, endothelial and negatively charged phospholipid-bearing MPs were seen in patients with severe diabetic foot ulcers. This demonstrated that MP characteristics might serve as a bio-marker for the pro- coagulant state and vascular pathology in patients with T2DM.

Accordingly, the results obtained in this study revealed the presence of significant increase in PMP (CD42+/31+&CD62P+) and EMP (CD42-

/CD31+&CD62E+) values in the diabetic patients compared to the control group. The ratio between the endothelial activation and apoptotic markers (CD62+/CD31+&CD42-), showed significant reduction of its value compared to the control group denoting the occurrence of endothelial injury and apoptosis accompanying the associated atherosclerosis.

The significant correlation between EMP (CD62+) and HbA1c indicates that the uncontrolled diabetic patients might be more prone to develop vascular injury and endothelial dysfunction, also the positive significant correlation detected between LDL and EMP(CD31+/CD42-) points to the importance of LDL in inducing arterial stiffness releasing EMP . Although, no significant difference was detected between diabetic patients without and those with vascular complication, yet it is worthy to follow up the patients having high values of MP in order to avoid the ongoing damage of the vascular wall.

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