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Increased Level of Circulating Endothelial Microparticles and Cardiovascular Risk Factors

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Abstract

Background: Formation of microparticles is part of normal cellular function. The number of microparticles increases in conditions that cause cell stress and is the marker of exposure of endothelial cells to unfavorable plasma molecules. In our study, we analyzed contribution of different risk factors (lipoproteins, triglycerides, cholesterol, and high glucose) in development of the increased level of endothelial microparticles.

Methods and Results: We included thirty-five participants who did not have the history of cardiovascular disease, diabetes, chronic inflammatory disease or cancer.

Lipid profile, glucose, C-reactive protein, circulating endothelial microparticles (EMPs) and platelet microparticles (PMPs) were measured for all subjects.

Microparticles in plasma samples of participants were phenotypes and quantified by flow cytometry. Measurements of the lipid profile, C-reactive protein and glucose were performed by established clinical laboratory tests.

The levels of circulating EMPs were compared to a variety of cardiovascular risk factors. As the result of our analysis, we found that the number of endothelial microparticles was elevated for subjects with increased level of cardiovascular risk factors such as cholesterol to HDL ratio, LDL to HDL ratio and the level of C-reactive protein.

Elevated number of endothelial microparticles was measured in subjects with increased level of glucose. In addition, the level of platelet microparticles was affected by the increased triglyceride and cholesterol content in plasma.

Conclusion: As the result of our analysis, we demonstrated that apoptotic microparticles were elevated at conditions of elevated risk factors. The increased level of EMPs in circulation might be an early indicator of endothelial dysfunction and cardiovascular risk.

Keywords: Endothelial microparticles; Cardiovascular risk factors; Triglycerides; Cholesterol; C-reactive protein

Introduction

Cellular microparticles are small plasma membrane vesicles composed primarily of lipids and proteins. Microparticles (MP) are heterogeneous, differing in size, phospholipids, and protein composition. They contain surface proteins that indicate their cellular origin, and their surfaces may also contain phosphatidylserine, a protein that provides a suitable site for tenase and prothrombinase complex assembly. Microparticles may also express tissue factor, a primary initiator of coagulation [1]. Microparticles are different from exosomes, since these latter are smaller and derived from endocytic compartments. Microparticles are formed as part of normal cellular function [2], they help maintain homeostasis, but their formation is increased when cells are stressed. In healthy subjects, most (> 90%) microparticles are of platelet origin, while fewer than 10% originate from granulocytes, endothelial cells, red blood cells or monocytes [3,4]. If produced in excess or when carrying pathogenic constituents or inflammatory signals, microparticles can initiate deleterious processes [5].

Endothelial cell microparticles (EMPs) were first described as derived from human umbilical vein endothelial cell (HUVEC) membranes [6] and induced by the activation of these cells with TNF-α [7]. They are shed from mature endothelial cells on stimulation by activating agents or during apoptosis [8]. EMPs carry membrane proteins and phospholipids of the parent cell and can be differentiated from microparticles derived from leukocytes, erythrocytes, or platelets [9-11]. Their relevance in various pathological conditions has been studied with respect to their pro-coagulant properties and their major role in inflammation and vascular dysfunction. They circulate at low levels in healthy individuals, but undergo phenotypic and quantitative changes that may play a role in inflammatory diseases. Elevated circulation of EMPs indicates exposure of endothelial cells to unfavorable plasma molecules [12]. EMPs are an emerging marker of endothelial cell dysfunction: their circulating numbers are elevated in a number of pathologic states.

Patients with illnesses such as lupus anticoagulant, thrombotic thrombocytopenic purpura, preeclampsia, paroxysmal nocturnal hemoglobinuria, cardiovascular disease, arterial hypertension, hypertriglyceridemia and multiple sclerosis show elevated microparticle levels, consistent with conditions of endothelial cell stress [6,12-17]. Increased levels of EMP have been reported in a variety of pathological situations including thrombosis [18], atherosclerosis [18,19], renal failure [20], diabetes [21], and hematopoietic cell transplantation [22].

EMPs may play a biological role in inflammation, vascular injury,

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angiogenesis and thrombosis [23,24]. EMPs can initiate atherosclerosis by promoting endothelial dysfunction and arterial wall inflammation, and they also may contribute to plaque progression and rupture. They contribute to the pathogenesis of cardiovascular disease because of their pro-inflammatory effect and their ability to promote thrombosis and endothelial dysfunction [25].

It has been demonstrated that EMPs reduce the vitality of endothelial progenitor cells (EPCs). According to the study [26], microparticles from hypercholesterolemic patients caused a significant in vitro EPCs apoptosis and reduced their colony forming capacity. Negative correlation was found between circulating microparticles and the number of circulating EPCs. The exposure of cultured endothelial cells to microparticles decreased endothelial nitric oxide release by almost 60%. Circulating MPs can provoke vascular dysfunction by reducing available nitric oxide (NO) and increasing the levels of reactive oxygen species, thereby promoting oxidative stress [27].

Endothelial cell dysfunction is an important factor in the pathogenesis of artherosclerosis. Currently, attempts to assess the degree of endothelial cell apoptosis *in vivo* have focused on the measurement of soluble factors such as ICAM, vascular cell adhesion molecule, and E-selectins, with contradicting results [28].

In our study, we aim to determine if EMPs can be used as early markers of endothelial dysfunction and, therefore, a predictor of cardiovascular risk and prognosis. Our hypothesis is that endothelial damage starts in early stages of metabolic dysfunction, so that EMP levels will increase along with increases in cardiovascular risk factors. We examine this by determining the correlation between EMP levels and classical risk factors such as hypercholesterolemia, dyslipidemia and hyperglycemia in subjects without history of cardiovascular disease or diabetes. Based on our results, we suggest that measurement of circulating EMP levels may provide a new option assessing a patient's risk of cardiovascular illness.

Study population and methods

Thirty-five adults ages 23-74 were included in our study. Participants did not have history of cardiovascular disease, diabetes, chronic inflammatory disease or cancer. Subjects represented the part of medical facility, had active life style and body mass index in range 21-40. The study was approved by the Institutional Review Board committee, and informed consent was obtained from all subjects.

EMP counting assay

To quantify circulating EMP levels, blood was collected in 5 ml tubes containing 3.2% citrate. Plasma was prepared within 30 min after blood collection by centrifugation for 20 min at 1500 g at room temperature to prevent platelet disappearance and concurrent formation of platelet-derived microparticles. Then plasma was centrifuged at 13000g 2 min to obtain platelet free plasma. Microparticles in these plasma samples were phenotyped and quantified by flow cytometry as described in previous studies [29-31]. Briefly, 50 µl of platelet poor plasma were incubated with 5 µl of antibodies: anti CD31, CD42a, CD51, and CD62E (all from BD Pharmingen), CD41a (Ancell) and lectin from Ulex europaeus UEA-1 (Sigma). After staining, plasma was diluted by 350 µl of PBS with 10 mmol citrate. 50 µl of 5 µm AccuCount standard beads (Spherotech) were added to the sample before analysis to allow calculating of MP absolute values without possible variations in the cytometer flow rate. Samples were analyzed on a Coulter flow cytometer (Quanta, Beckman Coulter).

Detection of particles was set to trigger by side-scattering signal. Particles <2 µm from that histogram were analyzed on a second histogram to distinguish platelet microparticles (PMPs) from EMPs. The distinction was made by dual staining of PE–labeled anti-CD31 with FITC-labeled anti-CD42b or CD41. EMPs were defined as CD31+/CD42b- (high PE fluorescence with low FITC fluorescence) while PMPs were defined as CD31+/CD42+. The rationale of two-color method (CD31 and CD42b) was that significant CD31 receptors occur on both EMP and PMP, whereas CD42b is restricted to platelets, allowing discrimination between them.

To evaluate the degree of endothelial insult, and distinguish mild and reversible activation from irreversible apoptosis, measured by marker PECAM (CD31), plasma EMPs were labeled with the alpha v beta 3 integrin (CD51) and E-selectin (CD62E), which are activation–induced markers.

Microparticles were incubated with different fluorochrome antibodies or isotype immunoglobulin.

As a positive control, we used endothelial microparticles derived from HUVECs. The culture medium of HUVECs was analyzed using a protocol described in [32]. HUVECs were incubated with recombinant Plasminogen Activator Inhibitor-1 (PAI-1) at the concentration 10 ng/mL for 3-6 hours. Culture media were collected and cleared from cells and cell debris by centrifugation at 5000*g* for 10 minutes. Supernatant was immediately used for microparticle immunolabeling followed by flow cytometry analysis.

Assay of lipid profile

The fasting serum was used for measurements of the lipid profile (total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoproteins (LDL), triglycerides, very low-density lipoproteins (VLDV)), glucose, and C-reactive protein (CRP) by established clinical laboratory tests. Cholesterol, HDL cholesterol, and triglycerides were

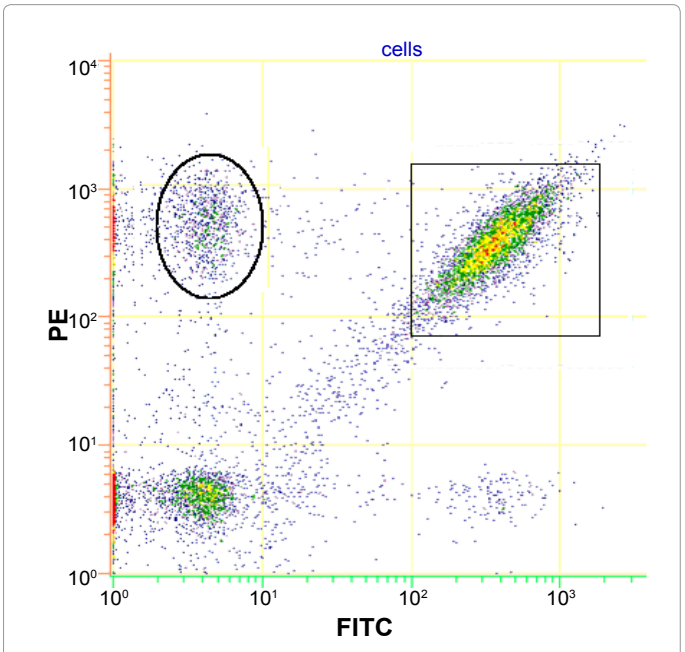

Figure 1: Flow cytometer output for circulating microparticles stained with anti-CD31 PE (vertical axis) and anti-CD42b FITC (horizontal axis) is shown in a two-color histogram.

	Min	Mean	Max	Normal (Upper)
Cholesterol (mg/dL)	123	199	273	200
Triglycerides (mg/dL)	46	124	366	150
HDL Cholesterol (mg/dL)	25	60	109	80
VLDL (mg/dL)	9	24	73	30
LDL (mg/dL)	48	115	174	100
Cholesterol/HDL Ratio	2	3.6	5.9	4.4
LDL/HDL Ratio	0.8	2.1	3.7	3.2
Platelet Count (K/ul)	200	287	426	–
C-Reactive Protein (mg/dL)	0.2	3.4	16.7	1.9
Glucose (mg/dL)	78	101	144	99
Systolic pressure mmHg	123.9	92	158	140
Diastolic pressure mmHg	73.8	58	96	90
Age	20	50	74	–

Table 1: Minimum, mean, and maximum values of several blood chemistry parameters and blood pressure along with the upper limit of the normal range for each. HDL = high density lipoprotein, LDL = low density lipoprotein, VLDL = very low density lipoprotein, and CRP =C-reactive protein.

Markers of EMPs	parameter	r	p-value
CD31+/CD41-	**HDL**	**-0.42**	**0.02**
	VLDL	**0.33**	**0.05**
	cholesterol/HDL	**0.46**	**0.002**
	CRP	**0.58**	**0.001**
CD62E	HDL	0.25	0.09 (NS)
	cholesterol/HDL	**0.43**	**0.01**
Lectin (UEA1)	CRP	0.25	NS
	glucose	**0.43**	**0.01**
	VLDL	0.64	0.2(NS)
	HDL/cholesterol	0.48	0.2(NS)
CD51	Cholesterol/HDL	0.222	NS
	VLDL	0.32	NS
CD41a	**HDL**	**-0.384**	**0.04**
	CRP	**0.4833**	**0.03**
	Cholesterol/HDL	**0.25**	**0.02**

Table 2: Correlation coefficients (r) and p-values for regressions between microparticles and tested values of lipid profile and glucose are given. NS = not statistically significant. Statistically significant values are bolded.

quantified by auto-analyzer by enzymatic method by using commercially available reagents (Genzyme Diagnostics). LDL cholesterol (in fasting samples) was determined by calculation. Level of CRP was determined by in vitro immunoassay (Genzyme Diagnostics) by detection on an automated clinical chemistry analyzer.

Statistical analysis

All data were analyzed by Systat software (Systat Inc) and KaleidaGraph software. Variables were presented as mean values ±SD, or as medians with corresponding 25th percentiles. Statistical significance was accepted if the null hypothesis could be rejected at $p<0.05$.

Results

An example of flow cytometry data on circulating microparticles from platelet-poor plasma is shown in Figure 1. Particles pre-selected based on side scatter and sizes are shown for CD31 and CD42b staining.

The circled section contains EMPs, and counts attained from this gate were used to determine the level of EMPs in plasma. The cluster in the upper right corner is PMPs, which highly outnumbers the EMPs.

EMP counts were compared to a variety of cardiovascular risk factors. Table 1 shows the average values, along with minimum and maximum values, for these factors in our sample. The data are summarized for high-density lipoproteins, low-density and very-low-density lipoproteins, triglycerides, C-reactive protein and glucose. The parameter values cover a wide range, and include both normal and 'abnormal' levels.

The EMP numbers were correlated with the risk factors listed above. In addition to correlation with microparticles determined as CD31+/CD42b- , we looked for correlation with CD62E, lectin, CD51 and CD41a positive microparticles. Correlation coefficients and p-values ($p \leq 0.05$) are shown in Table 2. Statistically significant correlations exist between circulating EMP numbers and HDL, VLDL, cholesterol/HDL, and CRP. The correlation with HDL was negative (increasing EMPs corresponded to decreasing HDL), while the EMPs show positive correlations with VLDL, cholesterol to HDL ratio. CD62E positive microparticles are considered to be caused by inflammation of endothelial cells. These were positively correlated with cholesterol levels. CD41a microparticles (considered of platelet origin) also correlated with cholesterol and CRP levels. No correlation with CD51 positive particles or lectin positive microparticles and the level of lipids in plasma was observed.

The correlations between EMPs and cholesterol and CRP levels are shown graphically in Figure 2, Figure 3 and Figure 4.

If subjects are divided into two groups based on LDL to HDL ratio, with an LDL/HDL of 2.5, which is accepted as a cutoff between the "healthy" group and the "average risk" group by the clinical laboratory risk classification [33], the difference in EMP levels in the two groups is statistically significant. These results are shown in Figure 5.

Discussion

The results of this study indicate a strong correlation between circulating EMP levels and cardiovascular risk parameters such as cholesterol levels and C-reactive protein levels in blood from healthy volunteers with no known cardiovascular issues. Our methods involved dual staining flow cytometry using PE labeled anti-CD31 with FITC labeled anti-CD42b or CD41, which allows separate PMPs from EMPs. The microparticles, which were defined as CD31+/CD42b- were considered released under the apoptotic stimuli and CD62E+ or CD51+ positive microparticles were caused by inflammation.

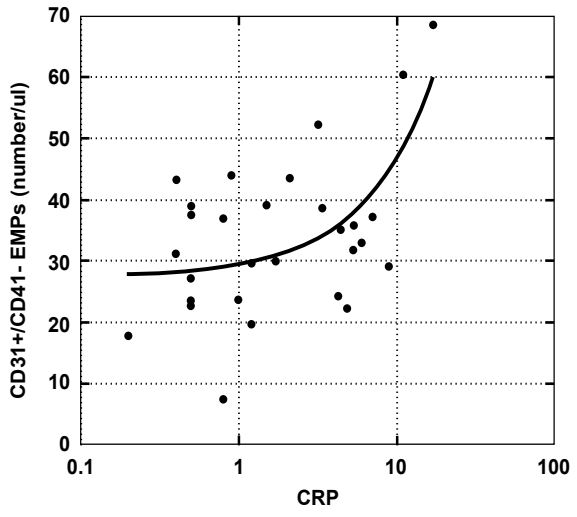

Figure 2: Numbers of circulating EMPs correlate with C-reactive protein.

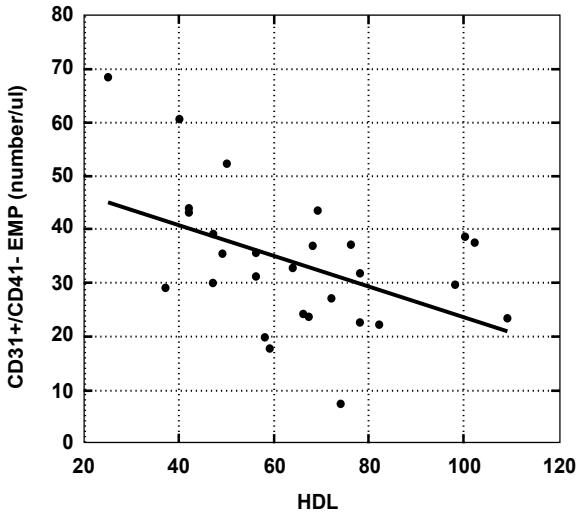

Figure 3: Inverse correlation of the numbers of circulating EMPs with high-density lipoprotein cholesterol (HDL).

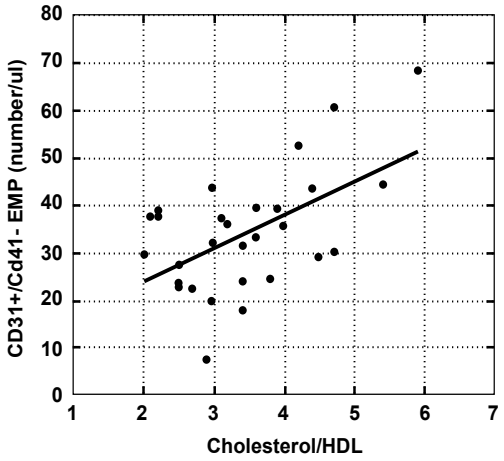

Figure 4: Correlation of the numbers of circulating EMPs with cholesterol to HDL ratio.

Triglycerides and lipoproteins are related to the impairment of endothelial function [34,35], and endothelial cell damage due to dyslipidemia plays a critical role in development and progression of atherosclerosis [36,37]. High-density lipoproteins, which transport cholesterol away from arteries, are protective. Low-density and very-low-density lipoproteins, in contrast, can penetrate the arterial wall and deposit cholesterol within the artery, contributing to heart disease.

According to literature, increase in triglyceride and cholesterol levels are associated with inflammatory state and enhanced production of tumor necrosis factor–a, interleukin -6, and C-reactive protein [38]. Triglyceride–rich proteins are able to induce inflammatory response and extensive retention of lipoproteins in the extracellular matrix and increased uptake by macrophages may initiate the atherogenic process [38].

Endothelial cell damage results in increased EMP levels, which in turn impair acetylcholine-induced vasodilatation and endothelial cell nitric oxide production [39]. In addition, previous studies have also demonstrated a procoagulant effect of EMPs by altering plasminogen activator inhibitor-1 levels [40]. It appears that EMPs represent a marker of endothelial dysfunction. Circulating MPs of endothelial origin may vary with respect to quantity and phenotype according to the endothelial response. For example, normal resting endothelial cells do not express E-selectin, but a soluble form of this molecule is released from activated cells [41]. Therefore, the presence of CD62E-positive microparticles may suggest endothelial activation.

The results of our analysis demonstrated that the circulating levels of CD31+/CD42b- EMPs are elevated for subjects with increased level of cardiovascular risk factors such as cholesterol/HDL ratio, LDL/HDL ratio and level of C-reactive protein. The correlation between cholesterol levels and EMPs in our study is consistent with that reported by Pirro et al. [26], who collected data at higher levels of hypercholesterolemia (serum level of low-density lipoprotein cholesterol >160 mg/dL) and the level of CD31+/CD42- microparticles. The results of this study demonstrated that hypercholesterolemic patients had more circulating CD31+/CD42- microparticles and less endothelial progenitors than controls. Our study shows correlations with a variety of cholesterol fractions and blood chemistry parameters.

Inverse correlation was found between HDL and CD31+/CD42b- circulating endothelial microparticles. Comparison of E-selectin-exposing EMP (CD62E positive) in plasma samples of subjects, with the level of serum lipoproteins showed correlation for cholesterol/HDL ($p<0.01$). In addition, microparticles were measured that expressed another marker of inflammation, vitronectin receptor CD51. There was no significant relation between level of fasting lipids and the level of microparicles expressing CD51. All these data suggest apoptotic stimuli rather than endothelial cell activation under the conditions of the increased lipids and lipoprotein levels.

Number of endothelial microparticles that was measured by lectin (UEA1) binding was elevated in subjects with increased level of glucose.

We found the dependence of CD31+/CD42b positive endothelial microparticles from the level of C-reactive protein. CRP is an acute-phase protein, and blood CRP raises from trace levels to high micrograms per milliliter during inflammatory diseases [42,43]. CRP synthesis is rapidly elevated by the liver as a result of an increase in interleukin IL-6 levels [44].

Considerable attention has been placed on CRP as a powerful

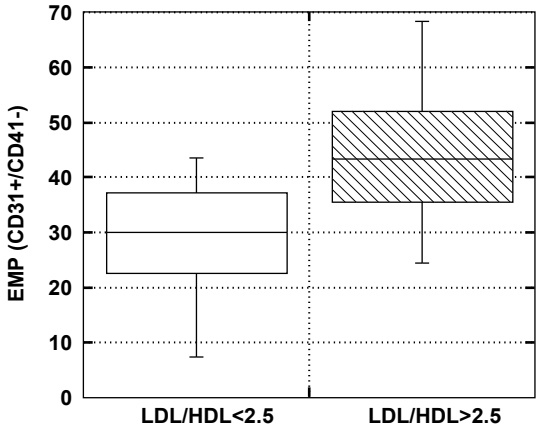

Figure 5: Distributions of EMP counts for subjects grouped according to LDL/HDL ratio.

indicator of cardiovascular risk [45,46]. The authors of [45] published the results of a large epidemiological study and concluded that circulating CRP levels were a better indicator of coronary artery disease than cholesterol. Similarly, it was concluded that elevated levels of CRP and IL-6 predict the development of type two diabetes and support a possible role for inflammation in diabetogenesis [47].

In our study, we found strong correlation between C-reactive protein and the level of apoptotic microparticles (CD31+/CD42b-). Furthermore, there was a correlation between levels of platelet microparticles (CD41a+) and the levels of C-reactive protein ($r=0.483$, $p<0.03$), cholesterol/HDL ratio ($r=0.25$, $p<0.02$), and HDL ($r=-0.384$, $p<0.04$).

In summary, we found that EMPs, determined via staining with CD31 and CD42b, provide an early indicator of cardiovascular risk.

We suggest that the future studies are needed to provide additional evidence of the contribution of risk factors in circulating microparticles and vascular dysfunction. Our study had limitations in the number of subjects and should be expanded with more subjects in different states of dyslipidemia.

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