Increased circulating platelet and endothelial-derived microparticles in patients with cardiac syndrome X

Fereshteh Ghaffari[(1)] , **Yousef Rasmi[(2)]** , **Mir Hossein Seyed Mohammadzad[(3)]**,
Shahram Seyedi[(4)], **Alireza Shirpoor[(5)]**, **Elmira Roshani-Asl[(1)]**, **Ehsan Saboory[(6)]**

Original Article

Abstract

BACKGROUND: Cardiac syndrome X (CSX) has been associated with endothelial dysfunction and inflammation. We conducted a case-control study to evaluate the association between platelet and endothelial-derived microparticles (PMPs and EMPs), as specific quantitative plasma markers of endothelial dysfunction, and the presence of CSX.

METHODS: The present study was conducted on 40 CSX patients and 19 healthy individuals. C-reactive protein (CRP), and hematological and biochemical parameters were evaluated. The MP concentration in platelet-poor plasma (PPP) was quantitatively determined through flow cytometry using specific anti-human CD31, CD41a, CD62E, and CD144 antibodies.

RESULTS: The mean platelet volume (MPV) and positive CRP rate (≥ 3.8 mg/l) were higher in patients compared to controls (P = 0.020 and P = 0.010, respectively). The CD62E+, CD144+, and CD31+41− EMPs, as well as CD41+ and CD31+CD41+ PMPs showed significant increase in CSX patients compared to controls (P < 0.050). There were direct correlations between the mean percentage of detected EMPs and PMPs as well as between their expression intensity; however, a reverse correlation was seen between the percentage of MPs and CD144 and CD41. Moreover, the MP level was reversely associated with prothrombin time (PT) and partial thromboplastin time (PTT) values. Only CD31+CD41+ PMP was correlated with CRP.

CONCLUSION: It seems that EMPs and PMPs increase in CSX, which may contribute to various processes involved in the development of this syndrome.

Keywords: Cardiac Syndrome X; Endothelium; Dysfunction; Inflammation; Microparticles

Introduction

Cardiac syndrome X (CSX) is microvascular angina characterized by chest pain due to reduced blood flow to heart tissue, positive exercise stress test, and electrocardiographic findings of ischemia; however, CSX patients present normal coronary artery function on invasive angiography.[1] Endothelial dysfunction, inflammation, and abnormal autonomic nervous system performance are the most likely underlying mechanisms for CSX development, although a multitude of factors has been implicated in the pathogenesis of the syndrome.[2] Microvascular endothelial dysfunction has been suggested as one of the most important causes of CSX.[3,4] For instances, Huang et al.[5] and Shmilovich et al.[6] found that circulating endothelial progenitor cell level was altered in patients with CSX that could be indicative of an endothelial dysfunction. Moreover, results of morphological and functional studies on the microvascular system along with increased levels of endocan, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and P-selectin in patients with CSX

1- Department of Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
2- Professor, Cellular and Molecular Research Center AND Department of Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
3- Associate Professor, Department of Cardiology, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
4- Assistant Professor, Department of Immunology, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
5- Professor, Department of Physiology, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
6- Professor, Zanjan Metabolic Diseases Research Center, Zanjan University of Medical Sciences, Zanjan, Iran
Address for correspondence: Yousef Rasmi; Professor, Cellular and Molecular Research Center AND Department of Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran; Email: rasmiy@umsu.ac.ir

are suggestive of chronic inflammatory processes leading to a progressive dysfunction in the vascular endothelium.[7-9]

Microparticles (MPs) are vesicles released from various cells, including leukocyte MPs (LMPs), erythrocyte MPs (ErMPs), platelet MPs (PMPs), and endothelial MPs (EMPs), and exhibit potent coagulatory and inflammatory activities.[10-12] There is an increasing line of evidence suggesting the involvement of MPs, particularly PMPs and EMPs, in driving coagulation and in inflammatory processes during the pathogenesis of cardiovascular diseases (CVDs).[13-16] Accordingly, alterations in the circulating EMPs due to endothelial cell activation, damage, or apoptosis have been proposed as a potential predictive value for endothelial dysfunction in CVDs.[16,17] Nozaki et al. showed that, in addition to endothelial dysfunction, future cardiovascular events can be predicted in high-risk patients through EMPs; however, the role of EMPs and PMPs in CSX remains unclear.[18] Characterization of circulating levels of EMPs and PMPs in patients with CSX may be essential for the elucidation of the role of this novel marker in the pathogenesis of CSX, as well as possible targeting of these markers as a therapeutic strategy in improving endothelial dysfunction, and the severity and progression of vascular disease. Therefore, herein, we aimed to investigate circulation levels of EMPs and PMPs in patients with CSX compared to healthy individuals. We implemented flow cytometry assay to detect the EMPs that displayed expression patterns of CD62E (E-Selectin) +, CD144 (VE-Cadherin) +, and CD31+41−, and the PMPs that exhibited CD41+ and CD31 (PECAM-1)+CD41+.[19] Moreover, the presence of a correlation between PMPs and EMPs as endothelial dysfunction biomarkers was also assessed.

Materials and Methods

The present study was conducted on 40 patients with CSX (51.7 ± 10.7 years of age) referring to the Department of Cardiology of Urmia University of Medical Sciences, Urmia, Iran, due to chest pain. The disease diagnosis was performed by a cardiologist following clinical examination, fitness testing, thallium (cardiolite) scanning, and angiography. Patients with typical angina pectoris, a positive exercise test or an abnormal thallium test, and a completely normal coronary angiogram were included in the study. Demographic and clinical information including age, gender, and body mass index (BMI) were recorded. The exclusion criteria included CVD (except CSX), diabetes, any endothelial dysfunction diseases, asthma, pulmonary-renal disorders, active infection, and malignant disease. Furthermore, 19 gender and BMI matched healthy individuals (37.9 ± 9.1 years of age) were recruited as controls. None of the controls had a previous history of chest pain or acute/chronic diseases. All participants signed informed consent forms. In addition, an ethical committee based at the hospital approved the study procedure, which met the ethical standards of the Helsinki Declaration.

A total of 10 ml of blood was taken from each subject into 2 tubes; 5 ml of the blood was collected into a tube containing 3.2% sodium citrate for blood count and plasma separation, and 5 ml of it was collected into the second tube without any anticoagulants for clotting analysis and serum separation. To produce platelet-poor plasma (PPP), essential for MP measurement,[20] the sodium citrate-containing blood samples were centrifuged at 1600 g for 15 minutes, and the obtained supernatant that consisted of platelet-rich plasma (PRP) was re-centrifuged at 1670 g for 10 minutes. Finally, the produced supernatant was collected as PPP and stored at -80 °C for a period of 2 weeks before use in order to let the concentration of MPs increase.[21] Sera were generated following centrifugation of the coagulated-blood samples at 500 g for 10 minutes and were then stored at -80 °C until use.

Complete blood count (CBC) was obtained using an automated cytometer (Sysmex KX-21N; Sysmex Corporation, Kobe, Japan). Prothrombin time (PT) and partial thromboplastin time (PTT) tests were carried out using HemosIL™ commercial kit (SynthASil-0020006800; Instrumentation Laboratory Co., Bedford, MA, USA) in an automatic coagulometer (Instrumentation Laboratory Co., Bedford, MA, USA). Routine fasting blood sugar (FBS), cholesterol, triglyceride (TG), high-density lipoprotein (HDL)-c, and low-density lipoprotein (LDL)-c level measurements were performed using commercially available kits (Pars Azmoon Co., Tehran, Iran) in an automated analyzer (model ALCYON 300; Abbott Laboratories, Chicago, IL, USA). Urea and creatinine serum levels were measured using commercial kits purchased, respectively, from Pars Azmoon Co. (Tehran, Iran) and Bionik Co. (Tehran, Iran), according to the manufacturers' instructions in an automated biochemical analyzer (BT3000; Biotecnica Instruments S.p.A., Rome, Italy). Serum C-reactive protein (CRP) was measured using a commercial CRP kit (ZK044.L.R, the Binding Site Group Ltd.,

Birmingham, UK) according to the manufacturer's instructions, using a semi-automated nephelometer (Minineph Modelo AD500, the Binding Site Group Ltd., Birmingham, UK).

The MP concentration of the PPP samples was quantitatively determined through flow cytometry equipped with a forward scatter (FSC) photomultiplier tube, using specific anti-human CD31-FITC, CD41a-PE, CD62E-PE, and CD144-PE monoclonal antibodies (eBioscience Inc., San Diego, CA, USA). The PPP samples were thawed on melting ice and incubated at 37 °C for 5 minutes. Then, 300 μl of PPP was added to 3 special tubes (D-51588; SARSTEDT AG & Co. KG, Germany) equally (100 μl per tube), and each tube was separately incubated in the dark with either CD144-PE, CD62E-PE, or both CD31-FITC and CD41a-PE antibodies at 4 °C for 45 minutes. Two distinct tubes containing anti-mouse PE-conjugated and FITC-conjugated IgG1 antibodies (eBioscience Inc., San Diego, CA, USA) were employed as isotype controls in parallel with the test. After incubation, l milliliter of phosphate-buffered saline (pH = 7.4) was added to the tubes. Finally, reading and analysis were performed using a flow cytometer (Sysmex Partec GmbH, Münster, Germany). Partec calibration beads (Product NO: 054008; Sysmex Partec GmbH, Münster, Germany) were used for adjusting the flow cytometer. The number and percentage of EMPs possessing a marker of interest were determined. The intensity of marker expression on particles was also displayed.

Data were analyzed using SPSS software (version 19; SPSS Co., Chicago, IL, USA). First, the Kolmogorov-Smirnov normality test was performed. The age effect, as a confounding factor, was checked for each quantitative variable, and mean of values was compared between patient and healthy groups using independent t-test. Nominal variables were examined using chi-square or Fisher's exact tests. The association between the variables was detected using the Pearson and Spearman correlation tests. The results are presented as mean ± standard deviation (SD). P-values of less than 0.050 were considered as statistically significant.

Results

A total of 40 patients with CSX and 19 healthy individuals were studied. The demographic features of the 2 groups are presented in table 1. The mean age in the CSX group was significantly higher than that in the control group (51.7 ± 10.7 vs. 37.9 ± 9.1 years; P = 0.001). There was no significant difference between the groups with respect to gender distribution, BMI, smoking, menopausal status, systolic and diastolic blood pressures, and risk of hyperlipidemia (for all: P > 0.050).

Table 1. Baseline demographic and clinical factors in patients with cardiac syndrome X and healthy control group

Variable	CSX (n = 40)	Control (n = 19)	P
Gender (male)	12 (32.5)	7 (42.1)	0.330
Smoker: non-smoker (%)	10.5: 89.5	0: 100	0.180
Menopausal women (%)	13 (33.3)	17 (9.1)	0.070
Hypertension (%)	23 (59.0)	0.0 (0.0)	0.001
Hyperlipidemia (%)	21 (5.4)	0.0 (0.0)	0.430
Systolic BP (mmHg) (mean ± SD)	120.50 ± 16.20	122.30 ± 16.40	0.110
Diastolic BP (mmHg) (mean ± SD)	72.80 ± 10.10	70.00 ± 12.20	0.950
Age (year) (mean ± SD)	51.70 ± 10.70	37.90 ± 9.10	0.001
BMI (kg/m^2) (mean ± SD)	27.90 ± 5.00	26.00 ± 0.70	0.440
Complete blood count (CBC)			
White blood cells (μl^{-1})	6902.00 ± 1825.00	7860.00 ± 1235.00	0.120
Red blood cells (10^5/μl)	47.00 ± 4.90	48.00 ± 5.30	0.004
Red cell distribution width (%)	13.60 ± 1.30	13.10 ± 1.40	0.260
Hemoglobin (g/dl)	13.00 ± 1.40	13.20 ± 1.20	0.670
Hematocrit (%)	40.60 ± 3.70	40.60 ± 3.00	0.990
Platelet (10^3/μl)	240.00 ± 71.00	250.00 ± 59.00	0.030
Mean platelet volume (fl)	9.90 ± 0.80	9.20 ± 1.20	0.020
Risk factors			
Cholesterol/HDL	3.15 ± 0.92	3.45 ± 0.36	0.464
TG/HDL	2.57 ± 1.48	3.09 ± 1.38	0.465
LDL/HDL	1.63 ± 0.86	1.84 ± 0.41	0.605

CSX: Cardiac syndrome X; BP: Blood pressure; HDL: High-density lipoprotein; TG: Triglyceride; LDL: Low-density lipoprotein; SD: Standard deviation
Continuous and categorical variables were reported as mean ± SD and Number (percentage)
Independent t-test and chi-square test (or Fisher's exact test) were used.

Hematological and biochemical measures of the study groups are presented in table 1. Red blood cells (RBC) count and platelet (Plt) count were significantly lower, but mean platelet volume (MPV) was greater in the patient group compared to the control group (P = 0.004, P = 0.030, and P = 0.020, respectively). Other hematological values did not show significant differences between the 2 groups (for all: P > 0.050). Furthermore, the concentration of LDL-c was significantly greater in the control group compared to the CSX group (P = 0.030). In addition, serum lipid risk factors for CVD did not exhibit a significant difference between the control and patient groups (for all: P > 0.050) (Table 1).

The CRP concentrations of less than 3.8 mg/l and equal to or greater than 3.8 mg/l were considered as negative and positive, respectively. Positive CRP was significantly more prevalent among the patients compared to the controls

(28.6% vs. 0.0%; P = 0.010).

Flow cytometry was carried out to assess and compare the EMP and PMP values, based on expression patterns of CD144, CD62E, CD31+CD41−, CD41+, and CD31+CD41+ (Figure 1A), in the PPP samples of the CSX patients and healthy controls. As depicted in figure 1B, the mean percentage of the MPs with expression pattern of CD62E+, CD31+, CD41+, CD31+CD41−, and CD31+CD41+ among the CSX patients was significantly higher compared to the control group (P = 0.040, P = 0.007, P = 0.030, P = 0.046, and P = 0.020, respectively), but CD144 did not differ between the groups (P = 0.100). However, considering the MP count per µl of PPP, only MPs with the expression pattern of CD31+, CD31+CD41−, and CD31+CD41+ revealed a significant increase in the patients compared to the controls (P = 0.040, P = 0.010, and P = 0.010, respectively).
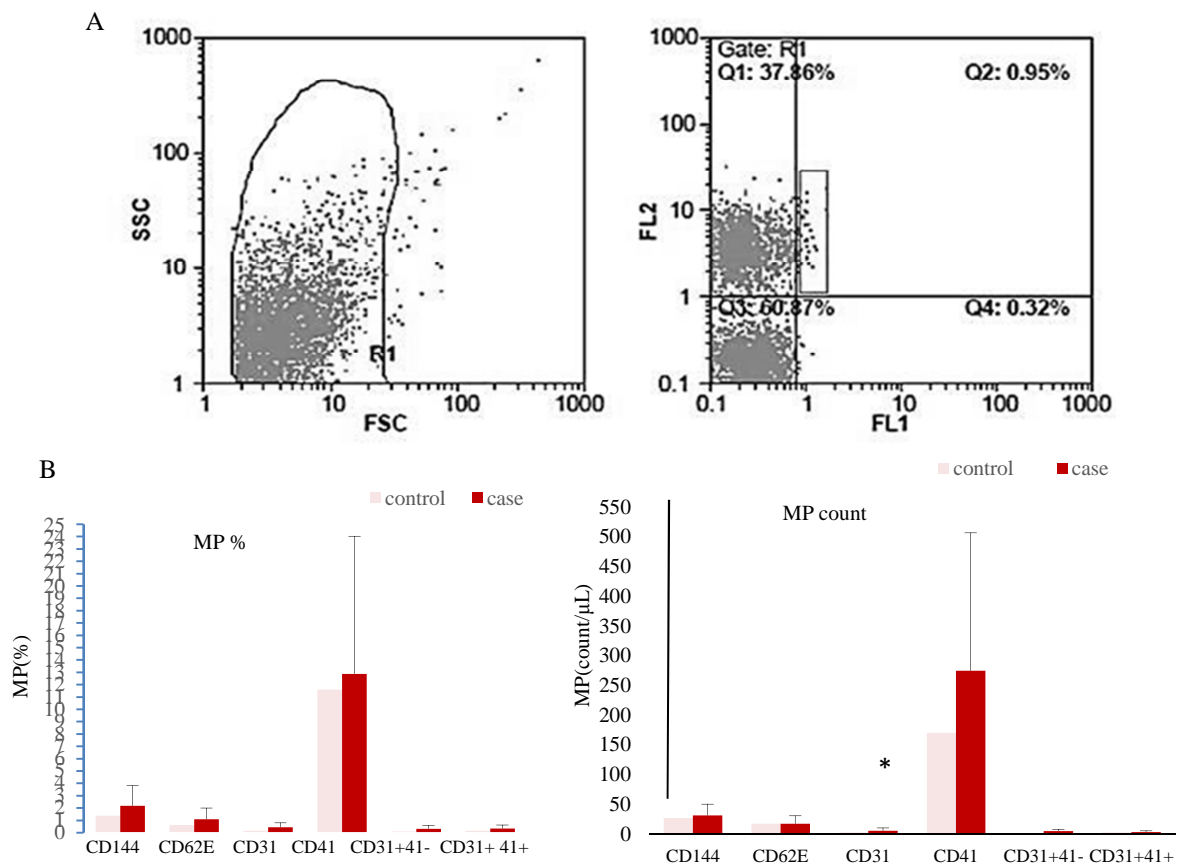

Figure 1. Flow cytometery analyses of circulating microparticles (MPs) in the patients with cardiac syndrome X (n = 40) and healthy control group (n = 19)
Particles were first separated by size on the Forward versus side scatter (FSC/SSC) graph (gate R1), then identified by expression pattern of CD31 and CD41, and the Q2 gate represents endothelial microparticles (EMPs) with expression pattern of CD31+CD41-. B) Average percentage of the EMPs expressing markers of interest as well as the particles count per µL of platelet-poor plasma (PPP) was calculated. Values are demonstrated as mean ± standard deviation. *: P < 0.050; **: P < 0.010

The expression intensity of CD31 (Y31) and CD62E (Y62) on a single EMP was greater in the patients than the control people (7.1 ± 16.0 *vs.* 3.0 ± 2.1, and 9.4 ± 14.6 vs. 3.3 ± 2.4, respectively); however, these differences were not statistically significant (P = 0.320, and P = 0.090, respectively). The expression intensity of CD41 (Y41) and CD144 (Y144) did not exhibit significant differences between the 2 groups (P = 0.940, and P = 0.660, respectively).

Additionally, the values of EMPs and PMPs in the CSX patients are illustrated in figure 2 by gender, menopausal status, and inflammatory CRP level; as can be seen, no significant differences were observed in the MP values by either of the factors.

Results of the Pearson correlation test on different MP values are demonstrated in figure 3.

There were positive correlations between mean percentages of EMPs with expression patterns of CD144 and CD62E (r = 0.521; P = 0.001), CD144 and CD31+CD41− (r = 0.536; P = 0.010), CD62E and CD31+CD41− (r = 0.522; P = 0.020). A negative correlation was seen between the percentages of MPs with CD144 and CD41 (r = -0.402; P = 0.010). Moreover, the mean count of EMPs with CD144 and CD31+CD41− revealed a positive correlation (r = 0.508; P = 0.040). In addition, the mean Y62 showed positive correlations with mean percentages of EMPs with CD31 (r = 0.338; P = 0.040) and CD144 (r = 0.342; P = 0.030), as well as with mean CD41 (r = 0.367; P = 0.010). The mean CD41 also positively correlated with mean count of PMPs with CD31+CD41+ (r = 0.501; P = 0.010).
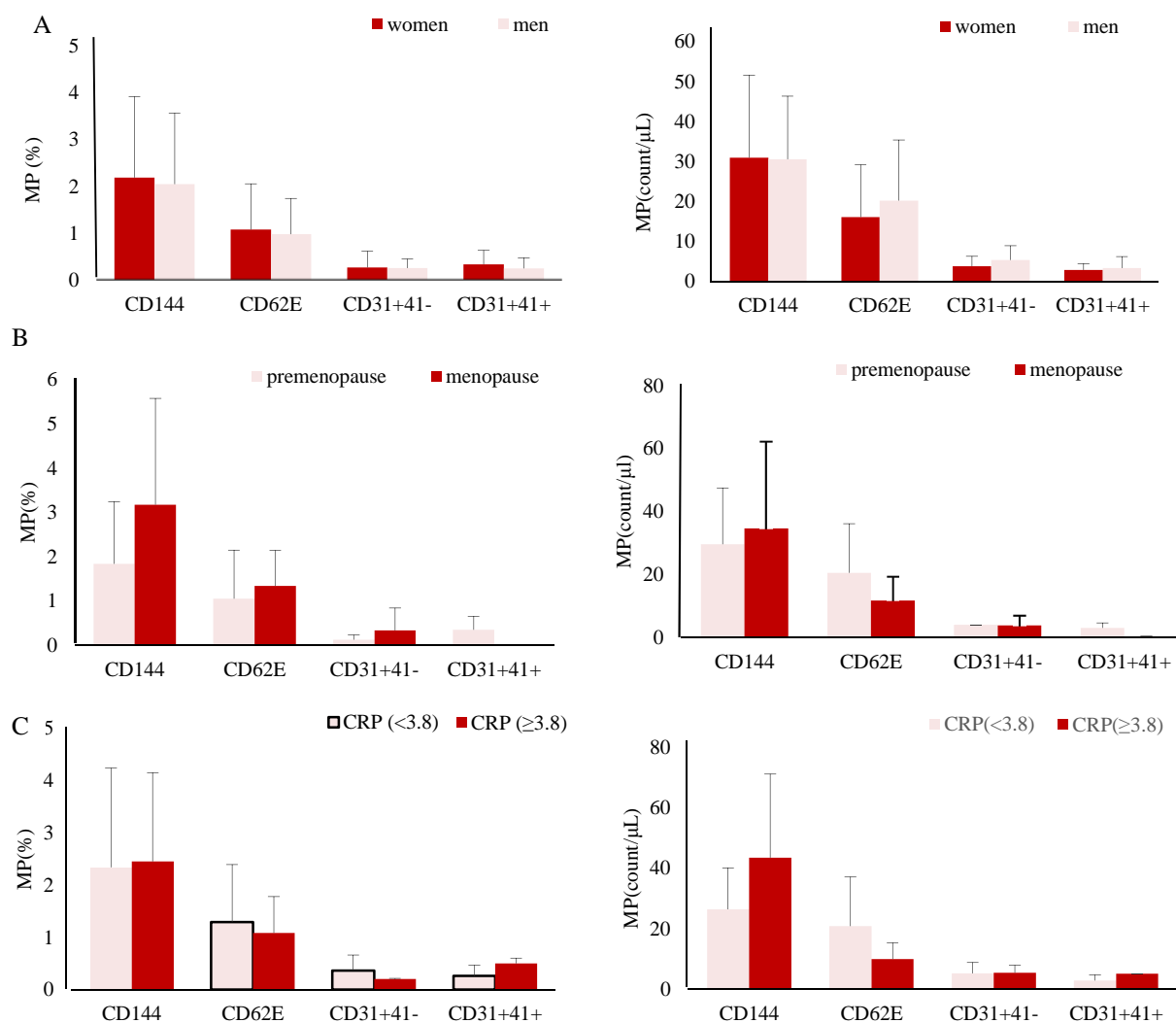

Figure 2. Comparison of values of circulating microparticles (MPs) in patients with cardiac syndrome X (n = 40) by gender (A), menopausal status (B), and inflammatory CRP level (C)
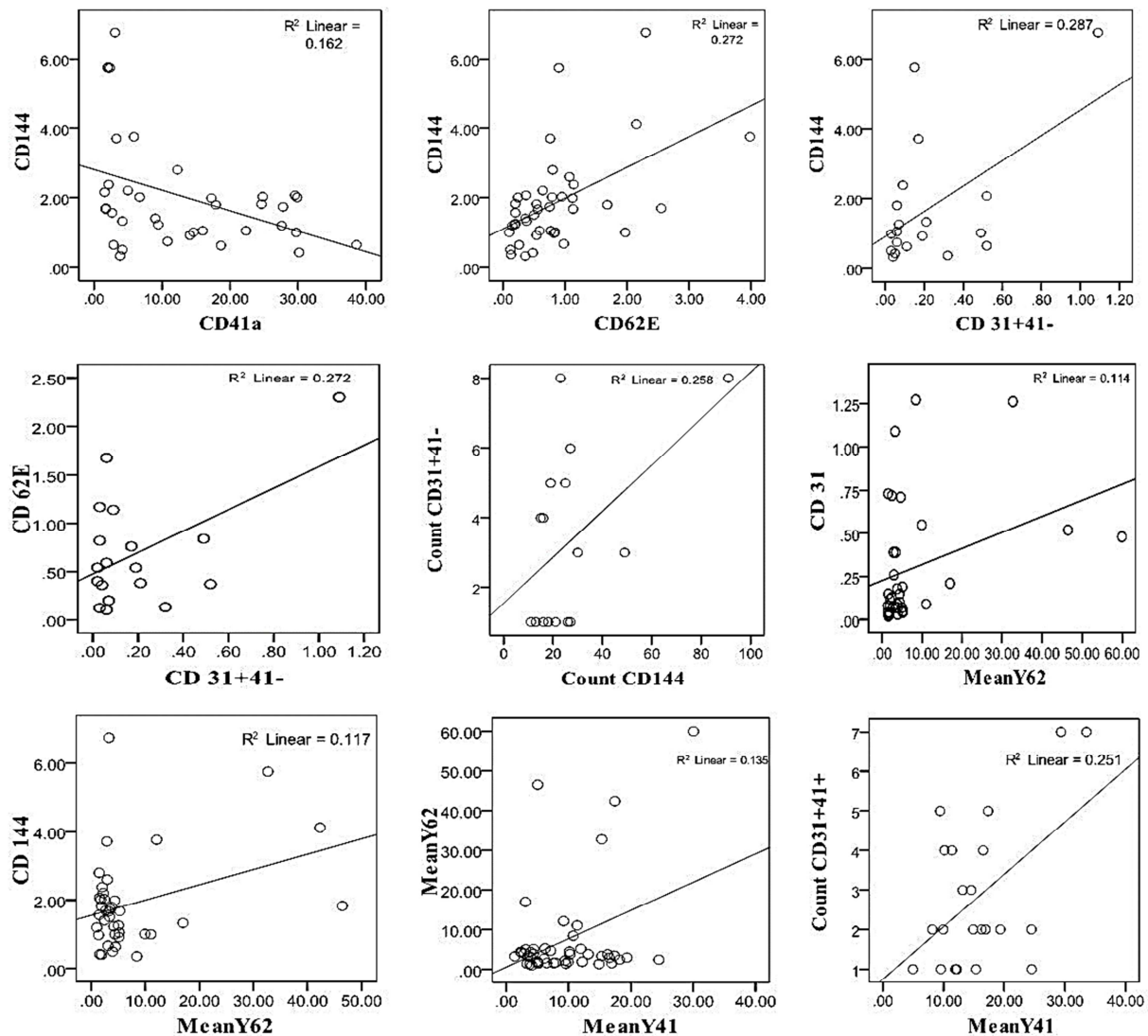Values are demonstrated as mean ± standard deviation.

Figure 3. Correlations between values of different circulating microparticles (MPs) in patients with cardiac syndrome X (n = 40)

As illustrated in figure 4, the mean percentages of EMPs expressing CD31 had a negative correlation with the PT (r = −0.416; P = 0.020) and the PPT (r = −0.344; P = 0.040) values. There was a negative correlation between the PT value and the mean percentage of EMPs expressing CD62E (r = −0.423; P = 0.008). Moreover, the PT value negatively correlated with the mean count of MPs with expression pattern of either CD144 (r = −0.419; P = 0.010), CD31+CD41− (r = −0.628; P = 0.010), or CD31+CD41+ (r = −0.561; P = 0.010). The mean percentage of EMPs with CD31+CD41− pattern showed a negative correlation with the Plt count (r = −0.515; P = 0.030), while the latter revealed a positive correlation with mean count of EMPs expressing CD144 (r = 0.323; P = 0.049). There was a negative correlation between the MPV

value and mean count of CD144-expressing EMPs (r = −0.383; P = 0.020).

Serum TG and cholesterol levels exhibited negative correlations with mean count of EMPs with expression patterns of CD144 (r = −0.308; P = 0.049) and CD31+CD41− (r = −0.481; P = 0.020), respectively. The creatinine level revealed positive correlations with both mean percentage (r = 0.498; P = 0.010) and mean count (r = 0.437; P = 0.040) of the EMPs with CD31+CD41− expression pattern.

Results of the Spearman correlation test on different MP values and serum CRP status revealed that serum CRP has significant positive moderate correlations with both mean percentage (r = 0.46; P = 0.040) and mean count (r = 0.48; P = 0.030) of the MPs concurrently expressing CD31+ and CD41+.
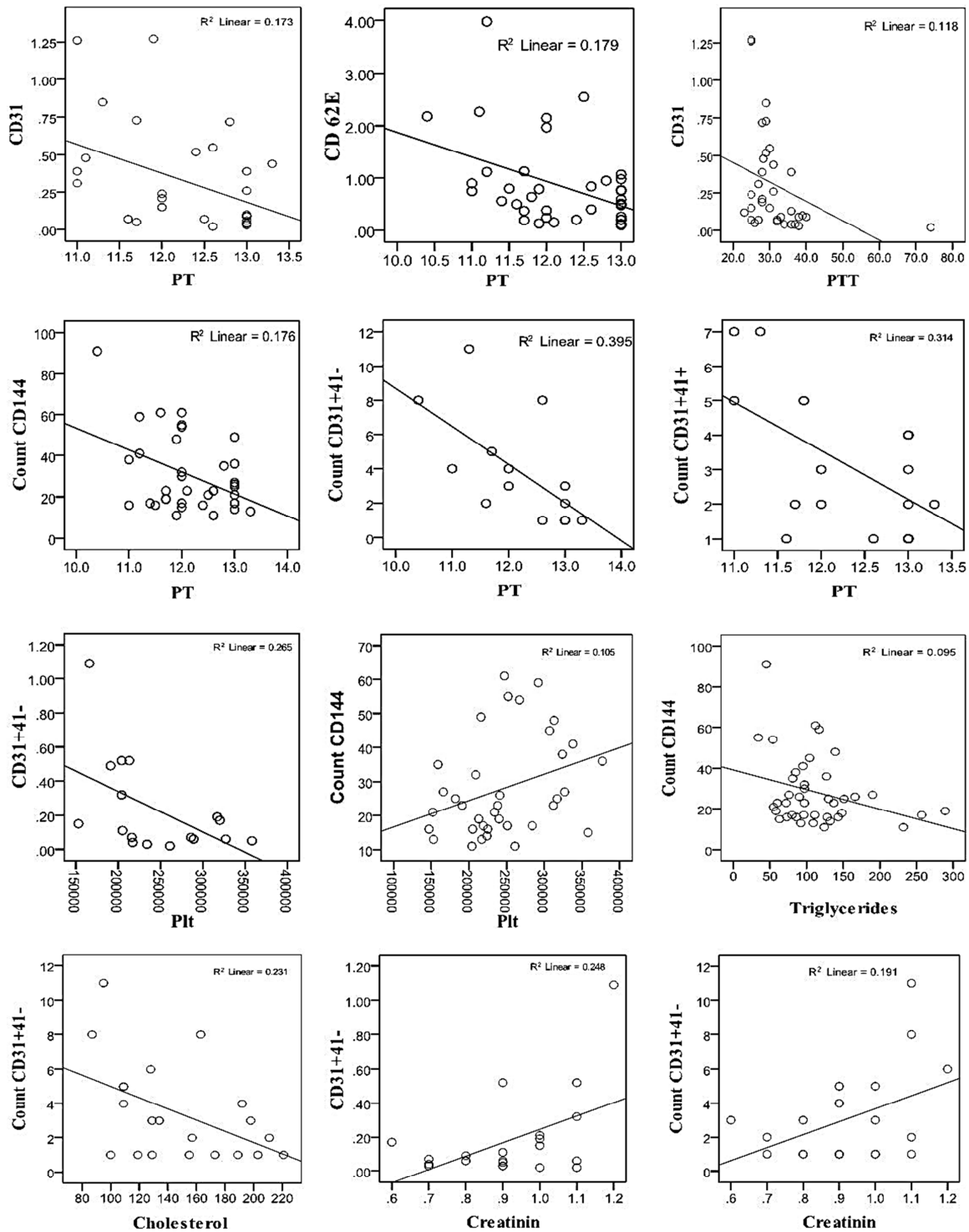
Figure 4 Correlations between values of different circulating microparticles with hematological and biochemical indices in patients with cardiac syndrome X (n = 40)
PT: Prothrombin time; PTT: Partial thromboplastin time; Plt: Platelet

Discussion

The present study results showed that PPP levels of CD31+, CD62E+, CD31+CD41−, and EMPs were markedly increased in the CSX group compared to the healthy group independent of age, gender, and menopausal effect. We observed an increased level of CD41+ and CD31+CD41+ PMPs in CSX patients, and notably we found direct

associations between almost all detected MP values. Furthermore, the CD144+ EMPs revealed an increase in the CSX group. This insignificant increase in CD144+ might be due to insufficient sample size in this study.

To the best of our knowledge, the present study is the first report of EMP alterations in CSX, although the role of EMPs in various diseases associated with CVDs has been demonstrated in numerous studies.[19,22-26] Consistent with our findings, Amabile et al. reported elevated levels of CD31+ and CD144+ EMPs in patients with microvascular angina.[22] Furthermore, Horn et al. observed that stenting of atherosclerotic lesions in the right coronary arteries led to substantial enhancement of CD62E+, CD144+, and CD31+CD41− EMPs in patients with stable angina.[19]

Dursun et al. found that increased circulating CD144+ EMP was related to abnormal heart pulse wave velocity, arterial stiffness, and atherosclerosis in patients with chronic renal disease.[23] Koga et al. proposed that the elevated level of CD144+ EMP in diabetes mellitus could be used as a specific marker to identify patients who are at risk of developing coronary artery disease (CAD).[24] Therefore, the present study findings are in agreement with that of Dursun et al.[23] and Koga et al.[24] However, the increment in CD144+ EMP level in our study was not statistically significant, which presumably is due to the small size of the study population. In addition, consistent with the present study, some studies have introduced CD31+41− EMP as a suitable biomarker for endothelial dysfunction in CVDs.[25,26] Wekesa et al. observed CD31+41− EMP elevation in carotid artery disease that was associated with plaque instability and transient ischaemic attack symptoms.[26] However, comparing CD62E+ and CD31+41− EMPs, Lee et al. found that only the former was capable of predicting the risk of cardiovascular morbidity in patients with stroke history.[27] Consequently, our findings are in line with evidence suggesting CD62E+, CD144+, and CD31+41− EMPs as potential biomarkers for CSX.

EMPs vary phenotypically and quantitatively, depending on their mechanism of genesis. For example, CD62E+ and CD31+ EMPs are frequently released from activated and apoptotic endothelial cells, respectively.[28,29] Thus, regarding the concurrent enhancement of EMPs, it seems that various mechanisms might be responsible for alterations in the EMPs in CSX. Inflammation plays a substantial role in the pathogenesis of endothelial dysfunction.[30] In vitro studies have demonstrated that endothelial cells release EMPs following stimulation particularly with inflammatory stimuli such as cytokines.[31,32] CRP is an inflammatory factor that has been shown to increase in CSX patients.[33] Consistent with this concept, we also observed that CRP level was remarkably augmented in CSX. However, we could not detect a significant association between EMPs and CRP levels in patients with CSX. Furthermore, the CD31+CD41+ PMP level considerably correlated with the CRP level in the CSX patients. Several studies have shown the involvement of PMPs in coagulation and inflammatory processes during the pathogenesis of CVDs.[14,15] In the present study, we observed associations between PMP and CRP levels, as well as between PMP and EMP levels. In addition, PMP and EMP levels revealed reverse associations with PT and PTT values, which was in accordance with the findings of Laresche et al.[34] All these observations suggest that EMPs along with PMPs may contribute to coagulation and inflammatory processes in developing CSX.

Moreover, we found that Plt count was markedly reduced, while the MPV was increased in CSX patients compared to healthy people. A low Plt count in the presence of an increased mean Plt volume has been implicated in the pathogenesis of non-cardiac chest pain. Furthermore, an enhancement in Plt aggregability and activity has been shown to be associated with CSX-associated atherosclerosis.[35] Notably, the Plt count and MPV had direct and reverse associations with the CD144+ EMP level, respectively. Nevertheless, in accordance with the findings of Salem et al.,[36] MPV showed reverse associations with CD144+ and CD31+CD41− EMP levels. At this time, there is no plausible explanation for this disagreement.

RBC count was significantly lower in patients. However, there are not enough data to suggest an association between the RBC count and CAD.

Unlike previous studies,[37,38] in our study, there was no considerable variation in lipid ratios between CSX patients and healthy individuals. In addition, the LDL-c levels showed a significant decrease in the patient group. This may be due to the use of anti-lipidemic drugs by the patients. However, more than 50% of the patients suffered from hyperlipidemia, which is an established risk factor for CVDs. Moreover, direct correlations have been previously found between EMPs, CRP, and cholesterol levels.[39] Controversially, in our study,

the CD144+ and CD31+CD41− EMP levels had reverse correlations with TG and cholesterol levels, respectively. Furthermore, increased serum level of creatinine has been implicated in microvascular endothelial dysfunction.[40] Consistent with this concept, in the present study, we found a direct association between serum creatinine and CD31+CD41− EMP levels.

Conclusion

To our knowledge, this is the first study that has evaluated the various markers of microparticles in CSX. The main limitation of this study was the small sample size. However, further investigations with larger sample size and long-term follow-up periods are required to pursue and confirm our findings in the future.

According to the present study findings, it seems that EMPs and PMPs may contribute to various processes involved in the development of CSX. In addition, the presence of endothelial dysfunction in patients with CSX is shown with the use of these novel markers. Moreover, our findings suggest that due to the procoagulant activity of EMPs and PMPs, they may be causally related to thrombus formation and coronary artery obstruction in CSX in the future. This assumption was further proven by the observation that EMP and PMP values were inversely correlated with PT.

Acknowledgments

Conflict of Interests

Authors have no conflict of interests.

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