Microparticles from Endothelial Cells and Immune Cells in Patients with Takayasu Arteritis

Xuesen Cheng¹, Aimin Dang¹, Naqiang Lv¹ and Tong Zhao²

¹Department of Special Care Center, National Clinical Research Center for Cardiovascular Diseases, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
²Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

Aim: This study was designed to analyze microparticles (MPs) from endothelial cells (EMPs) and immune cells from healthy individuals and patients with Takayasu arteritis (TA), and any possible relationships between MPs and TA activity.

Methods: MPs derived from the plasma of 51 subjects were analyzed, including 32 patients with TA and 19 healthy individuals. Flow cytometry was performed with Annexin (Anx)-V and antibodies against surface markers of endothelial cells (CD144), T cells (CD3), B cells (CD19), and monocytes (CD14).

Results: The concentrations of total EMPs, AnxV+ EMPs and AnxV- EMPs were significantly increased when comparing patients with TA and healthy controls (54 × 10³ vs. 32 × 10³ MPs /ml, P=0.0004; 22 × 10³ vs. 12 × 10³ MPs /ml, P=0.0006; and 31 × 10³ vs. 19 × 10³ MPs /ml, P=0.005), and comparing active TA patients with remission ones (85 × 10³ vs. 45 × 10³ MPs /ml, P=0.016; 39 × 10³ vs. 14 × 10³ MPs /ml, P=0.0092; and 47 × 10³ vs. 29 × 10³ MPs /ml, P=0.0371). In addition, the concentrations of total EMPs (odds ratio [OR] 1.024, 95% confidence interval [CI]: 1.001 to 1.048, P=0.037), AnxV+ EMPs (OR 1.089, 95%CI: 1.011 to 1.172, P=0.024), and AnxV- EMPs (OR=1.029, 95% CI: 1.002 to 1.056, P=0.034) were positively related to TA activity. With multiple linear regression analysis, platelet was associated with both total and AnxV- EMP concentrations independently, while erythrocyte sedimentation rate was independently correlated with AnxV+ EMPs.

Conclusion: Concentrations of endothelial microparticles are correlated with inflammation in Takayasu arteritis and may be useful markers to assess disease activity.

Key words: Takayasu arteritis, Microparticle, Endothelial cells, Disease activity

1. Introduction

Takayasu Arteritis (TA) is a chronic inflammatory condition with unclear etiology or pathology that mainly affects the aorta and its major branches, as well as pulmonary and coronary vessels, resulting in luminal stenosis and aneurismal dilation of large vessels¹. Once believed to be a rare disease affecting young women among Asian populations, the prevalence of TA has been established in both sexes, as well as ethnicities around the world², ³.

Immunohistochemical studies of aortic tissues from patients with TA have demonstrated an infiltration of CD4+ T cells, CD8+ T cells, γδ T cells, natural killer cells, macrophages, and neutrophils⁴. Previous studies indicated that leukocytes could affect endothelial cells through leukocyte integrins and endothelial cell adhesion molecules of the immunoglobulin superfamily. Blocking leukocyte α4β1 or α4β7 integrins or their endothelial receptors mucosal addressin cell adhesion molecule 1 (MAdCAM-1) or vascular cell adhesion molecule 1 (VCAM-1) could prevent or reverse inflammation in various models⁵. Vascular endothelial cell (EC) injury mediated by immunoreaction plays an essential role in the pathogenesis of TA. The involvement of apoptotic ECs in
vascular lesions indicates that apoptosis may be one of the major pathways in inflammatory damage to ECs. What is more, the externalization of plasma membrane phosphatidylserine (PS) is a fundamental feature of apoptosis.

Ranging from 100 nm to 1 µm in diameter, microparticles (MPs) are small vesicles generated from membranes by the process of exocytosis, resulting from either cell activation or apoptosis after a variety of stimuli. During the process of generating MPs, the plasma membrane PS transferred from inside layer to the outer layer with a tremendous increase in intracellular Ca²⁺ concentrations. The increase in the concentration of MPs under conditions of vascular injury or dysfunction reflects vascular health. Increased MPs from ECs (EMPs) and immune cells were found in inflammatory conditions, including rheumatoid arthritis (RA), sepsis, systemic sclerosis, and systemic lupus erythematosus (SLE).

Since endothelial dysfunction and inflammation are essential hallmarks of TA, we speculate that the concentrations of EMPs and MPs from immune cells might increase in the peripheral blood of patients with TA. To test this hypothesis, flow cytometry was used to analyze MPs isolated from patients with TA and healthy subjects, and the study determined the possible relationships between subpopulations of MPs and disease activity of TA.

2. Materials and Methods

2.1 Patients and Healthy Subjects

From December 2015 to June 2017, 32 consecutive, unselected patients with TA from our center were recruited for this study. All patients fulfilled the American College of Rheumatology criteria for TA, including at least 3 of the following criteria: 1) disease onset age ≤ 40 years, 2) claudication of extremities, 3) decreased brachial artery pressure, 4) a blood pressure difference between both arms ≥ 10 mmHg, 5) bruit over subclavian arteries or the aorta, and 6) abnormalities on arteriography. Patients with other connective tissue diseases, such as multiple sclerosis, RA, Behcet disease, ankylosing spondylitis, or rheumatic heart disease as comorbidities were excluded.

A patient with active TA was considered if two of the following criteria of the National Institutes of Health were presented new onset or worsening: 1) systemic signs or symptoms not attributable to other clinical conditions; 2) erythrocyte sedimentation rate (ESR) ≥ 20 mm/h and/or C-reactive protein (CRP) level ≥ 8 mg/l without infection or malignancy; 3) onset of signs or symptoms of vascular insufficiency; and 4) typical angiographic features.

Data on the clinical characteristics of all patients with TA were extracted from clinical medical records. After a rest of at least 5 minutes, blood pressure of each subject in a seated position was measured twice with a digital sphygmomanometer by a trained physician. The average value of these two measurements was used as the final blood pressure. A third measurement was not performed unless the difference between the first 2 measurements was more than 10 mmHg for systolic blood pressure. Hypertension was defined if the subject had a measured systolic blood pressure ≥ 140 mmHg, with or without diastolic blood pressure ≥ 90 mmHg, or if the patients were undergoing treatment for hypertension. Coronary heart disease was identified if the result of coronary angiography showed a reduction of more than 50% in the diameter of more than 1 major coronary artery. Two experienced interventional cardiologists reviewed the results of coronary angiography.

Healthy volunteers were recruited at our institute and comprised healthy check-up individuals. Subjects with hypertension, hyperlipidemia, or diabetes or subjects who were active smokers were excluded. The study complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Declaration of Helsinki and was approved by the ethics committee. All study subjects signed consent forms.

2.2 Laboratory Methods

Peripheral 12-h fasting venous blood samples were collected from subjects for the analysis of laboratory parameters and MPs. Laboratory exams were done within 2 hours of blood collection, and the parameters analyzed included white blood cell count, the counts and percentages of neutrophil granulocytes, lymphocytes, and monocytes, platelet (PLT), platelet crit (PCT) and levels of glucose (GLU), low-density lipoprotein cholesterol (LDL-C), high-sensitivity CRP (hs-CRP), ESR, and CRP. The level of CRP was detected with the Westergren method (Vacuette SRS100, Greiner Bio-One, Frickenhausen Germany). Hs-CRP and ESR levels were detected with immunoturbidimetry (Immage 800, Beckman Coulter Inc. Brea, USA). All analyses for different subjects were performed with the same instrument in the same clinical laboratory. The reference ranges for the counts and percentages of neutrophil granulocytes, lymphocytes, and monocytes were 1.8×10^9/l-6.3×10^9/l, 40%-75%, 1.1×10^9/l-3.2×10^9/l, 20.0%-50.0%, 0.10×10^9/l-0.60×10^9/l and 3.0%-10.0%, respectively.
2.2 Preparation of Platelet-free Plasma Samples

As reported by the International Society of Extracellular Vesicles in 2013\(^\text{17}\), samples for MP analysis were collected into EDTA-containing tubes, centrifuged (2,000 g for 10 minutes at 20°C) within 2 hours to deplete blood cells, followed by another round of centrifugation (14,400 g for 10 minutes at 20°C) of the plasma to remove residual platelets making platelet-free plasma (PFP) and pelleterizing MPs. All PFP samples were divided into 200-µl aliquots and stored at −80°C.

2.3 Labeling and Analysis of MPs by Flow Cytometry

To differentiate MPs from different origins, we used anti-human fluorescein isothiocyanate (FITC)-CD3 antibodies for T cells, anti-human phycoerythrin (PE)-CD19 antibodies for B cells and anti-human allophycocyanin (APC)-CD14 for monocytes. Considering the CD144 were indicated to be free of contaminating with a higher frequency\(^\text{18}\), we used anti-human FITC-CD144 antibodies for ECs. At the same time, the PS on the surface of MPs was measured using PerCP-Cy5.5 Annexin (Anx)-V in the presence of ~2.5 mM Ca\(^{2+}\).

PFPs for MP analysis were thawed and resuspended. All liquid reagents used were filtered through 0.2-µm pores (Pall Co., Ltd, USA) to decrease background signals. Then, 60 µl of each sample was divided into two equal parts. Each part was labeled with either 2.5 µl FITC-conjugated-anti-CD144 or a mix of 10 µl FITC-conjugated-anti-CD3, 10 µl PE-conjugated-anti-CD19 and 10 µl APC-conjugated-anti-CD14 antibodies at 4°C in the dark for 20 minutes. After labeling, samples were centrifuged with phosphate buffered saline (PBS) (20,000 g for 30 minutes at 4°C) to remove unbound antibodies. After that, each sample was mixed with 1 µl PerCP-Cy5.5-conjugated-AnxV and 50 µl binding buffer (2X) to detect whether the MPs were PS positive or not, followed by another labeling in the dark (20 minutes at 4°C). Unbound antibodies were removed with PBS as described above. Samples stained with isotype-matched non-specific antibodies (FITC-conjugated IgG1 kappa, PE-conjugated IgG1 kappa and APC-conjugated IgG1 kappa at the same concentration as the respective cell-marker antibodies) incubated under the same conditions were used as controls.

Beads at 1-µm diameter were used to mark the upper limit of MPs\(^\text{19}\). Each sample was mixed with 50 µl (concentration at 500 beads/µl) 500-nm counting beads and 500 µl binding buffer (1X), followed by resuspension. The beads were then counted by a flow cytometer within an hour with a “high” flow-rate mode Becton Dickinson (BD Co., Ltd, USA) Influx cell sorter, which was under the control of BD FACS software version 1.2.0.137. Analysis was stopped once 2,000 beads had been counted. Absolute MP counts per milliliter of plasma were then calculated. The formula for the calculation of MP concentration was as follows: \(C=N\times \frac{(x/y)}{30}\), where \(C\) = total concentration of MPs; \(N\) = number of MPs counted; \(x\) = total number of MPs counted (500); \(y\) = number of MPs counted (2,000); and 30 is the dilution factor. The concentration of each MP subpopulation was calculated by multiplying the concentration of total MPs by the proportion of total MPs. The flow rate was measured before each experiment. Both forward scatter (FSC) and side scatter (SSC) signals were recorded with logarithmic gain.

2.4 Statistical Analysis

Continuous data were presented as either the mean ± standard deviation or the median with inter-quartile range (IQR) depending on if data were normally distributed or not. Normality of continuous variables was assessed with Shapiro–Wilk tests. Two-tailed unpaired Student’s t tests and the Mann–Whitney U test were used to compare the difference between two groups. Qualitative data were presented as number of subjects with percentages. Frequencies between groups were compared using chi-square test and Fisher’s exact test.

Bivariate logistic regression analysis was used to assess the association between the concentrations of MP subpopulations and disease activity. The sensitivities and specificities were determined using receiver operating characteristic (ROC) curves. The Spearman approach was used to assess the associations between log-transformed MP subpopulation concentrations and other continuous variables, and \(P\) < 0.1 was considered statistically significant. Multiple linear regression was performed to analyze the independence of correlations with variables and MP subpopulation concentrations.

Scatter plots were used with median presented by lines. \(P\) < 0.05 was considered statistically significant. GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA) and SPSS version 25.0 (SPSS Inc., Chicago, IL, USA) were used for plotting and statistical analysis.

3. Results

3.1 Study Subjects

A cohort of 51 subjects were included in this study; among them, 32 were patients with TA, with a median age of 49.5 years, while the other 19 subjects were healthy controls, with a median age of 35.0 years.
phases.

3.3 Concentrations of MP Subpopulations in Different Groups

Concentrations of total EMPs (54 × 10^3 MP /ml vs. 32 × 10^3 MP /ml, P = 0.0004), AnxV+ EMPs (22 × 10^3 MP /ml vs. 12 × 10^3 MP /ml, P = 0.0006), and AnxV- EMPs (31 × 10^3 MP /ml vs. 19 × 10^3 MP /ml, P = 0.0005) were significantly higher in patients with TA than in healthy controls (Fig. 1).

Additionally, concentrations of total EMPs (85 × 10^3 MP /ml vs. 45 × 10^3 MP /ml, P = 0.016), AnxV+ EMPs (39 × 10^3 MP /ml vs. 14 × 10^3 MP /ml, P = 0.0092) and AnxV- EMPs (47 × 10^3 MP /ml vs. 29 × 10^3 MP /ml, P = 0.0371) in patients with active TA were significantly increased than in remission ones (Fig. 2).

No significant difference was observed regarding concentrations of MPs from T cells, B cells or monocytes neither between healthy controls and patients with TA (Table 2) nor patients with different phases of TA.

Table 1. Characteristics of patients with TA in remission and active phases

| Variables                      | Group      | P Value \(^{\dagger}\) |
|--------------------------------|------------|------------------------|
|                                | Remission (N=14) | Active (N=18) |       |
| Male, %                        | 1 (7.1)    | 1 (5.6)                | 1.000 |
| Age, y                         | 49.5 ± 14.1 | 43.1 ± 12.6            | 0.182 |
| WBC, 10^9/\ell                 | 8.0 (7.3, 10.4) | 8.0 (6.3, 11.1)       | 0.694 |
| N, %                           | 66 ± 8     | 62 ± 8                 | 0.207 |
| NC, 10^9/\ell                  | 5.4 (4.2, 6.8) | 4.5 (3.9, 6.5)       | 0.419 |
| L, %                           | 27 ± 7     | 30 ± 8                 | 0.241 |
| LC, 10^9/\ell                  | 2.3 ± 0.7  | 2.6 ± 1.1              | 0.334 |
| M, %                           | 5.2 (4.5, 7.1) | 5.6 (5.1, 6.0)        | 0.561 |
| MC, 10^9/\ell                  | 0.5 (0.3, 0.6) | 0.4 (0.4, 0.7)       | 0.985 |
| PLT, 10^9/\ell                 | 216 (169, 254) | 259 (215, 360)       | 0.037 |
| PCT, %                         | 0.23 (0.20, 0.27) | 0.26 (0.22, 0.35) | 0.045 |
| GLU, mmol/l                    | 4.8 (4.6, 5.7) | 4.4 (4.2, 5.0)       | 0.054 |
| LDL-C, mmol/l                  | 2.3 ± 0.7  | 2.3 ± 0.7              | 0.689 |
| hs-CRP, mg/l                   | 1.9 (0.9, 10.6) | 5.5 (0.7, 11.3)       | 0.837 |
| ESR, mm/h                      | 7.0 (4.8, 15.0) | 24.5 (11.5, 35.3)    | <0.001 |
| CRP, mg/l                      | 2.6 (1.5, 7.0) | 8.5 (2.3, 14.1)       | 0.037 |
| Hypertension, %                | 7 (50)     | 4 (22.2)               | 0.142 |
| CHD, %                         | 3 (21.4)   | 9 (50.0)               | 0.147 |
| Prednisone, %                  | 10 (71.4)  | 13 (72.2)              | 1.000 |
| Statins, %                     | 10 (71.4)  | 10 (55.6)              | 0.471 |

\(^{\dagger}\) Data are expressed as the median (IQR) or the mean (SD), unless otherwise indicated.

WBC, white blood cell; N, neutrophil percentage; NC, count of neutrophil; L, lymphocyte percentage; LC, count of lymphocyte; M, monocyte percentage; MC, count of monocyte; platelet volume distribution width; PLT, platelet; PCT, platelet crit; GLU, glucose; LDL-C, low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; CHD, coronary heart disease.

\(P<0.05\)
3.4 Relationships between Subpopulations of EMP and TA Activity

Single-factor bivariate logistic regression analysis indicated that concentrations of total EMPs (odds ratio \( OR = 1.024 \), 95% confidence interval \( [CI] = 1.001 \) to 1.048, \( P = 0.037 \)), AnxV+ EMPs (\( OR = 1.089 \), 95%CI: 1.011 to 1.172, \( P = 0.024 \)) and AnxV-
Table 2. Comparison of TMP, BMP, and MMP concentrations in controls and patients with TA

| Variables, MPs (×10^3)/ml | Group§ | Controls (N=19) | TA (N=32) | P values* |
|---------------------------|---------|-----------------|-----------|-----------|
| CD3+ MPs                  |         | 87 (67, 200)    | 89 (53, 140) | 0.965     |
| AnxV+ CD3+ MPs            |         | 21 (14, 34)     | 17 (13, 33)  | 0.828     |
| AnxV- CD3+ MPs            |         | 72 (48, 166)    | 74 (52, 121) | 0.836     |
| CD19+ MPs                 |         | 73 (54, 121)    | 86 (60, 114) | 0.736     |
| AnxV+ CD19+ MPs           |         | 14 (9, 19)      | 13 (8, 21)   | 0.996     |
| AnxV- CD19+ MPs           |         | 63 (44, 101)    | 73 (53, 89)  | 0.754     |
| CD14+ MPs                 |         | 229 (110, 514)  | 234 (131, 420) | 0.810     |
| AnxV+ CD14+ MPs           |         | 25 (17, 44)     | 29 (11, 46)  | 0.791     |
| AnxV- CD14+ MPs           |         | 205 (89, 426)   | 192 (101, 333) | 0.530     |

§Data are expressed as the median (IQR). MPs, microparticles; TMP, T cell microparticles; BMP, B cell microparticles; MMP, monocytes microparticles; TA, Takayasu arteritis. *P<0.05

Table 3. Comparison of TMP, BMP, and MMP concentrations in patients with different phases of TA

| Variables, MPs (×10^3)/ml | Group§ | Remission (N=14) | Active (N=18) | P values* |
|---------------------------|---------|------------------|--------------|-----------|
| CD3+ MPs                  |         | 89 (42, 143)     | 88 (68, 153) | 0.808     |
| AnxV+ CD3+ MPs            |         | 19 (12, 34)      | 16 (13, 32)  | 0.874     |
| AnxV- CD3+ MPs            |         | 76 (30, 124)     | 73 (54, 121) | 0.772     |
| CD19+ MPs                 |         | 78 (55, 110)     | 97 (66, 119) | 0.536     |
| AnxV+ CD19+ MPs           |         | 14 (8, 22)       | 13 (8, 22)   | 0.567     |
| AnxV- CD19+ MPs           |         | 64 (43, 85)      | 81 (54, 109) | 0.512     |
| CD14+ MPs                 |         | 223 (61, 386)    | 240 (143, 553) | 0.722     |
| AnxV+ CD14+ MPs           |         | 29 (14, 45)      | 29 (10, 54)  | 0.632     |
| AnxV- CD14+ MPs           |         | 192 (47, 319)    | 193 (124, 397) | 0.955     |

§Data are expressed as the median (IQR). MPs, microparticles; TMP, T cell microparticles; BMP, B cell microparticles; MMP, monocytes microparticles; TA, Takayasu arteritis. *P<0.05

Table 4. Concentrations of EMPs before and after relapse

| NO. | ESR (mm/h) before | CRP (mg/l) before | Total EMPs (×10^3 MPs/ml) before | AnxV+EMP (×10^3 MPs/ml) before | AnxV-EMP (×10^3 MPs/ml) before | ESR (mm/h) after | CRP (mg/l) after | Total EMPs (×10^3 MPs/ml) after | AnxV+EMP (×10^3 MPs/ml) after | AnxV-EMP (×10^3 MPs/ml) after |
|-----|------------------|-------------------|----------------------------------|---------------------------------|---------------------------------|------------------|------------------|----------------------------------|---------------------------------|---------------------------------|
| 1   | 7                | 10.8              | 17                               | 14                              | 3                               | 23               | 34.5             | 73                               | 26                              | 3                               |
| 2   | 12               | 1.13              | 62                               | 12                              | 50                              | 65               | 10               | 341                             | 225                             | 117                             |
| 12  | 7                | 8.92              | 27                               | 10                              | 18                              | 20               | 15.5             | 88                               | 38                              | 51                              |
| 14  | 4                | 1.46              | 29                               | 10                              | 20                              | 30               | 17.7             | 149                             | 46                              | 103                             |

§ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MPs, microparticles; ErMPs, erythrocyte microparticles.

EMP (OR = 1.029, 95% CI: 1.002 to 1.056, P=0.034) were positively associated with disease activity. Areas under the curve (AUCs) for the concentrations of total EMPs, AnxV+ EMPs and AnxV- EMPs were 0.788 (95% CI: 0.63 to 0.947, P=0.006), 0.794 (95% CI: 0.63 to 0.958, P=0.005), and 0.761 (95% CI: 0.594 to 0.927, P=0.012), respectively.
especially in patients with active TA. The concentrations of total EMPs, AnxV+ EMPs, and AnxV- EMPs were associated positively with TA activity. The concentration of total EMP was significantly increased in patients with TA than healthy subjects in this study. EMP is one of the most studied subpopulations of MPs, which released either from apoptotic ECs or ECs activated by a variety of triggers such as cytokines and complement activation. EMPs have been demonstrated to increase in various cardiovascular diseases, like CHD20, pulmonary hypertension21, diabetes, and rheumatic diseases such as RA10, SLE13, and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides in adults22. The concentrations of total MPs and EMPs were demonstrated to be higher in patients with RA than in healthy controls. In atherosclerosis disease, EMPs release after the stimulations of oxidative stress, endogenous molecules, and apoptosis inducer leading to vascular dysfunction23. However, as the pathology of atherosclerosis disease and TA are different, how EMPs are generated in TA is still unclear, which need further studies in the

3.5 Concentrations of EMPs with Inflammatory Markers of TA

With Spearman approach, age, PLT and PCT were correlated with concentrations of total EMPs and AnxV- EMPs. PLT, PCT, and ESR were correlated with AnxV+ EMPs. With multiple linear regression analysis, PLT was correlated with concentrations of total EMPs (Standardized B=0.419, P=0.016) and AnxV- EMPs (Standardized B=0.514, P=0.003) independent of age, gender, PCT, ESR, hs-CRP, and CRP. And ESR was correlated with AnxV+ EMPs (Standardized B=0.474, P=0.006) independent of age, gender, PLT, PCT, hs-CRP, and CRP (Table 5).

### Table 5. Independent predictors of EMPs by multiple linear regression analysis

|                         | $\rho$ | $P^*$ | Standardized $\beta$ | $P^*$ |
|-------------------------|--------|-------|-----------------------|-------|
| Total EMPs              |        |       |                       |       |
| age                     | −0.298 | 0.098 |                       |       |
| gender                  | −0.028 | 0.879 |                       |       |
| PLT                     | 0.499  | 0.004 | 0.419                 | 0.016 |
| PCT                     | 0.454  | 0.009 |                       |       |
| ESR                     | 0.292  | 0.105 |                       |       |
| hs-CRP                  | −0.110 | 0.548 |                       |       |
| CRP                     | 0.006  | 0.975 |                       |       |
| AnxV+ EMPs              |        |       |                       |       |
| age                     | −0.209 | 0.250 |                       |       |
| gender                  | −0.042 | 0.820 |                       |       |
| PLT                     | 0.345  | 0.053 |                       |       |
| PCT                     | 0.329  | 0.066 |                       |       |
| ESR                     | 0.337  | 0.059 | 0.474                 | 0.006 |
| hs-CRP                  | −0.061 | 0.741 |                       |       |
| CRP                     | 0.166  | 0.365 |                       |       |
| AnxV- EMPs              |        |       |                       |       |
| age                     | −0.305 | 0.089 |                       |       |
| gender                  | −0.056 | 0.761 |                       |       |
| PLT                     | 0.514  | 0.003 | 0.514                 | 0.003 |
| PCT                     | 0.426  | 0.015 |                       |       |
| ESR                     | 0.155  | 0.396 |                       |       |
| hs-CRP                  | −0.076 | 0.680 |                       |       |
| CRP                     | −0.112 | 0.542 |                       |       |

$^a$ MPs, microparticles; EMPs, endothelial microparticles; AnxV, annexin V; PLT, platelet; PCT, platelet crit; ESR, erythrocyte sedimentation rate; hs-CRP, high sensitive C reactive protein; CRP, C reactive protein.

$^* P<0.05$

4. Discussion

To the best of our knowledge, this is the first study on MPs from ECs and WBC, including T cells, B cells and monocytes from patients with TA. The results indicated that the concentrations of EMPs, including total EMPs, AnxV+ EMPs, and AnxV- EMPs were increased significantly in patients with TA, especially in patients with active TA. The concentrations of total EMPs, AnxV+ EMPs, and AnxV- EMPs were associated positively with TA activity. The concentration of total EMP was significantly increased in patients with TA than healthy subjects in this study. EMP is one of the most studied subpopulations of MPs, which released either from apoptotic ECs or ECs activated by a variety of triggers such as cytokines and complement activation. EMPs have been demonstrated to increase in various cardiovascular diseases, like CHD20, pulmonary hypertension21, diabetes, and rheumatic diseases such as RA10, SLE13, and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides in adults22. The concentrations of total MPs and EMPs were demonstrated to be higher in patients with RA than in healthy controls. In atherosclerosis disease, EMPs release after the stimulations of oxidative stress, endogenous molecules, and apoptosis inducer leading to vascular dysfunction23. However, as the pathology of atherosclerosis disease and TA are different, how EMPs are generated in TA is still unclear, which need further studies in the
future. In this present study, we’ve noticed that the EMP concentration in remission TA patients and healthy controls were similar, thus the significant difference between TA patients and healthy subjects on EMP levels should related to the high level of EMP in active TA patients. In addition, EMP concentrations in patients with other vasculitis were increased as well. With these considerations, the use of EMP in TA diagnosis might be limited.

The present study indicated that EMPs were positively related with TA activity and the concentrations of EMPs were increased when disease relapsed in four patients. A previous study reported that EMPs are related to disease specific features, including disease activity. In SLE, rather than anti-EC antibodies, EMPs related to disease specific features, including disease patients. A previous study reported that EMPs are increased when disease relapsed in four patients. EMPs released from the activated and apoptotic cells are the main cause of endothelial dysfunction. Assessed by flow-mediated dilatation (FMD), the levels of EMPs in patients with active SLE were significantly related to their vascular endothelium dysfunction. When the inflammation was suppressed, the absolute number of EMPs decreased with endothelial dysfunction of patients with SLE improved. In Kawasaki Disease, a kind of pediatric vasculitis, the concentrations of EMPs in the blood of patients were significantly higher than those in the blood of healthy controls and were negatively correlated with the values of FMD. After anti-inflammation treatment of patients with Henoch-Schönlein Purpura and ANCA-associated vasculitis, the concentrations of EMPs observed to significantly decrease, and the decrease in the concentrations of EMPs was much faster than EC numbers after disease remission. Thus, EMP could be considered as a measure to assess disease activity.

Blood MPs are derived from various vascular cells, such as monocytes, granulocytes, platelets, T cells, B cells, ECs and erythrocytes. In resting cells, plasma membranes are composed of two leaflets. The outer leaflet contains phosphatidylethanolamine, whereas the inner leaflet is rich in PS and phosphatidylcholine. The transbilayer lipid distribution occurs mainly under the conditions of significant and sustained increase of cytosolic Ca²⁺, which leads to the surface exposure of PS and release of MPs. Additionally, MPs may be released because of the mechanical destruction of cells or the loss of membrane integrity in necrotic cells. Considering the fact that not all MPs expose PS on their surface, PS usually binds to AnxV, the study analyzed AnxV⁺ and AnxV⁻ MPs and indicated that independent of PS exposure, EMPs were positively correlated with TA activity.

There are several possible pathogenic effects of EMPs, notably on inflammation. In a model of human umbilical vein ECs (HUVECs), after treatment with TNF-α, EMPs were induced in a time-dependent manner, under the regulation of TNF receptor-1 or nuclear factor-κB. By expressing pro-apoptotic molecules and increasing intercellular adhesion molecule-1, EMPs promote monocyte adhesion and contribute to inflammation in ECs. Some EMPs contains apoptosis-modified chromatin and regulates the levels of co-stimulatory surface molecules and pro-inflammatory cytokines such as IL-6, TNF-α and IFN-α, and initiate NETosis in blood-derived neutrophils. Certolizumab, a TNF-α inhibitor, could prevent the production of EMPs by activated ECs indicating a new mechanism of anti-TNF therapy and suggested that EMPs could be an important therapeutic target. EMPs also play a role in hemostasis and thrombosis. This is largely related to the exposure of PS and tissue factor (TF). Based on control studies with EMPs from anti-human TF antibody-treated and non-activated HUVECs, EMPs from activated ECs expose TF on their surface, which is responsible for coagulant activity both in vivo and in vitro. The role of EMPs in endothelium function is controversial. In the report by Brodsky, EMPs were shown to impair vasorelaxation and nitric oxide production by aortic ring cells in a concentration-dependent manner in Sprague-Dawley rats. In mice, vitro experiments indicated that EMPs promote EC migration and proliferation by delivering miR-126 and further regulating the expression of the target protein SPRED1. However, one study reported that EMPs released by cultured cells inhibit angiogenesis in mouse models of atherosclerosis. In addition, in clinical studies, EMPs were shown to protect vessels under conditions of acute vascular stress, like septic shock. The effects of EMPs on vessels are likely dependent on the particular stimuli or the microenvironment. Instead of being a functionless marker of injury, EMPs might thus be a pivotal factor delivering downstream pro-inflammatory factors that can protect vessels from acute vascular inflammation and maintain, or even ameliorate vascular dysfunction in chronic diseases.

In contrast with some previous MP studies in immunological diseases, the concentrations of MPs from lymphocytes and monocytes in patients with TA were similar to those in healthy controls in this present study. These observations may be related to the long-term disease course in these patients, as lymphocytes play a role in disease pathogenesis during the early stages of disease, whereas ECs come into play in late stages. Secondly, lymphocytes and monocytes were mainly observed within erosional vessel walls,
thus, it is possible that lymphocytes and monocytes may affect in TA pathophysiology at sites of inflammation, while ECs play a role via releasing MPs in peripheral blood. Last but not the least, a previous study proved that in the peripheral blood from active TA patients, the ratio of CD4+/CD8+ T cells and HLA-DR circulating T cells were increased. This present study only calculated CD3+ MPs from T cells, but MPs from different subpopulations of T cells or the ratios of them were not considered.

There are several limitations need to be considered in this study. First, the current study is cross-sectional and makes it difficult to make out a causal relationship between MPs and TA. Second, despite of the fact that there are no criteria for EMPs detecting, and previous studies indicated that CD144 positive EMPs were increased in other vasculitis and proved to be free of contaminating with a higher frequency than others. The study only examined CD144+ EMPs, which represent one subpopulation of EMPs, while other populations like CD62E+ and CD31+ EMPs were not included in this study. This is the same issue when examining MPs from T cells, B cells and monocytes, for which the study only considered CD3+ , CD19+ and CD14+ subpopulations, respectively. More studies on MPs from subpopulations of different parental cells, and functions of EMPs in patients with TA and the mechanisms by which the EMPs participate in TA pathophysiology are warranted both in vivo and in vitro. Third, some studies indicated antinuclear antibodies (ANA), IgG, IL-6 and TNF-α increased in TA patients, though the results were controversial and uncertain. The relationships between these potential markers with MPs were not included in this study and could be analyzed in the following research. Last, as integrins and adhesion molecules play essential roles in leukocytes and ECs interaction, MPs might be related to these molecules in pathology. Further studies on this issue could be considered in the future.

**Conclusion**

Concentrations of endothelial microparticles are increased significantly in active Takayasu arteritis patients comparing with remission ones and correlated positively with inflammation in Takayasu arteritis. EMPs could be useful markers in assessing disease activity.

**Disclosures**

None declared.

**Acknowledgments**

None.

**Author Contributions**

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

**Research Funding**

This study was supported by the National Natural Science Foundation of China (grant no. 81170285) and the Research Fund for the Doctoral Program of Higher Education of China (grant no. 20101106110012) in the decision to submit the article for publication.

**Honorarium**

None.

**Competing Interests**

The funding organizations played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

**References**

1) Subramanyan R, Joy J, Balakrishnan KG: Natural history of aortoarteritis (Takayasu’s disease). Circulation, 1989; 80: 429-437
2) Arnaud L, Haroche J, Limal N, Toledano D, Gambotti L, Costedoat Chalumeau N, Le Thi Huong Boutin D, Cacoub P, Cluzel P, Koskas F, Kieffer E, Piette JC, Amoura Z: Takayasu arteritis in France: a single-center retrospective study of 82 cases comparing white, north African, and black patients. Medicine (Baltimore), 2010; 89: 1-17
3) Lee GY, Jang SY, Ko SM, Kim EK, Lee SH, Han H, Choi SH, Kim YW, Choe YH, Kim DK: Cardiovascular manifestations of Takayasu arteritis and their relationship to the disease activity: analysis of 204 Korean patients at a single center. Int J Cardiol, 2012; 159: 14-20
4) Seko Y, Minota S, Kawasaki A, Shinkai Y, Maeda K, Yagita H, Okumura K, Sato O, Takagi A, Tada Y: Perforin-secretory killer cell infiltration and expression of a 65-kd heat-shock protein in aortic tissue of patients with Takayasu’s arteritis. J Clin Invest, 1994; 93: 750-758
5) Danese S, Panes J: Development of drugs to target interactions between leukocyte and endothelial cells and treatment algorithms for inflammatory bowel diseases. Gastroenterology, 2014; 147: 981-989
6) Stefanès T: Endothelial apoptosis: could it have a role in
the pathogenesis and treatment of disease? Chest, 2000; 117: 841-854
7) Bombeli T, Karsan A, Tait JF, Harlan JM: Apoptotic vascular endothelial cells become procoagulant. Blood, 1997; 89: 2429-2442
8) Freyssinet JM, Toti F: Formation of procoagulant microparticles and properties. Thromb Res, 2010; 125 Suppl 1: S24-28
9) Burger D, Kwart DG, Montezano AC, Read NC, Kennedy CR, Thompson CS, Touyz RM: Microparticles induce cell cycle arrest through redox-sensitive processes in endothelial cells: implications in vascular senescence. J Am Heart Assoc, 2012; 1: e001842
10) Rodriguez-Carrion J, Alperi-Lopez M, Lopez P, Alonso-Castro S, Carro-Esteban SR, Ballina-Carcia FJ, Suarez A: Altered profile of circulating microparticles in rheumatoid arthritis patients. Clin Sci (Lond.), 2015; 128: 437-448
11) Nieuwland R, Berckmans RJ, McGregor S, Boing AN, Romijn FP, Westendorp RG, Hack CE, Sturk A: Cellular origin and procoagulant properties of microparticles in meningocelepsis. Blood, 2000; 95: 930-935
12) Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, Gay RE, Pisetsky DS, Gay S, Matucci-Cerinic M, Distler O: The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. Arthritis Rheum, 2008; 58: 2845-2853
13) Parker B, Al-Husain A, Pemberton P, Yates AP, Ho P, Gorodkin R, Teh LS, Alexander MY, Bruce IN: Suppression of inflammation reduces endothelial microparticles in active systemic lupus erythematosus. Ann Rheum Dis, 2014; 73: 1144-1150
14) Arend WP, Michel BA, Bloch DA, Hunder GG, Calabrese LH, Edworthy SM, Fauci AS, Leavitt RY, Lie JT, Lightfoot RW Jr, Masi AT, McShane DJ, Mills JA, Stevens MB, Wallace SL, Zvaifler NJ: The American College of Rheumatology 1990 criteria for the classification of Takayasu arteritis. Arthritis Rheum, 1990; 33: 1129-1134
15) Ueno A, Awane Y, Wakabayashi A, Shimizu K: Successfully operated obliteratorive brachiocerebral arteritis (Takayasu) associated with the elongated coarctation. Jpn Heart J, 1967; 8: 538
16) Ryan TJ, Faxon DP, Gunnar RM, Kennedy JW, King SR, Loop FD, Peterson KL, Reeves TJ, Williams DO, Winters WL Jr, Fisch C, DeSanctis RW, Dodge HT, Reeves TJ, Weinberg SL: Guidelines for percutaneous transluminal coronary angioplasty. A report of the American College of Cardiology/American Heart Association Task Force on Assessment of Diagnostic and Therapeutic Cardiovascular Procedures (Subcommittee on Percutaneous Transluminal Coronary Angioplasty). Circulation, 1988; 78: 486-502
17) Witwer KW, Buzas EI, Bemis LT, Bora A, Lasser C, Lotvall J, Nolte-t Hoe EN, Piper MG, Sivaraman S, Skog J, Thery C, Wauben MH, Hochberg F: Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles, 2013; 2
18) Venable AS, Williams RR, Haviland DL, McFarlin BK: An analysis of endothelial microparticles as a function of cell surface antibodies and centrifugation techniques. J Immunol Methods, 2014; 406: 117-123
19) Dachary PJ, Freyssinet JM, Pasquet JM, Carron JC, Norden AT: Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulphydryl groups. Blood, 1993; 81: 554-565
20) Bernal-Mizrachi, Jr W, Fierro C, Macdonough R, Velazques HA, Purrow J, Jimenez JJ, Horstman LL, Ferreira A, de Marchena E, Ahn YS: Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes. Int J Cardiol, 2004; 97: 439-446
21) Amabile N, Heiss C, Real WM, Minasi P, McGlothlin D, Rame EJ, Grossman W, De Marco T, Yeghiazarians Y: Circulating endothelial microparticle levels predict hemodynamic severity of pulmonary hypertension. Am J Respir Crit Care Med, 2008; 177: 1268-1275
22) Brogan PA, Shah V, Brachet C, Harnden A, Mant D, Klein N, Dillon MJ: Endothelial and platelet microparticles in vasculitis of the young. Arthritis Rheum, 2004; 50: 927-936
23) Paudel KR, Panth N, Kim DW: Circulating endothelial microparticles: a new hallmark of atherosclerosis progression. Scientifica (Cairo), 2016; 2016: 8514056
24) Duval A, Helley D, Capron L, Youinou P, Renaudineau Y, Dubucquoi S, Fischer AM, Hachulla E: Endothelial dysfunction in systemic lupus patients with low disease activity: evaluation by quantification and characterization of circulating endothelial microparticles, role of anti-endothelial cell antibodies. Rheumatology (Oxford), 2010; 49: 1049-1055
25) Guiducci S, Ricci L, Romano E, Ceccarelli C, Distler JH, Miniati I, Calabri GB, Distler O, Matucci Cerinic M, Falcini F: Microparticles and Kawasaki disease: a marker of vascular damage? Clin Exp Rheumatol Suppl, 2011; 29 Suppl 64: 121-125
26) Dursun I, Dusunsel R, Poyrazoglu HM, Gunduz Z, Patiroglu T, Ulger H, Guroge MK: Circulating endothelial microparticles in children with Henoch-Schonlein purpura; preliminary results. Rheumatol Int, 2011; 31: 1595-1600
27) Erdbruegger U, Grossheim M, Hertel B, Wyss K, Kirsch T, Waywoadt A, Haller H, Haubitz M: Diagnostic role of endothelial microparticles in vasculitis. Rheumatology (Oxford), 2008; 47: 1820-1825
28) Jy W, Horstman LL, Jimenez JJ, Ahn YS, Bir? E, Nieuwland R, Sturk A, Dignat-George F, Sabatier F, Camoin-Jau L, Sampol J, Hugel B, Zobairi F, Freyssinet JM, Nomura S, Shet AS, Key NS, Hebbel RP: Measuring circulating cell-derived microparticles. J Thromb Haemos, 2004; 2: 1842-1851
29) Connor DE, Exner T, Ma DD, Joseph JE: The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemostoc, 2010; 103: 1044-1052
30) Lee SK, Yang SH, Kwon I, Lee OH, Heo JH: Role of tumour necrosis factor receptor-1 and unclear factor-κB in production of TNF-α-induced pro-inflammatory microparticles in endothelial cells. Thromb Haemost, 2014; 112: 580-588
31) Diker J, Tel J, Pieterse E, Thielen A, Rother N, Bakker M, Fransen J, Dijkmann HB, Berden JH, Det Vries JM, Hilbrands LB, van der Vlag J: Circulating apoptotic mic-
roparticles in systemic lupus erythematosus patients drive the activation of dendritic cell subsets and prime neutrophils for NETosis. Arthritis Rheumatol, 2016; 68: 462-472
32) Angelot F, Seilles E, Biichle S, Berda Y, Gaugler B, Plumas J, Chaperot L, Dignat-George F, Tiberghien P, Saas P, Gamache-Ottou F: Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases. Haematologica, 2009; 94: 1502-1512
33) Mooberry MJ, Key NS: Mipoparticle analysis in disorders of hemostasis and thrombosis. Cytometry A, 2016; 89: 111-122
34) Abid MN, Boing A, Biro E, Hoek FJ, Vogel GM, Meuleman DG, Sturk A, Nieuwland R: Phospholipid composition of in vitro endothelial microparticles and their in vivo thrombogenic properties. Thromb Res, 2008; 121: 865-871
35) Brodsky SV, Zhang F, Nasleetti A, Goligorsky MS: Endothelium-derived microparticles impair endothelial function in vitro. Am J Physiol Heart Circ Physiol, 2004; 286: 1910-1915
36) Jansen F, Yang X, Hoelscher M, Cattelan A, Schmitz T, Proebsting S, Wenzel D, Vosan S, Franklin BS, Fleischmann BK, Nickenig G, Werner N: Endothelial microparticle-mediated transfer of microRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation, 2013; 128: 2026-2038
37) Ou ZJ, Chang FJ, Luo D, Liao XI, Wang ZP, Zhang X, Xu YQ, Ou JS: Endothelium-derived microparticles inhibit angiogenesis in the heart and enhance the inhibitory effects of hypercholesterolemia on angiogenesis. Am J Physiol Endocrinol Metab, 2011; 300: e661-e668
38) Mostefai HA, Meziani F, Mastronardi ML, Agouni A, Heymes C, Sargentini C, Asfar P, Martinez MC, Andrianietsitohaina R: Circulating microparticles from patients with septic shock exert protective role in vascular function. Am J Respir Crit Care Med, 2008; 178: 1148-1155
39) McCarthy EM, Wilkinson FL, Parker B, Alexner MY: Endothelial microparticles: pathogenic or passive players in endothelial dysfunction in autoimmune rheumatic diseases? Vascul Pharmacol, 2016; 86: 71-76
40) Bulut D, Maier K, Bulut-Streich N, Borgel J, Hanefeld C, Mugge A: Circulating endothelial microparticles correlate inversely with endothelial function in patients with ischaemic left ventricular dysfunction. J Card Fail, 2008; 14: 336-340
41) Nomura S, Inami N, Ozaki Y, Kagawa H, Fukuhsara S: Significance of microparticles in progressive systemic sclerosis with interstitial pneumonia. Platelets, 2008; 19: 192-198
42) Sagar S, Ganguly NK, Koicha M, Sharma BK: Immuno-pathogenesis of Takayasu arteritis. Heart Vessels Suppl, 1992; 7: 85-90
43) Nityanand S, Giscombe R, Srivastava S, Sinha N, Grunewald J, Lefvert Ak: A bias in the alphabeta t cell receptor variable region gene usage in Takayasu’s arteritis. Clin Exp Immunol, 1997; 107: 261-268
44) Venable AS, Williams RR, Haviland DL, McFarlin BK: An analysis of endothelial microparticles as a function of cell surface antibodies and centrifugation techniques. J Immunol Methods, 2014; 406: 117-123
45) Uppal SS, Verma S: Analysis of the clinical profile, autoimmune phenomena and T cell subsets (CD4 and CD8) in Takayasu’s arteritis: a hospital-based study. Clin Exp Rheumatol, 2013; 21(6 Suppl 32): S112-116
46) Eichhorn J, Sima D, Thiele B, Lindschau C, Turoski A, Schmidt H, Schneider W, Halle H, Luft FC: Anti-endothelial cell antibodies in Takayasu arteritis. Circulation, 1996; 94(10): 2396-2401
47) Nava A, Senecal JL, Banares JL, Ralmond I, Reyes PA: Absence of antiphospholipid/co-factor antibodies in Takayasu arteritis. Int J Cardiol, 2000; 75 Suppl 1: S99-S104
48) Park MC, Lee SW, Park YB, Lee SK: Serum cytokine profiles and their correlations with disease activity in Takayasu’s arteritis. Rheumatology (Oxford), 2006; 45: 545-548
Supplemental Fig. 1.
A, C, E and G were the scatter diagrams of FITC, FITC, PE and APC; B was the scatter diagram of endothelial microparticles in samples, respectively; D, F and H were the scatter diagrams of microparticles from T cells, B cells and monocytes in samples, respectively.