Dynamic Release and Clearance of Circulating Microparticles During Cardiac Stress

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D.A. and L.V.A. contributed equally to this study.

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In September 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.2 days.
ABSTRACT

Rationale: Microparticles are cell-derived membrane vesicles, relevant to a range of biological responses and known to be elevated in cardiovascular disease.

Objective: To investigate microparticle release during cardiac stress and how this response differs in those with vascular disease.

Methods and Results: We measured a comprehensive panel of circulating cell-derived microparticles by a standardised flow cytometric protocol in 119 patients referred for stress echocardiography. Procoagulant, platelet, erythrocyte and endothelial, but not leucocyte, granulocyte or monocyte-derived microparticles were elevated immediately following a standardised dobutamine stress echocardiogram, and decreased after one hour. 25 patients developed stress-induced wall motion abnormalities suggestive of myocardial ischemia. They had similar baseline microparticle levels to those who did not develop ischaemia but, interestingly, their microparticle levels did not change during stress. Furthermore, no stress-induced increase was observed in those without inducible ischaemia but with a history of vascular disease. 14 patients subsequently underwent coronary angiography. A microparticle rise during stress echocardiography had occurred only in those with normal coronary arteries.

Conclusion: Procoagulant, platelet, erythrocyte and endothelial microparticles are released during cardiac stress and then clear from the circulation during the next hour. This stress-induced rise appears to be a normal physiological response that is diminished in those with vascular disease.

Keywords: Microparticles, vascular disease, dobutamine stress echocardiogram, cardiac stress

Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>EMP</td>
<td>Endothelial cell-derived microparticle</td>
</tr>
<tr>
<td>PMP</td>
<td>Platelet-derived microparticle</td>
</tr>
<tr>
<td>LMP</td>
<td>Leucocyte-derived microparticle</td>
</tr>
<tr>
<td>DSE</td>
<td>Dobutamine stress echocardiography</td>
</tr>
</tbody>
</table>

INTRODUCTION

Cell-derived microparticles are sub-cellular membrane vesicles, ranging in size from 0.1μm to 1.0μm, that ‘bud off’ during cell activation or apoptosis. They are identifiable in the circulation by their parent cell surface markers. Certain subtypes, particularly platelet-derived, increase after strenuous exercise in healthy individuals, which suggests microparticles may have a physiological function to remove stress-induced cellular by-products. Such a response could be relevant to cardiovascular disease pathogenesis because failure to clear microparticles could lead to raised circulating levels of proatherogenic factors. In addition, ineffective microparticle release could lead to localised cell damage. We studied whether there is a stress-induced, dynamic release and clearance of microparticles in patients exposed to cardiac stress during dobutamine echocardiography. The group included those with atypical symptoms in whom coronary disease is excluded as well as those with complex disease who require risk stratification, which allowed study of variation in response between those with and without vascular disease.
METHODS

Expanded methods can be found in the online data supplement.

Sample collection and analysis.
Participants were recruited sequentially from patients undergoing dobutamine stress echocardiography (DSE) at the John Radcliffe Hospital, Oxford. Citrate blood samples were taken peripherally prior to, immediately following and 1 hour after DSE. Circulating microparticles were measured by flow cytometry. Functional impact was assessed by procoagulant assay.

Cardiovascular disease assessment.
DSE identified patients with coronary disease sufficient to cause acute myocardial ischaemia to study microparticle response related to ischaemia. Medical history and coronary angiography identified patients with vascular disease to determine whether differences in microparticle response relates to vascular disease or ischaemia.

Statistical analysis.
Statistical analysis is detailed in the online supplement.

RESULTS

Microparticules and cardiac stress.

119 participants took part and 107 had measurements 1 hour after DSE. Immediately following DSE, procoagulant, platelet, erythrocyte and CD31+CD41- EMP levels rose significantly. One hour later, procoagulant and platelet microparticles levels had returned to baseline. Erythrocyte-derived microparticles had also started to decrease and CD31+CD41- EMPs had fallen below baseline (Figure 1). Other microparticles did not alter during stress (data not shown). The rise in microparticles was correlated with an increase in procoagulant activity consistent with a clinically relevant functional impact (Figure 2C).

Microparticules and myocardial ischaemia.

There were 94 negative and 25 positive DSEs. Participants with a positive DSE were significantly older and had slightly lower cholesterol, but other clinical and echocardiographic variables were similar (Online Table I). Baseline microparticle levels did not differ between groups (Online Table II). However, procoagulant, platelet, erythrocyte and CD31+CD41- EMPs increased in participants following a negative DSE (Figure 2A) with no change up to one hour in those with a positive DSE. Adjustment for risk factors and medication by regression analysis did not alter this finding.

Microparticules and vascular disease.

To investigate whether this lack of response related to myocardial ischemia or underlying vascular disease, we compared microparticle release in 72 participants with a negative DSE without evidence of vascular disease, to microparticle release in 11 with known vascular disease. Procoagulant, platelet and erythrocyte-derived microparticles had not increased in participants with vascular disease (Figure 2B). In 14 participants who subsequently underwent coronary angiography, procoagulant, platelet and erythrocyte-derived microparticles had increased only in individuals with normal coronary
arteries (Figure 3A). Interestingly, a stress-induced increase in procoagulant and erythrocyte-derived microparticles had occurred in a patient with a positive DSE subsequently found to have normal coronaries and no change in microparticles had occurred in a patient with a negative DSE found to have coronary disease (Figure 3B).

**DISCUSSION**

We demonstrate a dynamic, rapid rise and fall in a broad range of circulating microparticles in response to cardiac stress. Interestingly, this response was not apparent in those who developed myocardial ischaemia, which appeared to relate to their underlying vascular disease, as no increase was seen in those with a negative stress echo but known vascular disease. Subsequent analysis based on angiographic disease supported these findings.

Our findings are consistent with previous reports of a trend for PMPs to increase after DSE as well as significant rises in healthy individuals following exercise and a high fat meal. Our analysis shows procoagulant, erythrocyte and PMPs levels are related and all elevate immediately following DSE. Microparticle levels depend on speed of clearance mechanism, currently thought to involve direct receptor-binding of liver or spleen phagocytes to phosphatidylserine or opsonisation proteins on the microparticles. PMP levels are known to fall rapidly following injection into rabbits and within 30 min following infusion in mice but few studies have investigated human clearance. We found, within one hour, elevated circulating procoagulant, platelet and endothelial cell-derived microparticles are significantly reduced.

Clearance rate differed by cellular origin, so that erythrocyte-derived microparticles had not declined as much and CD31+CD41- EMPs fell below baseline. As EMPs carry von Willebrand factor they present a potential thrombotic risk, which might explain their apparently more rapid clearance mechanism. However, EMP response to cardiac stress, in general, differed. CD31+CD41- EMPs, indicative of endothelial apoptosis, increased, but with a relatively small proportional change. There was no change in CD62E+ EMPs, a marker of endothelial cell activation, or CD144+ EMPs, an independent predictor of future cardiovascular events. Lower concentrations of CD31+CD41- EMPs or the standard negative staining approach, which excludes PMPs, but may lead to inclusion of LMPs, could explain this. Alternatively, CD31+CD41- EMP release in response to stress may have distinct characteristics as different EMP subpopulations are known to be released in response to different triggers.

Although we found no difference in EMP levels between those with and without vascular disease, EMP levels are an independent predictor of cardiovascular events in coronary patients and in those with end-stage renal failure, so EMP stress response in vascular patients may vary from other cellular microparticles.

This significant variability in the degree of change in microparticle levels between individuals following cardiac stress raises the interesting possibility that this could be a predictive biomarker. One patient with a normal DSE subsequently had an out-of-hospital arrest and had not had a microparticle rise during stress. However, lack of microparticle release was an indicator of vascular disease rather than ischaemia. A larger study with more extensive angiographic and clinical follow up will be required to determine prognostic significance of this dynamic response and allow for calculation of positive and negative predictive values. Investigation of dynamic microparticle release from other cells, in particular cardiomyocytes, may be of interest as markers of ischaemic burden.

In summary, we demonstrate, in a large cohort, that procoagulant, platelet, erythrocyte and endothelial-derived microparticles are released and cleared rapidly in response to cardiac stress. This dynamic response was not evident in the group of subjects with vascular disease. Although provisional, pending
replication, these observations raise the possibility that microparticle release is a protective mechanism to remove cellular stress signals, which is diminished in those with vascular disease. Interventions to enhance release and clearance of microparticles may be relevant for disease prevention.

SOURCES OF FUNDING
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DISCLOSURES
None

REFERENCES

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FIGURE LEGENDS

Figure 1. Dot plots (Median with IQR) of annexin V+ (A), platelet (B), erythrocyte (C) and endothelial cell microparticles (D), at time-points pre DSE, immediately post DSE and 1 hour post DSE.

Figure 2. Dot plots (Median with IQR) of annexin V+, platelet, erythrocyte and endothelial cell microparticles in Negative DSE and Positive DSE patients pre and immediately post DSE (A), and in patients with a Negative DSE and No Vascular Disease or Vascular Disease pre and immediately post DSE (B). Microparticle procoagulant activity by Zymuphen assay (C).

Figure 3. Box and whisker plots of annexin V+ microparticles, CD31+CD41+ PMPs, glycophorin A+ erythrocyte-derived microparticles and CD31+CD41- EMPs pre and immediately post DSE in participants with a confirmed diagnosis by angiogram (A). Percentage change in microparticle levels during DSE in a false positive and a false negative participant (B).
Novelty and Significance

What Is Known?

- Microparticles are small membrane vesicles, which can be stimulated to ‘bud off’ from cells into the circulation, and can trigger a range of biological responses.
- The cellular origin and quantity of circulating microparticles can be measured.
- Certain subtypes have been shown to be elevated in those with cardiovascular disease but also in healthy individuals after exercise.

What New Information Does This Article Contribute?

- Cardiac stress induces a rapid, dynamic increase in a broad range of circulating microparticles.
- These microparticles are then cleared from the circulation during the next hour.
- The stress-induced rise appears to be a normal physiological response that is diminished in those with vascular disease.

Microparticles are sub-cellular membrane vesicles that ‘bud off’ from cells into the circulation in response to different stimuli and can be identified, and quantified, based on markers on their surface. Although circulating levels of certain microparticles are raised in people with cardiovascular disease, exercise has also been found to increase levels in healthy subjects. This study investigated whether microparticle release is a normal phenomenon in response to cardiac stress, what type of microparticles are released, how quickly they then clear from the circulation and whether vascular disease is associated with a different response. A dynamic, rapid rise in a broad range of circulating microparticles was demonstrated in patients undergoing a standardised cardiac stress protocol. These microparticles then cleared from the circulation within an hour. Interestingly, this dynamic rise and fall was not apparent in those who had vascular disease; diagnosed based on their medical history and, when available, evidence from coronary angiography. These observations raise the possibility that stress-induced microparticle release may be a biomarker of a physiological response to remove cellular stress signals, which is diminished in those with vascular disease. Interventions to enhance both release and clearance of microparticles may be relevant for disease prevention.
Figure 3
Dynamic Release and Clearance of Circulating Microparticles During Cardiac Stress
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Methods

Subjects
Participants were recruited sequentially from patients referred to the same cardiologist at the John Radcliffe Hospital in Oxford for dobutamine stress echocardiography. All patients over the age of 18 years were approached unless they had had a myocardial infarction in the prior 7 days or unstable cardipulmonary disease. All aspects of data and sample collection were approved by the Oxfordshire Research Ethics Committee (REC No. 08/H0604/127) and all participants gave written informed consent.

Blood Sample Collection & Storage
Citrate blood samples were taken peripherally prior to and immediately following dobutamine stress echocardiography in all patients. An additional time-point 1 hour after the test was added following initial review of the data. Citrated blood samples were centrifuged at 3000g for 15 minutes within 10 minutes of the blood collection. Plasma was then centrifuged again at 13,000g for 2 minutes to produce platelet-poor-plasma. Two 250μl aliquots of platelet-poor-plasma was frozen immediately and stored at -80°C. In 9 participants, citrate blood was also taken following the administration of a contrast agent (Sonovue, Bracco) used during echocardiography, to assess any potential impact on measured microparticle levels. There was no difference in the levels of any microparticle subtype detected following administration of the ultrasound contrast agent alone (annexin V+ MPs 249.0 vs. 219.2 (p=0.820), CD31+CD41+ platelet-derived microparticles (PMPs) 123.7 vs. 123.6 (p=0.820), glycophorin A+ erythrocyte-derived microparticles 40.3 vs. 36.1 (p=0.820), CD31+CD41- EMPs 6.54 vs. 4.0 (p=0.820)).

Dobutamine Stress Protocol
Participants were asked to avoid agents that could antagonise the effects of dobutamine (e.g. beta-blockers) for 24 hours prior to the test. Dobutamine infusion was commenced intravenously at 10μg/kg/min and increased by 10μg/kg/min at 3 minute intervals to a maximum of 40μg/kg/min until 85% of maximal age predicted heart rate was reached. If peak maximal heart rate was not reached with dobutamine, and the patient had no contraindication, additional atropine was given (maximum 1.2mg). The test was terminated early if the patient developed significant ischaemia or an arrhythmia, and, if necessary for clinical stability, beta-blocker administered to reverse these conditions.

Flow Cytometry Measurement of Microparticles
250μl aliquots of platelet-poor-plasma were thawed at room temperature, centrifuged at 18,000g for 30 minutes, 225μl of supernatant was removed and 225μl of PBS-Citrate 0.32% added. The sample was centrifuged again at 18,000g for 30 min, 225μl of supernatant was removed and 75μl of PBS-Citrate 0.32% was added. AnnexinV-fluoresceinisothiocyanate (FITC) was used to stain procoagulant microparticles. CD31-phycoerythrin (PE) and CD41-phycoerythrin-Cy5 (PE-Cy5) were used to differentiate between platelet microparticles (CD31+CD41+) and EMPs (CD31+CD41-). CD144-PE, CD62E-PE-Cy5 and CD106-PE-Cy5 were also used as markers for EMPs. Glycophorin-A (CD235a)-PE was used to stain erythrocyte-derived microparticles. CD45-allophycocyanin (APC) was used as a marker for total leucocyte-derived microparticles (LMPs). CD66B-FITC was used to stain granulocyte-derived microparticles. CD14-PE was used to identify monocyte-derived microparticles. All antibodies were supplied by BD (Oxford, UK), except for CD144 (eBioscience, Hatfield, UK). Appropriate FITC PE, PE-Cy5 and APC isotypes were used as negative controls.
10μl of sample was incubated with the appropriate monoclonal antibody or isotype control for 30 minutes at room temperature, protected from light, followed by addition of 900μl PBS-Calcium. Samples were acquired using a BD FACSCalibur® (Becton Dickinson, Oxford UK). The microparticle gate was checked with 1 μM beads (Sigma L-2778). The positivity gates were checked by fluorescence-minus-one staining. All microparticle analyses were performed blind to the patient’s stress echocardiography and angiogram results.

**Procoagulant Microparticle Assay**

A second aliquot of 250μl citrate plasma was used to measure the functional MP procoagulant activity. Plasma samples were diluted in sample diluent supplemented with calcium, Factor Xa and thrombin inhibitors, then added to a 96-well plate coated with streptavidine and biotinylated annexin V. Following one hour incubation and five washing steps, Factor Xa-Va mixture and prothrombin were added. After 10 min, a thrombin specific chromogenic substrate was added. Citric acid was used to stop the reaction after 3 min and the thrombin generation is measured at 405nm. MPs in the plasma bind to the annexin V on the plate and then provide the phospholipid surface for FXa-FVa to activate prothrombin to thrombin substrate. The phospholipid concentration on microparticles is the limiting factor and, therefore, the thrombin generation is directly related to the phospholipid concentration on the MPs in the plasma.

**Cardiovascular Disease Assessment**

**Stress Echocardiography** – Stress echocardiography was used to identify patients with coronary artery disease sufficient to cause acute myocardial ischaemia and, thereby, determine impact of ischaemia on microparticle release. All examinations were conducted using a Phillips iE33 system with X5-1 transducer (Philips Medical Systems, Zoetermeer, The Netherlands). Image quality was assessed prior to the start of the test and, if endocardial definition was reduced in two adjacent myocardial segments, a contrast infusion started at 0.7ml/min to improve left ventricular opacification (SonoVue, Braco, UK). 2D echocardiographic images were obtained of the left ventricular apical four, three and two chamber views as well as the parasternal long and short (mid ventricular) views at baseline, at an intermediate level of stress and then at peak stress. Wall motion was assessed off-line, based on systolic wall thickening and endocardial movement, by two experienced operators who evaluated the whole myocardium split into a 16 segment model. Scores were given for wall motion as 1 = normal; 2 = hypokinetic; 3 = akinetic and 4 = dyskinetic. A deterioration in segment score with increasing dobutamine was used as the indicator of ischemia and each participant defined as having a ‘positive’ i.e. evidence of inducible ischaemia, or ‘negative’ stress echocardiogram.

**Medical History** – Medical history was used to identify patients with a history of vascular disease but negative stress echocardiograms and, thereby, study the impact of vascular disease on microparticle release, independent of ischaemia. Medical records were reviewed and patients completed a questionnaire. Cardiovascular risk factors, such as hypertension, hypercholesterolaemia, diabetes or smoking, as well as treatment for these factors, was noted, but not used as evidence of established vascular disease. Patients were classified as having vascular disease if they had a history of biochemical (troponin rise) or ECG-proven myocardial ischaemia, or coronary disease on previous coronary angiography. In addition, those with previous coronary bypass surgery or vascular surgery as well as those with peripheral vascular disease, including ultrasound-documentied abdominal aortic aneurysms, were classified as having vascular disease.

**Follow up coronary angiography** – All patient medical records were reviewed in the year following their participation to determine whether they had undergone coronary angiography subsequent to the stress echo. Review of the clinical reports revealed patients who had undergone angiography either had significant coronary disease (greater than 70% luminal narrowing in at least one coronary artery) or normal coronary arteries (no more than 30%
luminal narrowing). This information was also used to identify false positive or false negative stress echocardiograms.

**Statistical Analysis**
Statistical analysis was performed using GraphPad Prism 5 and SPSS Version 20. Differences in demographics and baseline microparticle levels between patient groups were compared by unpaired t-tests or Mann Whitney tests (if non-parametrically distributed). Changes in microparticle levels following stress echocardiography were assessed by paired non-parametric T-tests (Wilcoxon matched-pairs signed rank tests). A p-value of <0.05 was considered statistically significant.
Online Table I. Demographics of Participants

<table>
<thead>
<tr>
<th></th>
<th>All Participants (n=119)</th>
<th>Negative DSE (n=94)</th>
<th>Positive DSE (n=25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (SD)</td>
<td>61.8 (12.5)</td>
<td>59.9 (12.2)</td>
<td>68.8 (10.9)</td>
<td>0.001**</td>
</tr>
<tr>
<td>Males/Females</td>
<td>54/65</td>
<td>39/55</td>
<td>15/10</td>
<td></td>
</tr>
<tr>
<td>Systolic BP</td>
<td>141.0 (23.0)</td>
<td>140.4 (23.2)</td>
<td>143.5 (23.0)</td>
<td>0.60</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>72.2 (15.0)</td>
<td>72.2 (15.1)</td>
<td>72.3 (14.9)</td>
<td>0.98</td>
</tr>
<tr>
<td>Resting HR</td>
<td>75.7 (15.2)</td>
<td>75.6 (16.3)</td>
<td>76.1 (11.4)</td>
<td>0.90</td>
</tr>
<tr>
<td>% Max Predicted HR</td>
<td>84.9 (6.3)</td>
<td>85.4 (6.1)</td>
<td>83.7 (6.7)</td>
<td>0.26</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.7 (1.2)</td>
<td>4.9 (1.1)</td>
<td>4.2 (1.3)</td>
<td>0.02*</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.3 (0.4)</td>
<td>1.3 (0.4)</td>
<td>1.2 (0.4)</td>
<td>0.39</td>
</tr>
<tr>
<td>Current Smokers</td>
<td>23.5%</td>
<td>24.3%</td>
<td>20.8%</td>
<td>0.17</td>
</tr>
<tr>
<td>Cholesterol lowering medication</td>
<td>54.8%</td>
<td>54.3%</td>
<td>56.5%</td>
<td>0.98</td>
</tr>
<tr>
<td>BP lowering medication</td>
<td>65.4%</td>
<td>63.8%</td>
<td>70.8%</td>
<td>0.53</td>
</tr>
<tr>
<td>Anti-platelet medication</td>
<td>40.9%</td>
<td>39.1%</td>
<td>45.8%</td>
<td>0.20</td>
</tr>
<tr>
<td>Diabetes</td>
<td>19.8%</td>
<td>20.0%</td>
<td>19.0%</td>
<td>0.85</td>
</tr>
<tr>
<td>Hypertension</td>
<td>64.8%</td>
<td>63.8%</td>
<td>68.0%</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Values are mean (SD) unless otherwise stated. P-values are derived from unpaired t-tests comparing Negative DSE participants with Positive DSE participants. BP = blood pressure, HR = heart rate.
### Online Table II - Baseline Levels of Circulating Microparticles

<table>
<thead>
<tr>
<th>Microparticles (MPs) per ul, median (10-90 Percentiles)</th>
<th>Negative DSE (n=94)</th>
<th>Positive DSE (n=25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V+ MPs</td>
<td>319.5 (132.8 – 728.9)</td>
<td>258.3 (111.8 – 1146.0)</td>
<td>0.50</td>
</tr>
<tr>
<td>CD31+CD41+ PMPs</td>
<td>170.1 (73.3 – 425.5)</td>
<td>170.0 (74.0 – 407.2)</td>
<td>0.99</td>
</tr>
<tr>
<td>Glycophorin A+ Erythrocyte MPs</td>
<td>85.4 (11.9 – 292.7)</td>
<td>48.7 (6.9 – 307.3)</td>
<td>0.34</td>
</tr>
<tr>
<td>CD31+CD41- EMPs</td>
<td>4.4 (1.2 – 19.7)</td>
<td>4.6 (0.8 – 22.9)</td>
<td>0.83</td>
</tr>
<tr>
<td>CD144+ EMPs</td>
<td>3.5 (0.6 – 15.3)</td>
<td>4.5 (1.6 – 16.0)</td>
<td>0.48</td>
</tr>
<tr>
<td>CD62E+ EMPs</td>
<td>3.2 (1.0 – 25.4)</td>
<td>4.7 (0.8 – 17.1)</td>
<td>0.90</td>
</tr>
<tr>
<td>CD106+ EMPs</td>
<td>3.4 (1.1 -16.5)</td>
<td>3.3 (0.9 – 16.0)</td>
<td>0.60</td>
</tr>
<tr>
<td>CD45+ LMPs</td>
<td>6.6 (0.7 – 20.8)</td>
<td>8.0 (0.3 – 25.6)</td>
<td>0.32</td>
</tr>
<tr>
<td>CD66B+ Granulocyte MPs</td>
<td>5.6 (1.3 – 20.0)</td>
<td>5.1 (1.3 – 26.8)</td>
<td>0.92</td>
</tr>
<tr>
<td>CD14+ Monocyte MPs</td>
<td>5.5 (0.6 – 30.2)</td>
<td>8.8 (0.5 – 35.5)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Microparticles (MPs) per ul, median (10-90 Percentiles). P-values are derived from non-parametric unpaired T-tests (Mann-Whitney Tests) between Negative DSE and Positive DSE participants.