Plasma-Derived Circulating Microvesicles Can Both Detect and Help Monitor Breast Cancer–Related Pathologies

*Daniel A. Holterman, Janet E. Duncan, Ta Deng, Shannon E. Smith, David B. Spetzler, Christine D. Kuslich
Caris Life Sciences, 4610 South 44th Place, Phoenix, AZ (www.carislifesciences.com)

**Abstract**
Circulating microvesicles (cMVs) are 40-100nm membrane-bound structures that play an important role in several biologic processes including angiogenesis and immune modulation. cMV levels are known to change in response to certain pathologies, and specific subpopulations of cMV can be isolated using antibodies targeted to membrane proteins specific for the secreting cells. We examined whether the levels of specific cMV subpopulations are altered in patients with a disease, specifically breast cancer. In order to characterize individual cancer-associated microvesicles (MVs), advanced breast cancer MVs were compared with normal control (NCM) MVs. Circulating microvesicles were isolated from breast cancer patients and non-cancer control patients, stained with fluorochrome-conjugated antibodies, and analyzed using flow cytometry. Tumor specific antibodies were paired with process specific markers, such as DLL4 and VEGFR2 for angiogenic cMVs, CTLA4 and Fasl for immunosuppressive cMV, and CD80 or CD83 for immunostimulatory cMV to identify and characterize cMV subpopulations.

Using this approach, distinct and informative cMV subpopulations were identified and quantified between breast cancer patients and healthy controls. For example, immunosuppressive MVs were elevated in the cancer patients compared with noncancer controls (68% vs 44% of CD45-cMV coexpressed CTLA4). Additionally, angiogenic MVs were elevated in cancer plasma, with 44% coexpressing DLL4 and CD31 compared with 2% of cMV from noncancer controls. This study suggests that in addition to potentially aiding in the diagnosis of breast cancer, monitoring specific subpopulations of cMVs may offer important information regarding biologic processes perturbed by breast cancer in individual patients. Without the need for an invasive biopsy or a standard pathology evaluation such as immunohistochemistry, cMVs therefore may also aid in prognosis of breast cancer.

**Methods**
Plasma-derived cMVs from 5 women with advanced-stage breast cancer (Stage IIIC) and 4 healthy women were labeled with panels of fluorochrome-conjugated antibodies according to the indicated panels in Table 1. The stained cMVs were analyzed using a Beckman-Coulter Mo-Flow XDP. Four-color staining was used to evaluate immunosuppressive cMVs (Tetraspanin+, CD45+, Fasl+, CTLA4+), angiogenic cMVs (Tetraspanin+, CD31+, DLL4+, VEGFR2+ or Tie2+, Ang-1+), and metastatic cMVs (Tetraspanin+, Mac1+, CD47+, TIMP1+, TIMP2+, MMP7+, MMP9+). Results were calculated as the percentage of positively stained particles as well as number of positive particles per ul of plasma.

**Results**
The results of these studies demonstrate increased percentage and number of immunosuppressive, angiogenic, and metastatic cMVs in late-stage breast cancer patients compared with healthy women. Immunosuppressive cMVs were >700-fold higher, metastatic cMVs were >3-fold higher and angiogenic cMVs were >21-fold higher by volume of plasma in advanced breast cancer plasma compared to controls.

**Conclusions**
CMVs were also evaluated to identify the presence of breast cancer in women. Two markers were most useful for this purpose. The sensitivity using these two markers in a training set of 80 breast cancer and 34 healthy controls was ~95%, the specificity was ~91% and the accuracy of this test was 93%. Additionally, 28 of the breast cancer cases were diagnosed as lobular carcinoma. Of these 28 cases 25 were correctly diagnosed in this assay for a sensitivity of ~90% for lobular carcinoma.

Because CMVs are known to play a role in cell-cell communication and to be involved with immunosuppression, metastasis and angiogenesis, flow cytometry was used to compare the protein expression of CMVs in breast cancer patients vs. controls. This staining is analogous to phenotyping of cells to evaluate these same processes in vivo. Phenotyping studies showed that typical “percent positive” analysis was not useful to identify subpopulations of biologically relevant CMVs. However, cancer patients tend to have elevated levels of CMVs compared to healthy controls so these percentage differences are magnified when we compare the total number of events in 1ul of plasma. For example the number of immunosuppressive CMVs derived from DCs (CD80+/TIMP1+) averages 22.5x10^6 in healthy controls and 68.6x10^6 in the breast cancer patients. Similarly 8 metastatic (CD147+/TIMP1+) CMVs/ul were detected in healthy volunteers compared to 28x10^6 in the breast cancer plasma. Finally angiogenic CMVs (VEGFR2+/Tie2+) increased from 10x10^6 to 221x10^6 in breast cancer plasma.

**Results continued**

**Table 1**

<table>
<thead>
<tr>
<th>Marker</th>
<th>N (standard cutoff)</th>
<th>CMVs (Tex-1)</th>
<th>CD80+/TIMP1+</th>
<th>VEGFR2+/Tie2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (2.38x10^6)</td>
<td>1% (22.5x10^6)</td>
<td>0.3% (28x10^6)</td>
<td>3% (221x10^6)</td>
</tr>
</tbody>
</table>

**Example Breast Cancer Metastatic**

**Example Breast Cancer Angiogenic**

**Example Healthy Metastatic**

**Example Healthy Immunosuppressive**

**Example Healthy Angiogenic**

**Example Breast Cancer**

**Flow cytometry analysis**

Can we use CarisCorrel™ (cMVs) to perform a “virtual biopsy” on a patient’s tumor from a simple blood draw?