Title

Identification of CD105+ Extracellular Vesicles as a Candidate Biomarker for Metastatic Breast Cancer

Authors

Douglas, Sasha R
Yeung, Kay T
Yang, Jing
et al.

Publication Date

2021-12-01

DOI

10.1016/j.jss.2021.06.050

Peer reviewed

HHS Public Access

Author manuscript

J Surg Res. Author manuscript; available in PMC 2022 December 01.

Published in final edited form as:

J Surg Res. 2021 December ; 268: 168–173. doi:10.1016/j.jss.2021.06.050.

Identification of CD105+ Extracellular Vesicles as a Candidate Biomarker for Metastatic Breast Cancer

Sasha R Douglas[a], **Kay T Yeung**[b], **Jing Yang**[c], **Sarah L Blair**[d], **Olga Cohen**[a], **Brian P Eliceiri**[a]

[a]Department of Surgery, University of California San Diego 9300 Campus Point Drive, #7220; La Jolla, CA 92037, USA

[b]Department of Medicine, Division of Hematology and Oncology, University of California San Diego, 9300 Campus Point Drive, #7220; La Jolla, CA 92037, USA

[c]Department of Pharmacology, Moores Cancer Center, University of California San Diego, 3855 Health Sciences Dr, La Jolla, CA 92093-0819, USA

[d]Division of Breast Surgery and The Comprehensive Breast Health Center, University of California San Diego, Mailbox 0819, 3855 Health Sciences Dr, La Jolla, CA 92037, USA

Abstract

Background: Extracellular vesicles (EVs) released by malignant tumor cells can mediate the immune response and promote metastasis through intercellular communication. EV analysis is an emerging cancer surveillance tool with advantages over traditional liquid biopsy methods. The aim of this pilot study is to identify actionable EV signatures in metastatic breast cancer.

Materials and Methods: Under an IRB-approved protocol for the analysis of patient plasma, samples were collected from women with newly diagnosed or progressive metastatic breast cancer and from women without cancer. Enriched EVs were analyzed via a bead-based multiplex assay designed to detect 37 distinct tumor-relevant epitopes. The mean fluorescent intensity (MFI) of EV epitopes meeting a minimum threshold of detectability was compared between groups via independent samples t-test. Subgroup analysis was conducted for metastatic breast cancer patients who were positive for estrogen and/or progesterone receptors and negative for HER2. Other variables potentially affecting CD105 levels were also analyzed.

Results: CD105 was found to have a significantly higher MFI in participants with metastatic breast cancer compared to control participants (p=0.04). ER/PR+ subgroup analysis revealed a

Corresponding Author: Brian Eliceiri, PhD, 9500 Gilman Drive; MC8236, La Jolla, CA 92037, USA, beliceiri@health.ucsd.edu, Phone: 619.543.1969.

Author Contributions: SD assisted with study design, analyzed and interpreted the data, wrote the manuscript, and approved the final version. KY assisted with study design, provided critical manuscript revisions, and approved the final version. JY provided critical manuscript revisions and approved the final version. SB provided critical manuscript revisions and approved the final version. OC analyzed the data, provided critical manuscript revisions, and approved the final version. BE conceived and designed the study, collected the data, analyzed and interpreted the data, provided critical manuscript revisions, and approved the final version.

DECLARATION OF INTEREST

Declarations of Interest: None.

similar pattern compared to control participants (p=0.01). Other analyzed variables were not found to have a significant correlation with CD105 levels.

Conclusions: CD105 EV levels were significantly higher in samples from participants with breast cancer compared to controls. Given that CD105 is known to mediate angiogenesis and promote metastasis, EV-associated CD105 in plasma represents a potential biomarker for diagnosis, surveillance and therapeutic targeting in patients with metastatic breast cancer.

Keywords

Endoglin; extracellular vesicles; exosomes; CD105; breast cancer; biomarkers

INTRODUCTION

Extracellular vesicles (EVs) are small membranous particles that are pre-formed in multivesicular bodies (MVBs) and released from the cell surface to carry cargo between cells [1, 2]. Analysis of EV content provides insight into cellular crosstalk in both normal physiologic and disease states [2, 3]. In cancer, this mode of intercellular communication can modify the tumor microenvironment by promoting malignant growth and dissemination of factors that suppress immunosurveillance [4, 5]. Because EVs are secreted by both normal and malignant cells, it can be challenging to identify markers specific to malignancy [6].

Despite this challenge, EV analysis represents a potentially superior alternative to more traditional biomarkers for several reasons. EVs can be harvested in a minimally invasive manner from multiple biofluids including blood, urine, and cerebrospinal fluid [1, 5]. The relative ease of sampling allows for serial analysis of EV content which has clinical utility for surveillance, treatment response, and recurrence monitoring [7]. In addition, EVs are relatively stable when frozen which allows for storage of samples for subsequent analysis at a later time point [8]. Perhaps most importantly in terms of setting them apart from more traditional biomarkers, EVs carry nucleic acids and proteins that are taken up by recipient cells which are subsequently altered [9]. For example, EVs from ovarian cancer cells have been shown to differentiate macrophages towards their more immunosuppressive phenotype [10]. Disruption of tumor promoting EVs has potential for intervention in the malignant process which may expand the population of individuals with breast cancer who could be amenable to surgical intervention. Taken together, the identification of EV proteins shed from tumors released into the peripheral blood constitutes a liquid biopsy approach with several advantages over other established methods.

In this study, we assessed an array of EV proteins relevant to tumor immunology with the goal of identifying proteins that correlate with disease in patients diagnosed with metastatic breast cancer. In a screen of 37 proteins, we identified CD105 (also known as endoglin) as a promising biomarker that was increased on EVs of cancer patients. CD105 is a transmembrane glycoprotein known to mediate angiogenesis and has been studied as a therapeutic target in tumor beds expressing high levels of CD105 [11–15]. Its normal physiologic function as a coreceptor for the transforming growth factor (TGF)-β super family is diverse and include embryonic development of vascular networks,

the differentiation of blood cells in hematopoiesis, and promotion of wound healing [16]. As a cancer stem cell marker, CD105+ cells have been shown to express greater levels of mesenchymal markers and are associated with promotion of tumor epithelial to mesenchymal transition (EMT), which allows for increased mobility of malignant cells [17]. Excess expression of CD105 has been shown to further promote tumor cell migration and intravasation via destabilization of the endothelium [18]. Increased plasma levels of CD105 are seen in patients with metastatic breast cancer but, to our knowledge has not yet been specifically traced to EVs [18]. Therefore, we focus on CD105+ EVs as translationally relevant tumor factors with potential for breast cancer detection, surveillance, and therapeutic intervention.

METHODS

Breast cancer patients and healthy individuals

Under a UCSD IRB-approved protocol for collection and banking of biological samples for use in biomedical research, plasma was collected from participants who provided informed consent. This included 16 women with stage IV metastatic breast cancer and 13 women without cancer. Female participants aged 18 and over were enrolled at UC San Diego medical center. Participants with metastatic cancer were included if newly diagnosed or progressing on current therapy and were excluded if diagnosed with any concurrent cancer within the past five years prior to enrollment. Samples were deidentified following collection and were correlated only with basic demographic data including age, hormone receptor status, treatment status, and metastatic location as applicable.

EV surface epitope assay

For each sample, 1 mL of plasma was centrifuged at 10,000 rpm at 4°C for 10 minutes and the top 80% of supernatant transferred to a fresh tube. This process was repeated, and samples were stored at −80°C until enough samples were accumulated for concurrent analysis. Each sample was then passed through a 0.2μm sterile low protein-binding syringe filter and centrifuged at 55,000 rpm for 70 minutes at 4°C. The pellet containing EVs was resuspended in PBS and analyzed via a multiplex analysis using bead-bound antibodies designed to measure proteins in pre-isolated EVs. The EV multiplex assay (MacsPlex Exosome Kit, Miltenyi Biotec #130-108-813) was performed in triplicate to detect 37 different target proteins (listed in Figure 1) relevant in tumor immunology, along with isotype antibody controls and analyzed on a MACSQuant10 flow cytometer according to the manufacturer’s recommendations [19].

Statistical analysis

Each EV protein was analyzed to assess whether it was consistently present above isotype control background and with an acceptable standard deviation between triplicate samples with the number of times a marker met these criteria expressed as a proportion out of the number of samples analyzed. The mean fluorescent intensity (MFI) of markers meeting these criteria greater than 50% of the time were compared between the cancer patient and healthy control group using an unpaired t-test with Welch correction so as not to assume equal variance. Subgroup analysis was also conducted for metastatic breast cancer patients

who were positive for estrogen and/or progesterone receptors and negative for HER2 (ER/PR+HER2−). Variables such as age, metastatic location, and newly metastatic or progressive disease were also considered as possible independent variables affecting CD105 levels. Statistical analysis was conducted using GraphPad Prism Version 8.0 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics for Macintosh, Version 26.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Mean age of participants was 56 (range 25–82, standard deviation (SD) 13) at time of enrollment for patients with cancer and 43 (range 26–57, SD 10) for controls. Though age difference between groups was significant ($p < 0.05$), it was not found to be correlated with CD105 levels on linear regression ($F(1,27)=1.076$, $p=0.31$) with $R^2 = 0.038$. Regarding breast cancer subtypes, eight were ER+/PR+, four were ER+/PR+ and HER2+, one was HER2+, and three were triple negative. Two (13%) participants had brain metastases, 7 (44%) had lung metastases, 9 (56%) had liver metastases, and 15 (94%) had bone metastases. Two (13%) participants had newly diagnosed metastatic disease and had not yet initiated therapy while 14 (87%) had established metastatic disease progressing on current therapy as confirmed by imaging (Table 1).

Our initial analysis revealed a difference in the rate of EV protein detectability within our samples, an indirect measure of the sensitivity of our assay that we used as the basis for inclusion or exclusion for further analysis. While some EV epitopes were either undetectable or rarely detectable, others were reliably detected (Figure 1). Proteins detected in more than 50% of samples included CD105, HLA-ABC, CD56, and SSEA4, but only CD105 had a significantly higher MFI in patients with metastatic breast cancer compared to controls (976 versus 586 respectively, $p<0.05$, Figure 2, Table 2). Subgroup analysis with only ER/PR+ breast cancer patients compared to controls revealed a similar pattern (875 versus 586 respectively, $p<0.05$, Figure 2, Table 3). Among cancer participants, variables such as metastatic location, multiple metastases, and whether the metastatic disease was newly diagnosed were considered without any significant differences detected (Table 4).

DISCUSSION

We demonstrate that CD105+ EVs are detectable in plasma from women with metastatic breast cancer and sex-matched volunteers without cancer. Significant differences were observed with higher levels of $CD105^+$ EVs in the cancer group and the ER/PR+HER2− cancer subgroup compared to the control group. This suggests an increase in angiogenesis activity related to progressive cancer metastases. Among the cancer group, none of the examined variables (i.e., metastatic location, number of metastases, or newly diagnosed disease) were significantly correlated with CD105 levels (Table 4). This may be related to small sample sizes, especially since variables such as brain metastases were less frequent in our cohort. However, it is interesting to note, that despite small sample size in the newly metastatic group compared to those progressing on therapy, there was a trend towards lower levels of CD105 in the former that may merit further investigation in larger groups. Although other EV proteins in our experimental panel were detected with no significant

differences, these unchanged EV proteins may serve as a control for common proteins in frozen human plasma EV samples in future studies.

There were several advantages in our methodology. The ease with which biofluids such as plasma can be collected and stored allows for multiple participant samples or serial samples from the same patient to be compared in a single multiplex analysis. As our study suggests, this could allow for examination of mediators of angiogenesis and immune surveillance compared between participants or over time with minimal technical variability. Use of a commercially available kit affords greater reproducibility for similar types of studies on other cancer subtypes, longitudinal studies, and other disease processes. Finally, the detection of differences in EVs is advantageous over other biomarkers given the increased potential for targeted intervention on intercellular crosstalk. EVs released by cancer cells can transfer functional mRNA to alter non-malignant cells; thus, disruption of this process could help prevent malignant spread and make other treatment such as surgery more durable and effective [20].

While our study design has many benefits in terms of sensitivity and compatibility with frozen samples, there are also several limitations. Small sample size was secondary to constraints in recruiting subjects meeting inclusion criteria from a single cancer center. Significant heterogeneity exists within this experimental group including differences in age, receptor status, and type and duration of treatment regimen. This may serve to improve external validity but reduces ability to examine the effect of individual variables. Use of a fixed panel improves reproducibility but is limiting in the sense that there may be differences in EV population that were no detected due to lack of inclusion in the panel.

Our analysis of CD 105+ EVs as a potential biomarker for metastatic breast cancer, in conjunction with what is already known about CD105, could serve as the foundation for several future research studies and clinical possibilities in both diagnostics and therapeutics. CD105 is well-established in vascular biology and previous studies have described that this transmembrane glycoprotein can promote malignant angiogenesis and metastases through regulation of VEGF expression in various types of cancer cells [21–25]. Regulation of this crosstalk by targeting CD105+ EVs, could interfere with the progression of this invasive process. Utilizing EVs in this manner would be especially helpful, as they exhibit organotropism for metastatic sites and can be used for targeted drug delivery [26, 27]. Anti-angiogenic therapy could be loaded into and carried by EVs directly to the site of metastatic angiogenesis. This strategy, if effective in animal models of metastatic breast cancer, could eventually be adopted as a targeted therapy in clinical trials.

As a diagnostic, future study with recruitment of participants with earlier breast cancer stages and serial sampling of plasma from both metastatic and non-metastatic breast cancer could improve our understanding of how CD105+ EV levels change in different disease states, during disease progression, and in response to therapy. Higher than normal levels of CD105+ EVs in patients with early-stage breast cancer, for example may allow for risk stratification for development of metastatic progression before it is detectable by traditional means. Normalization of CD105+ EV levels in patients on therapy or after surgery may

suggest efficacy of treatment, while failure of normalization could suggest the need to alter treatment.

Conclusions

We have identified CD105 as a promising EV protein to distinguish women with metastatic breast cancer from women without cancer using a commercial kit. Future directions include quantifying CD105+ EV levels in patients with different stages of breast cancer, using serial sampling to correlate levels with clinical improvement or progression, and targeting malignant EVs to disrupt the metastatic process. Such studies could lead to minimally invasive techniques for early breast cancer detection, recurrence monitoring, and treatments that could expand the population of women with breast cancer who could benefit from surgery.

ACKNOWLEDGEMENTS

We would like to acknowledge and thank Ann-Marie Hageny for her expertise using the MACSPlex Exosome Kit.

FUNDING

Specimen samples from participants with metastatic breast cancer were provided by Moores Cancer Center at UC San Diego Health Comprehensive Biorepository, which is partially funded by the National Cancer Institute (NCI P30CA23100).

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award Number T32CA121938 (S.D.), the NIH R01GM140137 (B.P.E.), the Academic Senate of UC San Diego (B.P.E and S.B.), DOD Breast cancer program award W81XWH-18-1-0055, METAvivor Research Award, and NIH R01CA174869 and R01CA236386 (J.Y.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

REFERENCES

1. Halvaei S, Daryani S, Eslami-S Z, et al. (2017) Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer. Mol Ther Nucleic Acids 10:131–141. 10.1016/j.omtn.2017.11.014 [PubMed: 29499928]
2. Ståhl A, Johansson K, Mossberg M, et al. (2019) Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. Pediatr Nephrol 34:11–30. 10.1007/s00467-017-3816-z [PubMed: 29181712]
3. Brahmer A, Neuberger E, Esch-Heisser L, et al. (2019) Platelets, endothelial cells and leukocytes contribute to the exercise-triggered release of extracellular vesicles into the circulation. J Extracell Vesicles 8:. 10.1080/20013078.2019.1615820
4. Grange C, Tapparo M, Collino F, et al. (2011) Microvesicles Released from Human Renal Cancer Stem Cells Stimulate Angiogenesis and Formation of Lung Premetastatic Niche. Cancer Res 71:5346–5356. 10.1158/0008-5472.CAN-11-0241 [PubMed: 21670082]
5. Xu R, Rai A, Chen M, et al. (2018) Extracellular vesicles in cancer — implications for future improvements in cancer care. Nat Rev Clin Oncol 15:617–638. 10.1038/s41571-018-0036-9 [PubMed: 29795272]
6. Bae S, Brumbaugh J, Bonavida B (2018) Exosomes derived from cancerous and non-cancerous cells regulate the anti-tumor response in the tumor microenvironment. Genes Cancer 9:87–100. 10.18632/genesandcancer.172 [PubMed: 30108680]
7. Osti D, Del Bene M, Rappa G, et al. (2019) Clinical Significance of Extracellular Vesicles in Plasma from Glioblastoma Patients. Clin Cancer Res 25:266–276. 10.1158/1078-0432.CCR-18-1941 [PubMed: 30287549]

8. Jeyaram A, Jay SM (2017) Preservation and Storage Stability of Extracellular Vesicles for Therapeutic Applications. AAPS J 20:1. 10.1208/s12248-017-0160-y [PubMed: 29181730]
9. Tao S-C, Guo S-C (2020) Role of extracellular vesicles in tumour microenvironment. Cell Commun Signal 18:163. 10.1186/s12964-020-00643-5 [PubMed: 33081785]
10. Chen X, Zhou J, Li X, et al. (2018) Exosomes derived from hypoxic epithelial ovarian cancer cells deliver microRNAs to macrophages and elicit a tumor-promoted phenotype. Cancer Lett 435:80–91. 10.1016/j.canlet.2018.08.001 [PubMed: 30098399]
11. Dallas NA, Samuel S, Xia L, et al. (2008) Endoglin (CD105): A Marker of Tumor Vasculature and Potential Target for Therapy. Clin Cancer Res 14:1931–1937. 10.1158/1078-0432.CCR-07-4478 [PubMed: 18381930]
12. Duff SE, Li C, Garland JM, Kumar S (2003) CD105 is important for angiogenesis: evidence and potential applications. FASEB J 17:984–992. 10.1096/fj.02-0634rev [PubMed: 12773481]
13. Zhuo H, Zheng B, Liu J, et al. (2018) Efficient targeted tumor imaging and secreted endostatin gene delivery by anti-CD105 immunoliposomes. J Exp Clin Cancer Res 37:42. 10.1186/s13046-018-0712-8 [PubMed: 29499713]
14. Ehlerding EB, Ferreira CA, Aluicio-Sarduy E, et al. (2018) 86/90Y-Based Theranostics Targeting Angiogenesis in a Murine Breast Cancer Model. Mol Pharm 15:2606–2613. 10.1021/acs.molpharmaceut.8b00133 [PubMed: 29787283]
15. Muñoz R, Arias Y, Ferreras JM, et al. (2013) In vitro and in vivo effects of an anti-mouse endoglin (CD105)–immunotoxin on the early stages of mouse B16MEL4A5 melanoma tumours. Cancer Immunol Immunother 62:541–551. 10.1007/s00262-012-1357-7 [PubMed: 23076642]
16. Schoonderwoerd MJA, Goumans M-JTH, Hawinkels LJAC (2020) Endoglin: Beyond the Endothelium. Biomolecules 10:289. 10.3390/biom10020289
17. Hu J, Guan W, Yan L, et al. Cancer Stem Cell Marker Endoglin (CD105) Induces Epithelial Mesenchymal Transition (EMT) but Not Metastasis in Clear Cell Renal Cell Carcinoma. Stem Cells Int 9
18. Li C, Guo B, Wilson PB, et al. (2000) Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. Int J Cancer 89:122–126. 10.1002/(SICI)1097-0215(20000320)89:2<122::AID-IJC4>3.0.CO;2-M [PubMed: 10754488]
19. Milentyi Biotec MACSPlex Exosome Kit, Human. 130–108–813:
20. Zomer A, Maynard C, Verweij FJ, et al. (2015) In Vivo Imaging Reveals Extracellular Vesicle-Mediated Phenocopying of Metastatic Behavior. Cell 161:1046–1057. 10.1016/j.cell.2015.04.042 [PubMed: 26000481]
21. Nogués A, Gallardo-Vara E, Zafra MP, et al. (2020) Endoglin (CD105) and VEGF as potential angiogenic and dissemination markers for colorectal cancer. World J Surg Oncol 18:99. 10.1186/s12957-020-01871-2 [PubMed: 32434528]
22. Tian H, Huang JJ, Golzio C, et al. (2018) Endoglin interacts with VEGFR2 to promote angiogenesis. FASEB J 32:2934–2949. 10.1096/fj.201700867RR [PubMed: 29401587]
23. Li Y, Zhai Z, Liu D, et al. (2015) CD105 promotes hepatocarcinoma cell invasion and metastasis through VEGF. Tumor Biol 36:737–745. 10.1007/s13277-014-2686-2
24. Grigore D, Simionescu CE, Stepan A Assessment of CD105, α-SMA and VEGF expression in gastric carcinomas. 7
25. Chien C-Y, Su C-Y, Hwang C-F, et al. (2006) High expressions of CD105 and VEGF in early oral cancer predict potential cervical metastasis. J Surg Oncol 94:413–417. 10.1002/jso.20546 [PubMed: 16967447]
26. Xie X, Lian S, Zhou Y, et al. (2021) Tumor-derived exosomes can specifically prevent cancer metastatic organotropism. J Controlled Release 331:404–415. 10.1016/j.jconrel.2021.01.030
27. Batrakova EV, Kim MS (2015) Using exosomes, naturally-equipped nanocarriers, for drug delivery. J Controlled Release 219:396–405. 10.1016/j.jconrel.2015.07.030

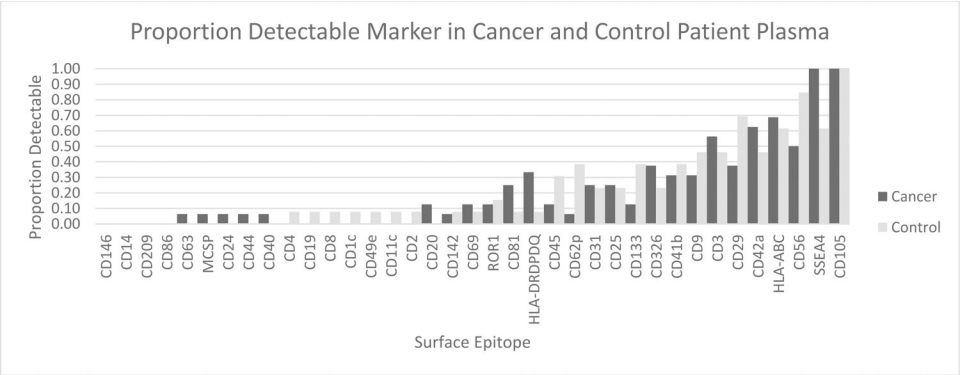

Figure 1:

Of the 37 EV surface epitopes examined, four proteins (CD105, SSEA4, CD56, and HLA-ABC) were consistently detected above background in both groups.

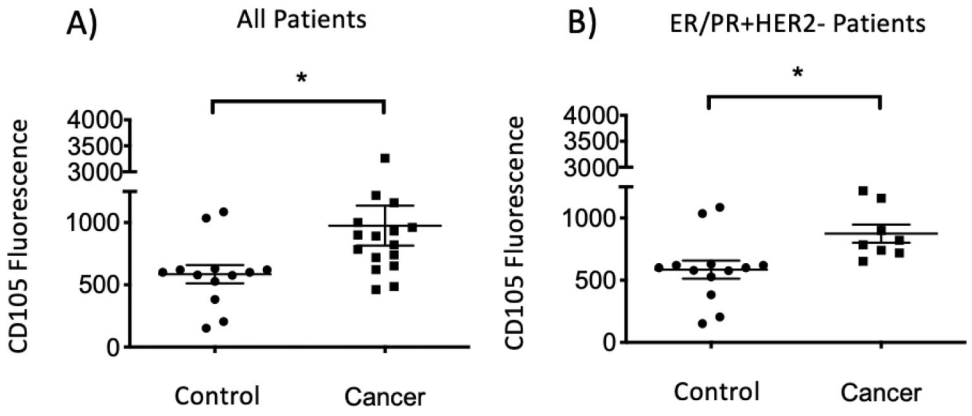

Figure 2:

CD105 is present in significantly higher levels in metastatic breast cancer patients compared to healthy controls. (A) Mean fluorescent intensity (MFI) of CD105+ surface epitopes on EVs of patients with metastatic breast cancer was significantly higher than controls ($p<0.05$). (B) Subgroup analysis with inclusion of only ER/PR+HER2− metastatic breast cancer patients again demonstrated a significantly higher MFI of CD105 positive surface epitopes on EVs compared to those in the control group ($p<0.05$).

Table 1:

Baseline data for participants with metastatic breast cancer including hormone receptor status, metastatic location, and whether the metastatic diagnosis was new or progressive on therapy.

	Number	Percent
Receptor Status		
ER/PR+HER2−	8	50
ER/PR+HER2+	4	25
ER/PR−HER2−	3	19
ER/PR−HER2+	1	6
Metastasis Location		
Bone	15	94
Brain	2	13
Liver	9	56
Lung	7	44
Multiple Metastatic Sites	12	75
Metastatic Diagnosis		
New	2	13
Progressive on treatment	14	8
Total Participants	16	100

Table 2:

Detectable marker mean fluorescent intensity (MFI) compared between groups using Welch's independent samples t-test.

Marker	MFI		p-value
	Cancer	Control	
CD105	976	586	0.04*
SSEA4	309	104	0.22
CD56	131	31	0.30
HLA-ABC	195	39	0.23

Table 3:

Subgroup analysis of ER/PR+HER2− cancer participants only. Detectable marker mean fluorescent intensity (MFI) compared between this subgroup and the control group using Welch's independent samples t-test.

Marker	MFI		p-value
	Cancer	Control	
CD105	875	586	0.01*
SSEA4	171	104	0.19
CD56	43	31	0.39
HLA-ABC	75	39	0.08

Table 4:

Variables affecting participants with metastatic breast cancer were individually assessed for correlation with CD105 levels using Welch's independent samples t-test.

	Number (percent)	MFI	p-value
Bone Metastasis			
Yes	15 (94)	964	N/A *
No	1 (6)	1159	
Brain Metastasis			
Yes	2 (13)	812	0.52
No	14 (87)	1000	
Liver Metastasis			
Yes	9 (56)	844	0.45
No	7 (44)	1146	
Lung Metastasis			
Yes	7 (44)	768	0.23
No	9 (56)	1137	
Multiple Metastatic Sites			
Yes	4 (25)	835	0.44
No	12 (75)	1401	
Newly Metastatic			
Yes	2 (13)	591	0.09
No	14 (87)	1031	

* insufficient n to calculate