Colocalization of Cancer Associated Biomarkers on Single Extracellular Vesicles for Early Cancer Detection

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Abstract
Early detection of cancer remains a significant challenge. Existing liquid biopsy approaches to detect early-stage cancer show promise but suffer significant limitations including the abundance and stability of the analyte, large sample requirements, and insufficient specificity to distinguish biomarkers as originating from benign or cancerous cells. Extracellular vesicles (EVs) from cancer cells exhibit both stability and abundance in human biofluids which makes them an attractive target for early-cancer detection. Measurement of multiple, colocalized cancer-associated biomarkers on single EVs may improve upon existing methods for the non-invasive detection of cancer. To demonstrate this, we developed Mercy Halo™, an immunoassay designed to capture and detect cancer-specific biomarkers on the surface of EVs derived from cancer cells. Our platform shows promise for the sensitive and specific detection of early-stage ovarian tumors in human plasma, outperforming a single biomarker (CA125), for the detection of stage I and II high-grade serous ovarian cancer (HGSOC).

Introduction
Cancer is more treatable when detected early, especially when found before it has started to spread[1]. Despite this, approximately 71% of cancer deaths in the United States occur in malignancies for which there are no United States Preventive Service Task Force (USPSTF) recommendation for screening[2]. Currently, three cancer types are recommended for screening in asymptomatic, average-risk populations: breast, cervical, and colorectal[2]. This discrepancy between screening utility and availability of an adequate test is due to the lack of effective, reliable, and inexpensive screening modalities. Current diagnostic assays such as imaging or fine-needle biopsies have proven effective at detecting tumors, yet remain invasive, expensive, low throughput, and are inadequate for detecting early-stage cancers. Liquid biopsies offer the advantage of being minimally invasive, relatively inexpensive, high-throughput, offer molecular mechanistic insights, and can be more readily used for screening or response monitoring[3,4]. Numerous technologies have been proposed as liquid biopsies to detect early-stage cancer from a blood sample, utilizing circulating tumor DNA or tumor cells, proteins, miRNAs, metabolites, and/or, more recently, extracellular vesicles (EVs)[5]. However, these technologies suffer from a lack of sensitivity or high false positive detection rates, which makes early detection of malignancies challenging. Given the low prevalence of cancer in asymptomatic, average-risk individuals, detection of early-stage cancer in this population requires a test with extremely high specificity (>98%), while providing a sensitivity sufficient to achieve at least a positive predictive value of 10%[6].

EVs represent a diverse class of lipid-bilayer bounded nanoparticles ranging from 50 nanometers to 1 micrometer[7,8]. They are released by all living cells and serve an array of biological functions from cell-to-cell communication to facilitating cancer progression by establishing a metastatic niche and suppressing the immune response. EVs offer a unique analyte for diagnostic tests given their abundance, stability relative to other liquid biopsy-based analytes (ctDNA, mRNA), and their representation of the cell of origin. EVs have been shown to contain numerous biomarkers derived from their cell of origin. The high plasma EV concentration of ~10^{10} EVs per mL and estimated tumor EV (tEV) shedding rates per cubic millimeter of tumor volume make EVs an abundant source of tumor-derived biomarkers to target in cancer screening assays designed for detection of smaller, early-stage tumors.

We hypothesized that detection of colocalized, cancer-associated biomarkers on the surface of individual tEVs could be a powerful approach for detection of early-stage cancer. Herein, we introduce Mercy Halo™, a platform technology that interrogates billions of individual EVs within a plasma or serum sample to selectively capture and detect tEVs from cancer cells[9]. We systemically demonstrate that Mercy Halo detects single EVs displaying up to three colocalized, cancer-associated biomarkers. This approach, coupled

with the rational design of biomarker combinations, enables detection of dilute tEVs from serum or plasma. We demonstrate the clinical feasibility of Mercy Halo by applying the technology to high-grade serous ovarian cancer detection, the most prevalent and deadliest subtype of ovarian cancer[10].

Results

Biomarker colocalization using Mercy Halo. Mercy Halo demonstrated the ability to detect single EVs containing up to three distinct colocalized surface biomarkers. A schematic of the assay is depicted in Figure 1. EVs enriched using size exclusion chromatography (SEC) are captured by antibody-functionalized beads targeting a cancer-associated surface biomarker (*Biomarker 1*) (Figure 1, panels 1-2). After removing unbound EVs, immunocaptured vesicles are incubated with two detection antibodies that recognize up to two additional biomarkers (*Biomarker 2* and *Biomarker 3*) (Figure 1, panel 3). Both detection antibodies are conjugated to double-stranded DNA oligonucleotides that contain complementary, single-stranded overhangs. These complementary oligonucleotides can hybridize and ligate if two DNA-conjugated antibodies are bound in proximity on the same EV (Figure 1, panel 4). The ligated DNA contains primer sites serving as a template for qPCR to quantify the number of colocalization events in the sample (Figure 1, panel 5). Based on this assay design, the complete set of Biomarkers 1, 2, and 3—termed a "biomarker combination"—must be expressed on the surface of a single EV to generate signal.

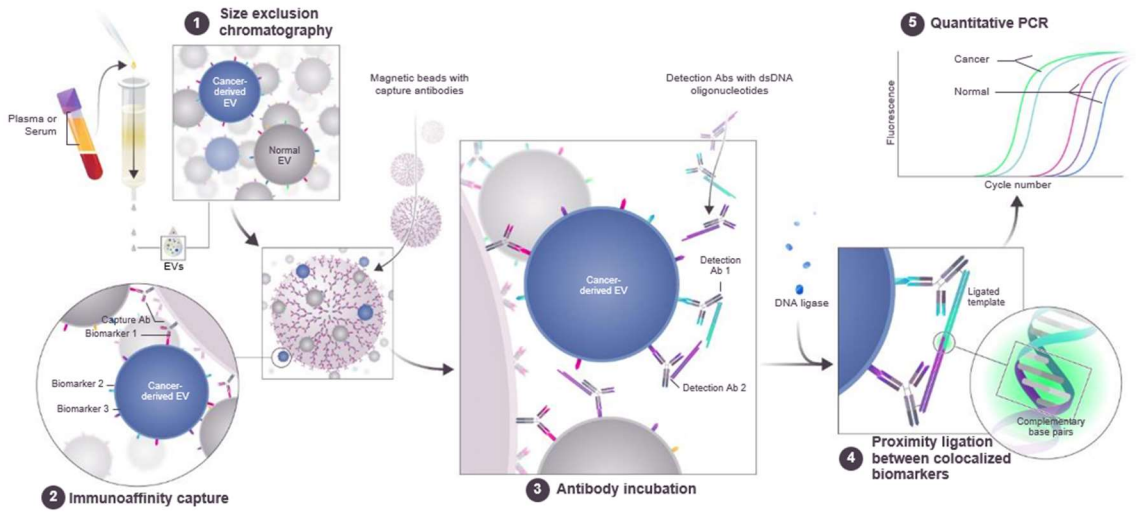

Figure 1. Overview of Mercy Halo™. (1) Extracellular vesicles purified from plasma or serum by size-exclusion chromatography are captured in solution with antibody-functionalized magnetic beads targeting a cancer-associated surface biomarker (2). Immunoaffinity captured EVs are incubated with additional cancer-specific biomarker detection antibodies conjugated to double-stranded DNA probes (3). The dsDNA oligonucleotides contain single-stranded overhangs which ligate only when in proximity to a complementary probe to generate a template for PCR (4). The abundance of biomarkers captured on the EVs is read out using quantitative PCR (5).

Characterization of the Mercy Halo analyte. In accordance with the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines[11], we completed a series of analytical experiments to demonstrate the detection of EVs in Mercy Halo. EVs from conditioned media (CM) released by COV413A cells were purified as described and characterized by nanoparticle tracking analysis (NTA), UV absorbance spectroscopy, western blot (WB), and transmission electron microscopy (TEM). Figure 2A compares

nanoparticle and total protein concentration in the void volume (Pre-EV) and six fractions of SEC-purified EVs (EV1-EV6). Fractions EV1-EV3, which comprise the Mercy Halo purified collection volume (PCV), contained high nanoparticle concentrations and low protein contamination. The particle size distribution from the combined PCV confirmed nanoparticles within the expected size range of 50 – 400 nm (Supplementary Figure 1A). Immunoblotting the pre-EV and EV fractions demonstrated the presence of well-characterized EV markers, and absence of non-EV proteins[11]. WB analysis confirmed the presence of CD9, CD63, CD81, and Flotillin in all three Mercy Halo PCV fractions (Figure 2B), while non-EV proteins (RPS3 and Tom20) were not detected in any purified fraction (Supplementary Figure 1G and 1H, respectively). Purified EVs were processed for TEM and images collected for the combined COV413A PCV, with representative images shown in Figure 2C. The observed morphology is consistent with membranous nanoparticles in the expected size range of single EVs[12].

To demonstrate compatibility of EVs with Mercy Halo, we purified EVs from CM derived from an array of human cancer cell lines and measured assay signal using single-biomarker combinations targeting CD9, CD63, and CD63 independently (e.g., CD9 capture, CD9 detection). Cell line EVs assayed at a constant nanoparticle concentration exhibited signal that generally correlated with the relative mRNA and protein expression of each marker. Figures 2D-2F provide heatmaps comparing normalized Mercy Halo signal and tetraspanin gene expression from the Cancer Cell Line Encyclopedia (CCLE)[13–15]. To further confirm that Mercy Halo detects intact EVs, we evaluated the detergent sensitivity of the assay signal for all three tetraspanin combinations. CD9-, CD63-, or CD81-Captured COV413A EVs were exposed to 0.075% Triton X-100 to disrupt the vesicular lipid bilayer while preserving antibody-antigen binding interactions prior to incubation with the DNA-conjugated detection antibodies. Incubation of captured EVs with Triton X-100 caused a significant reduction in assay signal ($\Delta Ct > 5$) for CD9, CD81, and CD63 when used for both capture and detection (Figures 2G-2I, respectively), consistent with membrane solubilization and subsequent disruption of colocalization events. CD9 and CD63 signal exhibited less detergent sensitivity than CD81, suggesting the presence of a subset of detergent-insensitive particles consistent with published results[16].

Similar analytical characterization of purified EV fractions from healthy human plasma (Supplementary Figure 3) confirmed the presence of nanoparticles between 50 and 400 nm, and WB analysis indicated an enrichment of tetraspanin markers in the PCV. TEM analysis demonstrated EV-like nanoparticles, but we observed increased levels of non-membranous particles relative to cell line CM, likely due to the heterogeneity of nanoparticles in plasma versus those in conditioned media[17].

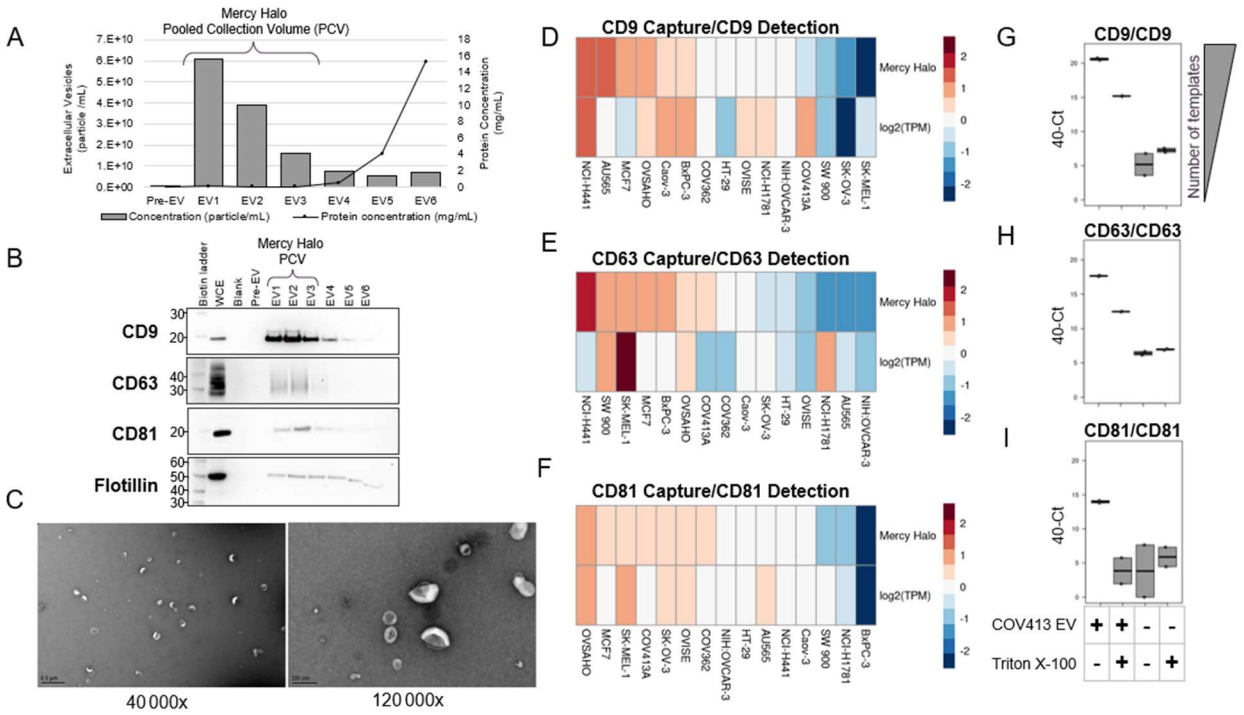

Figure 2. Characterization of Human Cancer Cell Line Extracellular Vesicles. (A) SEC Purified EV fractions used for Mercy Halo analyzed by nanoparticle tracking analysis (NTA, purple bars) and protein quantification (black line). The pre-EV fraction represents the void volume of the column. Purified collection volume (PCV), fractions EV1-EV3, used in Mercy Halo are highlighted. (B) Individual SEC fractions (Pre-EV, elution fraction collected prior to the EV elution fractions, and EV elution fractions EV1-EV6) from COV413A conditioned media were characterized alongside whole cell extracts (WCE) of COV413A cells demonstrating PCV-specific expression of EV-related biomarkers, CD9, CD63, CD81, and Flotillin. (C) Transmission electron microscopy of EVs derived from the PCV (fractions EV1-EV3) from COV413A conditioned media at two different magnifications demonstrates intact EVs ranging from 50-300 nm. Scale bars are 500 nm (40,000x) and 200 nm (120,000x), respectively. (D-F) Heat maps demonstrating performance of Mercy Halo readout (top row) using CD9 (D), CD63 (E), and CD81 (F) antibodies for both capture and detection correlates with mRNA expression (log2 TPM, bottom row) in an array of human cancer cell lines. Expression data was sourced from the CCLE, and all data was row scaled to allow comparison across data types. (G-I) Detergent sensitivity of Mercy Halo using CD9 (G), CD63 (H), or CD81 (I) antibodies for both capture and detection in COV413A EVs treated without (-) or with (+) 0.075% Triton X-100 after capture to disrupt intact EVs. Data is presented as 40 minus the cycle threshold (40-Ct) such that a higher 40-Ct value represents greater abundance of ligated dsDNA templates.

Assay signal correlates with cell line gene expression and protein expression. We analyzed gene expression data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) project to identify cancer-associated surface biomarkers that are likely expressed on EVs. Using ovarian cancer as a proof of concept, we selected four surface proteins overexpressed by ovarian cancer relative to healthy tissues: Tetherin/BST2, Folate receptor alpha/FOLR1, Mucin-1/MUC1, and Mucin-16/MUC16. All four biomarkers were confirmed by WB to be present in the PCV (Supplemental Figure 2). In addition, Mercy Halo results confirmed these markers are colocalized with CD81 and CD63 on EVs from COV413A (Supplementary Figure 4) and other cell lines (data not shown), offering additional support that Mercy Halo biomarkers are present on EVs. To evaluate the correlation between Mercy Halo signal and cell line expression, we characterized purified EVs from 32 human cancer cell lines at a constant nanoparticle

concentration using single-biomarker capture/detect combinations directed towards BST2, FOLR1, MUC1, and MUC16. Mercy Halo signals for each biomarker are summarized in Figure 3 along with published gene expression and protein expression values (when available). Mercy Halo signal strength was positively correlated with gene and protein expression for all four biomarkers (Spearman's correlation coefficients between 0.47 and 0.87), suggesting that cell line biomarker expression is predictive of EV protein composition. Scatterplots summarizing pairwise correlations are provided in Supplementary Figure 5.

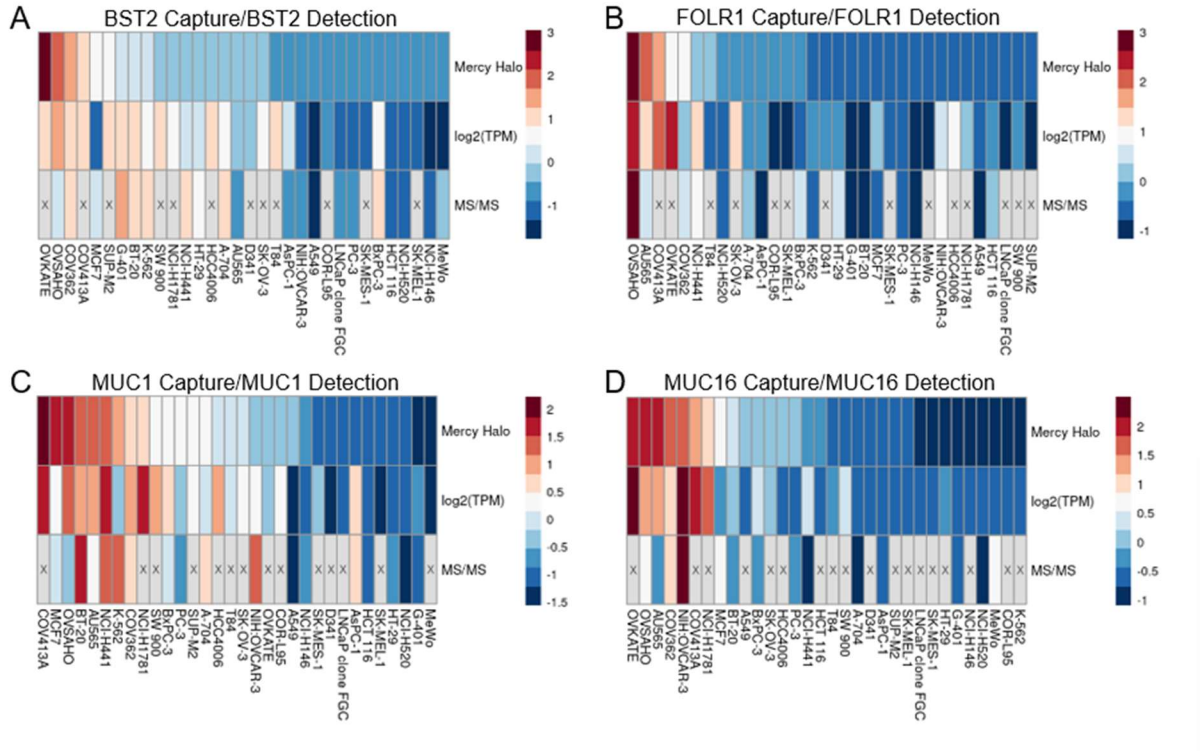

Figure 3. Comparison of Mercy Halo Signal with Biomarker Expression in Cell Lines. Heat maps comparing Mercy Halo signal (top row), mRNA expression (middle row, log2 TPM), and protein abundance as determined by mass spectroscopy (bottom row, MS/MS) for the four biomarkers tested in this study, Tetherin/BST2 (A), Folate receptor alpha/FOLR1 (B), MUC1 (C) and MUC16 (D). Gene expression and MS/MS data was sourced from the Cancer Cell Line Encyclopedia (CCLE) from the Broad Institute. All data was row scaled to allow comparison across data types.

Mercy Halo detects colocalized biomarkers on individual EVs. Mercy Halo signal operates akin to a Boolean logic "AND gate" whereby all three surface epitopes must be confined to the same EV for DNA template generation (Figure 4A). To demonstrate this, we characterized EVs from four human cancer cell lines (COV413A, OVKATE, SW900, and G-401) exhibiting a range of BST2, FOLR1, and MUC1 expression. The assay was configured to detect individual EVs that are BST2+/FOLR1+/MUC1-, BST2+/FOLR1-/MUC1+, and BST2+/FOLR1+/MUC1+ by modifying the capture and detection antibodies accordingly. Cell line EVs were based on the data summarized in Figure 3 and were tested at an equivalent concentration of 1.6E9 particles/mL. Figure 4 illustrates that Mercy Halo signal correlates with biomarker colocalization. BST2/FOLR1 (Figure 4B) and BST2/MUC1 (Figure 4C) generated strong signals for the cell lines that express both biomarkers (COV413A/OVKATE and COV413A/SW900, respectively). Weak BST2/FOLR1 and BST2/MUC1 signals were observed for cell lines missing at least one marker (SW900/G-401 and OVKATE/G-401, respectively). The three-biomarker combination

BST2/FOLR1/MUC1 showed a clear separation between COV413A EVs (triple-positive) and cell lines missing at least one biomarker (Figure 4D), suggesting Mercy Halo signal is only robust when all biomarkers are colocalized on the same EV.

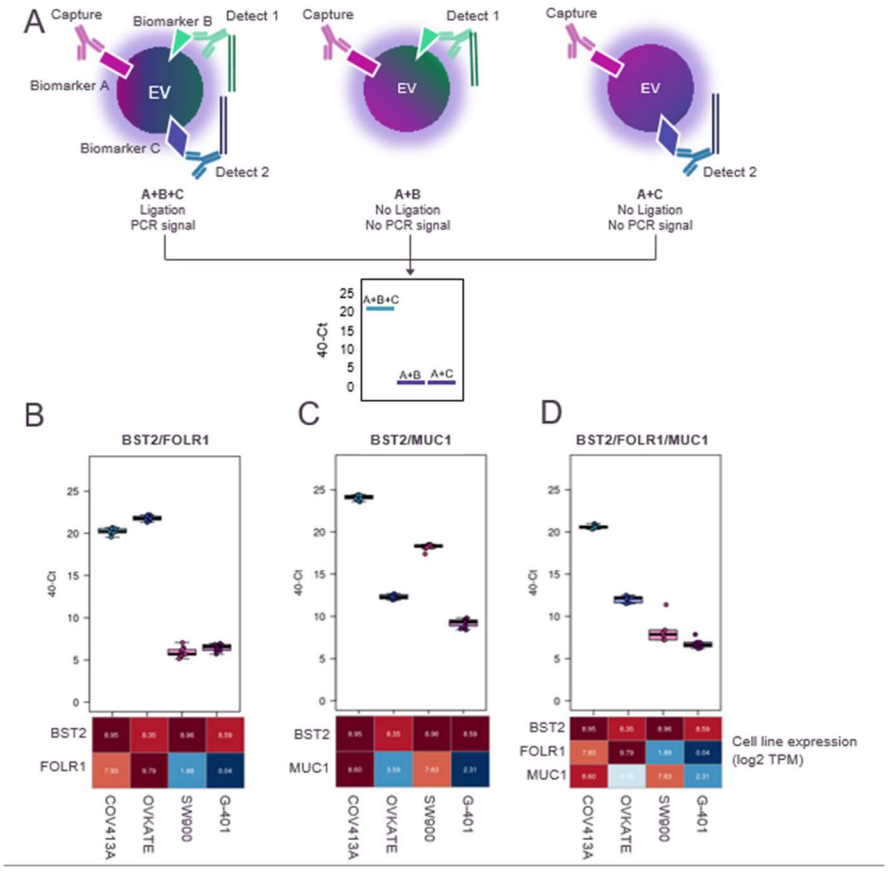

Figure 4: Colocalization of Biomarkers is Required for Mercy Halo Readout. (A) Schematic illustrating the concept that all three antibodies must be present on the same EV for productive readout. Individual EVs that contain all three biomarkers of interest can be captured and detected successfully by Mercy Halo (high 40-Ct value). If only two biomarkers are present (one capture + one detect), no ligation occurs, and 40-Ct is low. (B-D) Mercy Halo using doublet or triplet combinations of antibodies tested on cell line EVs from COV413A (BST2+, FOLR1+, MUC1+), OVKATE (BST2+, FOLR1+, MUC1-), SW900 (BST2+, FOLR1-, MUC1+), and G-401 (BST2+, FOLR1-, MUC1-) showing that doublets of BST2/FOLR1 (B), BST2/MUC1 (C), or a triplet of BST2/FOLR1/MUC1 (D) only yield positive qPCR signal in cell lines with matching biomarker expression. Data are presented as 40 minus the cycle threshold (40-Ct).

Assay signal not due to bridging. Theoretically, complementary DNA-conjugated antibodies located on adjacent EVs could generate a DNA template if within sufficient proximity ("bridging"). We characterized a mixture of EVs from two different cancer cell lines, OVKATE and SW900, each predicted to express two of the three biomarkers (BST2+/FOLR1+/MUC1-, and BST2+/FOLR1-/MUC1+, respectively). To assess bridging, we characterized the individual cell line EVs (4E8 particles/mL) and a 1:1 mixture (2E8 particles/mL each) using the triplet biomarker combination BST2/FOLR1/MUC1. Double-positive EVs from OVKATE or SW900 generated assay signals of 13.3 and 9.5, respectively, while the 1:1 mixture

generated a signal of 11.8 (Supplementary Figure 6). The mixture signal was consistent with the arithmetic mean of the individual cell line EVs, suggesting that bridging does not take place in the assay.

Detecting multiple colocalized biomarkers on EVs improves signal-to-noise ratio. We hypothesized that the colocalization of multiple cancer-associated biomarkers could improve the specificity of distinguishing dilute cancer EVs from normal EVs, as illustrated in Figure 5A. We spiked healthy plasma with ovarian cancer cell line EVs and evaluated discrimination performance using combinations of one, two, and three unique biomarkers. Spiked plasma was prepared by mixing purified COV413A EVs with a female plasma pool derived from ninety healthy women at a final concentration of 1E8 COV413A particles/mL. Unspiked and spiked plasma pools were characterized in Mercy Halo using biomarker combinations containing BST2, FOLR1, and/or MUC1 in various capture/detection assay configurations.

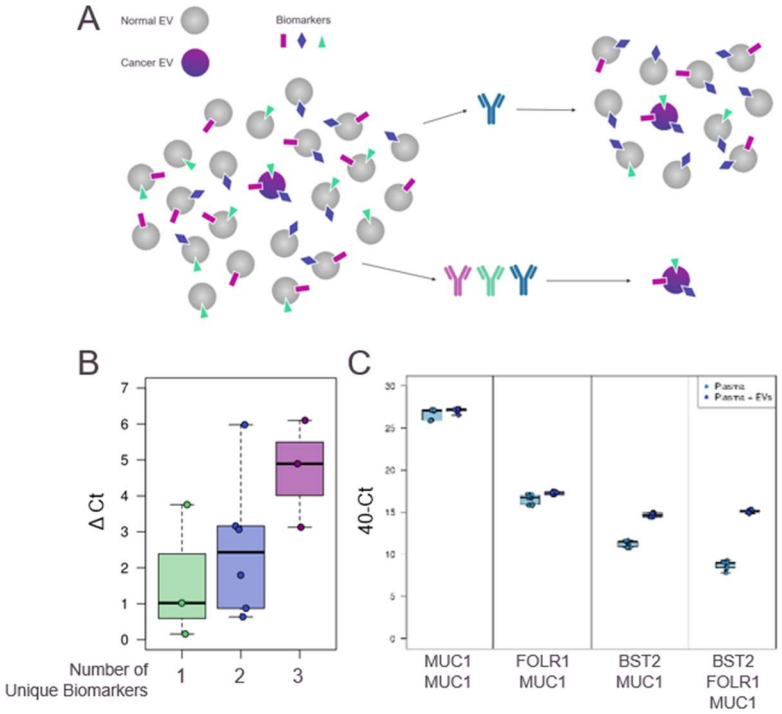

Figure 5: Detection of Colocalized Biomarkers Improves Signal over Background. (A) Detection of co-expressed biomarkers on individual EVs improves discrimination of cancer EVs versus normal EVs relative to single biomarker measurements. (B) Detection of COV413A EVs spiked into healthy plasma using combinations of one, two, and three unique biomarkers. Each data point represents a distinct biomarker combination targeting BST2, FOLR1, and/or MUC1 for capture and detection. The difference (ΔCt) between healthy background (unspiked) and spiked plasma improves with the number of unique biomarkers. Data points represent the median of 12 assay replicates. (C) Mercy Halo signal from unspiked and spiked plasma for a representative set of one-biomarker (MUC1), two-biomarker (FOLR/MUC1 or BST2/MUC1), and three-biomarker (BST2/FOLR1/MUC1) combinations. Increasing the number of unique biomarkers results in reduced healthy background and improved discrimination of spiked plasma.

Single-biomarker combinations, comprising capture and detection antibodies targeting BST2-only, FOLR1-only, or MUC1-only, generally exhibited poor discrimination between unspiked and spiked plasma samples (Figure 5B; median ΔCt = 1.0). However, discrimination performance improved for biomarker

combinations requiring the colocalization of two or three unique cancer-associated biomarkers (Figure 5B; median ΔCt values of 2.4 and 4.9, respectively). Figure 5C compares the assay signal from unspiked and spiked plasma for a representative set of biomarker combinations containing BST2, FOLR1, and/or MUC1. A single-biomarker "combination" targeting MUC1-positive EVs was unable to discriminate between unspiked and spiked plasma due to high healthy background signal from normal tissues. However, a triplet assay configuration capture and detect BST2/FOLR1/MUC1 resulted in 100,000X reduction of unspiked plasma signal and enabled robust detection of spiked COV413A EVs.

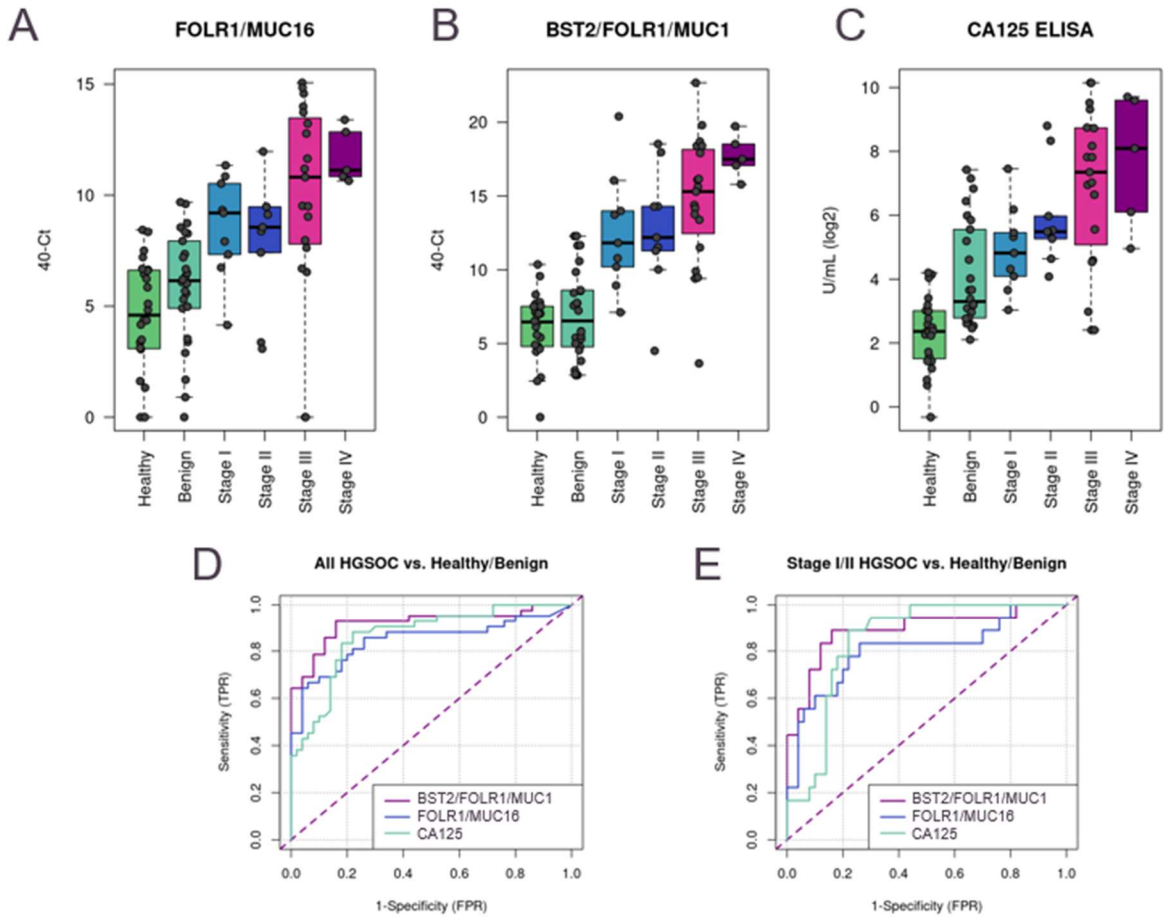

Figure 6. Mercy Halo Discriminates High-Grade Serous Ovarian Cancer (HGSOC) from Healthy Controls and Benign Ovarian Masses. Performance of (A) Mercy Halo biomarker combination FOLR1/MUC16, (B) Mercy Halo biomarker combination BST2/FOLR1/MUC16, and (C) CA125 ELISA in a cohort of healthy controls (n=24), patients with benign ovarian masses (n=26), and patients with stage I (n=9), II (n=9), III (n=15), and IV (n=4) HGSOC. All plots are presented as 40 minus the cycle threshold (40-Ct). (D) ROC curves for distinguishing all HGSOC cases from controls (patients with no symptoms or benign ovarian masses) using Mercy Halo and CA125 ELISA. (E) ROC curves for distinguishing early-stage HGSOC cases from controls.

Discrimination of high grade serous ovarian cancer (HGSOC) in patient samples. To assess the feasibility of Mercy Halo for early-stage cancer detection, we applied the technology to a clinical cohort of 92 patients comprising 42 HGSOC cases (18 stage I/II, 24 stage III/IV), 26 females with benign adnexal masses, and 24 female healthy controls. Plasma from each subject was characterized by two distinct Mercy

Halo biomarker combinations targeting FOLR1/MUC16-positive and BST2/FOLR1/MUC1-positive EVs. Each biomarker combination required 100 μL of plasma. We also measured the plasma concentration of CA125—the most widely used diagnostic and prognostic protein marker for ovarian cancer[18–21]—using a commercially available ELISA kit.

Figures 6A and 6B summarize the results of this feasibility study for the biomarker combinations FOLR1/MUC16 and BST2/FOLR1/MUC1, respectively. FOLR1/MUC16 achieved 50% sensitivity (95% CI: 26%-74%) for stage I/II HGSOC at 95% specificity relative to healthy controls and benign adnexal masses. BST2/FOLR1/MUC1 achieved a 55.6% sensitivity (95% CI: 30.8%-78.5%) for stage I/II disease. Both combinations outperformed CA125 ELISA, which generated an early-stage sensitivity of 16% (95% CI: 4%-41%) at 95% specificity (Figure 6C). In this proof-of-concept study, the improved performance of Mercy Halo over CA125 is most notable at high (>85%) specificity ranges, as shown in the receiver operating characteristic (ROC) curves for all-stage HGSOC (Figure 6D) and early-stage HGSOC (Figure 6E). This improvement is primarily driven by a reduced false positive rate in patients with benign adnexal masses and has been further improved in subsequently developed assay configurations.

Discussion

Cancer screening is a proven approach to reduce cancer-associated mortality because localized disease is amenable to treatment. Early detected cases exhibit a five-year mortality rate of 11% versus 79% for late-stage disease[22], underscoring the clinical need for more accurate and accessible screening tests. Despite considerable effort developing blood-based screening approaches, several technical challenges have limited test performance. Early-stage tumors are small, limiting the abundance of the available analytes in liquid biopsies. Cancer mis-regulates normal physiological processes, which means the analytes produced by cancers are often also produced by normal tissues, either in the tissue of origin or other parts of the body. Even when arising from the same tissue type, cancer utilizes blind evolutionary opportunism and therefore the mechanisms that lead to carcinogenesis can vary widely. Measuring colocalized cancer-associated biomarkers on intact EVs addresses each of these challenges.

Tumor EVs are a promising analyte for early cancer detection due to their abundance in blood and molecular similarity with their cell of origin. Unlike ctDNA-based detection methods, cell death is not required for the release of EVs. As a result, tEVs outnumber ctDNA copies in circulation by orders of magnitude in early-stage disease[23,24]. This enables the utilization of smaller sample volumes (~100 μL) relative to ctDNA liquid biopsy assays, which require several mLs of blood per run. Mercy Halo measures EV surface markers and thereby leverages the natural enrichment of membrane-bound epitopes relative to cargo proteins due to the 100-fold increase in surface area-to-volume ratio between an EV and its cell-of-origin. Moreover, it can interrogate billions of EVs simultaneously which enables the detection of dilute (<1 in 100,000)[23] cancer-derived EVs originating from small tumors. The qPCR-based readout enhances assay technical sensitivity relative to conventional enzyme-amplified sandwich immunoassays, similar to other approaches using immuno-PCR[25,26], proximity ligation assays[27,28], and proximity extension assays[29,30].

EV composition represents a stochastic sampling of membrane-associated and intracellular biomarkers expressed by the parent cell. Compositional changes in cancer cells are reflected in EVs that are shed into the TME (Figure 3). While most biological mechanisms exploited by cancer are also found in healthy tissues, Mercy Halo feasibility testing has demonstrated that it can detect colocalized, cancer-associated biomarkers representing distinct mechanisms rarely found in the same normal cell. This "AND-gate" functionality enables highly specific detection of dilute EV subpopulations with an improved signal-to-noise ratio over bulk EV biomarker measurements. As a demonstration of this concept, measuring multiple

colocalized biomarkers improves the ability of Mercy Halo to discriminate between normal plasma and plasma spiked with cancer cell line EVs (Figure 5).

The development of cancer screening tests is hindered by the heterogeneity of the disease. Our results (Figure 3 and 4) suggest we can perform EV biomarker discovery by leveraging existing experimental work characterizing tissue biomarker expression such as TCGA or GTEx to address this issue and we can rationally design Mercy Halo to target biomarkers that are highly expressed the cancer of interest. Combinations of capture and detection biomarkers may be strategically chosen to detect cancer derived EVs while excluding those originating from healthy tissues. The detection of three colocalized biomarkers implies a design space comprising billions of unique combinations based on membrane-associated protein coding targets, post-translational modifications, and other surface markers. While this increases the complexity of combination design, it also increases the likelihood of finding combinations that are unique to all or large subsets of cancers.

We note that there are biological and technical limitations to this approach. EVs derived from hematopoietic lineage cells comprise 99.8% of all EVs in human plasma, so biomarker targets highly expressed in blood may generate assay background. Assay performance is dependent on the quality and availability of compatible affinity reagents. Promising cancer-associated biomarkers may not have viable affinity reagents available, limiting potentially useful biomarker combinations. Among biomarkers with compatible affinity reagents, we have observed a range in discrimination performance among two- and three-biomarker combinations tested (Figure 5B). Using ovarian cancer cell line EVs spiked into healthy plasma, the combination FOLR1/MUC1 exhibited weaker discrimination than BST2/MUC1, likely due to a ~32-fold increase in healthy background signal. Variability in cancer specificity may be driven by several factors including biomarker expression, antibody affinity and specificity, and the orientation of the assay design (i.e., capture versus detection biomarkers).

In a preliminary study, we demonstrate that single EV analysis using Mercy Halo is a feasible approach for early-stage cancer detection. Biomarker combinations FOLR1/MUC16 and BST2/FOLR1/MUC1 exhibited promising early-stage sensitivity for HGSOC relative to benign ovarian masses and healthy female controls. Assay signal positively correlated with cancer stage, suggesting an increase in tEVs containing colocalized biomarkers. This result is consistent with predicted correlations between EV shedding and tumor burden[23]. In addition, both combinations exhibited improved separation between benign and malignant ovarian masses relative to CA125 ELISA. The elevated CA125 levels observed among women with benign adnexal masses is consistent with previous studies[20,31] and highlights the clinical need for improved blood-based biomarkers. This proof-of-principle study has several limitations. Samples from ovarian cancer cases and benign ovarian masses were collected at a cancer treatment facility, while healthy female controls were collected at a primary care clinic. Variability in preanalytical sample handling could bias study results when comparing HGSOC cases to healthy controls. The blood samples from HGSOC cases were drawn at clinical diagnosis and may not represent asymptomatic cases that would benefit from screening. While the results from this study are encouraging, we plan to refine the Mercy Halo biomarker combinations and validate test performance in larger, independent cohorts.

Measuring colocalized biomarkers on single EVs using Mercy Halo is a promising approach for early-stage cancer detection. This versatile platform addresses the biological abundance limitations of ctDNA-based approaches while offering improved specificity over bulk EV measurements. We anticipate this assay format is readily extensible to a broad array of clinical indications substitution of the biomarkers and capture/detection reagents. Advances in affinity reagent development and EV biomarker discovery may be leveraged to improve existing assays by incorporating new reagents or designing orthogonal combinations to boost assay performance.

Materials and Methods

Cell culture. All human cancer cell lines used in this study, and their respective sources, are indicated in Supplemental Table 4. SK-MEL-1, and SK-MES-1 were used under license from the Memorial Sloan Kettering Cancer Center. NCI-H146, NCI-H441, NCI-H520, and NCI-H1781 were developed by Dr. Gazdar and Dr. Minna and used under license from the National Cancer Institute. COV362 and COV413A were used under license from the European Collection of Authenticated Cell Cultures (ECACC). OVSAHO and OVISE were obtained from Sekisui XenoTech (Kansas City, KS) under license from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). All cells were thawed, subcultured, and maintained in complete growth media as recommended by the supplier. For EV production, cells were passaged into large capacity tissue culture-treated flasks or dishes until 70-80% confluent. The flasks or dishes were then transitioned to EV-depleted FBS (Thermo Fisher Scientific, A2720801) or serum-free Cancer Cell Line Media XF (CCLM XF, PromoCell, C-28077), depending on the individual cell line as follows; growth media was removed and the cells were rinsed twice with 1/2 volume of either complete media supplemented with exosome-depleted (EV-) FBS or CCLM XF serum free media prior to being replaced in basal media with 10% EV- FBS, or CCLM XF. After 48 hours, the conditioned media (CM) was collected and cellular debris removed by centrifugation at 300×*g* for 5-minutes followed by two subsequent centrifugations at 1500×*g* for 10 minutes each. All centrifugations were done at 21°C. The cleared CM was transferred to 50 mL conical tubes in ~45 mL aliquots and stored at -80°C prior to purification.

Concentration of conditioned media. Typically, 200 mL of CM was thawed at room-temperature and centrifuged at 1,300×*g* for 10 minutes to remove any insoluble debris. The supernatant was transferred via serological pipet to fresh 50 mL conical tubes then re-centrifuged at 2,000×*g* for 30 minutes. The supernatant was carefully transferred to a fresh tube and concentrated using Amicon Ultra Centrifugal filter units with a 10,000 molecular weight cut-off (Millipore-Sigma). This process was repeated until all the retained material was concentrated to approximately 1 mL. All centrifugations were done at 21°C unless specified otherwise.

EV Isolation from concentrated conditioned media or plasma. EVs were purified from either 1 mL of concentrated CM or patient potassium EDTA plasma. In each instance, 1 mL of concentrated CM or plasma was applied to an Izon qEV original 70 nm Legacy SEC column (ICO-70, Izon Science US, Medford, MA, USA) following the vendor-recommended protocol. Briefly, qEV columns were allowed to equilibrate to room temperature and washed in 10 mL room-temperature 1x PBS. Concentrated CM (described above) was applied to the top of the column and the flow through material was discarded. Three milliliters of 1x PBS were applied to the column and collected as the pre-EV fraction (void volume) and analyzed as described. EV-elution fractions (EV1, EV2…EV8) were then collected by applying 0.5 mL 1x PBS sequentially until all desired fractions were collected. The PCV was determined based on physical and functional characterization of each EV fraction. Purified plasma EVs were immediately run on the Mercy Halo assay. The concentrated CM EVs were quantified using either an nCS1 instrument (Spectradyne, Signal Hill, CA, USA) or ZetaView Classic S model nanoparticle tracking analysis (NTA) instrument (Particle Metrix, Amersee, Germany) prior to aliquoting and freezing at -80°C for future use in MH assays. The protocol for purifying plasma and CM EVs was identical, with the exception that 0.2 µM filtered 1X PBS, pH 7.4 was used for the concentrated CM EV purification (so particles in the PBS will not confound the downstream quantification) while unfiltered 1X PBS, pH 7.4 was used to purify EVs from plasma (which is not quantified).

Western blot analysis of human cell line or plasma extracellular vesicle fractions. Whole-cell extracts (WCE) of COV413A cells were prepared by scrape-harvesting 90%-95% confluent cells in 1x #9803 Cell lysis buffer (Cell Signaling Technology, Danvers, MA). Lysates were incubated at 4°C for 30 minutes prior to ultrasonic cavitation (3 x 15 seconds at 33% power) using QSonica Q125 Ultrasonic Disruptor (QSonica, Newtown, CT) fitted with a 2 mm titanium probe tip. Protein concentrations were determined, and the lysate was further diluted to 1mg/mL in 2X LDS Sample Buffer (NP0008, ThermoFisher Scientific). Human plasma lysate was prepared by mixing whole human plasma 1:1 with 4X LDS Sample Buffer followed by ultrasonic cavitation to homogenize the sample. Individual EV fractions from concentrated CM was diluted 1:1 in 4X LDS Sample Buffer and human plasma EVs were concentrated 10:1 prior to 1:1 dilution in 4X LDS Sample Buffer. In all cases, samples were heated to 70°C for five minutes prior to use. For polyacrylamide gel electrophoresis (PAGE) and western blot experiments, 10-20 µL of WCE, plasma lysate, or EV fraction was loaded alongside biotinylated ladder and/or prestained protein molecular weight markers (Catalog numbers 7727 and 59329, respectively, Cell Signaling Technology) on NuPAGE™ Bis-Tris™ pre-cast gels (ThermoFisher Scientific). Gel percentage, running buffer, and transfer conditions was dictated by the apparent molecular weight of the proteins being immunoblotted. In most cases, we used 1.0 mm, 4-12% Bis-Tris minigels with MOPS-based running buffer (ThermoFisher Scientific) according to the manufacturer's instructions. After PAGE, gels were either stained with Gel-Code™ Protein Stain (ThermoFisher Scientific) or transferred to 0.2-micron PVDF membranes (1620174, Bio-Rad) using a XCell SureLock Mini-Cell (ThermoFisher Scientific) according to the manufacturer's recommendations. After electrophoretic transfer, all membranes were blocked at room temperature for one hour in 1X TBST (#9997, Cell Signaling Technology) supplemented with 5% non-fat dry milk (AB10109, American Bioanalytical). Primary antibodies (Supplemental Table 3) were incubated overnight (16 hours) in 1X TBST supplemented with either 5% non-fat dry milk or bovine serum albumin (#9998, Cell Signaling Technology) at 4°C with constant rocking according to the manufacturer's recommendation. Each blot was washed thrice for 5 minutes in 1X TBST at room temperature prior to addition of HRP-conjugated secondary antibodies and HRP-anti-biotin antibody (Supplemental Table 6), both diluted in 1X TBST + 5% non-fat dry milk, for one hour at room temperature. Each blot was then washed thrice in 1X TBST for 5 minutes and proteins were detected using either LumiGLO/Peroxide Reagent or SignalFire™ ECL reagent (7003 and 6883, respectively, Cell Signaling Technology) and an iBright™ imaging system (ThermoFisher Scientific). All images are presented with no image adjustments (other than cropping to highlight the area of interest) and are typically between 5 and 15-minute exposures, depending on the signal strength.

Characterization of EV size distribution and concentration. A ZetaView (Particle Metrix, Amersee, Germany) nanoparticle-tracking analysis (NTA) instrument or a nCS1 instrument (Spectradyne, Signal Hill, CA, USA) was used to determine the size distribution and concentration of EVs after purification by SEC. ZetaView software 8.05.14 SP7 (Particle Metrix, Amersee, Germany) acquired and exported the data. The instrument was calibrated as recommended by manufacturer prior to all measurements. Following SEC isolation, prior to NTA measurement, the isolated particles were diluted 1:250 - 1:2000, which allowed the instrument to accurately measure the particle distribution (50-200 particles per field of view). As recommended by manufacturer for the quantification of EVs, the sensitivity was set at 80, shutter at 100, min brightness 20, min area 10, and max area 10000. All 11 positions of the instrument were analyzed, with outlier positions excluded automatically by the instrument. The size distribution results were binned in 1nm intervals from 1 to 1000 nm.

Mercy Antibody Panels. All antibodies used for Mercy Halo assay development were obtained from commercial sources. Initially, around 500 different antibodies against ~80 biomarker targets were screened for functionality and compatibility using cell line EVs. Antibody clones that passed primary screening were further optimized to determine the optimal conjugation concentration of both probes and beads in

biomarker-expressing cell lines vs biomarker-negative cell lines or buffer alone controls. The top antibodies and combinations were then validated in human plasma and further down-selected based on those antibodies and combinations that produced the best discrimination of HGSOC from healthy women and those with benign adnexal masses. The final combinations include antibodies against MUC1, BST2, FOLR1, MUC16, and other biomarkers to be described elsewhere. For more information on assay performance and Mercy Halo antibody panels see https://mercybio.com/partnership-opportunities/.

Plasma Samples. A healthy donor collection of blood samples was conducted under an IRB-approved protocol (MB-CLN-001). Each donor provided 2 tubes of blood which were collected using a 10-mL collection volume K_2EDTA tube (Thermo Fisher Scientific, 0265732). This plasma was collected from 24 women with no history of cancer, including 12 women 26-55 years old (pre-menopausal cohort) and 12 women 52-71 years old (post-menopausal cohort) After collection, each tube of K_2EDTA plasma was inverted 6-8 times, centrifuged at the collection site (1500 RCF at room temperature for 15 minutes) within 60 minutes collection to separate the plasma from the other blood components. The plasma was divided into 1-mL aliquots in 2 mL cryovials, and then frozen and stored at -80ºC. The frozen plasma aliquots were shipped from the collection site to Mercy BioAnalytics on dry ice and after arrival were stored at –80°C. A plasma pool from ninety healthy women was prepared in-house using samples purchased from ProteoGenex Inc. Late- and early-stage HGSOC cases were purchased from ProteoGenex, Inc. and Reprocell. All ProteoGenex plasma samples were sourced from Moscow, Russia and collected in either K_2EDTA or K_3EDTA blood collection tubes. Reprocell, Inc. samples were sourced from the United States and collected in acid citrate dextrose (ACD) blood collection tubes. Plasma samples from women with benign ovarian masses were purchased from ProteoGenex were also sourced from Moscow, Russia and collected in K_2EDTA blood collection tubes.

Antibody-functionalized magnetic beads. Antibodies containing bovine serum albumin (BSA) were purified using protein A and G Spintrap purification kits (Cytiva, GE28-9031-34 or GE28-9031-32) according to manufacturers' instructions. All antibodies used in the Mercy Halo Assay underwent a buffer exchange process to ensure that any carriers or impurities that might interfere with conjugation were removed. Up to 500 µg of antibody material was added to 2 mL of sterile 1X PBS (Thermo Fisher Scientific, 10010031) in 30K MW Amicon Ultra Filter Units (Millipore, UFC203024) and centrifuged for ten minutes at 4,375*xg*. This process was repeated 6 times, after which the antibodies were recovered and sterile filtered by centrifugation in 0.22 µM Spin-X tube filters (Costar, 8160) for 2 minutes at 12,000*xg*. Antibody concentrations were measured using the NanoDrop spectrophotometer. Immunoaffinity magnetic beads were prepared by coating antibodies onto Dynabeads® M-270 Epoxy beads using the Dynabeads Antibody Coupling Kit (Thermo Fisher Scientific, 14311D) according to the manufacturer's instructions. Briefly, the beads were suspended at 10 mg mL^{-1} in C1 buffer and mixed thoroughly by vortexing. 1 mL aliquots of the beads were transferred to 1.5 mL tubes, which were then placed on a DynaMag™-2 magnetic rack (Thermo Fisher Scientific, 12321D) for 1 minute to separate the beads from the solution and the supernatant was removed. C1 buffer and 20-160 µg of capture antibody, totaling a volume of 500 µL, were added and the beads were resuspended by gentle vortexing. After adding 500 µL C2 buffer, the beads were gently vortexed, and the tubes were placed on a HulaMixer™ (Thermo Fisher Scientific) for conjugation at 37°C for 19 ± 0.5 h. The tubes were briefly centrifuged at 300×*g* for 10 seconds, placed on the magnetic rack for 1 minute to separate the beads from the solution, and the supernatant was removed. The beads were washed using four cycles of resuspension and pulse centrifugation in Dynabeads wash solutions containing 0.05% Tween 20, followed by three washes in 1X PBS) (10010072, Thermo Fisher Scientific, Waltham, MA) with 0.1% BSA and 0.1% Pluronic F-68. The antibody-functionalized magnetic beads were resuspended in 1 mL of 1X PBS, pH 7.4 with 50% glycerol, 0.1% BSA, and 0.1% Pluronic F-68 and stored at -20°C.

DNA-conjugated antibody preparation Antibodies were buffer exchanged and purified, if needed, as described above. Antibodies were subsequently conjugated to DNA using a copper-free click chemistry

reaction. Oligonucleotides (Supplementary Table 2) were synthesized and purchased as single-stranded DNA (ssDNA) from Integrated DNA Technologies, Inc. (Coralville, IA, USA) or Bio-Synthesis, Inc. (Lewisville, TX, USA). Before performing the conjugation, the ssDNA was annealed to form a double-stranded product. Complementary ssDNA strands (Strands 1+3 and Strand 2+4, Supplementary Table 2) were mixed to a final concentration of 50 µM in 1X PBS and annealed using a standard thermal cycler (Applied Biosystems, ProFlex PCR System). The annealing protocol was as follows: 95°C for 2 minutes, 90°C for 3 minutes, and then reducing the temperature by 5°C in 3-minutes intervals until 25°C was reached. Monoclonal antibodies at 1 mg mL^{-1} in 1X PBS were incubated with DBCO-PEG5-NHS Ester linker (A102P, Click Chemistry Tools) at a 4:1 molar ratio of crosslinker to antibody for at least 2 h at room temperature. Following incubation, the antibody-linker solution was washed once using a 30 kDa Amicon Ultra-2 centrifugal filter (Sigma-Aldrich, UFC203024). Annealed DNA oligonucleotides with a 5′ azide modification (Integrated DNA Technologies) were added to the activated antibodies at a 3:1 molar ratio of oligonucleotide to antibody and were allowed to react overnight at room temperature. The conjugate concentrations were measured using the Qubit® protein assay kit (Q33211, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, and the DNA-conjugated antibodies were stored at 4°C in 1X PBS.

Mercy Halo assay protocol. Following EV isolation by SEC, a concentrated buffer solution was added to the purified EVs to obtain working concentrations of 0.1% BSA, 0.1% Pluronic F-68, 100 µg mL^{-1} salmon sperm DNA, and 0.1% ProClin 950. Antibody-functionalized magnetic beads directed against Biomarker 1 were diluted 1:2 with 0.1% BSA, 0.1% Pluronic F-68, 100 µg mL^{-1} salmon sperm DNA, and 0.1% ProClin 950, and 4.8 µL aliquots of the diluted beads per well (corresponding to 16 µg of beads/well) were added to the wells of a 96 deep-well KingFisher plate. 250 µL of the diluted, purified EVs was added to each well of a deep-well KingFisher plate. The sample plate was covered with an adhesive plastic film, was placed on an Eppendorf ThermoMixer C (Eppendorf, Enfield CT, USA) and incubated at 21°C for 2 hours, mixing at 1100 RPM. During this incubation, all the wash and reaction plates required for Mercy Halo were prepared and are described in the table below:

Reaction plate type	#	Volume/well	Components
Antibody plate	1	100 µL	DNA-conjugated antibody, 0.1% BSA, 0.1% Pluronic F-68, 100 µg mL^{-1} salmon sperm DNA
TEPBS wash	2	1 mL	1X PBS, pH 7.4, 0.05% Tween 20, 5 mM EDTA
PBST wash	1	1 mL	1X PBS, pH 7.4, 0.05% Tween 20
Ligation plate	1	100 µL	75,000 units mL^{-1} of T7 DNA ligase (New England Biolabs, M0318), 33 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT 3.75% PEG 6000, 0.5% Tween 20
PBST transfer plate	1	150 µL	1X PBS, pH 7.4, 0.05% Tween 20
qPCR plate	1	30 µL	TaqMan Fast Advanced Master Mix (Applied Biosystems, 4444557), 500 nM primers, 200 nM TaqMan probe

After the 2-hour EV-bead-binding incubation was complete, a KingFisher Flex System (Thermo Fisher Scientific, Waltham, MA, USA) was loaded with the sample plate, the antibody-probe plate, and the wash plates, the ligation reaction plate and the PCR plate and the custom KingFisher program is run. Briefly, the beads are magnetically captured and incubated in the antibody-probe plate for 32 minutes and 50 seconds, then washed twice in TEPBS for three minutes and 30 seconds, then washed in PBST for three minutes and 30 seconds and placed in the ligase reaction plate for 21 minutes and 40 seconds. Finally, the beads are transferred into a shallow plate containing PBST. The beads are then manually transferred to a qPCR plate using a KingFisher Flex 96 PCR magnetic head. qPCR plates were covered with a clear plastic adhesive

and placed into QuantStudio Dx Real-Time PCR instruments (Thermo Fisher Scientific, Waltham MA, USA) run using the following amplification conditions: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, and 60°C for 20 seconds. See Supplementary Table 2 for primer and TaqMan probe sequences. All samples were run in either duplicate or triplicate and those showing a Ct spread >3 among the replicates were excluded from subsequent data analysis.

Detergent disruption of EVs. To demonstrate that the Mercy Halo assay is EV-based, a detergent-sensitivity experiment was performed. Exposure of EVs to detergent would disrupt their membranes, resulting in loss of MH assay signal. As described in the paragraph above, EVs purified with SEC were diluted in a buffer containing 0.1% BSA, 0.1% Pluronic F-68, 100 µg mL^{-1} salmon sperm DNA, and 0.1% ProClin 950. Antibody-functionalized beads were used to capture the purified EVs in a 2-hour incubation under agitation. The beads with the captured EVs were then transferred, using the KingFisher Flex system, to deep-well plates containing 0.1% BSA, 0.1% Pluronic F-68, 100 µg mL^{-1} salmon sperm DNA, 0.1% ProClin 950, and 0.075% Triton X-100. The plates were incubated for 30 minutes, at 21°C, under agitation (1100 rpm) in a ThermoMixer C instrument to allow for EV lysis. After incubation with detergent, the deep-well plate containing the beads were washed and read out with detection antibodies to either tetraspanins or Mercy Halo biomarkers as described above (Mercy Halo assay protocol).

Transmission Electron Microscopy of cell-line and plasma EVs.
Cancer cell-line and HGSOC cancer EVs were shipped to Alpha Nano Tech (400 Park Offices Drive, STE 108, Morrisville, NC, 27709) in 1X PBS, on dry ice, for transmission electron microscope imaging using a Gatan Orius SC1000 CCD camera with Gatan Microscopy Suite 3.0 software. Copper-carbon formvar grids were glow discharged immediately prior to loading with the sample. Samples were processed undiluted. Grids were floated on 10 µL sample drop for 10 minutes, washed two times with water by floating on the drop of water for 30 seconds, and negatively stained with 2% uranyl acetate by floating on the drop of stain for 30 seconds. The grid was blot dried with Whatman paper and imaged with Jeol 1230 electron microscope. Pixel sizes for 40,000X, 80,000X, and 120,000X are 1.28 nm, 0.7 nm, and 0.47 nm, respectively.

CA125 ELISA. A commercial CA125 ELISA kit (R&D SYSTEMS, Human CA125/MUC16 Quantikine ELISA kit, cat # DCA 125) was used to gauge the relative sensitivity and specificity of Mercy Halo. Blinded human plasma samples and known high, medium, and low CA125 controls (Bio-Rad, Cat#: 94900) were diluted 1:10 in 1X PBS, pH7.4. Samples were run in duplicate following the manufacturer's instructions. The VarioSkan Lux (Cat #: VL0000D0) was used to measure the resultant optical density of the plate at 450 nm, 540 nm, and 570 nm. If the measured human plasma CA125 concentration exceeded the detection range of 5-320U/mL as determined from the standard curve generated using the standards provided with the kit, the human plasma samples were diluted 1:100 and reprocessed.

Statistical Analysis

Mercy Halo data was presented as 40 - Cycle threshold (Ct). When assay data was collapsed to a single value, the median of replicates was displayed. Cell line gene expression and mass spectrometry data was obtained from the Cancer Cell Line Encyclopedia (CCLE). Mass spectrometry data was not available for all cell lines. Heatmaps containing Mercy Halo data were ordered by signal strength. Heatmaps containing multiple data types were row-scaled with a cap of +/- 3 standard deviations to enable comparisons between different data modalities. When only gene expression was shown, heatmaps were ordered to match the cell lines displayed in the figure, log2 transcripts per million (TPM) displayed, and no scaling was used. Correlation analysis between Mercy Halo and gene expression or mass spectrometry data was done using Spearman's Rank Correlation. Assay sensitivity at 95% specificity was calculated

using the epiR R package (version 2.0.40) and ROC curves were generated with the R package ROCit (version 2.1.1). All analyses were performed in R v.4.0.5.

Data Availability

The datasets and code generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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