Supporting Information

Nanoscale Flow Cytometry for Immunophenotyping and Quantitating Extracellular Vesicles in Blood Plasma.

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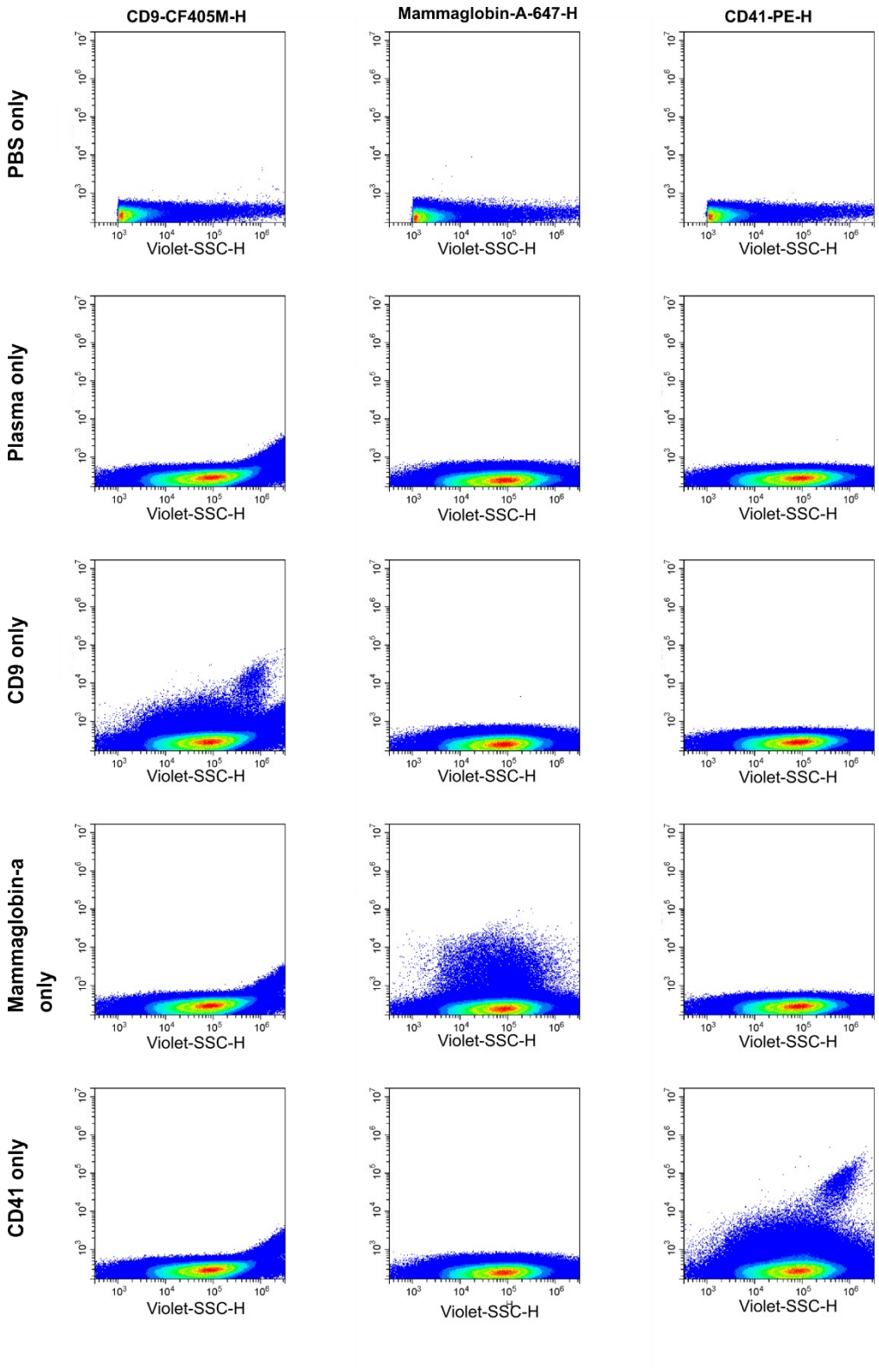

Figure S1: To ensure the absence of autofluorescence, noise, non-specific antibody signal and bleedthrough into other channels the following controls were carried out on the nanoscale flow cytometer: PBS alone, plasma alone and single antibody tests were ran independently of one another. The signal in all detectors of interest were assessed. A compensation value of 3 was applied to the PE channel to counteract bleedthrough into the FITC channel. Controls were carried out on three independent plasma samples. Representative images are shown.

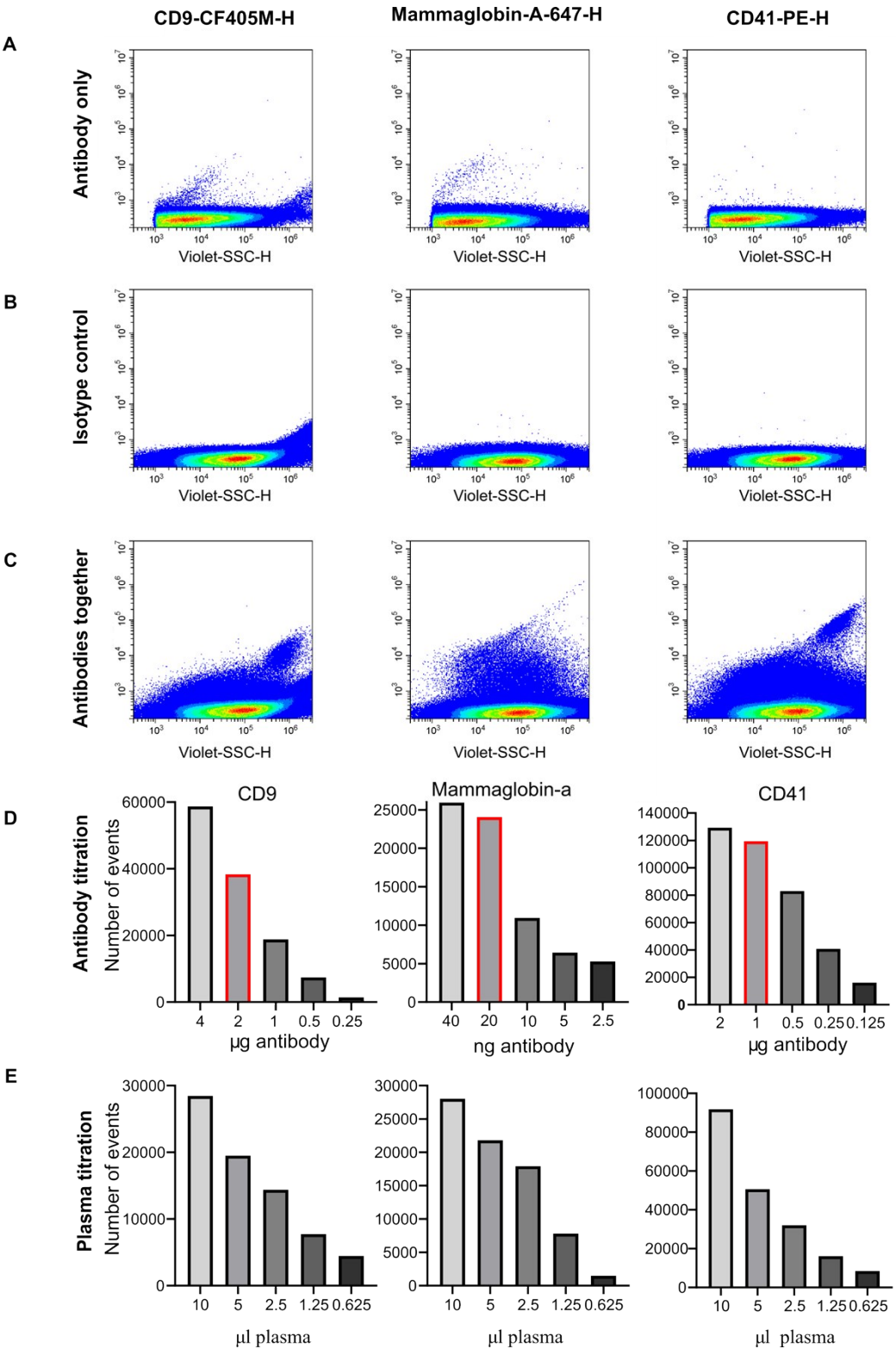

Figure S2: Further controls were carried out: (A) antibodies only, (B) isotype controls, (C) and all the antibodies together. (D) To ensure the correct concentration of CD9, CD41 and mammaglobin-a antibodies were being used to acheive saturated and specific signal, the antibody concentration was titrated. (E) The antibody concentration at which the signal begun to decrease was used to label decreasing volumes of plasma to ensure the antibody signal was specific; detected signal decreased by approximatley half upon plasma titration.

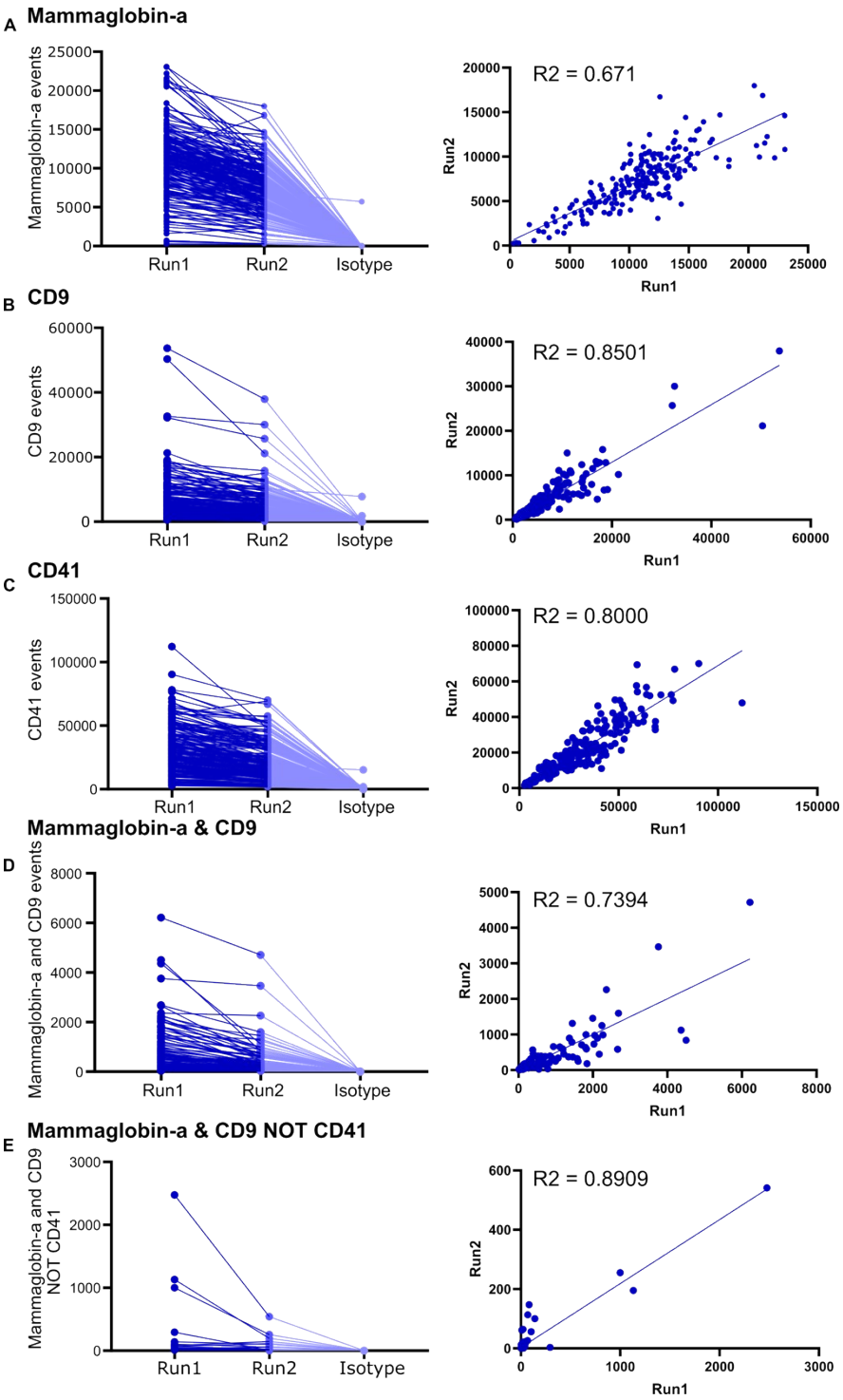

Figure S3: Graphs in column 1 show the reproducibility between two different experiments using 219 breast cancer plasma samples (run 1 and run 2), and the specificity (isotype control) of nanaoscale flow cytometry for high throughput analysis of breast cancer plasma sample analysis of mammaglobin-a (A), CD9 (B) and CD41 (C) levels. CD9 and mammaglobin-a dual positive EV population reproducibility and specificity is also demonstrated (D & E). Graphs in column 2 show the linear regression analysis for correlation of the values obtained during run1 and run2, the r^2 value is shown as a readout of correlation where a 1.0 would represent a perfect and strong correlation.

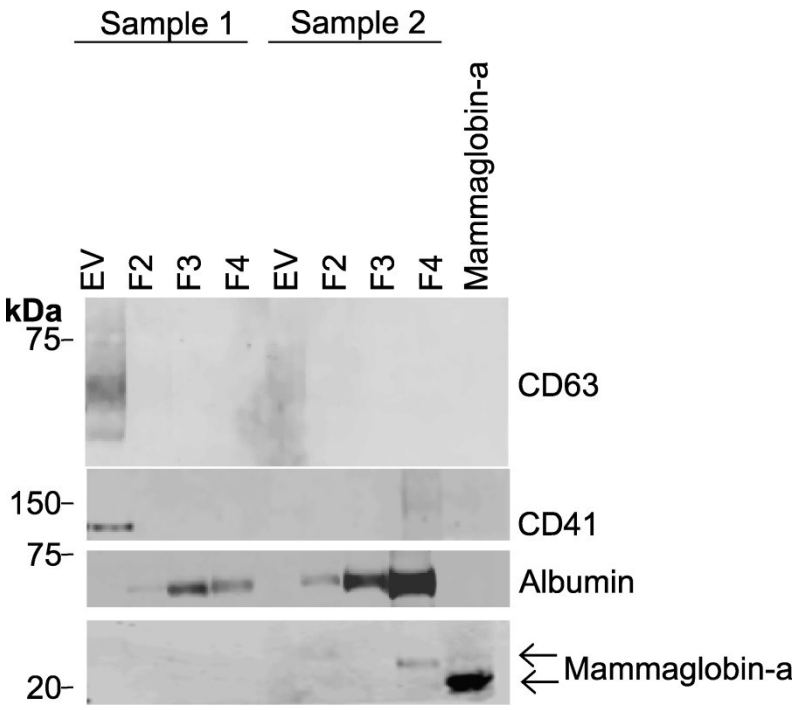

Figure S4: Two plasma samples were subjected to size exclusion chromatography and the EV fraction was collected followed by fractions (F) 2, 3 and 4. 20 ug of each fraction, and 1.2 µg of recombinant mammaglobin-a protein was loaded onto bis/tris gels and Western blotted for EV marker CD63, EV contaminant protein albumin, platelet derived EV marker CD41 and breast cancer marker mammaglobin-a.

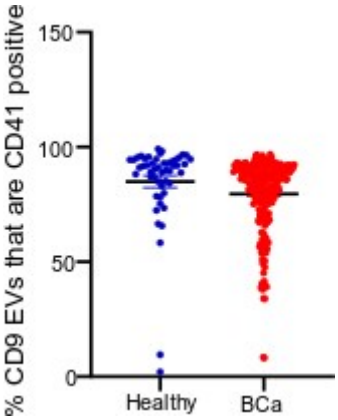

Figure S5: (B) The percentage of CD9 EVs that were also positive for platelet marker CD41 was analyzed in healthy (50) and breast cancer (219) patient plasma by nanoscale flow cytometry. n=2.

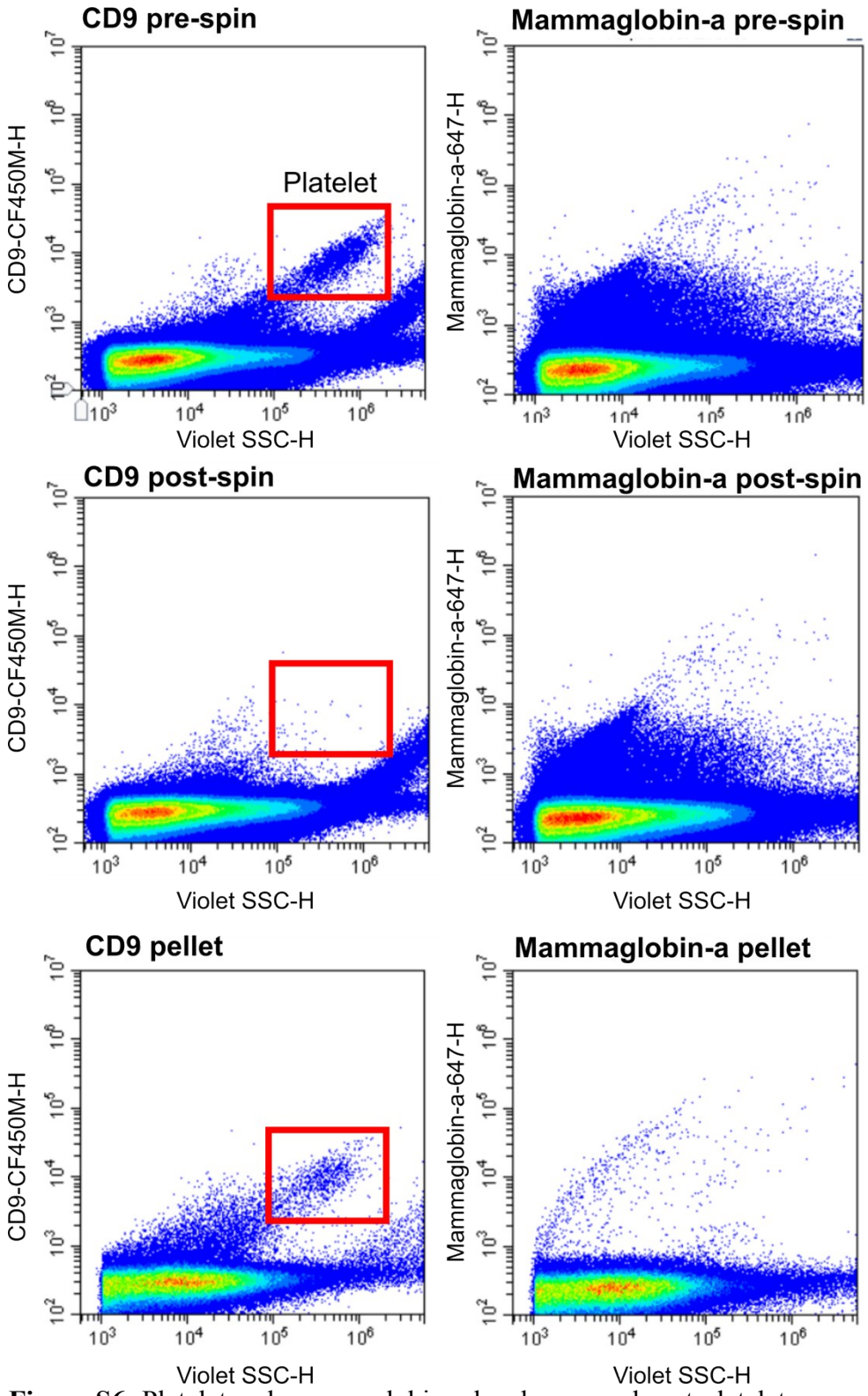

Figure S6: Platelet and mammaglobin-a levels pre- and post-platelet removal. Representative flow plots of plasma before 2,800 x g spin, after 2,800 x g spin, and the resuspended pellet from the 2,800 x g spin.

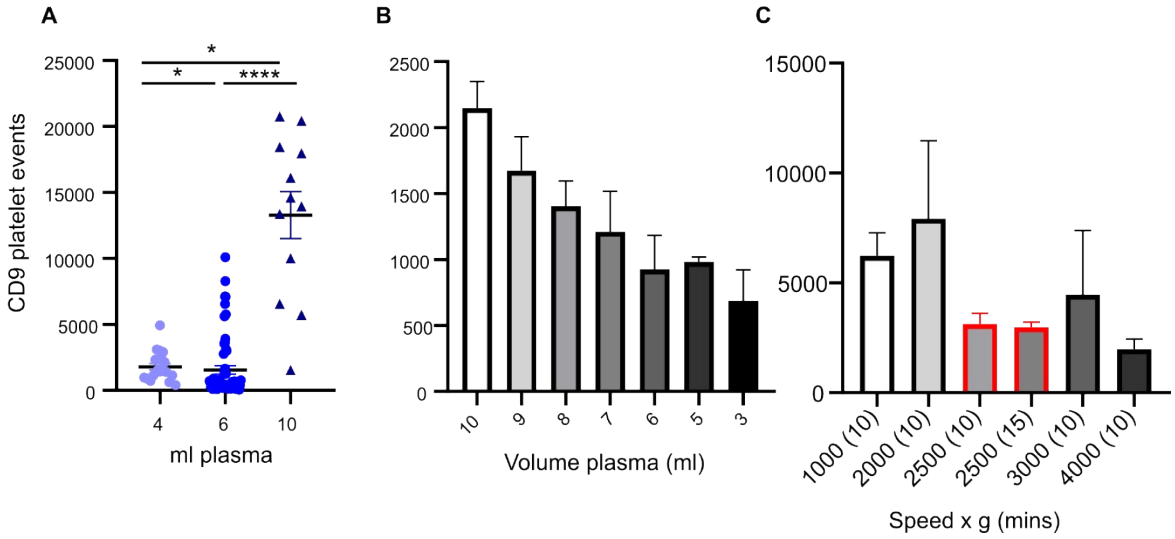

Figure S7: (A) Breast cancer patient plasma samples were labeled for CD9, and the number of platelets in each sample was quantified and associated with the blood draw volume information from the biobank. (B) Decreasing volumes of whole blood, starting from 10 ml, were spun at 2500 x g for 15 minutes. Resultant plamsa was labelled for EVs using CD9 and the number of platelets in the sample analyzed. (C) 10 ml of plasma was centrifuged at different speeds and times. The number of platelets in the resultant plasma was analysed by labelling the plasma EVs with CD9. Outlined in red are the most common plasma isolation protocols used by our biobanks. 1 whole blood bag was used for this study (B & C) and two technical repeats were done.

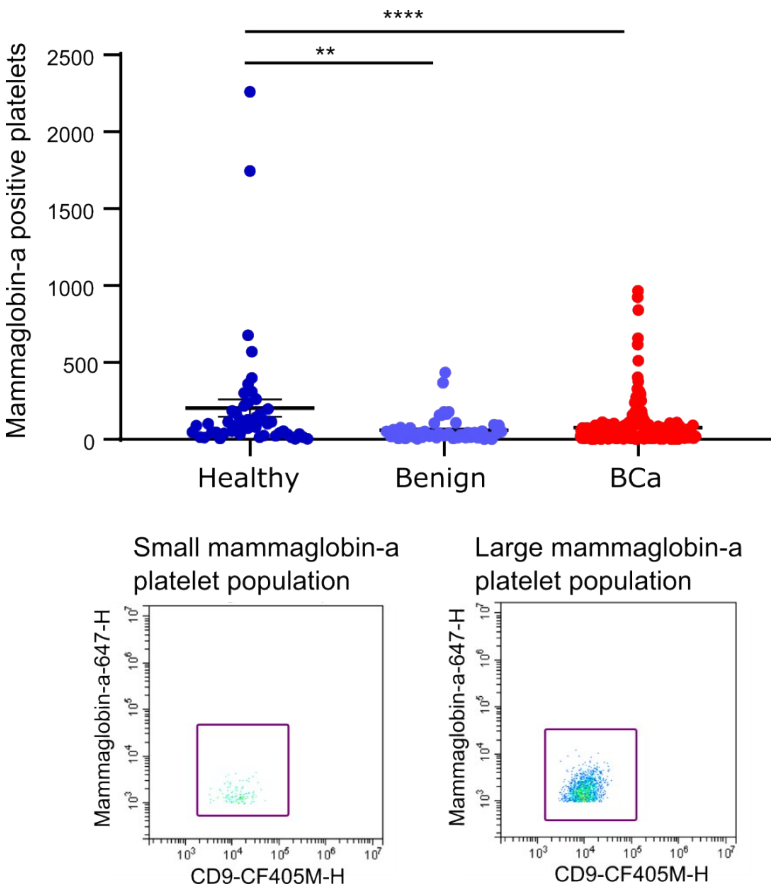

Figure S8: Flow cytometry analysis of mammaglobin-a and CD9 positive platelets in 50 healthy, 56 benign and 219 breast cancer plasma samples. The average of two independent runs was taken for analysis. Representative images of the mammaglobin-a and CD9 positive platelet populations detected by nanoscale flow cytometry.

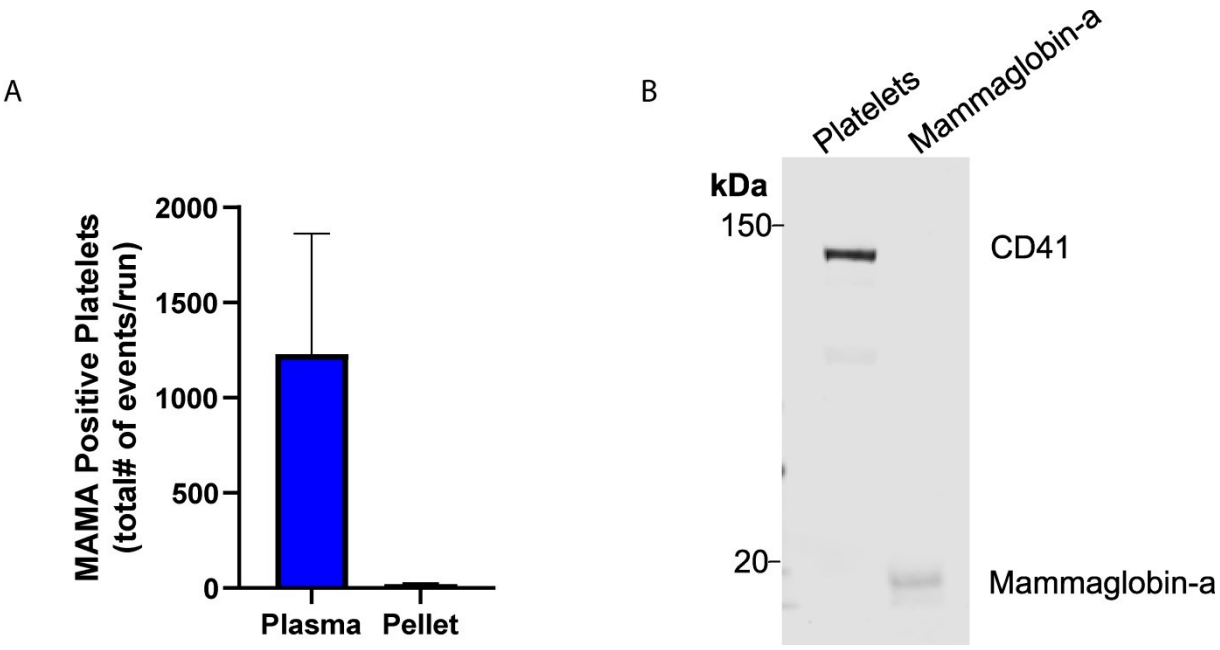

Figure S9: Mammaglobin-a positive platelets in whole plasma samples and platelet isolated samples. (A) Nanoscale flow cytometry based quantification of mammaglobin-a positive platelets in plasma and in resuspended pellets from post 2800xg plasma centrifugation. (B) 20 µg of platelet pellet was Western blotted for CD41 and mammaglobin-a alongside 1.2 µg recombinant mammaglobin-a protein as a positive control.

Table S1: Antibody product information and concentrations used in the study.

Antibody	Catalogue number	Company	Concentration
CD9 CF405M	ab123624	Abcam	Flow 4 µg/sample
CD41 PE	ab134372	Abcam	Flow 1 µg/sample
CD63 FITC	Ab18235	Abcam	Flow 1.2 µg/sample
CD42a FITC	11-0428-42	Thermo Fisher Scientific	Flow 0.89 µg/sample
Mammaglobin-a 647	DCABH-L4031AF647	Creative diagnostics	Flow 20 ng/sample
Isotype CD9: mouse IgG2a CF405M	ab126036	Abcam	Flow 4 µg/sample
Isotype mammaglobin-a: Rabbit IgG 647	3452S	Cell Signalling	20 ng/sample
Isotype CD41: mouse IgG1 PE	ab91357	Abcam	Flow 1 µg/sample
Isotype CD42a: mouse IgG1 FITC	11-4714-42	Thermo Fisher Scientific	0.89 µg/sample
CD63	ab59479	Abcam	Western 1:1000
Mammaglobin-a	TA313104	Origene	Western 1:500
CD41	ab83961	Abcam	Western 1:1000
Human albumin	ab10241	Abcam	Western 1:2000
Secondary anti mouse IgG IRdye® 680RD/800CW	926-68070	LI-COR	Western: 1:10000

All antibody concentrations were established using nanodrop quantification.