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TITLE: Automation of a Blood Test for Methylation Markers to Reliably Predict Response to Therapy and Prognosis of Outcome in Patients with Metastatic Breast Cancer

PRINCIPAL INVESTIGATOR: Saraswati Sukumar

CONTRACTING ORGANIZATION: THE JOHNS HOPKINS UNIVERSITY

3400 N CHARLES ST

BALTIMORE MD 21218-2608

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Saraswati Sukumar

E-Mail: saras@jhmi.edu

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Johns Hopkins University 733
N.Broadway, Suite 117,
Baltimore, MD 21205-1832

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14. ABSTRACT. Reliable and rapid tests for the prediction of response to an initiated therapy, and prognostication of the outcome of metastatic breast cancer are sorely needed. Our goal is to develop a 3-4-hour, automated blood test based on quantitative, multiplexed assessment of methylated, tumor specific, cell-free DNA in the blood. We have completed the goals set out in the SOW: that is, to devise the PCR conditions in which 6 genes (5 methylated genes plus a control ACTB) could be amplified using 6 different fluorophores in the same reaction, using templates of 200-600 copies of methylated DNA in 1 ml of plasma, while producing no false signals in the absence of methylated template DNA. More improvements to the assay and completion of training, and test sets showed that the test is performing at 95% sensitivity and 92% specificity with a ROC/AUC of 0.909. Validation analysis was accomplished in samples from a prospective TBCRC005 trial. At week 4 MBC patients with high cumulative methylation (CM) had a significantly shorter median PFS (2.88 months v 6.60 months, p = 0.001) and OS (14.52 months v 22.44 months, p=0.005) compared to those with low CM (accepted for publication). Blood tests that function with accuracy will minimize morbidity from ineffective therapy, reduce costs from additional imaging studies, and improve clinical outcomes.

15. SUBJECT TERMS

Methylated, DNA, blood, test, automated, cartridge, metastatic, breast, cancer, quantitative

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INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The major cause of the loss of nearly 600,000 women worldwide to breast cancer each year is the development of treatment refractory distant metastases. Intensive research in the recent past has resulted in a growing number of treatment options for patients with metastatic breast cancer (MBC) (2-4). However, many months are often needed to determine effectiveness of an initiated treatment regimen, a decision usually guided by clinical findings and imaging studies. Often, the patient is treated with toxic chemotherapies for months before discontinuation of the ineffective agent and the beginning of a new treatment regimen. We are addressing the overarching challenge to r*educe morbidity and mortality associated with metastatic breast cancer.* Reliable and rapid tests for the prediction of response to an initiated therapy, and prognostication of the outcome of metastatic breast cancer are sorely needed. Blood tests that function with accuracy will minimize morbidity from ineffective therapy, reduce costs from additional imaging studies, and improve clinical outcomes. We will address this unmet need by developing a 3-4 hours automated blood test based on quantitative, multiplexed assessment of methylated, tumor specific, cell-free DNA in the blood.

1. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Cell-free, circulating tumor DNA, CTC, blood, methylated, DNA, quantitative, multiplex, PCR

2. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Optimize the automated GeneXpert cartridge assay. Obtain IRB approval**; Month 1**; Resubmitted protocol specific to DOD grant: Approved **Month 8; Months 4-12 (100% completed)**

Specific Aim 2: Establish and validate thresholds for detecting methylated tumor DNA in serum using training and test set of sera using GeneXpert cartridges and compare with results already obtained using the lab test, cMethDNA. **Months 12-25 (100% completed, paper published in Cancer Research Communication, 2022)**

Specific Aim 3. Test the utility of the automated cartridge assay in the metastatic setting (Trial TBCRC 005) using blinded serum samples collected at baseline, 3-4 weeks after treatment and at 8/12 weeks coinciding with clinical restaging, and compare change in cumulative methylation of the 10-gene panel to clinical response. Cartridge-based assay utility will be assessed and compared to blood cMethDNA and to CTC tests performed on the same patients**. Months 22-36 (100% completed, paper published in Clin Cancer Res., 2023)**

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 1: Optimize the automated GeneXpert cartridge assay.

Major Task 1: Optimization of minimum detectable levels of methylated DNA in blood.

Subtask 1-Establish the minimum detectable level of methylated ctDNA. To achieve this goal, in the GeneXpert cartridge, we will assay 9 replicate samples at each dilution level of methylated *plasmid DNA* carrying methylated gene sequences for each gene diluted into normal serum, determine methylation index MI, (percent methylation relative to spiked BGE gene copies) for each marker, cumulative methylation index, CMI (the sum of percent methylation of each gene) CV and confidence intervals (CI). 25, 50, 100, 500, 1000 plasmid copies will be spiked into 300 ul normal serum. If assay does not perform with sensitivity similar to cMethDNA, change design of the cartridge with new fluorophores to maximize sensitivity.

Subtask 2-Establish the foundation of a quantitative assay by mapping measured methylation index (MI: % methylation of each gene) levels to known input quantities of fully methylated cell line DNA spiked into normal serum. 1, 10, 100, 1000, 10,000 cells (lower cell numbers 1, 10, 100 will be added to 300 ul normal serum. A stock of 50 ml spiked normal serum will be prepared for repeated use in subtask 2 and 3We will fit a linear model to estimate the slope of curve describing the relationship between quantities of input DNA and measured MI levels for each individual marker, as well as for the cumulative index based on all 6-10 markers. This model will establish the basis for accurate quantification of methylation levels

Subtask 3- To evaluate the reproducibility of the assay when repeated measurements are made on the same sample, including *inter-operator reliability*. with 3 independent operators completing 3 replicates each. Mixtures of cells into serum from subtask 1, and metastatic serum samples (n=13) will be used. We use coefficient of variation (CV) as our measure of reproducibility, reporting the CV for each marker, as well as the CMI (cumulative methylation index, addition of % methylation present in each gene in the panel), at each dilution, along with approximate 95% confidence intervals based on the normal distribution. Team members meet every week.

Milestone: 1. Reproducibility confirmed by replicate analysis of methylated plasmid spiked DNA, cell line DNA and 2. Interuser and Interassay reproducibility established. All milestones met- subtasks completed

Major achievements.

1. We developed the BCM cartridge system, consisting of an assay accomplished in 5.0 hours. It consists of one bisulfite conversion cartridge, and two methylation cartridges that amplify 6 genes, including ACTB reference per cartridge. BCM requires 30 minutes of hands-on time per sample

2. The analytical sensitivity of BCM was determined. The minimum detectable level of spiked methylated DNA was 75 copies. Descriptive statistics for 300 copies and a schematic indicated the relationship between Ct and copy number in BCM

A. Analytical testing results of spiking/reproducibility and inter-user reproducibility.

B. The BCM cumulative methylation algorithm was developed to compute the sum of methylation for the entire 9-gene panel (Table 2).

C. The relationship between Ct and copy number in BCM was described.

Methods.

Materials and Methods

Study design and sample collections. We used prospective blood collections from studies that followed women with metastatic breast carcinoma at Johns Hopkins (JH): 1) A training set obtained from the Johns Hopkins Breast Program Repository (J0888, NCT01937039, collected from March 2015 to December 2015), 2) a test set obtained from patients with MBC enrolled in the IMAGE II study (Individualized Molecular Analyses Guide Efforts in Breast Cancer, J16146 JNCT02965755) and control normal/benign samples from J0888 collected from 2016 to 2020 and, 3) two longitudinal studies (J0214, NCT00080665 and J0425, NCT00274768), collected at JH from 2004-2008. All J0888 samples used in training and test studies were from different donors. We obtained written patient consent and approval from the Johns Hopkins institutional review board for samples used in this study.

DNA marker selection. To ensure good breast cancer coverage for the LBx-BCM prototype assay we chose nine CpG DNA markers from among a larger panel of cMethDNA genes that, together, recognized all four histological subtypes of breast cancer.

The prototype LBx-BCM. The GeneXpert® System (Cepheid, Sunnyvale, CA) is a closed, automated PCR-based molecular diagnostic testing platform using self-contained cartridges to perform nucleic acid extraction and PCR. The LBx-BCM prototype was developed to meet the increased technical sensitivity required for detection of picograms of free ctDNA in blood. One cartridge is used for bisulfite conversion of unmethylated cytosine residues to uracil, which changes the DNA sequence specifically for unmethylated DNA, but not for methylated DNA (the conversion cartridge). Two additional cartridges are used for the performance of methylation-specific PCR (the methylation detection cartridges); these two detection cartridges contain reagents, in each cartridge, for nested multiplex real-time quantitative PCR of 4-5 target genes and ACTB as the internal reference, using 6 different fluorophores. Primer and probe sequences are presented in Table S1. The entire assay is completed within 4.5 hours and requires approximately 15 minutes of hands-on time. LBx-BCM is a research use only (RUO) prototype in development, not for use in diagnostic procedures, and has not been reviewed by any regulatory body.

LBx-BCM algorithm for methylation. The method of calculating cumulative methylation (CM) is described in Table S2. Step 1: GeneXpert software assigns the Ct at the end of the run; the user assigns Ct = 45 if no signals were detectable during the run; Δ Ct (Ct gene – Ct ACTB) is calculated to normalize all results to the ACTB reference DNA. If some samples have negative Δ Ct (Ct gene – Ct ACTB) for a gene, all samples are transformed by adding a constant value to give positive integers for that gene. Step 2: If Δ Ct (Ct gene – Ct ACTB) is higher than the historical replicate median of 300 copies + 13 Δ Ct units, the user adjusts to Δ Ct (Ct gene – Ct ACTB) = 0, thereby removing signals from the analysis that are too low to quantitate (less than 0.04 copies of target) (Fig. S1, Table S2) (appended paper, Cancer Res Commun; 2(6) June 2022). Step 3: Gene methylation (M) = [1 / Δ Ct (Ct gene – Ct ACTB)] * 1200. This is a robust transformation intended to raise the methylation values from baseline and increase the assay dynamic range. Step 4: Calculate cumulative methylation as follows, where CM = sum of M in the 9 gene-panel.

Sample processing. Plasma from STRECK Cell-free DNA BCT tubes (STRECK, Omaha, NE, #218962) was collected, harvested and frozen at -80ºC within 5 days. Two sequential centrifugation steps ensured that the plasma was free of cells prior to freezing. Serum was harvested from serum-separation tubes (SST; BD, Franklin Lakes, NJ, #367988), and frozen at -80ºC within 4 hours. Plasma and serum were stored frozen at -80ºC in aliquots. Before using, the samples were thawed at room temperature, inverted ten times, then micro-centrifuged at 14,000 rpm for 15 min at room temperature.

The LBx-BCM assay. For the LBx-BCM assay, plasma or serum (1.0 ml) was mixed with 50 µl proteinase K (600 units/ml; PK; Roche Diagnostics Corp., Indianapolis, IN), 2.0 ml Lysis Buffer (Cepheid, Sunnyvale, CA) and incubated for 10 minutes at room temperature. After incubation, absolute ethanol (1.5 ml) was added and the sample was loaded into the bisulfite conversion cartridge for processing (2.5 hr). The bisulfite-converted DNA sample was divided equally into two LBx-BCM methylation detection cartridges and methylation specific-PCR was performed (1 hour and 45 minutes; *AKR1B1*, *TM6SF1*, *ZNF671*, *TMEFF2*

target genes and *ACTB* reference gene in Cartridge A; *COL6A2*, *HIST1H3C*, *RASGRF2*, *HOXB4*, *RASSF1* target genes and ACTB in Cartridge B). The following reactions were run in each methylation detection cartridge: #1- a methylation-independent, nested multiplexed PCR that pre-amplified the 9-gene panel for 20 cycles, and #2- a methylation-specific, nested quantitative 6-plex real-time PCR that uses internal primers and 6 fluorophores (one per marker) to quantitate amplicons generated in the first PCR. The assay is completed within 4.5 hours including approximately 15 minutes of preparation time.

Preparation of analytical replicates. We spiked 600, 300, 150 or 0 copies of a laboratory stock of methylated human control DNA (#N2131, Promega Corp., Madison, WI) that was previously quantified by digital droplet PCR into 1.0 ml of commercial normal plasma or serum (female human pooled plasma, K_2EDTA anticoagulated or pooled serum, BioIVT, Hicksville, NY). After adding PK (50µl), lysis buffer (2 ml) and absolute ethanol (1.5 ml), each sample was transferred to a bisulfite cartridge. Within this cartridge, DNA was extracted, then converted with sodium bisulfite (D5030-1, Lightning Conversion Reagent, Zymo Research, Irvine, CA) and afterwards transferred in equal amounts to each of two detection cartridges for quantitative nested methylation-specific real-time PCR.

Interuser reproducibility. J0888 repository samples obtained from patients with MBC ($N = 11$) and normal controls ($N = 4$) were aliquoted into duplicate sample sets. One set was tested by User A and the other set was tested by User B on separate days using the same reagents. Users were blinded to the origin of the samples. LBx-BCM was performed as described above. Interuser concordance was evaluated using the Spearman correlation coefficient.

Statistical analysis. Analyses of independent groups were performed and data was visualized using box-whisker plots. Differences between groups was evaluated using the non-parametric Mann-Whitney test. The performance of the 9-gene panel was characterized by estimating the area under the Receiver Operating Characteristic curve (AUC), sensitivity, specificity, and likelihood ratio along with the 95% confidence intervals. Classification Accuracy = TP + TN / TP + TN + FP + FN using ROC derived laboratory methylation cutoffs (38.5 CM units for LBx-BCM; 1.5 CM units for cMethDNA). All statistical tests were two-sided and considered statistically significant at $P < 0.05$. Spearman correlation was performed to compare the CM of the reference laboratory assay, cMethDNA, to CM obtained in the LBx-BCM system in the test set samples (40 cancer and 26 control non-cancer samples). GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA) was used for all analyses.

Results. (all the figures and tables in the same order are also in appended Cancer Res Commun; 2(6) June 2022).

The Liquid Biopsy for Breast Cancer Methylation (LBx-BCM) prototype assay.

The LBx-BCM's quantitative PCR workflow is depicted in Fig. 1A. Steps 1-4 involve pre-processing of the sample for DNA extraction. In Step 5, the mixture is placed in the bisulfite conversion cartridge. In Step 6, the bisulfite treated DNA is divided equally into two LBx-BCM methylation detection cartridges to amplify and detect 9 methylated genes (up to 5 methylated genes plus ACTB per cartridge). At the end of the assay (4.5 h), the cycle threshold (Ct) for each gene is provided.

For LBx-BCM marker development we selected a 9-marker panel. We selected primer/probe combinations that performed optimally in the presence of the other markers and fluorophores in the 6-plex reaction in each cartridge. The final 9-gene panel consisted of *HOXB4, RASGRF2, AKR1B1, TM6SF1, COL6A2, HIST1H3C, TMEFF2, RASSF1,* and *ZNF671*. Primer and probe sequences for the gene panel are shown in Table S1.

Evaluation of the analytical performance of the LBx-BCM prototype.

Interassay reproducibility

The challenge in development of an automated assay for ctDNA is the ability to detect only a few hundred picograms or less of target DNA in a vast abundance of normal cell-free DNA. We developed the cartridge-based LBx-BCM assay (Fig. 1A), including the method for calculating CM (Fig. S1; Table S2).

The analytical performance of the assay was evaluated by spiking replicates of 300, 150, 75 and 0 copies (1 ng – 250 pg) of fully methylated target DNA into 0.5 ml of either commercial pooled normal plasma (Fig. 1B, Fig. 1C, Table S3) or normal serum (Fig. S2A, Fig. S2B, Table S3). Based on the Δ Ct (Ct Gene – Ct ACTB), scatter diagrams showed that nearly all replicates of 300 – 75 copies of target DNA were detected. In the absence of target DNA (0 spiked copies), either the input DNA was too low to quantitate, or no PCR amplification was observed (Fig. 1B, Fig. S1, Table S3). The Δ Ct increased with decreasing number of copies of target for each gene. CM of the 9-gene panel was significantly different for 0 versus 75 copies ($P < 0.0001$), 75 versus 150 copies ($P = 0.0003$), and 150 versus 300 copies ($P < 0.0001$) (Mann-Whitney Analysis, Fig. 1C) in spiked normal plasma. Similar results were observed in spiked normal serum (Fig S2A, Mann-Whitney analysis, Fig. S2B). For calculation of gene methylation (M) and CM of all genes (Table S2) we used the replicate control median ΔCt of 300 copies, as shown in Table S3.

Interuser reproducibility

We evaluated LBx-BCM reproducibility between users to determine whether the method gave similar results independent of the operator. A total of 15 samples, including patients with MBC ($N = 11$), and healthy controls ($N = 4$), were divided into duplicate sets and assayed on differnet days using cartridges from the same batch. The Spearman $r = 0.887$ indicated a high level of interuser reproducibility (Fig. 1D).

Table 1. Patient characteristics

Patient characteristics	**J0888**	**J16146 (IMAGE II) - J0888**	**J0214 -J0425**
A. Metastatic Breast Cancer	**Training set**	**Test set**	**Longitudinal set**
Patient characteristics	*n* = 20	*n* = 40	*n* = 22
Race			
Caucasian	17	25	16
Black	1	12	5
Other	2	3	1
Location of disease:			
Visceral	0	4	3
Non-visceral	8	5	1
Both	12	28	18
Unknown	0	3	0
Receptor status:			
ER/PR-positive, HER2-negative	17	29	12
ER/PR-positive, HER2-unknown	0	1	0
HER2-positive	2	1	4
Triple-negative (ER,PR,HER2 negative)	1	8	6
Unknown	0	1	0
Received prior chemotherapy for MBC	8	33	9
No. prior treatment regimens (all, incl. hormone)			
0	1	1	13
1	6	8	2
2	2	9	5
3	3	9	1
≥ 4	8	13	1
Age:			
Median	58	59	54
Range	29-82	27-80	28-73
B. Benign/Normal Controls	**Training set**	**Test set**	**Longitudinal set**
Patient Characteristics	*n* = 20	*n* = 26	*n* = 0
Race:			
Caucasian	10	16	
Black	10	9	
Other	0	1	
Age:			
Median	58	53	
Range	29-82	28-76	

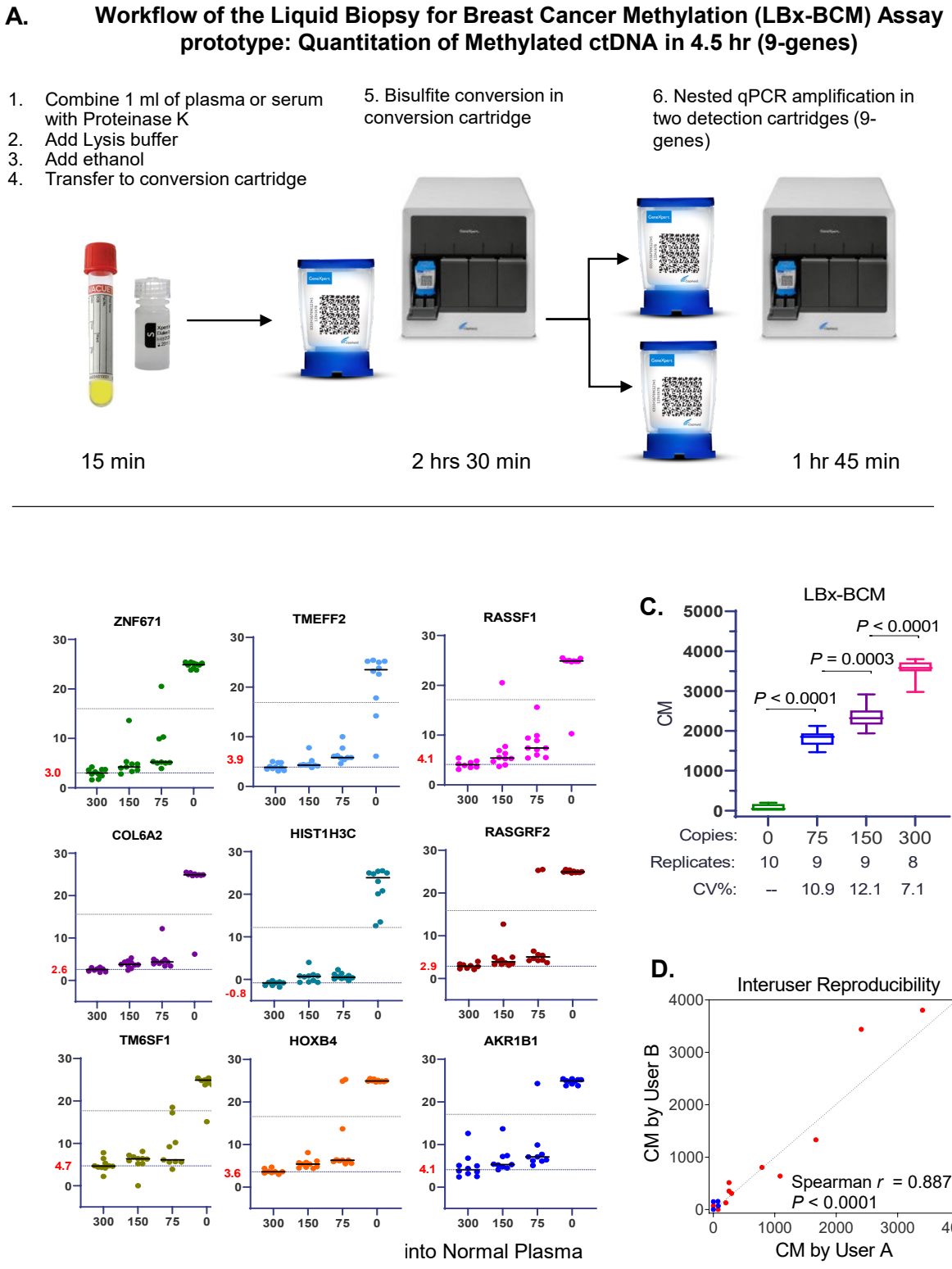


Fig. 1 LBx-BCM and its technical validation. **A**. Workflow of the Liquid Biopsy - Breast Cancer Methylation (LBx-BCM) assay**. B**. Analytical sensitivity of LBx-BCM for each gene in the 9-gene panel relative to ACTB. We spiked 300, 150, 75 and 0 copies of fully methylated DNA into 0.5 ml aliquots of pooled commercial normal plasma.

Fig 1 (continued)

Replicate LBx-BCM assays were performed. The ΔCt (Ct Gene – Ct ACTB) of samples is plotted on the Y axis for each gene. Number of copies of methylated DNA spiked into plasma is indicated on the X axis. For each gene, the median ΔCt (Ct Gene – Ct ACTB) for 300 copies of spiked DNA is indicated numerically in red to the left of the Y-axis and by the lower dotted line. The upper dotted line indicates median ΔCt for 300 copies + 13 for each marker. **C**. Cumulative methylation (CM). The CM of 9-genes was calculated for each run of 300, 150, 75 and 0 copies as described in the Methods. Box plots represent the median and range of CM of the 9-gene panel among spiked-in replicates. Mann-Whitney analysis was performed to determine whether methylation was significantly different as indicated by *P*-values. The coefficient of variation, expressed as percent (CV %) is indicated. For the calculation of gene methylation (M) and CM we utilized the replicate median $\Delta Ct_{300\ \text{DNA copies}}$ indicated in Table S2, DNA spiked into serum for training and longitudinal sets and spiked into plasma for the test set. **D.** Interuser Reproducibility. To test reproducibility between users, a total of 15 plasma samples including 11 from patients with MBC, and 4 from healthy controls were aliquoted in duplicate and each set was assayed for LBx-BCM by two users on different days. Data from User A (X-axis) and User B (Y-axis) are plotted (Spearman $r = 0.887$, $P < 0.0001$).

SUPPLEMENTARY FIGURES AND TABLES

Table S1. Sequences of primers and probes used for amplification of gene panel in LBx-BCM assay

Primer Name	Primer Sequence (5' To 3')	Orientation	Methylation - specific	Size
AKR1B1_Ext_F	GYGTAATTAATTAGAAGGTTTTTT	Forward External	No	216 bp
AKR1B1_Ext_R	AACACCTACCTTCCAAATAC	Reverse External	No	
AKR1B1_FM	GCGCGTTAATCGTAGGCGTTT	Forward	M	84 bp
AKR1B1_RM	CCCAATACGATACGACCTTAAC	Reverse	M	
AKR1B1_M_Probe	CGTACCTTTAAATAACCCGTAAAATCGA	Reverse	M	
COL6A2 _Ext_F	AGGTTTAGGAGAAGTTGTAGA	Forward External	No	154 bp
COL6A2 _Ext_R	TACCAACAATAAAAACCCAAAC	Reverse External	No	
COL6A2_FM	ATTCGGGTTGATAGCGATTCGT	Forward	M	81 bp
COL6A2_RM	CGATTCCACCAACGCCCCG	Reverse	M	
COL6A2_M_Probe	CCCAAAACGAATATAAACGACCCG	Reverse	M	
HIST1H3C_Ext_F2	GTGTGTGTTTTTATTGTAAATGG	Forward External	No	139 bp
HIST1H3C_Ext_R2	ATAAAATTTCTTCACRCCACC	Reverse External	No	
HIST1H3C_FM2	AATAGTTCGTAAGTTTATCGGCG	Forward	M	92 bp
HIST1H3C_RM2	CTTCACGCCACCGATAACCGA	Reverse	M	
HIST1H3C_M_Probe	TACTTACGCGAAACTTTACCGCCGA	Reverse	M	
HOXB4_Ext_F	TTAGAGGYGAGAGAGTAGTT	Forward External	No	228 bp
HOXB4_Ext_R	AAACTACTACTAACCRCCTC	Reverse External	No	
HOXB4_FM	CGGGATTTTGGGTTTTCGTCG	Forward	M	94 bp
HOXB4_RM	CGACGAATAACGACGCAAAAAC	Reverse	M	
HOXB4_M_Probe	AACCGAACGATAACGAAAACGACGAA	Reverse	M	
RASGRF2_Ext_F	GAGGGAGTTAGTTGGGTTAT	Forward External	No	284 bp
RASGRF2_Ext_R	CCTCCAAAAAATACATACCC	Reverse External	No	
RASGRF2_FM	GTAAGAAGACGGTCGAGGCG	Forward	M	93 bp
RASGRF2_RM	ACAACTCTACTCGCCCTCGAA	Reverse	M	
RASGRF2_M_Probe	AACGAACCACTTCTCGTACCAACGA	Reverse	M	
RASSF1A_Ext_F	GTTTTATAGTTTTTGTATTTAGG	Forward External	No	198 bp
RASSF1A_Ext_R	AACTCAATAAACTCAAACTCCC	Reverse External	No	
RASSF1A_FM	GCGTTGAAGTCGGGGTTC	Forward	M	74 bp
RASSF1A_RM	CCCGTACTTCGCTAACTTTAAACG	Reverse	M	
RASSF1A_M_Probe	TGGTTTCGTTCGGTTCGCGTTTGT	Reverse	M	
TM6SF1_Ext_F	AGGAGATATYGTTGAGGGGA	Forward External	No	253 bp
TM6SF1_Ext_R	TCACTCATACTAAACCRCCAA	Reverse External	No	
TM6SF1_FM	CGTTTAGCGGGATGCGGTGA	Forward	M	78 bp
TM6SF1_RM	ACACGAAAACCCCGATAACCG	Reverse	M	
TM6SF1_M_Probe	AAACACTCATCGCAACCGCCGCG	Reverse	M	
TMEFF2_Ext F	TTATGGTAGTAGTTTTTYGYGTT	Forward External	No	276 bp
TMEFF2_Ext R	CCCACAACACCATAACTAATTC	Reverse External	No	
TMEFF2_FM	TTTCGTTTCGGGGTTGAGTTTAG	Forward	M	101 bp
TMEFF2_RM	ACGATAACAATAACACCCGACGA	Reverse	M	
TMEFF2_M_Probe	CAAACCCGCGCATAATCTCGAAAATT	Reverse	M	
ZNF671_Ext_F	TAGGTGGAGGTGTTGGGAAA	Forward External	No	298 bp
ZNF671_Ext_R	CTATCCTAAAACACAAAAACTAC	Reverse External	No	
ZNF671_FM1	GTGTTTCGAGACGCGTTTGATG	Forward	M	81 bp
ZNF671_RM1	AACTACCGAAAACGACAAACGTC	Reverse	M	
ZNF671_M_Probe	ATCGAAAACGCAAACACTTCCGTCC	Reverse	M	
ACTB_Ext_F	GATAGGATAGTTTTATTTTTAGGAGG	Forward External	No	161 bp
ACTB_Ext_R	CCACAACCTAATAAAAAAAATAACC	Reverse External	No	
ACTB_F	TAGGGAGTATATAGGTTGGGGAAGTT	Forward	No	102 bp
ACTB_R	AACACACAATAACAAACACAAATTCAC	Reverse	No	
ACTB_Probe	CTAAACCTCCTCCATCACCACCCCAC	Reverse	No	

Step	Marker	Sample (Serum,ST173-1)	Replicate Controls (Table S2)
STEP 1:	**Calculate ΔCt = Ct Gene - Ct ACTB**	**ΔCt**	**ΔCt median (of 300 copies DNA in serum replicate controls)**
	AKR1B1	7.9	5.7
	TM6SF1	6.7	4.9
	ZNF671	27.9	3.5
	TMEFF2	9.0	4.8
	COL6A2	7.7	3.9
	HIST1H3C	3.5	0.8
	RASGRF2	6.2	4.1
	HOXB4	28.8	4.8
	RASSF1A	7.0	5.9
STEP 2:	**Transform to zero all background signals > ΔCt median + 13, (shown in Fig. S1)**	**ΔCt, adjusted**	**ΔCt median + 13, threshold**
	AKR1B1	7.9	18.7
	TM6SF1	6.7	17.9
	ZNF671	~~27.9~~ **0**	16.5
	TMEFF2	9.0	17.8
	COL6A2	7.7	16.9
	HIST1H3C	3.5	13.8
	RASGRF2	6.2	17.1
	HOXB4	~~28.8~~ **0**	17.8
	RASSF1A	7.0	18.9
STEP 3:	**Calculate [(1/ΔCt) * 1200]**	**Gene methylation (M)**	
	AKR1B1	152	
	TM6SF1	179	
	ZNF671	0	
	TMEFF2	133	
	COL6A2	156	
	HIST1H3C	343	
	RASGRF2	194	
	HOXB4	0	
	RASSF1A	171	
STEP 4	**Calculate cumulative methylation (CM) = sum of M values for the 9 genes**	**CM = 1328**	

Table S2. Calculation of LBx-BCM Methylation, Algorithm Example*

*Step 1: Obtain the Ct for each gene and the reference DNA from the GeneXpert software. If no signal was detected assign Ct = 45. Calculate Δ Ct (Ct Gene – Ct ACTB) to normalize all genes to the reference (ACTB). Separately for each gene, transform all ΔCt to positive integers. If a negative ΔCt value is present for any sample, add a constant across all samples for that gene to return only positive integers (add that constant to the replicate control median ΔCt of 300 copies too). Step 2: Remove signals too low to quantitate. Change to Δ Ct = 0 if the Δ Ct is higher than the replicate control median ΔCt of 300 copies + 13 units (i.e, less than 0.04 copies of target DNA, Fig. S1). Step 3: Calculate the inverse of Δ Ct (Ct gene – Ct ACTB) so that high values have high methylation. Multiply by a factor of 1200 to increase the dynamic range of the data. This value now represents gene methylation (M). Step 4: Sum all M for the 9 genes in the marker panel to get the cumulative methylation (CM).

Table S3. Analysis of technical replicates by the LBx-BCM assay

A. Serum*	AKR1B1	TM6SF1	ZNF671	TMEFF2	COL6A2	HIST1H3C	RASGRF2	HOXB4	RASSF1
Methylated Copies	300	300	300	300	300	300	300	300	300
Number of values	11	11	11	11	11	11	11	11	11
Minimum**	4.3	4.1	2.2	4	2.5	0.2	3.3	4.1	4.9
25% Percentile	5	4.4	3.2	4.6	3.3	0.6	3.8	4.6	5
Median***	**5.7**	**4.9**	**3.5**	**4.8**	**3.9**	**0.8**	**4.1**	**4.8**	**5.9**
75% Percentile	6.7	5.8	4.3	5.7	4.6	0.9	4.7	5.1	7
Maximum	10.6	16.9	4.9	6.1	5	2	6.1	5.6	7.2
Coefficient of variation	30.1%	60.4%	20.6%	13.9%	20.3%	55.8%	18.8%	9.12%	15.5%

B. Plasma*	AKR1B1	TM6SF1	ZNF671	TMEFF2	COL6A2	HIST1H3C	RASGRF2	HOXB4	RASSF1
Methylated Copies	300	300	300	300	300	300	300	300	300
Number of values	10	10	10	10	8	8	8	8	8
Minimum**	2.4	2.2	1.6	3.1	1.9	-1.8	2.1	3.0	3.1
25% Percentile	3.0	4.3	2.2	3.4	2.1	-1.4	2.3	3.3	3.5
Median***	**4.1**	**4.7**	**3.0**	**3.9**	**2.6**	**-0.8**	**2.9**	**3.6**	**4.1**
75% Percentile	5.5	5.6	3.7	4.8	2.9	-0.6	3.4	4.2	4.7
Maximum	12.6	7.8	4.2	5.0	3.1	-0.3	4.0	4.7	5.4
Range	10.2	5.6	2.6	1.9	1.2	1.5	1.9	1.7	2.3
Coefficient of variation	60.7%	30.2%	29.5%	17.2%	17.9%	51.6%	21.9%	15.3%	18.3%

*Fully methylated DNA (300 copies) was spiked into 0.5 ml of serum or plasma.

**The LBx-BCM ΔCt of each replicate was calculated.

***The replicate median ΔCt = 300 copies of spiked DNA (bolded) for each gene was used to calculate cumulative methylation. Table supports data shown in Table S1, Fig. 1B and Fig. 2A.

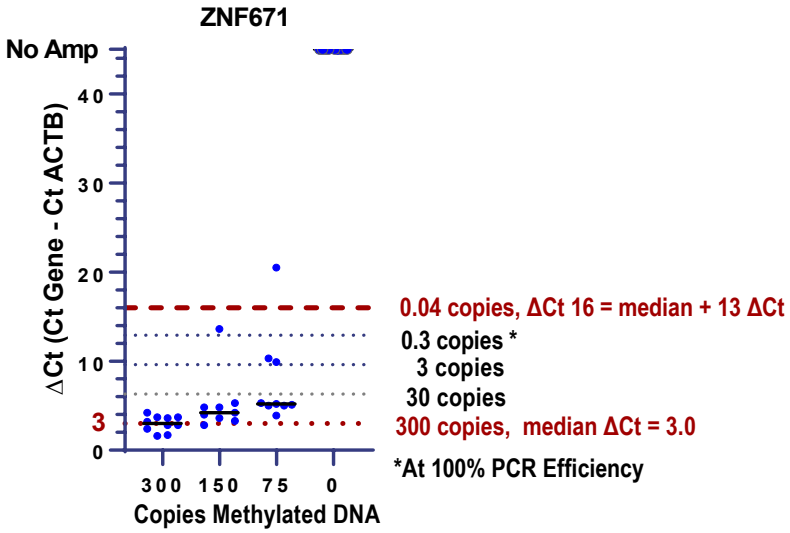


Fig. S1 Relationship between PCR Cycle threshold (Ct) and target DNA copy input. In this example, for ZNF671 the median ΔCt for ten replicates of 300 copies = 3.0 when methylated DNA was spiked into normal pooled plasma (Table S2). At 100% PCR reaction efficiency each 3.33 ΔCt increase is a 10-fold decrease in DNA copies, as indicated by the dotted lines. The heavy dashed threshold at ΔCts = 16 indicates the 3.0 Median value + 13 ΔCt units. A change in 13 Δ Ct units is approximately 2^{13}- fold less than 300 copies in this example, or equal to 0.04 copies, a value below the limit of detection of LBx-BCM. Therefore, for the LBx-BCM methylation algorithm any individual gene methylation value higher than this dashed threshold is considered as a non-specific signal and the sample value adjusted to equal zero in step 2 of the LBx-BCM methylation algorithm (Table S1). No Amp: no signal seen during the run.

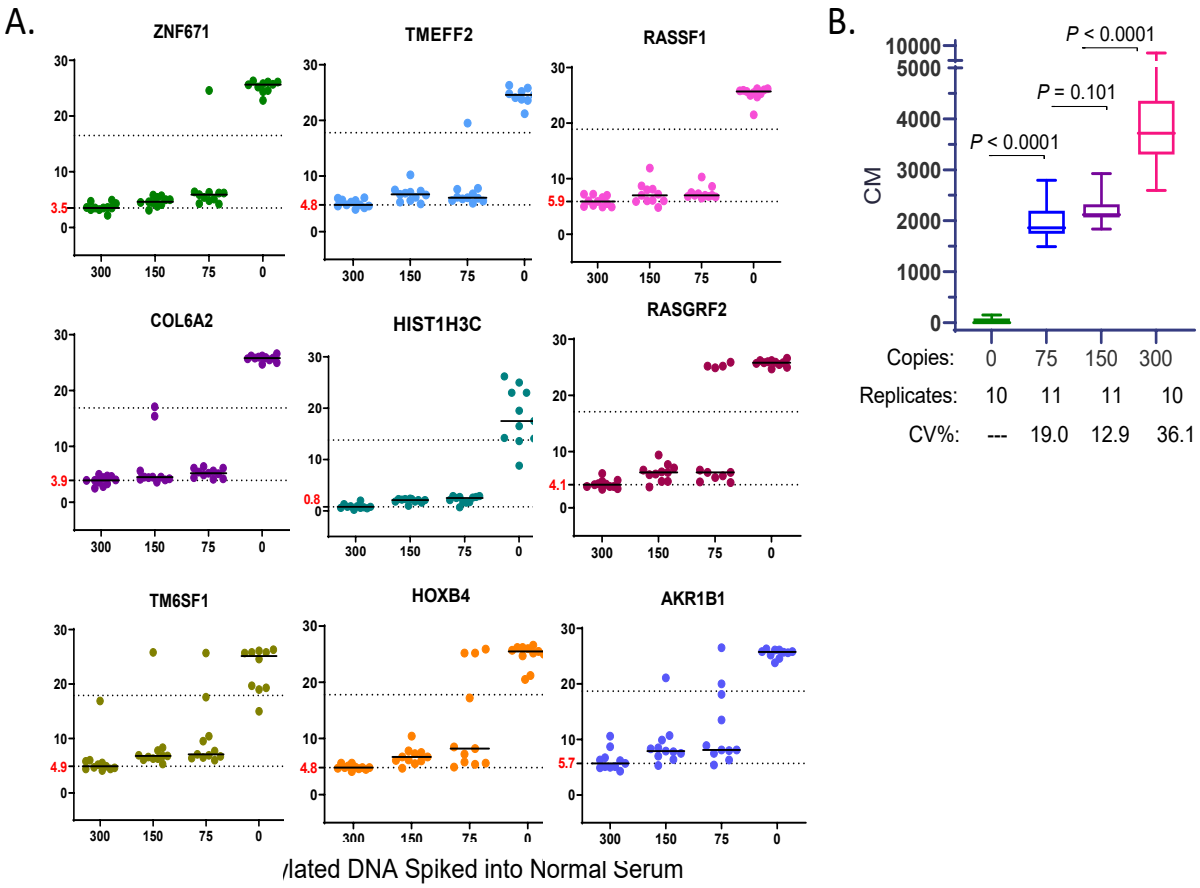


Fig. S2 Analytical sensitivity of LBx-BCM. Fully methylated DNA (300, 150, 75 and 0 copies) was spiked into 0.5 ml aliquots of pooled commercial normal serum in each of 10-11 replicate LBx-BCM assays. **A.** The ΔCt (Ct Gene – Ct ACTB) of all test samples is plotted on the Y axis for each gene. The number of copies of methylated DNA spiked into serum is indicated on the X axis. For each gene, the median ΔCt (Ct Gene – Ct ACTB) for 300 copies of spiked DNA is indicated numerically in red to the left of the Y-axis and by the lower dotted line. The upper dotted line indicates ΔCt for 300 copies + 13 for each marker. **B.** Cumulative methylation of the 9-gene panel for replicates. Mann-Whitney analysis shows significance (*P*-values) between groups of serum samples containing different numbers of spiked methylated DNA copies. The number of replicates performed and the coefficient of variation expressed as percent (CV %) is indicated.

Specific Aim 2. Establish and validate thresholds for detecting methylated tumor DNA in serum using training and test set of sera using GeneXpert cartridges and compare with results already obtained using the lab test, cMethDNA.

Major task: Establish and validate thresholds for detecting methylated tumor DNA in serum using training and test set of sera using GeneXpert cartridges and compare with results already obtained using the lab test, cMethDNA. (completed)

Subtask 1. Establishing thresholds for detectability. Analyze sera from Training set of serum from 28 healthy patients and 24 stage IV breast cancer patients. In prior studies using cMethDNA in serum and plasma, it was determined that a CMI of > 7 distinguishes between MBC and normal sera with both sensitivity and specificity above 90% and area under the curve (AUC) of higher than 0.95. Set threshold for normal sera using cartridge assay.

Subtask 2. Testing markers and method of analysis. Analyze test set of serum from 27 healthy subjects and 33 stage IV breast cancer patients. Using the 6 or 10 gene set, analyze samples in Test set. Use threshold set in subtask 1, and determine sensitivity, specificity and ROC. ROC analysis will characterize the discriminatory power of the test and select appropriate thresholds for detectability in the training data. If the number of sera is not adequate, we will request use of sera from blood bank from the Breast Cancer Program serum repository, collected in study J0888

Subtask 3. Exploratory analysis of Stage 4 sera from JH clinical trials. To confirm performance of the cartridge in clinical trial samples, two sets of sera will be tested. Baseline sera is available form 29 women, and serial samples from 13 women. These will be tested in an exploratory analysis in the cartridge

Subtask 4. Data analysis of the Test set. The Breast cancer Program has an ongoing collection of blood from all consenting patients with all stages of breast cancer under study J0888. In case our study gets underpowered, we will petition access to that repository for our use, and repeat the analysis. Team members meet every week

Milestone(s) Achieved: 1. Established thresholds for detectability. 2. Tested markers and method of marker analysis3. Exploratory analysis of Stage 4 sera from JH clinical trials Performance criteria of the cartridge was investigated using clinical study samples. Performance criteria of the cartridge known, justification for accessing samples from TBCRC005 was presented to committee members

Major achievements:

i. Training set sera (J0888 repository serum, 20 breast cancer stage IV and 20 normal individuals) were used to assess the newly developed cumulative methylation (CM) algorithm and determine if it could distinguish cancer versus normal sera. Using this algorithm, BCM distinguished circulating tumor DNA (CtDNA) in cancer from normal controls (P = 0.002, Mann-Whitney). Optimal sensitivity/specificity was achieved at a CM threshold = 38.5 and this cutoff value was then locked for the duration of the study.

ii. Test set sera (J16146/J0888 EDTA plasma, 40 breast cancer stage IV and 26 normal individuals). With these samples we performed an exploratory analysis of stage IV sera from the IMAGE II (J16146) JH trial. We confirmed performance of the cartridge in clinical trial samples, with sensitivity of 85%, specificity of 92% and ROC AUC = 0.909. BCM distinguished CtDNA from normal controls (P < 0.0001, Mann-Whitney). Most cancer patients (N = 40) with high disease burden had high CM (odds ratio 20.86, P = 0036; Fisher's Exact).

iii. Cross-Platform validation of Test set sera. cMethDNA was performed on the same test samples as in monitoring set (J0214/J0425 serum, 22 stage IV cancer patients, blood was serial collected at the start and throughout treatment. Methylation results generally corresponded to or preceded changes in disease burden.

iv. A paper reporting the findings in Aim 1 and 2 was published in Cancer Res Commun; 2(6) June 2022 (App)

LBx-BCM-based detection of metastatic breast cancer in the training set

The LBx-BCM ctDNA method was initially evaluated in JH repository J0888 samples (patient characteristics and sample sets presented in Tables 1, 2) to verify that LBx-BCM could distinguish between MBC versus normal serum using circulating cell-free DNA. For many of these patients, blood was collected while they were undergoing chemotherapy. We examined cumulative methylation (CM) of the 9-marker panel in serum samples (MBC, $N = 20$; control normal, $N = 20$), and observed significantly higher methylation in the cancer sera compared to normal controls as shown in the histogram (Fig. S3A) and in box-whiskers plot (Fig. S3B, Mann-Whitney test $P = 0.002$). The Receiver Operating Characteristic (ROC)-derived threshold that provided the highest combined sensitivity and specificity was 38.5 CM units (Fig. S3C). At this threshold the ROC AUC = 0.766 (95% CI 0.616 – 0.916; $P = 0.004$), with 75% sensitivity (95% CI 53.1 – 88.8) and 65% specificity (95% CI 43.3 – 81.9).

Accuracy of LBx-BCM to detect metastatic breast cancer in the test set. We then locked existing assay parameters and tested an independent, well-annotated and prospectively collected set of plasma samples. The cancer samples were from the IMAGE II trial (MBC $N = 40$, and controls from the J0888 repository (benign breast disease, $N = 17$; healthy normal, $N = 9$). Patient characteristics and sample sets are shown in Table 1, 2. Consistent with the results in the training set, LBx-BCM detected significantly more methylation in plasma samples from breast cancer than in normal or benign samples as shown in the histogram (Fig. 2A) and box-whiskers plots (Fig. 2B; Mann-Whitney test $P < 0.0001$). At the training set CM threshold of 38.5 units (Fig. S3C), the test set ROC AUC = 0.909 (95% CI = 0.836 - 0.982, $P < 0.0001$), with a sensitivity of 83% (95% CI = 68.1-91.3) and a specificity of 92% (95% CI =75.9 - 98.6) (Fig. 2C). The endogenous reference gene ACTB Ct in the test set for Stage IV samples ranged from 16.0 - 27.8, and in the normal samples ranged from 21.0 - 27.4; the difference between cancer and normal was statistically significant (Fig. S4, Mann Whitney $P < 0.0001$).

Interplatform concordance between LBx-BCM and cMethDNA

LBx-BCM and cMethDNA assays utilize nearly identical primer/probe sequences and similar nested quantitative multiplex methylation-specific PCR strategies. However, cMethDNA normalizes methylation to a gene-specific recombinant standard of 50 methylated copies spiked into 300 µl of plasma/serum prior to purification of DNA, while LBx-BCM normalizes methylation to the endogenous actin reference in the DNA present in 500 µl plasma/serum. As a technical verification step to determine if LBx-BCM achieved a similar level of performance as cMethDNA, we performed cMethDNA on the entire IMAGE II/J0888 test set (Fig. 3). Consistent with the LBx-BCM results, cMethDNA detected significantly more methylation in plasma samples from breast cancer than from normal or benign individuals as shown in histogram and box-whiskers plots (Fig. 3A, Fig. 3B; Mann-Whitney test $P < 0.0001$). At the CMI (cumulative methylation index) threshold of 1.5 units, for cMethDNA the ROC AUC = 0.896 (95% CI = 0.817 - 0.974, $P < 0.001$), with a sensitivity of 83% (95% CI = 68.1 – 91.3) and a specificity of 92% (95% CI = 75.9 – 98.6) (Fig. 3C). LBx-BCM and cMethDNA methylation results were highly concordant (Spearman $r = 0.891$, $P < 0.0001$; $N = 66$ paired samples) (Fig. 3D).

Changes in LBx-BCM methylation during treatment of MBC

We had previously reported results of longitudinal studies in serial blood collections for cMethDNA. Because LBx-BCM demonstrated excellent concordance with this assay, we predicted that LBx-BCM methylation levels would also change during the course of chemotherapy. We analyzed cumulative methylation by LBx-BCM in serum samples obtained from MBC patients in two prospective clinical studies conducted at Johns Hopkins - J0214 and J0425. Serum was collected prior to the initiation of treatment (baseline), 18–49 days (median 21 days) after starting a new line of treatment, and upon completion of additional cycles. Patients received either 28-day cycles of docetaxel or 21-day cycles of capecitabine. Representative plots of LBx-BCM methylation are shown in Fig. 4 and Fig. S5. In these heavily pretreated patients with stage IV breast cancer, changes in CM occurred frequently during the course of treatment. For many patients there was an initial reduction in methylation after the initiation of therapy. Increased methylation was observed among patients who progressed on treatment (PD) and among some patients with stable disease (SD) (Fig. 4 and Fig. S5).

Table 2. Study design and sample sets used for evaluating performance of LBx-BCM

Sample sets					
A. Performance in Training and Test Sets					
Sample sets	Blood				
	Serum		Plasma		
	Cancer	Normal	Cancer	Benign	Normal
Training Set for BCM - J0888 Metastatic Breast Cancer, Healthy Controls, Figure S3	20	20	0	0	0
Test Set - for BCM and cMethDNA - IMAGE II Trial Metastatic Breast Cancer and J0888 Benign and Healthy Controls, Figures 2-3	0	0	40	17	9
B. Changes in methylation during chemotherapy-longitudinal study					
Sample sets, Figures 4 and S5	Serum, Baseline + follow-up				
	Patients		Total Samples		
J0425 Metastatic Breast Cancer	13		46		
J0214 Metastatic Breast Cancer	9		28		
IMAGE II - Individualized Molecular Analyses Guide Efforts in Breast Cancer, J16146					
All normal samples are from different individuals					

Table 3. Interplatform reproducibility between LBx-BCM and cMethDNA

A. Cumulative Methylation (9 genes) in MBC and normal sera, Test Set Samples*	**LBx-BCM**		**cMethDNA****	
	Control	**MBC**	**Control**	**MBC**
N	26	40	26	40
Minimum	0	0	0	0
25% Percentile	0	79	0	10
Median	0	428	0	85
75% Percentile	0	1651	0	258
Maximum	231	11729	17	801
Mean	12	1117	1	166
Lower 95% CI of mean	0	490	0	103
Upper 95% CI of mean	31	1744	3	229

B. ROC Analysis	**LBx-BCM**	**cMethDNA**
Area under the curve	0.909	0.896
95% confidence interval	0.836 to 0.982	0.817 to 0.974
P-value	< 0.0001	< 0.0001
Sensitivity	83%	83%
Specificity	92%	92%
ROC CM threshold***	≥ 38.5	≥ 1.5
Likelihood ratio	11.1	10.7
Classification Accuracy	89%	85%

*The test set samples from the J16146 (IMAGE II)/J0888 studies were used
** cMethDNA was used as a reference assay
***Positive for methylation is defined as ≥ the ROC CM (Cumulative Methylation) threshold

Fig. 2

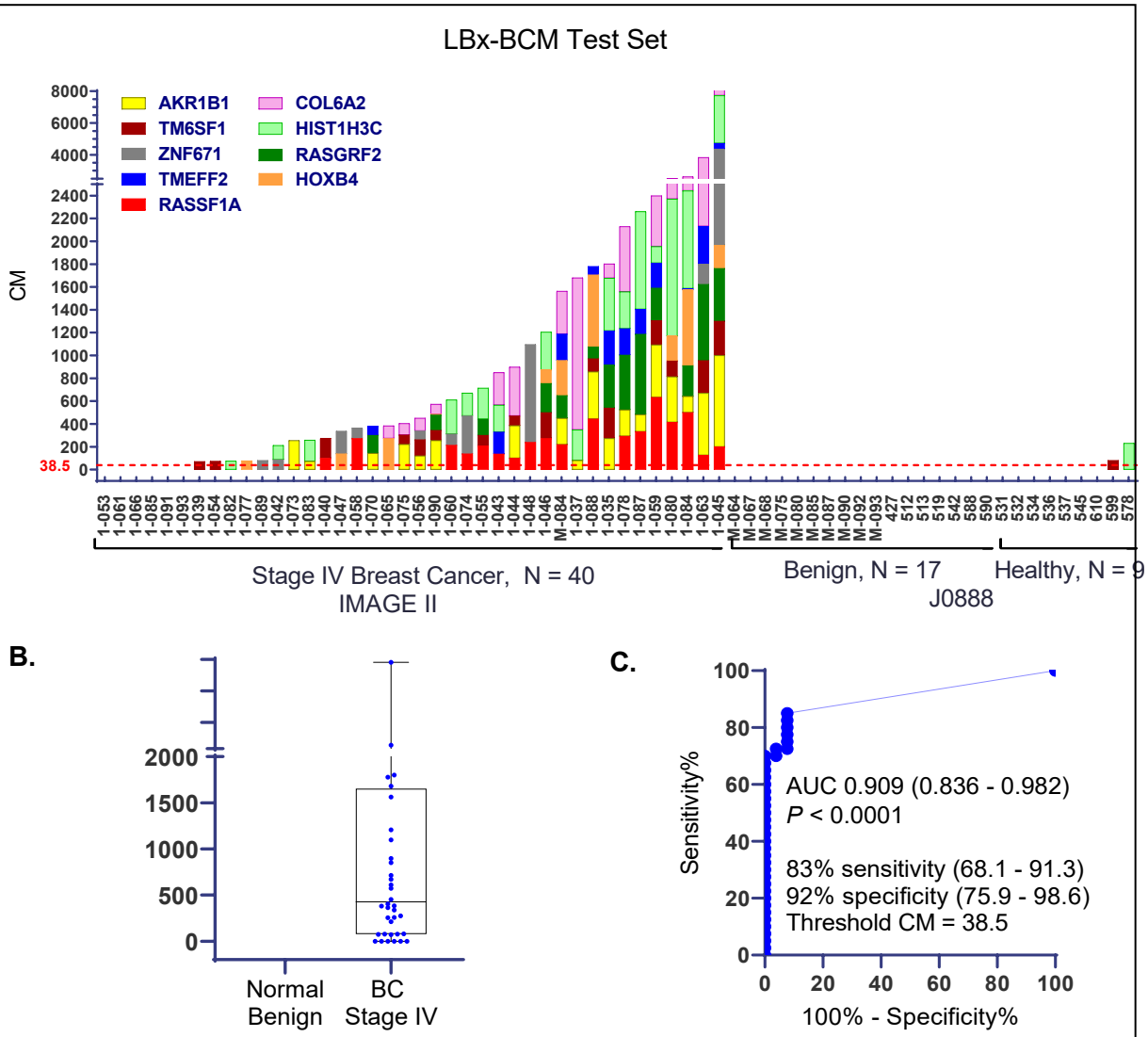


Fig 2. Performance of LBx-BCM in test set of IMAGE II/J0888 study samples.

A. Histograms indicate the magnitude of cumulative methylation (CM) (Y-axis) in each plasma sample (X-axis). Each colored segment indicates the extent of methylation for each individual gene. **B.** A box plot of CMs shows significant differences in ctDNA methylation between cancer and benign/normal samples ($P < 0.0001$, Mann-Whitney). **C.** Detection sensitivity and specificity. The ROC analysis indicated LBx-BCM had 83% sensitivity and 92% specificity to detect cancer with an AUC of 0.909. The ROC analysis utilized the 38.5 CM unit cutoff established in training set samples (Fig. S3).

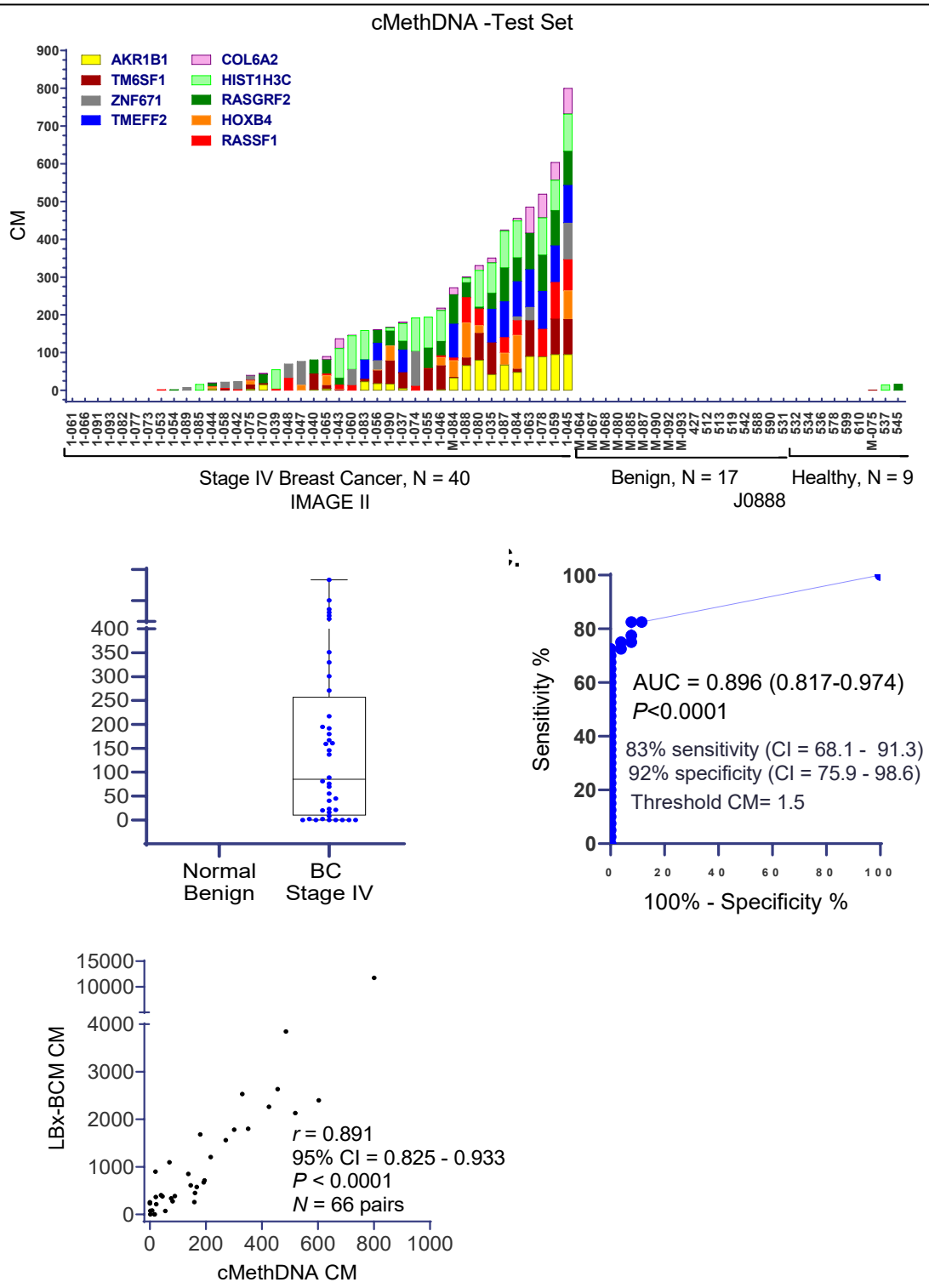


Fig. 3 Assay concordance between LBx-BCM and cMethDNA. We also tested the IMAGE II/J0888 test set samples using the reference cMethDNA assay to directly compare the methylation measures by both platforms when tested on the same samples. **A.** Histogram analysis. cMethDNA histogram indicates the magnitude of cumulative methylation (CM) (Y-axis) for each sample (X-axis). Each colored segment indicates the extent of methylation for individual genes. **B.** Box plot shows CM in samples of normal/benign versus cancer ctDNA methylation ($P < 0.0001$, Mann-Whitney). **C.** Detection sensitivity and specificity. ROC analysis indicated cMethDNA had 83% sensitivity and 92% specificity to detect cancer using a cutoff of 1.5 CM units. **D.** Interplatform assay concordance. CM was plotted for LBx-BCM (Y-axis) and cMethDNA (X-axis) for individual samplesThe Spearman $r = 0.891$ indicated high level of concordance between these two platforms.

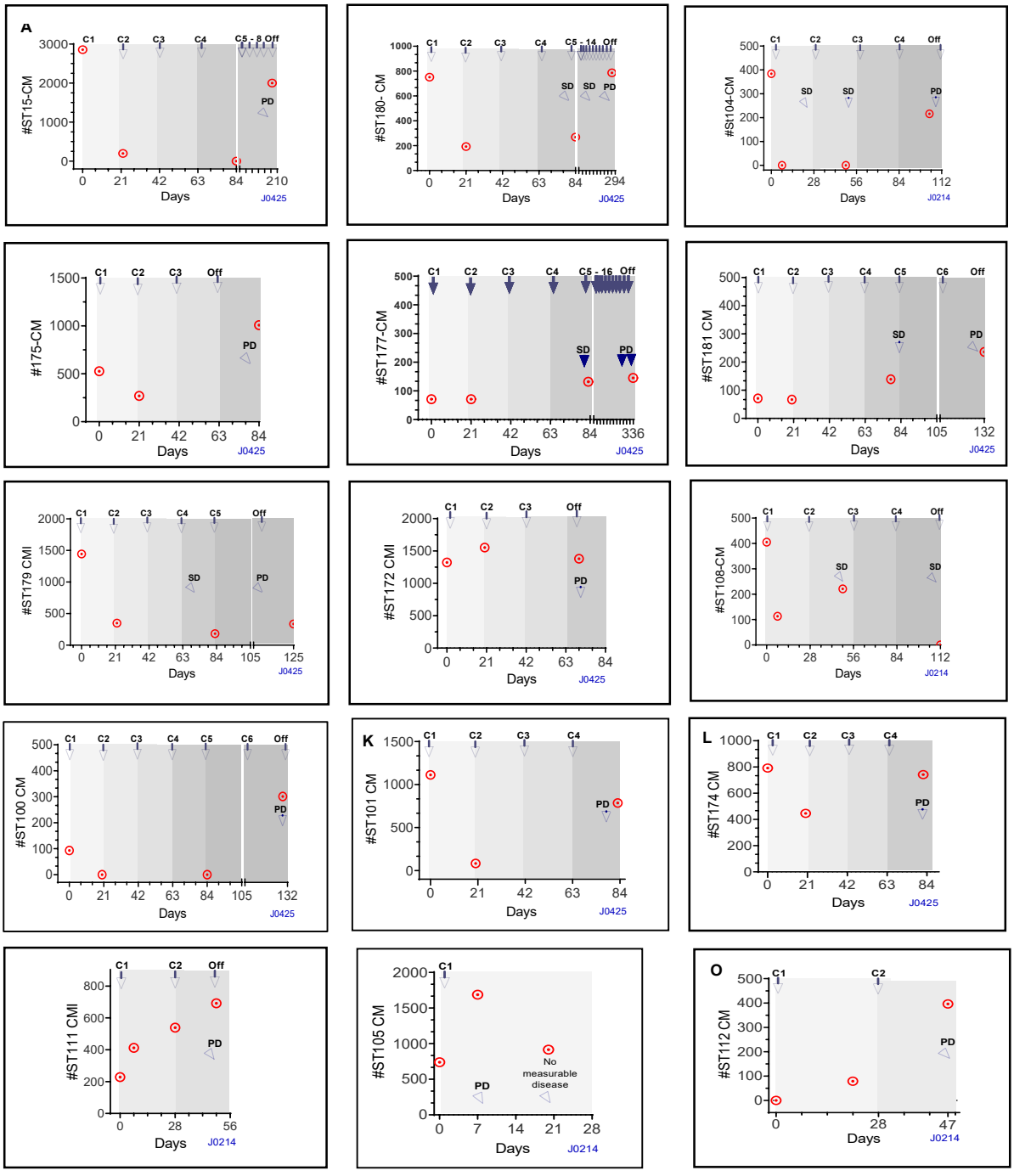


Fig. 4 Changes in methylation in ctDNA by LBx-BCM in response to chemotherapy. Metastatic breast cancer patients (MBC) were enrolled in the J0214 and J0425 studies and received either 21-day cycles of capecitabine or 28 days of docetaxel, indicated by C1, C2 and so on, and the shaded background. **A-O:** Blood was collected from each patient at baseline, and on days indicated in the plots after the start of the new chemotherapy. Cumulative Methylation (CM) measured by LBx-BCM in each patient sample is shown on the X-axis, indicated by dots. Additional diagrams are presented in Fig. S5. PD: progressive disease, SD: stable disease

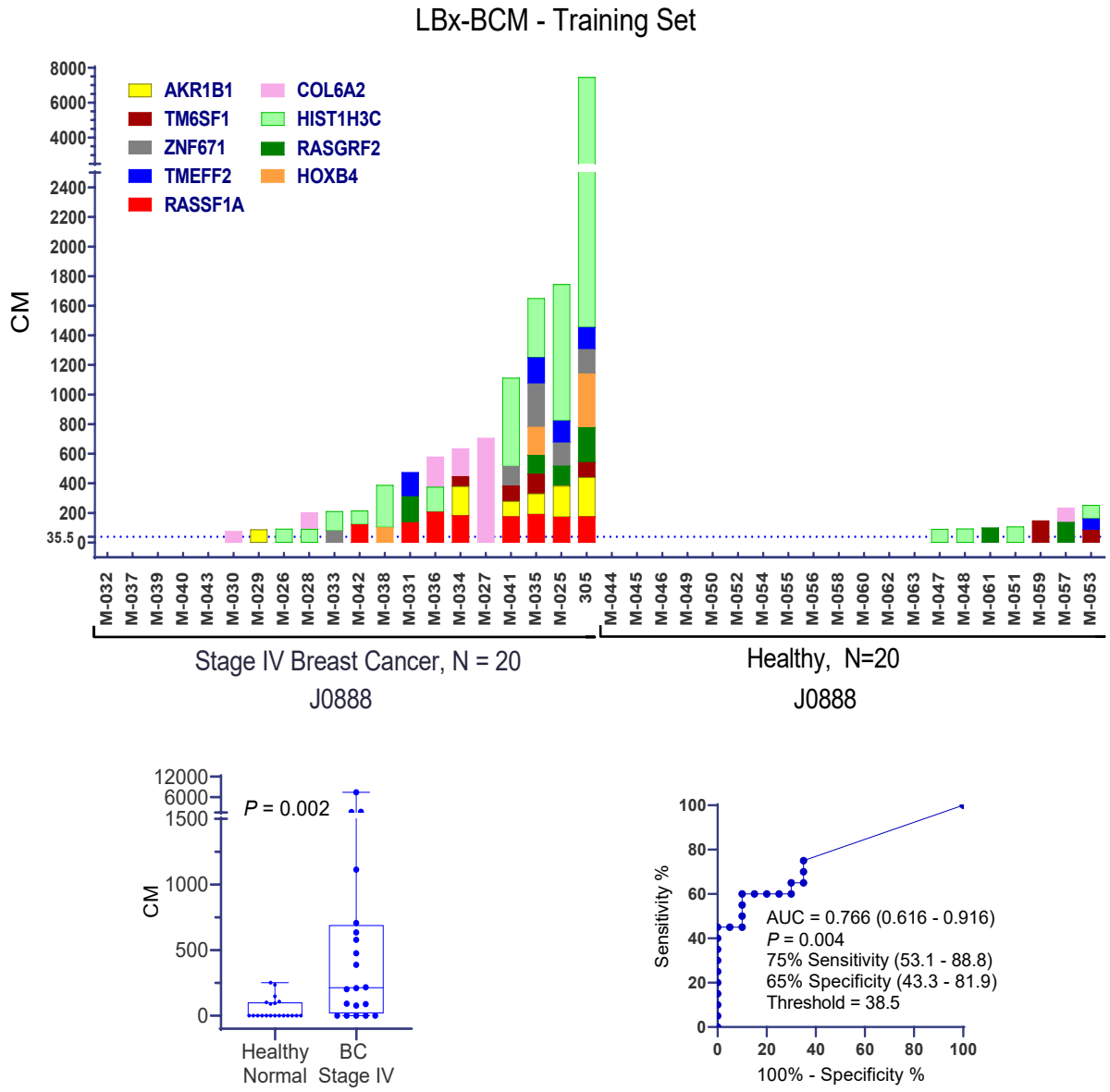
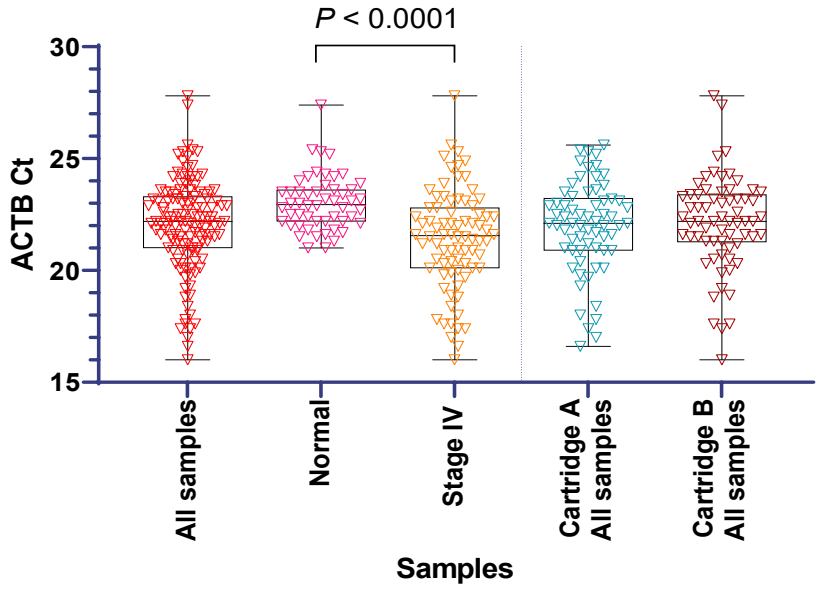


Fig. S3 Performance of LBx-BCM in the J0888 training set. The LBx-BCM was performed on a set of 40 serum samples for the purposes of training the assay and the cumulative methylation (CM) algorithm (Methods and Table S1) on patient sera from the J0888 Repository. CM is plotted as a histogram, showing the extent of methylation (Y-axis) within individual samples (X-axis). The height of the colored segment of each bar indicates the relative amount of methylation present in individual genes. The dotted line indicates the ROC-derived threshold of CM = 38.5 units which provides the highest combined sensitivity and specificity. **B.** Shown in the box plot, the Mann-Whitney analysis indicates significantly ($P = 0.002$) higher methylation in metastatic breast cancer patients undergoing treatment compared to normal individuals.



B.

ACTB Ct	All samples	Normal	Stage IV	Cartridge A All samples	Cartridge B All samples
Number of values	132	52	80	66	66
Minimum	16.0	21.0	16.0	16.6	16.0
25% Percentile	21.0	22.2	20.1	20.9	21.3
Median	22.2	23.0	21.6	22.1	22.2
75% Percentile	23.3	23.6	22.8	23.2	23.4
Maximum	27.8	27.4	27.8	25.6	27.8
Range	11.8	6.4	11.8	9.0	11.8
5% Percentile	17.6	21.2	17.4	17.5	17.6
95% Percentile	25.2	25.3	25.1	25.3	25.2
Mean	22.0	23.0	21.4	22.0	22.1
Std. Deviation	2.1	1.2	2.2	2.0	2.1
Std. Error of Mean	0.2	0.2	0.3	0.3	0.3
Coefficient of variation	9.36%	5.34%	10.50%	9.27%	9.52%

Fig. S4 ACTB levels in the LBx-BCM assay study samples. **A**. Equal amounts of bisulfite treated DNA from 1 ml plasma was used to quantitate target gene methylation in each of two marker cartridges; Cartridge A: AKR1B1, TM6SF1, ZNF671, TMEFF2 normalized to ACTB reference; Cartridge B: COL6A2, HIST1H3C, RASGRF2, HOXB4, RASSF1 normalized to ACTB reference. All ACTB Ct levels were < 28.0 Ct. The difference in ACTB levels between cancer and normal plasma was statistically significant (Mann Whitney $P <$ 0.0001. **B**. Descriptive statistics show that the endogenous reference gene ACTB Ct for Stage IV samples ranged from Ct 16.0 - 27.8, and for the normal samples ranged from Ct 21.0 - 27.4. Median and mean of ACTB Cts in the cancer and normal plasma, and in all samples ($N = 132$) are presented. Coefficient of variation expressed as percent (CV %) is indicated.

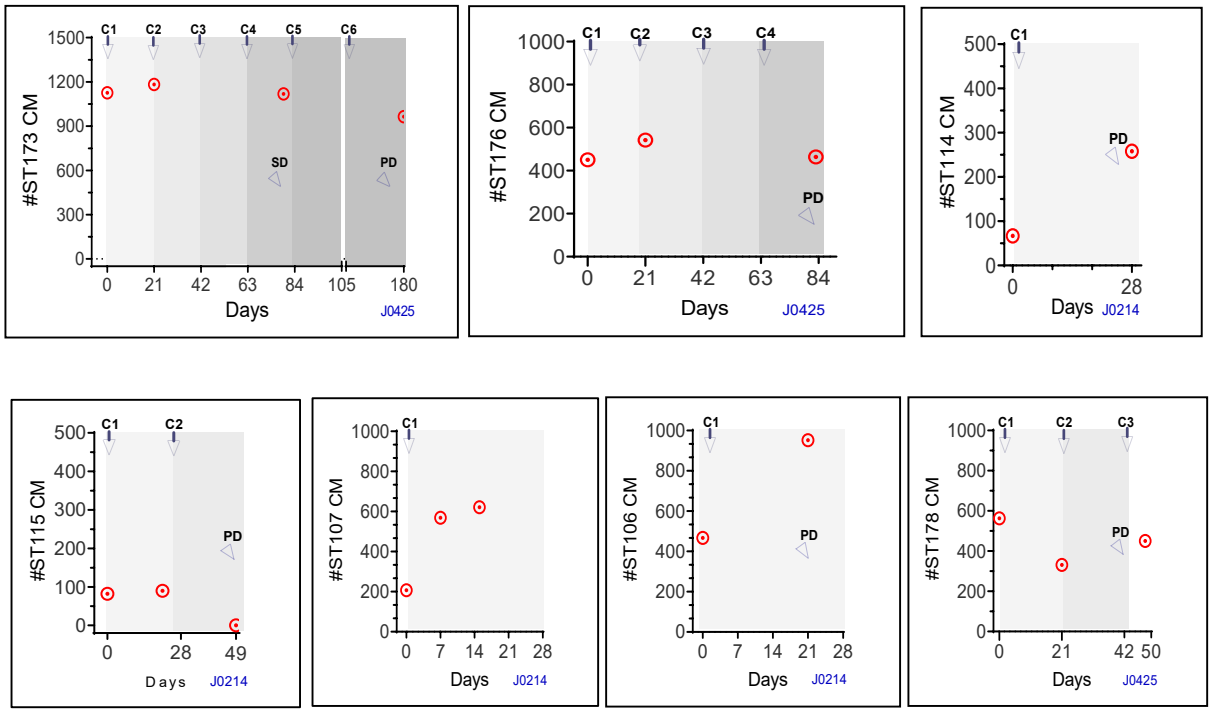


Fig S5. Changes in LBx-BCM methylation in response to chemotherapy. In a continuation of Fig. 4, shown **(A-G)** are LBx-BCM analyses of seven additional longitudinal serial samples (J0214 and J0425 studies) of blood from patients undergoing treatment for MBC. LBx-BCM was performed and cumulative methylation (CM) (Y-axis) is plotted from serum samples drawn at baseline (0 days) and immediately before each treatment cycle (X-axis). For each patient, treatment cycles (21-28 days; C1, C2 etc.) are shown as a shaded area. PD, progressive disease; SD, stable disease (SD).

Impact of the work in Aims 1, 2.

We developed, to the best of our knowledge, the first automated ctDNA methylation assay capable of simultaneously quantitating methylation levels in a panel of markers. Our goal was to develop an assay that would be sophisticated yet simple to perform in underserved regions worldwide, and could be used at point of care to provide same-day feedback to clinicians and patients. We reported the development of a nested, quantitative, multiplexed methylation-specific PCR assay, called Liquid Biopsy for Breast Cancer Methylation assay (LBx-BCM) run on the GeneExpert® system. It can be performed in approximately 4.5 hours sample-to-answer and it uses many of the same markers and principles as the highly sensitive manual cMethDNA method that served as its foundation. LBx-BCM is a prototype for research use only.

In the current study we report technical development and validation of LBx-BCM. We chose a ctDNA marker panel consisting of *HOXB4, RASGRF2, AKR1B1, TM6SF1, COL6A2, HIST1H3C, TMEFF2, RASSF1,* and *ZNF671* and then developed LBx-BCM using the training set of J0888 repository samples. LBx-BCM was validated by performing interassay, interuser and interplatform reproducibility studies comparing LBx-BCM to the reference method cMethDNA (7). Interassay reproducibility studies demonstrated that LBx-BCM was able to detect statistically significant differences in CM between cartridges spiked with 75, 150 and 300 copies of methylated DNA ($P < 0.0001$ - $P = 0.0003$). Interuser reproducibility studies showed that the LBx-BCM assay run by two different users on different days performed consistently at a high level of concordance ($N = 15$; Spearman $r = 0.887$, $P < 0.0001$). Most importantly, with the test set of prospectively collected IMAGE II MBC trial samples, the interplatform reproducibility study demonstrated impressive overall concordance between LBx-BCM cumulative methylation (CM) and cMethDNA CMI results run on the same samples (Spearman $r = 0.891$, $P < 0.0001$, $N = 66$ paired test set samples). ROC performance and diagnostic accuracy were nearly identical between LBx-BCM and cMethDNA. LBx-BCM achieved high sensitivity (83%) and specificity (92%) for an overall diagnostic accuracy of 89%, and a ROC AUC of 0.909. By comparison, for cMethDNA, the sensitivity was 83%, specificity was 92%, for an overall diagnostic accuracy of 85% and ROC AUC = 0.896.

Our automated LBx-BCM system has several important strengths. It is highly sensitive and specific, is technically simple and convenient, has a fast turn-around time, and performs with a high level of accuracy. The detection sensitivity of LBx-BCM is as good or better in advanced breast cancer as other reported quantitative methylation – specific PCR assays, reviewed in Constancio et al. For example, Shan et al used six methylated markers to reported could discriminate between breast cancer patients and healthy women with a sensitivity of 79.6% and a specificity of 72.4% (AUC, 0.727 (95% CI, 0.712 to 0.742), $P < 0.001$. By comparison, Klein et al. report a multi-cancer early detection (MCED) blood test that uses methylated cfDNA sequencing combined with machine learning detected cancer signals and predicted its origin in multiple cancer types with high accuracy. Based on large study sets, for cancer signal detection, an overall sensitivity of 51.5% (49.6% to 53.3%) at a high specificity of 99.5% was achieved. Analyzing a relatively small set of stage IV breast cancers (N = 20), signal detection reached a sensitivity of 90.9% at the same high level of specificity, although the sensitivity to predict tumor origin was only 29.6%. Shen et al (24) used 1-10 ng cfDNA to perform methyl-DNA immunoprecipitation followed by high throughout sequencing to profile methylation patterns typical of tumor cfDNA in several tumor types. However, validation of the method based on differentially methylated regions was not performed in breast cancer.

A second strength of LBx-BCM is the minimal number of off-board steps. The short time requirement of only 4.5 hours is unique to this assay. Another strength is the fact that, unlike many other blood-based assays based on genomic sequencing (25-27), a sample of the primary or metastatic tumor is not required, resulting in substantial savings in time and cost. Taken together, these considerations suggest that our assay could be widely applied at the point of care. We also believe it can be easily adapted to a variety of cancer types.

However, the main limitation of our study is its small sample size. Also, in our studies we noted that LBx-BCM performed less well on serum in comparison to plasma. In reproducibility analyses of 75 – 300 copies of spiked exogenous methylated DNA, the coefficient of variation (CV) was tighter in plasma (CV = 7.1% – 10.9%, Fig. 1D) than in serum (CV = 19.0% – 36.1%, Fig. S2). Consistent with these observations, plasma

samples in the test set showed better sensitivity and specificity (83% and 92%, respectively) than serum in the training set (75% sensitivity and 65% specificity). However, this small training set of sera was from patients who were currently on treatment. We cannot definitively determine if it was the quality of the sample or the assay itself that contributed to the lower performance in the training set. In addition, although our results are promising, our study cohort was primarily from patients (29/39) with ER+/PR+/HER2- breast cancer. The LBx-BCM assay needs to be evaluated in large, prospectively designed studies which include a balanced representation of all histological subtypes of breast cancer and a greater ethnic diversity. Such a cohort has already been identified in the prospective TBCRC 005 trial of stage IV breast cancer patients for whom serial blood sampling was performed at baseline, 3-4 weeks after initiation of a new chemotherapy treatment and 8-12 weeks later. It would also be important to evaluate the performance of this automated system in detecting disease in patients with earlier stages of breast cancer.

In conclusion, we have developed and technically validated a quantitative multiplexed and automated assay for methylated markers in the GeneExpert® system for assaying cell-free DNA from a liquid biopsy which can be implemented at the point of care.

Specific Aim 3. BCM performance in TBCRC005, a prospective clinical trial of metastatic breast cancer patients. This BCM study builds on prior cMethDNA assay data generated by our group from TBCRC005 that was published in Journal of Clinical Oncology (JCO) in 2017 (Visvanathan et al., J Clin Oncol **2017**;35:751-8). The JCO study was the first to demonstrate an association between circulating DNA methylation of tumor-associated genes and subsequent disease outcomes and treatment response.

For this aim we evaluated EDTA plasma from both baseline and week 4 in 142 women. In addition, for 125 women a third sample was collected at the time of restaging (week 8-12) and processed for BCMA. To evaluate within and between batch variability two QCs samples from non-TBCRC005 samples were included in each of the batches.

Major Task 3. Our primary goal of this aim is to test if a change in cumulative methylation levels based on BCM tracks with treatment response in women with metastatic disease. Our secondary goal is to characterize the dynamics of this new assay at multiple time points to inform the development of the **first predictive model** for early treatment response based on DNA methylation in women with metastatic disease. Additional goals include confirming that the new assay has prognostic utility for clinical outcomes.(completed)

Subtask 1. Obtain permission to access Stage 4 serial serum samples collected in prospective trial, TBCRC005

Subtask 2. To verify that the cartridge preserves the predictive power demonstrated by cMethDNA we will repeat these analyses using circulating tumor DNA (ctDNA) methylation levels as measured using the GeneXpert cartridges, Assay setup: The cartridge assay will be used to measure duplicate samples of a methylation 6-10-gene panel in 300 mL of serum. In addition, a set of identical quality control pooled specimens approximately 5% of the total samples will be inserted into every batch to assess inter- and intra - batch reproducibility. All samples from one individual will be run in the same batch to minimize bias from interassay variability.

Subtask 3. Data analysis: Cox proportional hazards models will befit to absolute ctDNA methylation levels to estimate the relationship to PFS and OS. Covariates will include age, ethnicity, prior treatment, stage, grade, ER, PR and Her2 status, and CTC levels. Results will be visualized using Kaplan-Meier curves. Individual gene methylation (M) will be calculated as a methylation
index (MI): The MI of each sample will be averaged across duplicates. The cumulative methylation index (CMI) is the sum of the MI for all gene

Subtask 4. Prepare data for publication and write draft. Team members meet every week. coinciding with clinical restaging done. Comparing change in cumulative methylation of the 6 or ten gene panel to clinical response completed. Comparing performance of cartridge assay with data already obtained using

cMethDNA and CTC assay completed. Paper draft prepared. Paper published. Clin Cancer Res. 2022 Dec 19; doi: 10.1158/1078-0432.

Major Achievements: Utility of the GeneXpert cartridge assay in the metastatic setting (TBCRC005) using blinded serum samples collected at baseline, 3-4 weeks after treatment and at 8-12 weeks was demonstrated. We previously showed that high levels of circulating methylated DNA are associated with subsequent disease progression in women with metastatic breast cancer (MBC). In this study, we evaluated the clinical utility of a novel Liquid Biopsy-Breast Cancer Methylation (LBx-BCM) prototype assay using the GeneXpert® cartridge system for early assessment of disease progression in MBC.

1. The 9-marker, LBx-BCM prototype assay was evaluated in TBCRC-005, a prospective biomarker study, using plasma collected at baseline, week 4 and week 8 from 144 MBC patients.
2. At week 4 MBC patients with high cumulative methylation (CM) had a significantly shorter median PFS (2.88 months v 6.60 months, p = 0.001) and OS (14.52 months v 22.44 months, p=0.005) compared to those with low CM. In a multivariable model, high versus low CM was also associated with shorter PFS (HR = 1.90, 95%CI 1.20-3.01; p =0.006).
3. Change in CM from baseline to week 4 (OR = 4.60, 95%CI 1.77, 11.93; p = 0.002) and high levels of CM at week 4 (OR = 2.78, 95%CI 1.29, 5.99; p = 0.009) were associated with progressive disease at the time of first restaging.
4. A robust risk model based on week 4 circulating CM levels was developed to predict disease progression as early as 3 months after initiating a new treatment.

We showed that the automated LBx-BCM prototype assay is a promising clinical tool for detecting disease progression a month after initiating treatment in women with MBC undergoing routine care. The next step is to validate its clinical utility for specific treatments.

Milestone(s) Achieved: The utility of the GeneXpert cartridge assay in the metastatic setting (TBCRC005) using blinded serum samples collected at baseline, 3-4 weeks after treatment and at 8-12 weeks coinciding with clinical restaging done Comparing change in cumulative methylation of the 6 or ten gene panel to clinical response completed. Comparing performance of cartridge assay with data already obtained using cMethDNA and CTC assay completed. Paper draft prepared

Methods.

Study Population and Design: A total of 144 out of 185 study participants enrolled in TBCRC 005 were included in the current study (see Supplement 1 for study schema). TBCRC 005 was the first prospective study designed to test the clinical utility of circulating DNA methylation biomarkers in blood from individuals with MBC. Patients were enrolled between January 2007 and June 2009. Plasma and serum were collected at baseline immediately prior to starting a new regimen, at 4 weeks, and at first restaging which was between 8-12 weeks. Treatment response was documented at first restaging by the treating physician. Eligible participants for TBCRC005 were females 18 years of age or older with histologically confirmed MBC, measurable disease, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, who were starting a new systemic therapy and being treated at one of seven participating US academic medical centers. To evaluate the potential utility of novel assays we chose to design the study to reflect real world clinical practice and therefore the choice of therapy was at the discretion of the treating physician.

Measurable/evaluable disease was defined as a lesion 1 cm or greater on computed tomography scan or magnetic resonance imaging or a superficial/palpable lesion 1 cm or greater. Patients with a diagnosis of a second cancer in the previous 5 years were excluded, except for basal or squamous cell carcinoma of the skin and/or cervical carcinoma in situ. An additional criterion for this study was that all participants had to have 1 ml of available plasma collected at both baseline and 4 weeks. All patients provided written informed consent. The institutional review board at each study site approved this study.

To evaluate within and between batch reproducibility, EDTA plasma from 36 MBC patients enrolled in SKCCC Johns Hopkins Breast Cancer Repository (a similar study population to TBCRC 005) was pooled, and 1.0 ml aliquots were made. Two aliquots were included in each of 22 batches.

Sample Preparation. Laboratory personnel were blinded to any sample details or associated clinical data. Samples were processed in random order ensuring that repeated samples from the same individual were analyzed in the same batch. Individual samples were pre-processed for LBx-BCM by mixing 1.0 ml of plasma with proteinase K and lysis buffer. Ethanol was added and the sample was transferred in its entirety to a single cartridge and treated with sodium bisulfite as previously described[15]. Here in a two-hour step, unmethylated cytosine was converted to uracil (a DNA sequence change that is detectable by PCR), while methylated DNA is protected and remains unchanged. The bisulfite treated DNA sample was then split equally and transferred to two methylation detection cartridges where two separate PCR reactions occurred in series: PCR 1 is methylation-independent, nested multiplexed PCR that serves as a pre-amplification step, and PCR 2 is a methylation-specific quantitative real-time PCR reaction that measures amplicons generated in the first PCR. PCR 2 uses 6 fluorophores in multiplex to quantify 4-5 methylated targets/cartridge and a beta actin (ACTB) reference gene, 9 targets in a total of two cartridges. A dilution series is used to provide reference levels for each marker. Briefly, target DNA is diluted to 0, 75, 150, and 300 copies and spiked into commercial, normal pooled plasma to provide a reference dilution curve. Replicates (n=10-12) are evaluated in the cartridge.

Calculation of DNA Methylation. Methylation in the following nine target genes was analyzed: *AKR1B1, TM6SF1, ZNF671,* and *TMEFF2,* plus *ACTB* reference in Cartridge A; *COL6A2, HIST1H3C, RASGRF2, HOXB4,* and *RASSF1* plus *ACTB* reference in Cartridge B. Normalized methylation levels were calculated in several steps. **1. Calculate Ct values:** The GeneXpert software assigns Ct values describing the PCR cycle threshold at which fluorescence signal exceeds background, defaulting to Ct = 45 if no signals were detectable during the run; 2. **Normalize to total amount of DNA:** Ct values are normalized to the total abundance of DNA in each sample, represented by Beta Actin levels (ACTB). The resulting $\Delta Ct = Ct_{gene} - Ct_{ACTB}$ is the difference between the Ct of the individual target gene and the Ct of ACTB within the cartridge for each marker. If some samples have negative ΔCt because the estimated abundance of the gene was slightly higher than actin, all samples were similarly transformed by a constant value to give positive integers for that gene. **3. Standardize individual markers to a common level to adjust for gene-specific biases in PCR measures.** The ΔCt value representing standard 300 copies of DNA was estimated from the dilution experiment described above, and ΔCt values for each marker was scaled to a common level. **4. Determine lower limit of detection**. If ΔCt (gene – ACTB) was higher than the replicate median of 300 copies + 13 ΔCt units, then we adjusted to ΔCt (gene – ACTB) = 0, thus removing signals from the analysis that were too low to reliably quantitate (less than 0.03 copies of target) (fig. S1, table S2). 5. **Invert and scale measures.** Scaled ΔCt were inverted so that higher values corresponded to greater abundance of DNA and multiplied by a factor of 1200 for compatibility with measures made using the laboratory based cMethDNA technique. Gene methylation (M) = [1 / ΔCt (gene – ACTB)] * 1200. Finally, cumulative methylation CM (CM) was calculated as the sum of all M for the 9 genes in the panel.

Data Analysis. Inter- and intra-batch variation was evaluated using the coefficient of variation (CV) for CM and each of the nine markers[16]. Median progression-free survival (PFS) and overall survival (OS) with 95% CIs were estimated using the Kaplan-Meier method. Survival distributions were compared between patients with high and low CM at week 4 using the log rank test, controlling for age, ethnicity, body mass index (BMI), prior therapy, tumor subtype, and visceral/non -visceral disease. High and low CM levels were based on the median. Change in CM from baseline to week 4 was also assessed. Change was defined simply as CM week 4 - CM baseline. Response at first restaging was categorized as progressive disease (PD), partial response/complete response (PR/CR), or stable disease (SD) based on treating physician assessment. Spearman's rho was used to detect monotonic trends. Similar multivariable models were used to evaluate the independent effect of CM after adjustment for CTC, CA27-29 and CEA. Predictive models of early progressive disease were assessed by area under an ROC curve (AUC) and reported with bootstrap 95% confidence intervals (95% CI). Time dependent ROC [17] was used to estimate area under the curve when predicting progression at specific time points (3, 6, and

9 months). Models requiring training were evaluated in leave-one-out cross-validation to achieve unbiased estimates of performance. Several models using a binary threshold, random forest and linear discriminant were tested to evaluate combinations of individual markers, cumulative methylation for all 9 genes, and both absolute methylation levels at week 4 as well as change in methylation at week 4 vs. baseline. All tests were two-sided and considered statistically significant at $p < 0.05$ and were performed using the R statistical software suite (available at http://www.r-project.org) with both standard packages and custom code.

Results.

Table 1 describes the patient characteristics for 144 females with MBC enrolled in TBCRC-005. In 124 of the 144 patients a third plasma sample was available at the time of first restaging, which was between 8-12 weeks after the start of treatment. The median age of participants was 56 years, 19% were Black, and 87% were postmenopausal. Most patients were diagnosed with an ER-positive/PR-positive/HER2-negative tumor; 23% were HER2-positive, and 19% triple negative. Twenty-six percent of patients had no prior chemotherapy and/or endocrine-based therapy and fifty percent had elevated CTCs (≥5 cells/mL of blood at baseline CellSearch CTC System). The median CM at baseline was 562.63, at week 4 was 229.69 and at week 8 was 8216.61. All patients were followed until death or last follow-up. The median follow-up time of the cohort was 71.1 months (about 6 years). The inter and intra-batch CV for CM were 0.06 and 0.05 respectively. The overall CV for individual genes ranged from 0.07 to 0.55 and the within batch CV for individual genes ranged from 0.05 to 0.33. See Supplementary Table S1 for further details.

CM and Disease Outcomes

Figure 1A and 1B display the Kaplan Meier curves for progression free survival (PFS) and overall survival (OS) by high versus low methylation at week 4 based on a median CM of 229.69. We confirmed that the median progression free survival (PFS) was significantly shorter for patients with high CM (2.88 months; 95%CI 2.52-4.08 months) versus low week 4 CM (6.60 months; 95% CI 5.76-8.52 months; $p = 0.001$) based on the new LBx-BCM assay. Median overall survival (OS) was also significantly shorter for patients with high CM (14.52 months; 95%CI 11.16-19.80) compared to low CM (22.44 months; 95%CI 20.16- 30.60; $p = 0.005$). In multivariate analyses, patients with high versus low week 4 CM levels had a shorter PFS (HR = 1.90, 95% CI 1.25- 3.01; $p = 0.006$) after adjustment for age, ethnicity, menopausal status, BMI, tumor phenotype, visceral tumor burden, and prior systemic therapy. A similar result was observed for overall survival (OS) (HR = 1.90, 95% CI 1.25- 3.01; $p = 0.006$) after adjustment for the same covariates, although the association was not statistically significant. Details of both analyses are described in Supplementary Table S2.

CM and Disease Status.

The association between CM and disease status at first restaging was evaluated next. At baseline 5% (7/144) of patients had no detectable CM. We observed that both the absolute level of CM at week 4 and the change in CM from baseline to week 4 were associated with response as shown in **Figure 2A**. In 77% of patients, absolute CM levels decreased in the first 4 weeks and then remained stable through time to first restaging. In 18% of patients with stable disease and 37% of patients with progressive disease, an increase in CM was observed from baseline to week 4. There was no increase in CM levels among responders. CM levels were stable from week 4 to week 8.

Figure 2B displays week 4 CM by disease status using box plots. The median CM at week 4 was highest in women with PD (CM=57.37) compared to women with SD (CM=41.48) and responsive disease (CM=12.81), which includes individuals with either PR or CR. A significant trend in CM levels was observed based on the response at first restaging (Spearman rho=0.33, $p = 6.76e-05$). A significant trend was also observed for change in CM from baseline to week 4 by disease status at first restaging as shown in **Figure 2c** (Spearman rho = 0.22, $p= 0.01$). In addition, a significant trend was observed for week 8 CM level but not for change in CM from week 4 to week 8 (Supplementary Table S2).

In univariate analysis shown in **Table 2**, high versus low CM at week 4 was associated with PD at first restaging (OR = 3.25, 95%CI 1.57-7.87, $p = 0.001$). The odds ratio was attenuated in a multivariable model (OR = 2.78,

95%CI 1.29-5.99, p = 0.01), also shown in **Table 2**. A change in CM from baseline to week 4 independent of CM at week 4 was also associated with PD at first restaging in a univariate analysis (OR = 3.81, 95% CI 1.64, 8.82, p = 0.002). The odds ratio was increased to 4.60 (95%CI 1.77-11.93, p = 0.002) in a multivariable model. Similar patterns were observed for the association between CM levels at week 8 and PD [OR = 5.12, 95% CI. (2.02 - 12.94, p = 0.001)] and change in CM between week 4 and week 8 and PD (OR = 3.273, 95% CI. 1.36 – 7.85, p= 0.01) (see Supplementary Table S3). Supplement 6 displays baseline CM levels by disease status using box plots. The median CM was highest for PD (CM = 713.43), compared to SD (CM= 533.65), and responsive disease (CM=425.48), which includes PR and CR. There was no significant trend observed (Spearman rho = 0.03, p = 0.70).

We also assessed whether high CM levels were independently associated with progressive disease in the presence other circulating markers that have been studied or used for monitoring disease progression in MBC patients (see Supplementary Tables S4–S6).The association between Week 4 CM levels at first restaging and progressive disease remained strongly significant in multivariable models that either included CTC (OR=6.93, p =0.002) , CEA (OR=5.48, p = 0.003), or CA27-29 (OR=4.18, p =0.012) (see supplement xx). Of note, association between circulating CTC (OR=1.04, p =0.952) and CEA (OR=1.16, p =0.747) and progressive disease were not statistically significant. However, circulating CA2729 remained statistically significant after adjustment for CM (OR=4.18, p = 0.004).

Development of New Predictive model for Early Disease Progression

Lastly, we developed a robust model in women with MBC after initiating a new treatment to predict disease progression at 3 months, 6 months, 9 months and first restaging based on CM measurements**. Figure 3a and 3b** display time updated ROC curves predicting disease progression based on high versus low week 4 CM and change in CM from baseline to week 4 respectively. On each graph, the black curve shows progressive disease at first restaging. The red, blue, and green ROC curves on each panel display the ROC curve for disease progression at 3, 6, and 9 months. Week 4 CM outperformed a change in CM based on disease progression at each time point. The combined model that includes both week 4 CM (high versus low) and change in CM yielded the best performance across the multiple end points (AUC = 0.668- 0.733). Alternative models, ranging from random forest models to a simple threshold model in which individual markers were binarized to low vs. high methylation yielded AUCs ranging from 0.557-0.679 (similar to naive week 4 CM), reflecting the robustness of the overall model. Time updated ROC analyses combining high versus low week 8 CM and PD as well as change in CM from week 4 to week 8 and PD, was slightly weaker (see Supplementary Figure S5).

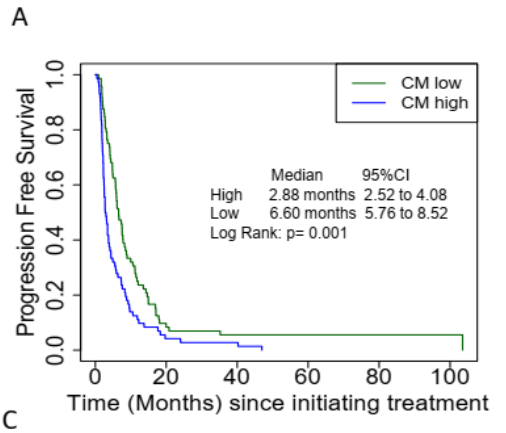
Table 1: Baseline Patient Characteristics of the Analytic Population (N = 144)			
Characteristic		N	%
	Age, years, median (range)	56	(28-84)
Ethnicity			
	White *	117	(81%)
	Black	27	(19%)
Menopausal status			
	Postmenopausal	122	(87%)
	Perimenopausal/Premenopausal	18	(13%)
	BMI kg/m2 , median (range)	26	(18-44)
Tumor phenotype of initial diagnosis			
	ER positive/PR positive/HER2 negative	84	(58%)
	HER2 positive (any ER)	33	(23%)
	Triple negative	27	(19%)
Disease burden			
	Visceral only (liver, lung, brain	25	(17%)
	Nonvisceral only (bone and/or soft tissue	49	(34%)
	Both	70	(49%)
Prior therapy			
	None	38	(26%)
	Chemotherapy only	32	(22%)
	Hormone therapy only	34	(24%)
	Chemotherapy and hormone therapy	40	(28%)
Other			
	Elevated CTC level(>= 5)	63	(50%)
	Progression-free survival, Mos, median (95% CI)	4.8	(3.72-6)
	Follow Up, Mos, median (95% CI)	71.1	(66.7 – NA)
CM levels			
	Baseline, median (range)	562.63	(0.00 -- 2706.77)
	Week 4, median (range)	229.69	(0.00 -- 2075.94)
	Week 8, median (range)	216.61	(0.00 -- 3086.98)

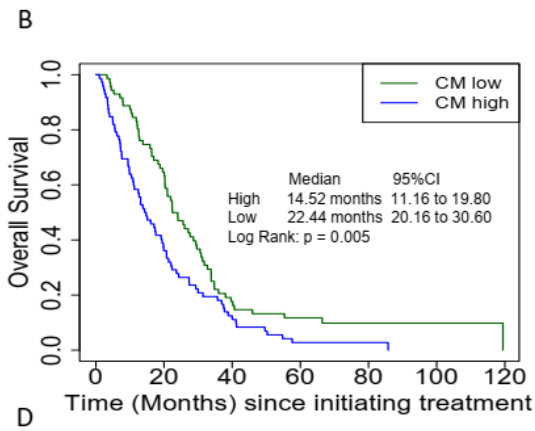
Table 2. Association between week4 CM changes in CM and disease status at first restaging.

Predictor	[1]CM level at week4			[2]Change in CM level btw baseline and week4		
	OR	95% CI	*P*	OR	95% CI	*P*
Univariate Analysis						
CM	3.25	1.57-7.87	0.001	3.81	1.64-8.82	0.002
Age (continuous)	0.97	0.94-1.00	0.086	0.97	0.94-1.00	0.070
Multivariate Analysis						
CM	2.78	1.29-5.99	0.009	4.60	1.77-11.93	0.002
Age (continuous)	0.97	0.94-1.01	0.186	0.97	0.94-1.01	0.183
Race						
[3]White (ref)	1.00			1.00		
Black	1.09	0.39-3.06	0.870	1.23	0.43-3.53	0.697
Tumor Phenotype						
ER +ve/PR +ve/ HER2 -ve (ref)	1.00			1.00		
HER2 (any ER) +ve	0.80	0.29-2.23	0.675	1.30	0.46-3.72	0.623
Triple -ve	0.67	0.20-2.27	0.523	0.75	0.21-2.62	0.650
Prior therapy						
None (ref)	1.00			1.00		
Chemotherapy	1.44	0.48-4.37	0.519	1.29	0.42-4.02	0.656
Endocrine-based Therapy (ET)	0.67	0.20-2.24	0.514	0.78	0.23-2.67	0.698
Chemo/ ET	0.83	0.26-2.59	0.744	1.05	0.33-3.32	0.932
Disease burden						
Visceral/Both (ref)	1.00			1.00		
Non visceral	0.69	0.30-1.54	0.362	0.65	0.28-1.47	0.301
BMI, kg/m^2						
< 25 (ref)	1.00			1.00		
25-30	0.44	0.17-1.18	0.104	0.38	0.14-1.00	0.051
> 30	1.34	0.51-3.56	0.555	0.87	0.31-2.45	0.796
Menopausal status						
Postmenopausal (ref)	1.00			1.00		
Premenopausal	0.91	0.25-3.31	0.887	0.92	0.25-3.39	0.895

Abbreviations: OR, odds ratio; CI, confidence intervals; CM, cumulative methylation; +ve, positive; -ve, negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Chemo, chemotherapy; ET, endocrine=based therapy; BMI, body mass index. [1]: CM binary measure: level > median level versus CM level ≤ median level. [2]: ΔCM binary measure > 0 versus ΔCM ≤ 0. [3]: Includes one Asian.

Figure 1





C

Time (Months)	12	24	36	48	60	72	84	96
CM low	18	5	4	4	4	1	1	1
CM high	8	3	2	0	0	0	0	0

D

Time (Months)	12	24	36	48	60	72	84	96	108
CM low	59	34	15	9	7	1	1	1	1
CM high	42	20	13	6	2	1	1	0	0

Figure 1A and 1B display Kaplan-Meier curves for Progression-free survival and Overall survival among women with metastatic breast cancer stratified by CM level beginning at week 4. High versus low CM was based on the median (CM > median versus <= median.) The corresponding multivariate Cox regression models are shown in Table 2. CM, cumulative methylation

Figure 2

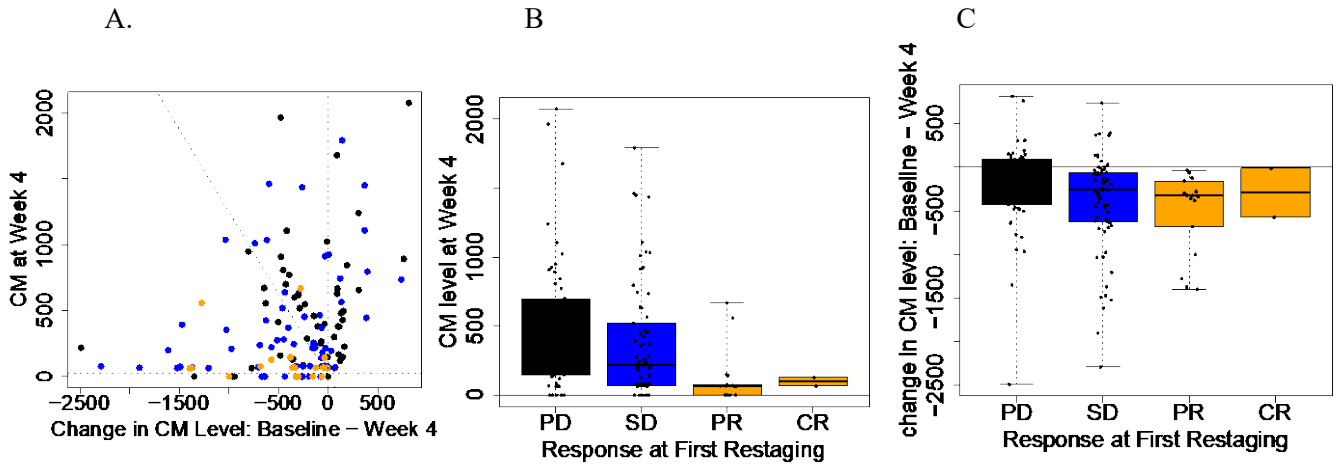


Figure 2A displays the relationship between CM level at week 4 and the Change in CM level between baseline and Week 4. The black dots represent PD, blue dotes SD and orange dots PR or CR.
Figure 2B and 2C uses box plots to display the relationship between disease status at first restaging and week4 CM level or change in CM level (baseline – week 4). CM, cumulative methylation; PD, progressive disease, SD, stable disease, PR, partial response, CR, complete response

Figure 3

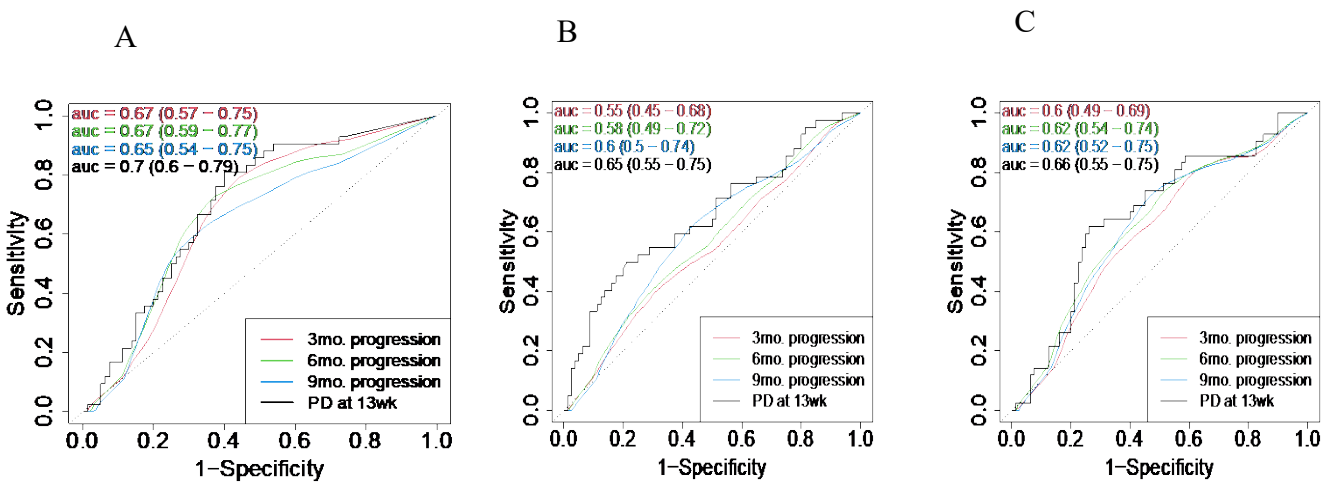


Figure 3
Time updated ROC curves for (A) CM level at week 4, (B) change in CM level (Week 4 – Baseline), and (C) the Combination of week 4 CM level and Change in CM level (Week 4 – Baseline) using linear discriminant analysis. On each graph, the black curve shows PD at first restaging. The red, blue, and green ROC curves on each panel display the ROC curve for disease progression at 3, 6, and 9 months, respectively.

Supplementary Table S1**.** Overall and within batch Coefficient of Variation for CM and each Gene.

	Overall CV			Within Batch CV		
	Mean	SD	CV	Mean	SD	CV
CM	**185.67**	**8.28**	**0.04**	**185.67**	**8.28**	**0.04**
Gene(s)						
AKR1B1	254.74	33.4	0.13	254.74	26.94	0.11
TM6SF1	242.28	31.49	0.13	242.28	25.82	0.11
ZNF671	103.69	57.27	0.55	103.69	33.96	0.33
TMEFF2	187.46	16.59	0.09	187.46	8.45	0.05
COL6A2	169.73	13.77	0.08	169.73	13.08	0.08
HIST1H3C	235.58	17.29	0.07	235.58	11.03	0.05
RASGRF2	192.71	19.46	0.1	192.71	12.05	0.06
HOXB4	126.89	35.97	0.28	126.89	21.49	0.17
RASSF1A	157.93	25.43	0.16	157.93	16.77	0.11

Abbreviations: CV, coefficient of variation; SD, standard deviation; CM, cumulative methylation.

Supplementary Table 2 Association between week 4 CM and PFS and OS

Predictor	Progression Free Survival (PFS)			Overall Survival (OS)		
	HR	95% CI	*P*	HR	95% CI	*P*
Multivariate Analysis						
CM	1.90	1.20-3.01	0.006	1.19	0.74-1.91	0.482
Age (continuous)	0.97	0.95-0.99	0.000	1.00	0.98-1.02	0.961
Race						
[1]White (ref)	1.00			1.00		
Black	0.66	0.43-1.02	0.060	0.67	0.44-1.04	0.071
Tumor Phenotype						
ER +ve/PR +ve/ HER2 -ve (ref)	1.00			1.00		
HER2 (any ER) +ve	1.25	0.77-2.04	0.366	0.97	0.57-1.64	0.895
Triple -ve	0.74	0.41-1.34	0.326	1.52	0.84-2.76	0.168
Prior therapy						
None (ref)	1.00			1.00		
Chemo	1.24	0.72-2.13	0.443	1.50	0.85-2.66	0.162
Endocrine-based therapy (ET)	0.99	0.56-1.76	0.985	1.15	0.64-2.06	0.631
Chemo/ET	1.12	0.63-1.98	0.703	1.62	0.91-2.88	0.103
Disease burden						
Visceral/Both (ref)	1.00			1.00		
Non visceral	0.97	0.66-1.43	0.885	1.09	0.74-1.60	0.661
BMI, kg/m^2						
< 25 (ref)	1.00			1.00		
25-30	1.08	0.64-1.82	0.775	0.94	0.56-1.56	0.799
> 30	0.72	0.37-1.39	0.323	1.01	0.51-1.99	0.975
Menopausal status						
Postmenopausal (ref)	1.00			1.00		
Premenopausal	1.14	0.70-1.85	0.595	2.16	1.29-3.63	0.003

Abbreviations: O=HR, hazard ratio; CI, confidence intervals; CM, cumulative methylation; +ve, positive; -ve, negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Chemo, chemotherapy; HT, hormone therapy; BMI, body mass index. [1]: Includes one Asian.

Supplementary Table 3
Association between week8 CM and change in CM and disease status at first restaging.

Predictor	[1]CM level at week8			[2]Change in CM level between week4 and week8		
	OR	95% CI	*P*	OR	95% CI	*P*
Univariate Analysis						
CM	5.18	2.21-12.15	0.000	2.95	1.32-6.58	0.008
Age (continuous)	0.97	0.93-1.01	0.089	0.97	0.94-1.01	0.109
Multivariate Analysis						
CM	5.12	2.02-12.94	0.001	3.27	1.36-7.85	0.008
Age (continuous)	0.98	0.93-1.02	0.271	0.98	0.94-1.02	0.279
Race						
[3]White (ref)	1.00			1.00		
Black	1.26	0.42-3.78	0.679	1.58	0.54-4.59	0.403
Tumor Phenotype						
ER +ve/PR +ve/ HER2 -ve (ref)	1.00			1.00		
HER2 (any ER) +ve	0.73	0.23-2.32	0.594	1.03	0.34-3.15	0.962
Triple -ve	0.54	0.14-2.09	0.372	0.55	0.15-2.06	0.374
Prior therapy						
None (ref)	1.00			1.00		
Chemo	1.43	0.42-4.90	0.566	1.16	0.35-3.88	0.806
Endocrine-based therapy (ET)	0.69	0.18-2.64	0.588	0.75	0.20-2.85	0.667
Chemo/ET	0.80	0.23-2.81	0.728	0.78	0.23-2.70	0.701
Disease burden						
Visceral/Both (ref)	1.00			1.00		
Non visceral	0.64	0.25-1.60	0.335	0.54	0.22-1.34	0.183
BMI, kg/m^2						
< 25 (ref)	1.00			1.00		
25-30	0.47	0.16-1.34	0.156	0.38	0.13-1.08	0.069
> 30	1.27	0.40-4.01	0.681	1. 03	0.33-3.19	0.958
Menopausal status						
Postmenopausal (ref)	1.00			1.00		
Premenopausal	1.61	0.39-6.61	0.508	1.24	0.32-4.79	0.753

Abbreviations: OR, odds ratio; CI, confidence intervals; CM, cumulative methylation; +ve, positive; -ve, negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Chemo, chemotherapy; HT, hormone therapy; BMI, body mass index. [1]: CM is a binary measure CM level > median level versus CM level ≤ median level. [2]: ΔCM is also a binary measure > 0 versus ΔCM ≤ 0. [3]: Includes one Asian.

Supplementary Table 4
Association between week 4 CM and disease status at first restaging after adjusting for CTC

Predicator	OR	95% CI	*P*
Multivariable Analysis			
CM	6.93	1.99-24.16	0.002
Age (continuous)	0.99	0.94-1.03	0.604
Race			
[1]White (ref)	1.00		
Black	1.26	0.33-4.78	0.738
Tumor Phenotype			
ER +ve/PR +ve/ HER2 -ve (ref)	1.00		
HER2 (any ER) +ve	1.02	0.24-4.34	0.979
Triple -ve	0.91	0.16-5.23	0.914
Prior therapy			
None (ref)	1.00		
Chemo	3.16	0.64-15.65	0.159
Estrogen-related Therapy (ET)	1.65	0.29-9.39	0.570
Chemo/ET	1.80	0.35-9.25	0.480
Disease burden			
Visceral/Both (ref)	1.00		
Non visceral	0.63	0.21-1.89	0.409
BMI, kg/m2			
< 25 (ref)	1.00		
25-30	0.37	0.11-1.28	0.116
> 30	0.60	0.15-2.43	0.473
Menopausal status			
Postmenopausal (ref)	1.00		
Premenopausal	1.35	0.23-7.90	0.741
CTC	1.04	0.29-3.75	0.952

Abbreviations: OR, odds ratio; CI, confidence intervals; CM, cumulative methylation; +ve, positive; -ve, negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Chemo, chemotherapy; HT, hormone therapy; BMI, body mass index; CTC, circulating tumor cells. [1]: Includes one Asian.

Supplementary Table 5. Association between week 4 CM and disease status at first restaging after adjusting for CEA.

Predicator	OR	95% CI	*P*
Multivariable Analysis			
CM		1.82-16.53	0.003
Age (continuous)	0.98	0.94-1.02	0.267
Race			
[1]White (ref)	1.00		
Black	1.42	0.42-4.85	0.571
Tumor Phenotype			
ER +ve/PR +ve/ HER2 -ve (ref)	1.00		
HER2 (any ER) +ve	0.80	0.24-2.71	0.719
Triple -ve	0.49	0.11-2.08	0.331
Prior therapy			
None (ref)	1.00		
Chemo	1.49	0.41-5.46	0.544
Estrogen-related Therapy (ET)	0.53	0.13-2.18	0.376
Chemo/ET	0.79	0.23-2.70	0.708
Disease burden			
Visceral/Both (ref)	1.00		
Non visceral	0.68	0.27-1.70	0.406
BMI, kg/m2			
< 25 (ref)	1.00		
25-30	0.44	0.15-1.28	0.131
> 30	0.98	0.31-3.14	0.977
Menopausal status			
Postmenopausal (ref)	1.00		
Premenopausal	0.83	0.18-3.77	0.813
CEA	1.16	0.47-2.86	0.747

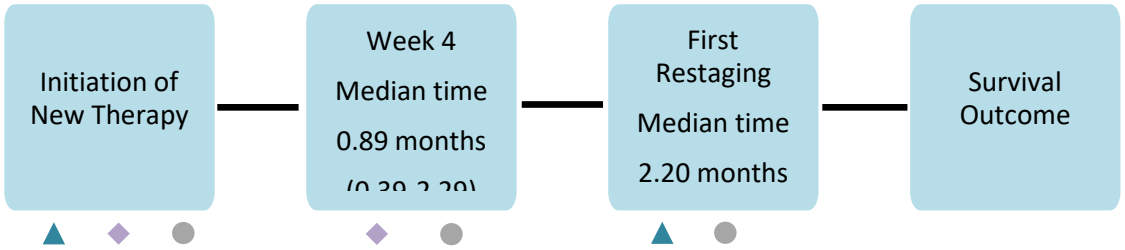
Abbreviations: OR, odds ratio; CI, confidence intervals; CM, cumulative methylation; +ve, positive; -ve, negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Chemo, chemotherapy; HT, hormone therapy; BMI, body mass index; CEA, carcinoembryonic antigen. [1]: Includes one Asian.

Supplementary Table 6
Association between week 4 CM and disease status at first restaging after adjusting for CA27-29.

Predicator	OR	95% CI	*P*
Multivariable Analysis			
CM	4.18	1.38-12.69	0.012
Age (continuous)	0.98	0.93-1.02	0.275
Race			
[1]White (ref)	1.00		
Black	1.47	0.40-5.37	0.557
Tumor Phenotype			
ER +ve/PR +ve/ HER2 -ve (ref)	1.00		
HER2 (any ER) +ve	1.11	0.31-3.99	0.874
Triple -ve	0.47	0.11-2.06	0.314
Prior therapy			
None (ref)	1.00		
Chemo	1.81	0.48-6.82	0.380
Endocrine-related therapy (ET)	0.52	0.12-2.27	0.383
Chemo/ET	0.98	0.26-3.62	0.970
Disease burden			
Visceral/Both (ref)	1.00		
Non visceral	0.63	0.24-1.65	0.343
BMI, kg/m2			
< 25 (ref)	1.00		
25-30	0.41	0.13-1.24	0.114
> 30	0.93	0.28-3.12	0.903
Menopausal status			
Postmenopausal (ref)	1.00		
Premenopausal	0.98	0.21-4.58	0.979
CA27-29	4.18	1.56-11.18	0.004

Abbreviations: OR, odds ratio; CI, confidence intervals; CM, cumulative methylation; +ve, positive; -ve, negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Chemo, chemotherapy; HT, hormone therapy; BMI, body mass index. [1]: Includes one Asian.

Supplementary Figure S1- Study Schema



Disease assessment (clinical examination and imaging

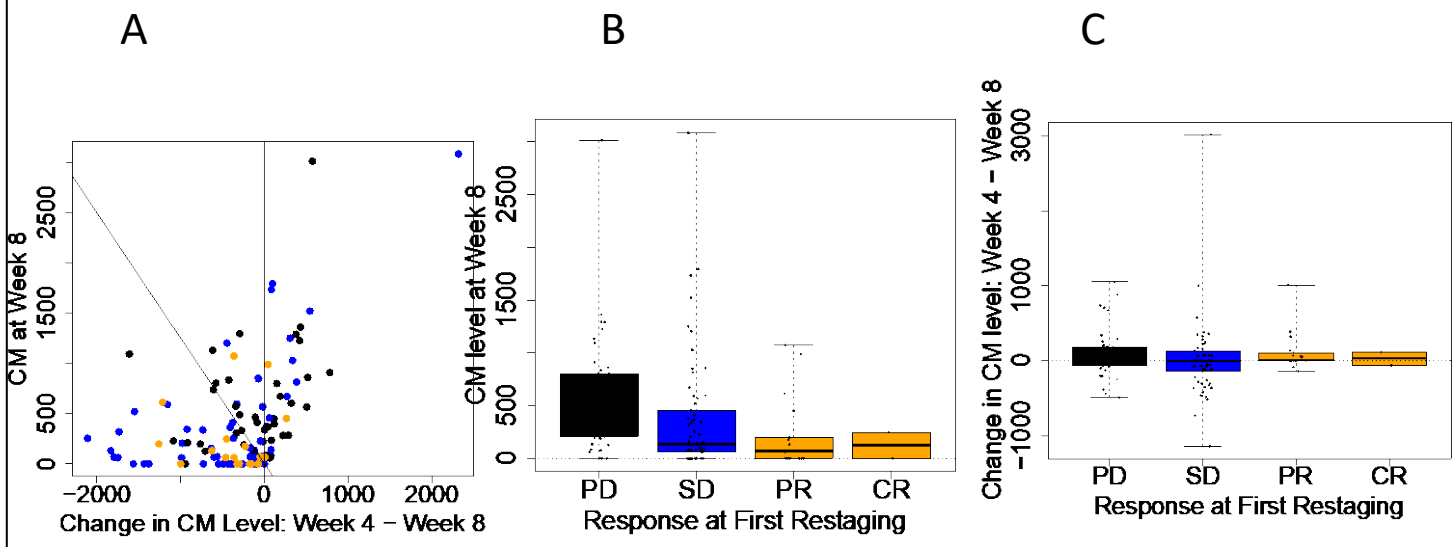
Whole blood

Serum

Inclusion criteria: Patients with stage IV metastatic breast cancer, measurable

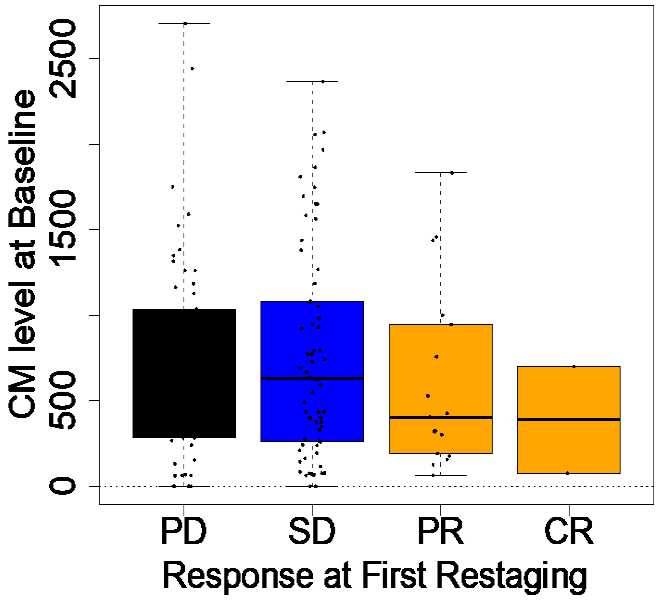
144 subjects, with baseline and week 4 blood samples
139 of which were assessed for 3mo progression
124 had a 3rd blood samples at first restaging
Treatment response was assessed by each treating physician at first restaging,

Supplementary Figure S2



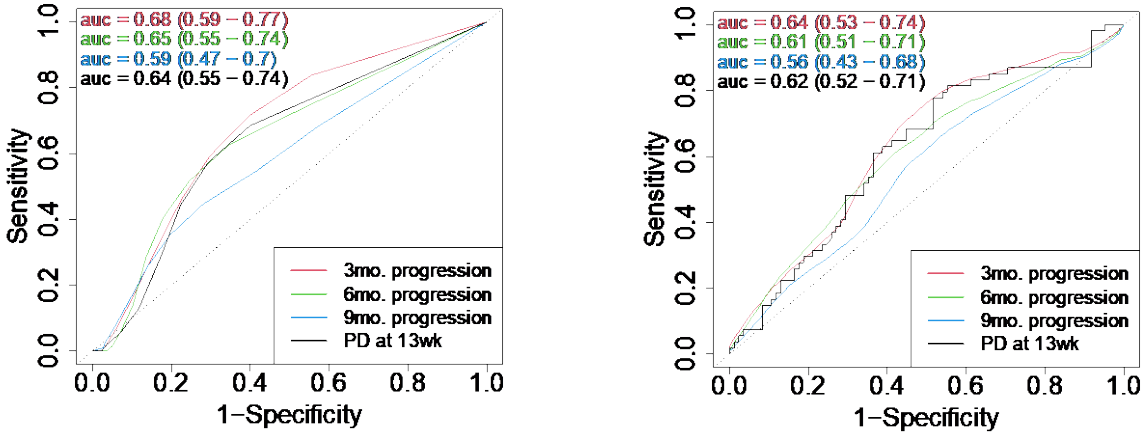
Supplementary Figure 2 displays the relationship between the week 8 CM level and the change in CM level (Week 8 – baseline). Figure S4B and C use box plots to display the relationship between disease status at first restaging and week 8 CM level or change in CM level (Week 8 – baseline).

Supplementary Figure S3



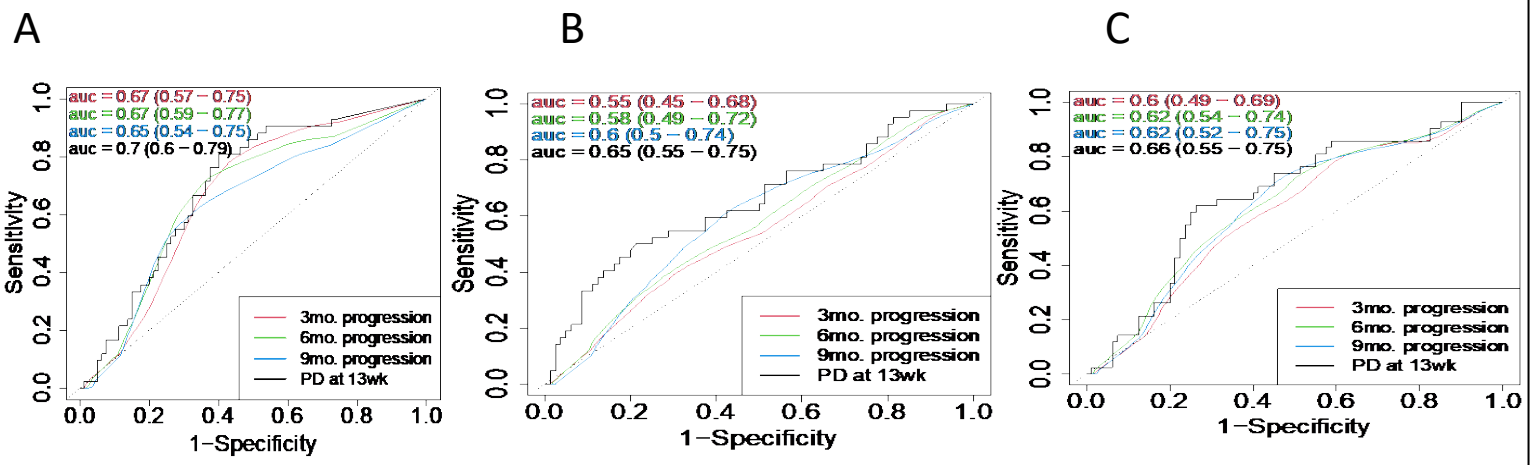
Supplementary Figure S3 displays baseline CM level by disease status at first restaging.

Supplementary Figure S4



Supplementary Figure S4 displays time updates ROC curves for week 4 CM level using two additional methods (A) binary thresholding and (B) random forest.

Supplementary Figure S5



Supplementary Figure S5 displays time updated ROC curves for (A) CM level at week 8, (B) Change in CM level (Week8 – Week4) and (C) the Combination of week 8 CM level and Change in CM level (Week 8-Week 4) using linear discriminant analysis. ROC, receiver operating characteristic; CM, cumulative methylation; auc, area under the ROC curve; PD, progressive disease; mo., months; wk, week.

Impact. The results of this real-world clinical study support the continued development and validation of this new DNA methylation LBx-BCM prototype assay in women with MBC, as a tool to predict early disease progression within one month of initiating therapy. This is an unmet need given the increasing armamentarium of drugs with similar response rates available for use in patients with MBC. This assay could enable medical oncologists to change less effective therapies early after the initiation of treatment and thereby minimize unnecessary toxicity for patients. Using a novel DNA methylation assay that could be used in the clinical setting, we confirmed our 2017 findings that higher week 4 CM levels are associated with worse disease outcomes in women with MBC. We identified that both week 4 CM levels and change in CM from baseline to week 4 are independent determinants of disease progression. CM continued to be an independent predictor of progressive disease after adjustment for CTC, CEA and CA27-29, which are frequently measured molecular markers of disease status in women with MBC. A new risk model with good discriminatory power was also developed to predict disease progression as early as 4 weeks after initiating a new treatment.

There is a paucity of data on the dynamics of circulating methylation levels over time in cancer patients. The results of our study suggest that in most MBC patients, methylation decreases immediately after treatment is initiated which is likely due to an initial response to treatment. Methylation levels then either remain at a new baseline or increase. More frequent measurements could inform whether there is an optimal time to measure CM over a 6-week period after initiating treatment. The methylated marker panel used in this study includes nine of the ten genes evaluated in the original TBCRC 005 study and a new gene *ZNF671* (associated with ER-negative breast cancer). In addition, actin was added as a reference control in each of the two cartridges for a total of 4-5 genes of interest plus one reference gene per cartridge. In developing our panel, we sought markers that were robustly and consistently methylated in breast tumors, in order to detect tumor-derived DNA in as many women as possible, and our findings here suggest that this is true in practice as well. We believe that our markers are largely interchangeable indicators of the presence of tumor, rather than specialized indicators of the state of the tumor, so that differences in CM reflect differences in overall tumor burden.

To our knowledge, this is the first methylation-based prediction model focused on early disease progression in patients with MBC. The risk model was robust when tested under many different statistical assumptions. Alternative models from binary threshold to random forest did not perform better than using CM values based on week 4 or change in CM. The discriminatory performance of our prediction model was well within the range of breast cancer risk models being used in clinical practice that report AUCs ranging from 0.53-0.66. The model prediction was also in line with a recent model developed to predict survival outcomes in women with early-stage triple negative breast cancer[20] reporting an AUC of 0.59-0.61.

The strengths of our study include the multicenter prospective design, the repeated samples, the careful selection of genes and the automated assay and the ability to adjust for other tumor molecular markers. Limitations of the study include the lack of central adjudication for outcomes at first restaging, the lack of repeated measures before week 4, and a large enough sample size to evaluate significant differences for specific treatment used in various breast cancer phenotypes.

In conclusion, the automated easy to use LBx-BCM prototype assay, a novel DNA methylation test was able to successfully identify early disease progression after initiation of therapy, in women with MBC. Further development of the LBX-BCM assay will include evaluation of weekly CM level after treatment initiation in women with metastatic disease to identify the optimum time to measure CM and subsequent validation and refinement of our model in similar patient populations as well as in early stage disease.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Our technician, 3 postdoctoral fellows and a visiting scientist (Associate Prof.) have been trained by Mary Jo Fackler, Senior Research Associate, very painstakingly on these methods in the lab and have been mentored by Dr. Sukumar with weekly meetings and teleconferences with our corporate partner, Cepheid.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Posters were presented at SABCS, 2020, 2022 and at the Breast Cancer Retreat in 2019.
Dr. Sukumar spoke at the International Pathology conference at Madagascar in 2018 and CHBAH, Johannesburg, South Africa in 2019, 2022, and in Hyderabad India in 2019, in EDRN conference, Bethesda MD in 2019.

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Sara Sukumar spoke on this subject at the "A woman's journey" an open forum for women to understand all issues pertaining to women's health

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

All the stated goals were completed. Sara Sukumar was invited to apply for an extension grant by DOD. She has done so, and is awaiting the results.

Our next steps will extend the TBCRC005 studies to a large scale independent clinical validation study. We will collaborate with Cepheid and the Oregon Health and Science University (OHSU) on SMMART (Serial Measurements of Molecular and Architectural Responses to Therapy) program at OHSU. The goal of the SMMART program is to develop new treatments for cancer for patients with advanced disease. Our objectives will include an independent validation study of BCM in stage IV breast cancer and monitoring response to therapy in longitudinal plasma samples from patients with metastatic breast cancer. Successful outcome potentially will lead to product commercialization.

IMPACT: D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report.

There has been a great interest from commercial enterprises like TakaRa, Quest Diagnostics and similar organizations to collaborate to pursue more liquid biopsy methods. Discussions are in progress.

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The goal of this project was to develop an automated cartridge that measures cell free DNA in plasma or serum of metastatic breast cancer patients undergoing therapy. In our previous clinical study, we used our lab developed method called cMethDNA, which had many innovations. The two principal strengths are that it is a nested PCR reaction, thereby enhancing signals from very low copy numbers of methylated DNA shed by the tumors and floating in the blood. Second was that signals from the blood were compared to a finite amount of methylated DNA that we spiked into the plasma or serum before extracting DNA. This allowed us to compare the floating DNA in the blood of patients to more realistic "molehill" controls rather than to large quantities or "mountains" of unmethylated DNA from dead cells that is dumped into the blood by all normal organs. However, the method requires expert technical prowess, and can take up to one to two weeks to complete. We have now partnered with Cepheid, a diagnostics company, to automate this "lab in a cartridge"- based method for a 5 hr assay, not days or weeks, with need for minimal technical expertise. During the project period we have tested our gene panel one by one in the cartridge, and in combinations, to optimize the performance of the panel. We developed a system of reporting cumulative methylation with Cepheid cartridge multiplex PCR real-time method. We have evaluated Training, Test, pilot Monitoring, and clinical TBCRC005 Sample sets, and plan for a major collaborative effort and an outside institution to utilize BCM in the SMMART program at OHSU. It is our goal that these steps will lead to commercial development.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

We have now determined that the same set of genes, with minor changes in the panel can detect colon cancer (n=180) cervical cancer (N=300) and lung cancer (n=256) in FFPE We have extended our findings to development of plasma tumor specific methylated marker panels for colon cancer and cervical cancer. Marker panels have been tested successfully in tissue and show high sensitivity and specificity by QM-MSP (tumor tissue) and in preliminary studies of colon cancer sera (ctDNA) compared to normal controls (>90% sensitivity/specificity). This automated test for metastatic breast cancer, once developed, could be examined for utility in other stages of breast cancer. Developing an early detection test will be our final achievement, but we are proceeding step by step: metastatic, Stage 4 to Stage 3 to Stage 2 and Stage 1. Because of the very low amounts of DNA shed by early stage tumors this will require technical improvements.

We have now determined that the same set of genes, with minor changes in the panel can detect colon cancer (n=180) cervical cancer (N=300) and lung cancer (n=256) in FFPE sections of tumors with higher than 98 percent sensitivity/specificity. Devising a cartridge that can detect these genes in PAP smears or blood would be a great step forward. Parallel work on these cancer types which will be extended to prostate cancer, is going on in the lab. Each of these areas are of interest to DOD Research programs.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

What was the impact on society beyond science and technology?

The cMethDNA patent, which forms the basis of this cartridge assay has been filed and has been granted. The work, which is an academic-industrial collaboration with Cepheid has led to great interest in other companies as well who are interested in liquid biopsy.

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*

- *improving social, economic, civic, or environmental conditions.*

It is our goal that the steps outlined in #4 above will lead to Cepheid commercialization of the BCM cartridge method. Implementation in clinical labs, including those in less resourced settings, should expedite diagnosis of advanced breast cancer, and provide rapid feedback for response to treatment.

1. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to

All specific aims have been completed. All the goals have been met. Two papers are now published

resolve them.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None. Despite COVID challenges, work continued at a near normal pace and was completed with minimal delay

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

None

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

1. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

The prototype BCM cartridge was developed. This will undoubtedly undergo further iterations over time.

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. **Fackler MJ, Tulac S,** Venkatesan N, Aslam AJ, de Guzman TN, Mercado-Rodriguez C, Cope LM, Downs BM, Vali AH, Ding W, Lehman J, Denbow R, Reynolds J, Buckley ME, Visvanathan K, Umbricht CB, Wolff AC, Stearns V, Bates M, Lai EW, Sukumar S. Development of an automated liquid biopsy assay for methylated markers in advanced breast cancer. Cancer Res Commun. 2022 Jun;2(6):391-401. doi: 10.1158/2767-9764.crc-22-0133. Epub 2022 Jun 1. PMID: 36046124

   Acknowledgement: This work was supported by DOD grant (W81XWH-18–1-0018) and a Cepheid research agreement (#90066820, to S. Sukumar). Sample collections in J0425, IMAGE II, J0888 were supported by Avon/NCI Partnership for Progress Grant (3P40 CA006973–41S), and Susan G. Komen (BCTR0504444, to A.C. Wolff)

   Visvanathan, K, Cope, L, Fackler MJ, Considine M, Sokoll L,Carey LA, Forero-Torres A, Ingle JN, Lin NU, Nanda R,, Storniolo AM, Tulac S, Venkatesan N, Wu NC, Marla S, Campbell S, Bates M, Umbricht CB, Antonio Wolff AC, and Sukumar, S. Evaluation of a Liquid Biopsy-Breast Cancer Methylation (LBx-BCM) Cartridge Assay for Predicting Early Disease Progression and Survival: TBCRC 005 Prospective Trial. Clin Cancer Res. 2022 Dec 19; doi: 10.1158/1078-0432.
2. Acknowledgement: This work was supported by a DOD grant (W81XWH-18–1-0018) and a Cepheid research agreement (#90066820) to S. Sukumar as well as the funding support to the TBCRC from The Breast Cancer Research Foundation and Susan G. Komen. CellSearch reagents were provided by Veridex, LLC.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Conference proceedings published the abstracts online

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Abstracts presented at two San Antonio Breast Cancer Symposium, 2020, 2022

Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None

Technologies or techniques

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Cepheid prototype BCM cartridge

Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

This work capitalized on existed JHU patented technologies for cMethDNA and QM-MSP methylation specific PCR licensed by Cepheid. However, two patents are pending (US 20220226809 A1 and US 20220364177 A1) for use of the markers in the automated cartridge

Other Products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

None

2.PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Saraswati Sukumar, PhD Project Role: PI
Researcher Identifier (e.g. ORCID ID): is https://orcid.org/0000-0002-5656-6703
Nearest person month worked: 4
Contribution to Project: Dr. Sukumar has spent 25%; 2 hours daily on examining progress, analyzing raw data, and planning and execution of the following day's work, and making arrangements for procuring reagents needed for the study, and writing, submitting and answering rebuttals from the JH IRB.

Name: Jeffrey Reynolds
Project Role: Research Coordinator Researcher Identifier (e.g. ORCID ID): Nearest person month worked: 3
Contribution to Project: Mr. Reynolds has spent 10% on writing the protocols and writing, submitting and answering rebuttals from the JH IRB.

Name: Mary Jo Fackler, Ph. D
Project Role: Senior Research Associate
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-7249-3807
Nearest person month worked: 10
Contribution to Project: Dr. Fackler has devoted 80 percent of her time to this project. She has devoted her time to redesigning oligos for specific genes, designing experiments, conducting the work and analyzing data.

Left for residency in late 2019 Name: Abdul Hussain Vali, MD Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked:
12
Contribution to Project: Dr. Vali has devoted 100 percent of his time to this project. He has devoted his time to getting training, and designing experiments, conducting the work and analyzing data.

Wanjun Ding, MD Project Role:
Visiting Scientist Researcher
Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Dr. Ding has devoted 25% percent of his time to this project. He has devoted his time to getting training, and recently designing experiments, conducting the work and analyzing data.
During this 2nd NCE period, 2/15/2022-12/14/2022 support was as follows;
Dr. Sukumar : 1%
Mary Fackler : ~8.5%
Bradley Downs : 30%

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

o *Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

Cartridges and reagents are supplied by a team at Cepheid after performing quality control. Cepheid personnel worked with us to optimize the cartridge. Two weekly teleconferences allow sharing of data, and implementation of changes when needed.

o *Provide the following information for each partnership:*

o *<u>Organization Name</u>*: **Cepheid**

o *<u>Location of Organization: (if foreign location list country) Partner's contribution to the project</u> (identify one or more)*

- *Financial support;Yes*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff)*
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- **Yes, cartridges and reagents**

- *Facilities (e.g., project staff use the partner's facilities for project activities) ;***No**
- *Collaboration (e.g., partner's staff work with project staff on the project);* **Yes, staff at Cepheid worked on optimizing reagents**
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)**;*** **No**
- *Other.*

2. **SPECIAL REPORTING REQUIREMENTS**
3. **COLLABORATIVE AWARDS:** Not applicable

QUAD CHARTS: Not applicable

9. Publications:

1. San Antonio Breast Cancer Symposium, Poster #PS4-03, Abstract #1361, December 8-11,202

2. Cancer Res Commun. 2022 Jun;2(6):391-401. (Appended)

Development of an automated liquid biopsy assay for methylated markers in advanced breast cancer

Mary Jo Fackler [1], Suzana Tulac [2], Neesha Venkatesan [2], Adam J Aslam [2], Timothy N de Guzman [2], Claudia Mercado-Rodriguez [1], Leslie M Cope [1], Bradley M Downs [1], Abdul Hussain Vali [1], Wanjun Ding [3 1], Jennifer Lehman [1], Rita Denbow [1], Jeffrey Reynolds [1], Morgan E Buckley [1], Kala Visvanathan [1 4], Christopher B Umbricht [5], Antonio C Wolff [1], Vered Stearns [1], Michael Bates [2], Edwin W Lai [2], Saraswati Sukumar [1]

PMID: 36046124 PMCID: PMC9426415 DOI: 10.1158/2767-9764.crc-22-0133

Abstract

Current molecular liquid biopsy assays to detect recurrence or monitor response to treatment require sophisticated technology, highly trained personnel, and a turnaround time of weeks. We describe the development and technical validation of an automated Liquid Biopsy for Breast Cancer Methylation (LBx-BCM) prototype, a DNA methylation detection cartridge assay that is simple to perform and quantitatively detects nine methylated markers within 4.5 h. LBx-BCM demonstrated high interassay reproducibility when analyzing exogenous methylated DNA (75-300 DNA copies) spiked into plasma (Coefficient of Variation, CV = 7.1 - 10.9%) and serum (CV = 19.1 - 36.1%). It also demonstrated high interuser reproducibility (Spearman $r = 0.887$, $P < 0.0001$) when samples of metastatic breast cancer (MBC, $N = 11$) and normal control ($N = 4$) were evaluated independently by two users. Analyses of interplatform reproducibility indicated very high concordance between LBx-BCM and the reference assay, cMethDNA, among 66

paired plasma samples (MBC $N = 40$, controls $N = 26$; Spearman $r = 0.891$; 95% CI = 0.825 - 0.933, $P < 0.0001$). LBx-BCM achieved a ROC AUC = 0.909 (95% CI = 0.836 - 0.982), 83% sensitivity and 92% specificity; cMethDNA achieved a ROC AUC = 0.896 (95% CI = 0.817 - 0.974), 83% sensitivity and 92% specificity in test set samples. The automated LBx-BCM cartridge prototype is fast, with performance levels equivalent to the highly sensitive, manual cMethDNA method. Future prospective clinical studies will evaluate LBx-BCM detection sensitivity and its ability to monitor therapeutic response during treatment for advanced breast cancer.

2.

3. **Clin Cancer Res. 2022 Dec 19;CCR-22-2128. doi: 10.1158/1078-0432.CCR-22-2128.(Appended)**

Evaluation of a Liquid Biopsy-Breast Cancer Methylation (LBx-BCM) Cartridge Assay for Predicting Early Disease Progression and Survival: TBCRC 005 Prospective Trial.

Kala Visvanathan*#, Leslie Cope*#, Mary Jo Fackler*, Michael Considine, Lori Sokoll, Lisa A. Carey, Andres Forero-Torres, James N. Ingle, Nancy U. Lin, Rita Nanda, Anna Maria Storniolo, Suzana Tulac, Neesha Venkatesan, Natalie C. Wu, Sudhakar Marla, Scott Campbell, Michael Bates, Christopher B Umbricht, Antonio
C. Wolff, and Saraswati Sukumar#.

Abstract

Purpose: We previously demonstrated that high levels of circulating methylated DNA are associated with subsequent disease progression in women with metastatic breast cancer (MBC). In this study, we evaluated
the clinical utility of a novel Liquid Biopsy-Breast Cancer Methylation (LBx-BCM) prototype assay using the GeneXpert® cartridge system for early assessment of disease progression in MBC.

Experimental Design: The 9-marker, LBx-BCM prototype assay was evaluated in TBCRC-005, a prospective biomarker study, using plasma collected at baseline, week 4 and week 8 from 144 MBC patients.

Results: At week 4 MBC patients with high cumulative methylation (CM) had a significantly shorter median PFS (2.88 months v 6.60 months, p = 0.001) and OS (14.52 months v 22.44 months, p=0.005) compared to those
with low CM. In a multivariable model, high versus low CM was also associated with shorter PFS (HR = 1.90, 95%CI 1.20-3.01; p =0.006). Change in CM from baseline to week 4 (OR = 4.60, 95%CI 1.77, 11.93; p = 0.002) and high levels of CM at week 4 (OR = 2.78, 95%CI 1.29, 5.99; p = 0.009) were associated with progressive disease at the time of first restaging. A robust risk model based on week 4 circulating CM levels was developed to predict disease progression as early as 3 months after initiating a new treatment.

Conclusions: The automated LBx-BCM prototype assay is a promising clinical tool for detecting disease progression a month after initiating treatment in women with MBC undergoing routine care. The next step is to validate its clinical utility for specific treatments.

4. Submitted poster for San Antonio Breast Cancer, Dec 2022.