

Circulating Tumor DNA Profiling From Breast Cancer Screening Through to Metastatic Disease

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PURPOSE We investigated the utility of the Oncomine Breast cfDNA Assay for detecting circulating tumor DNA (ctDNA) in women from a breast screening population, including healthy women with no abnormality detected by mammogram, and women on follow-up through to advanced breast cancer.

MATERIALS AND METHODS Blood samples were taken from 373 women (127 healthy controls recruited through breast screening, 28 ductal carcinoma in situ, 60 primary breast cancers, 47 primary breast cancer on follow-up, and 111 metastatic breast cancers [MBC]) to recover plasma and germline DNA for analysis with the Oncomine Breast cfDNA Assay on the Ion S5 platform.

RESULTS One hundred sixteen of 373 plasma samples had one or more somatic variants detected across eight of the 10 genes and were called ctDNA-positive; MBC had the highest proportion of ctDNA-positive samples (61; 55%) and healthy controls the lowest (20; 15.7%). *ESR1*, *TP53*, and *PIK3CA* mutations account for 93% of all variants detected and predict poor overall survival in MBC (hazard ratio = 3.461; 95% CI, 1.866 to 6.42; *P* = .001). Patients with MBC had higher plasma cell-free DNA levels, higher variant allele frequencies, and more polyclonal variants, notably in *ESR1* than in all other groups. Only 15 individuals had evidence of potential clonal hematopoiesis of indeterminate potential mutations.

CONCLUSION We were able to detect ctDNA across the breast cancer spectrum, notably in MBC where variants in *ESR1*, *TP53*, and *PIK3CA* predicted poor overall survival. The assay could be used to monitor emergence of resistance mutations such as in *ESR1* that herald resistance to aromatase inhibitors to tailor adjuvant therapies. However, we suggest caution is needed when interpreting results from a single plasma sample as variants were also detected in a small proportion of HCs.

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INTRODUCTION

Breast cancer is the most common cancer in women worldwide, with more than 600,000 people dying annually largely because of metastatic recurrence occurring more than 5 years after diagnosis.¹ Up to 30% of patients will relapse, and the risk of developing distant metastases potentially spans decades.² Current imaging methods such as mammography and positron emission tomography-computed tomography scans have limited sensitivity and accuracy,³ so following a standard regime of adjuvant treatment, further screening to enable detection of micrometastases could significantly reduce the risk of recurrence and may be clinically beneficial.

Recent studies have shown the utility of circulating tumor DNA (ctDNA)-based blood tests for detecting minimal residual disease and predicting relapse.⁴⁻⁶ This ctDNA, a minor fraction of the total circulating cell-free DNA (cfDNA; < 0.1%-10%),⁷ reflects the mutational signature

of the primary tumor and heterogeneity of metastatic lesions. We recently used patient-specific ctDNA analysis to detect preclinical metastases across breast cancer subtypes up to two years ahead of imaging, providing a possible window for therapeutic intervention.⁸

In patients with estrogen receptor (ER)-positive breast cancer, following prolonged exposure to adjuvant endocrine therapy, resistance mutations can emerge on metastatic progression.⁹ This is particularly true for aromatase inhibitors, given as first-line treatment in postmenopausal women with ER-positive breast cancer¹⁰ where around 30% of these cancers acquire activating *ESR1* gene mutations that confer resistance to treatment.¹¹ With endocrine therapy considered as effective as chemotherapy,¹² it is a preferred treatment by many women, given the lower incidence of adverse side effects. It is therefore important to develop reliable and sensitive methods to detect these acquired mutations enabling a switch in treatment at an earlier stage.

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

To evaluate the prevalence of circulating tumor DNA (ctDNA) in blood samples from women with early- and late-stage breast cancer, using the commercially available Oncomine Breast cfDNA Assay and explore, to our knowledge, for the first time the spectrum of mutations detected in healthy women with no evidence of any breast lesion by mammogram.

Knowledge Generated

Mutations in *ESR1*, *PIK3CA*, and *TP53* genes accounted for 93% of all observed variants detected across 10 breast cancer driver genes. The frequency of ctDNA-positive samples was significantly higher in patients with metastatic disease compared with healthy controls, with overall survival significantly worse in patients with detectable ctDNA.

Relevance

The assay accurately detects key breast cancer mutations of clinical importance such as in *ESR1* and *PIK3CA*, and could be used to stratify use of targeted therapies and monitor emergence of resistance mutations such as in *ESR1* to tailor adjuvant therapy.

There are multiple commercially available mutation panels for ctDNA profiling; however, there is a lack of standardization and no agreement on a defined methodology.¹³ Importantly, raw data arising from ctDNA deep sequencing studies can include sequencing artifacts, germline variants, and occasional variants derived from the expansion of clonal populations of blood cells, known as clonal hematopoiesis of indeterminate potential (CHIP).¹⁴ As cfDNA is derived from blood, potential CHIP mutations should be evaluated and excluded by comparison with matched germline DNA (gDNA).¹⁵

Here, we report the interim analysis of the first 187 samples from the Breast Screening and Monitoring Study (BSMS, IRAS 118701) and compare results with a symptomatic breast cancer cohort. The BSMS study is looking at samples from women who have been recalled after a screening mammogram, to see whether blood tests could be used to help screen for and monitor breast cancer. We tested a single blood sample from 373 women, including 127 healthy controls (HC) recruited from BSMS and 218 women with different stages of breast cancer with the Oncomine cfDNA Breast Assay. The assay enables detection of single nucleotide variants (SNVs) or insertions or deletions (INDELs) across 10 breast cancer driver genes to a variant allele frequency (VAF) of 0.1% with approximately 81% sensitivity and 99.9% specificity.¹⁶ We investigate the prevalence of detectable ctDNA across the disease course, define the most common mutations at each stage of BC, and explore, to our knowledge, for the first time the spectrum of mutations detected in healthy women who had no evidence of any breast lesion by mammogram. We demonstrate that the prevalence of *ESR1* and *PIK3CA* mutations increases as the disease progresses, and mutations in *ESR1*, *TP53*, and *PIK3CA* predict poor overall survival (OS) in metastatic breast cancer (MBC) and finally identify a low proportion (12.4%) of CHIP-derived mutations present.

MATERIALS AND METHODS

Patients and Samples

The study protocols were approved by the Riverside Research Ethics Committee (Imperial College Healthcare NHS Trust; Tissue Bank Ethics or REC reference numbers: 12/LO/2019; 13/LO/1152; R10015-16A; 07/Q0401/20), Leeds (East) MultiResearch Ethics Committee (MREC 07/H1306/164), and East Midlands Local Research Ethics Committee (REC: 13/EM/0196), conducted in accordance with Good Clinical Practice Guidelines and the Declaration of Helsinki. All 373 participants provided written informed consent before participation and were older than age 18 years. Study participants included women from a breast screening cohort and a symptomatic cohort (Fig 1, Data Supplement). Twenty milliliter blood was collected to plasma as described previously.¹⁷

Extraction and Quantitation of DNA

Total cfDNA was isolated from 4 mL of plasma with the MagMAX Cell-free DNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instructions, and gDNA was isolated from white blood cells as described previously.¹⁸ Quantitation and integrity checks were performed using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) and Agilent TapeStation HS D5000 (Agilent) according to manufacturer's instructions.

Targeted Next-Generation Sequencing

Library reactions were set up on the Ion Chef system according to the manufacturer's protocol, using the Oncomine Breast cfDNA v1 Assay and run on either a 530 or 540 chip on the Ion S5 XL sequencing platform (all Thermo Fisher Scientific). Alignment of sequencing raw data was performed by the Torrent Suite Software version v 5.12 (Thermo Fisher Scientific). All high-confidence variant calls (those with an allele molecular coverage of ≥ 2 and Allele Mol Freq [MAF %] \geq the limit of detection for each

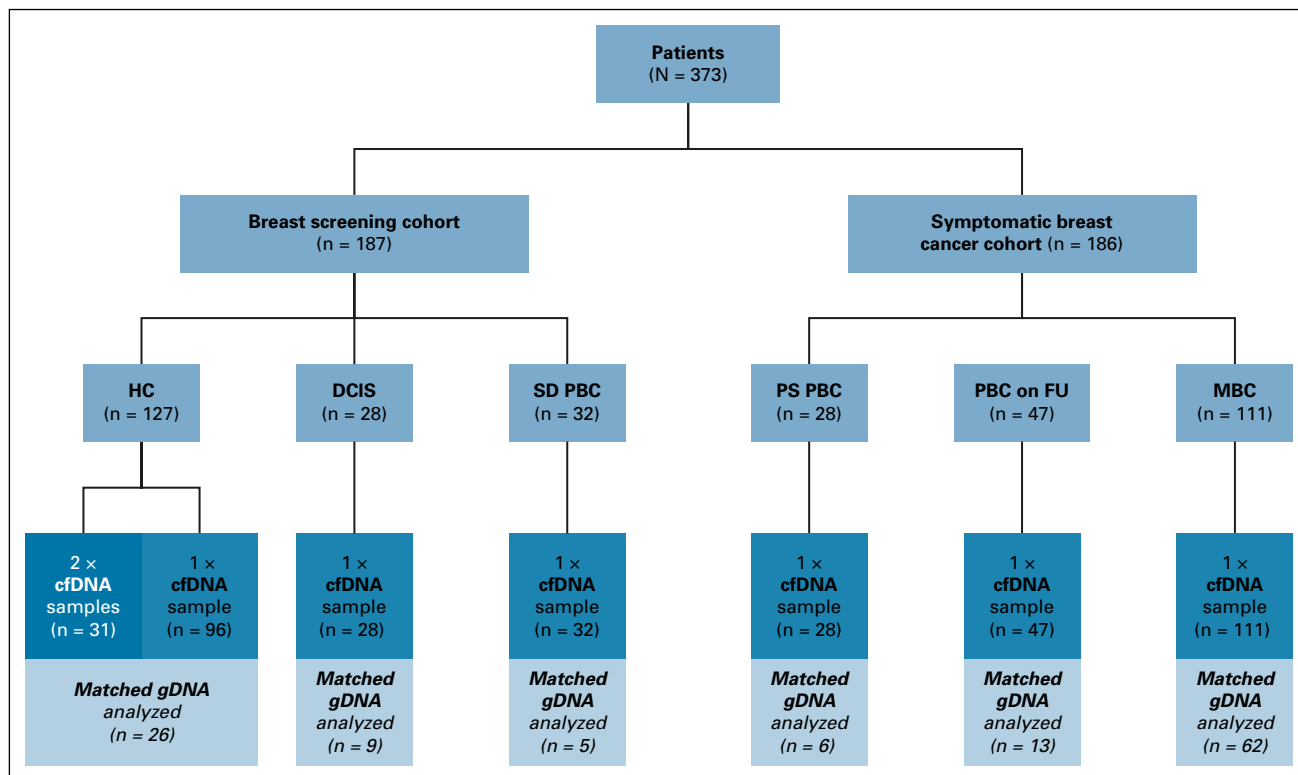


FIG 1. Study summary with participant groups and samples included for analysis. CONSORT diagram summarizing individuals and groups enrolled in the study, with the total number of plasma cfDNA and gDNA samples analyzed with the OncoPrint Breast cfDNA Assay. Participants were from a screening cohort who were recalled because of a suspicious mammogram including HC, patients with screen detected DCIS, and patients with SD-PBC and symptomatic primary breast cancer patients who had a blood sample taken before surgery (PS-PBC), patients with primary breast cancer on follow-up, and patients with radiologically confirmed MBC. cfDNA, cell-free DNA; DCIS, ductal carcinoma in situ; gDNA, germline DNA; HC, healthy controls; MBC, metastatic breast cancer; PBC, primary breast cancer; PBC on FU, primary breast carcinoma on follow-up; PS PBC, presurgical primary breast carcinoma; SD-PBC, screen-detected primary breast cancer.

variant) were reviewed manually using the Integrated Genomics Viewer package (v2.3.25) by two observers.¹⁹

Statistical Analysis

Mann-Whitney, Wilcoxon, and Kruskal-Wallis nonparametric tests were used to examine differences between patients regarding presence of gene mutations, and VAF of specific genes. One-way analysis of variance tests were followed by Dunn's multiple comparison test. Spearman's rank correlation coefficient was used to investigate the correlation between cfDNA quantity and the mutational VAF. These analyses were carried out using GraphPad Prism v7 software. All *P* values were two-sided and those < .05 were considered statistically significant.

Kaplan-Meier estimator and Cox regression models were used to assess OS. Survival model was constructed using the counting process notation (start, end, event)^{20,21} where the date of blood collection was taken as the start and the date of death was considered the end, with an agreed administrative censoring date of November 30, 2020. Survival curves were compared using the log-rank test. Cox proportional-hazards regression analysis was used to

estimate hazard ratios for OS. Analyses were performed using R version 4.0.1, using survival (version 3.2-7) and survminer (version 0.4.8) packages.

RESULTS

Plasma cfDNA Levels Increase From Primary to Metastatic Disease

Here, we report the analysis of plasma cfDNA in 373 women. We performed an interim analysis in the BSMS of the first 187 women who were recalled because of an unsatisfactory mammogram, comprising 127 HC, 28 patients with ductal carcinoma in situ (DCIS), and 32 with screen-detected primary breast cancer (SD-PBC; Table 1). We compared result with 186 patients with symptomatic breast cancer including 28 unselected PBC who had a blood sample taken before surgery (PS-PBC), 47 PBC on follow-up who were receiving adjuvant therapy and were free of recurrent disease at the time of blood sampling (median follow-up time after blood sampling of 63 months [range 49-80 months]), and 111 patients with radiologically confirmed MBC (Fig 1; Data Supplement). The median age was 56 years (range of 34-91 years). One hundred ninety

TABLE 1. Prevalence of SNVs Detected in the Natural History of Breast Cancer

Cohort	No.	No SNVs Detected, No. (%)	1 SNV, No. (%)	≥ 2 SNVs, No. (%)
HC	127	107 (84)	18 (14)	2 (2)
DCIS	28	17 (61)	10 (35.5)	1 (3.5)
SD PBC	32	25 (78)	4 (12.5)	3 (9.5)
PS PBC	28	22 (79)	6 (21)	0 (0)
PBC on FU	47	36 (77)	10 (21)	1 (2)
MBC	111	50 (45)	35 (32)	26 (23)
Total	373	257 (69)	93 (25)	35 (9)

NOTE. Number of ctDNA-positive and -negative samples, illustrated by either none, one, or two or more SNVs.

Abbreviations: ctDNA, circulating tumor DNA; DCIS, ductal carcinoma in situ; HC, healthy controls; MBC, metastatic breast cancer; SNV, single nucleotide variant; PBC, primary breast cancer.

eight of the 218 (91%) patients with breast cancer were ER-positive, 36 (16.5%) were human epidermal growth factor receptor 2 (HER2)-positive, and 10 (4.5%) had triple-negative breast cancer (TNBC). An additional blood sample was collected from 31 of the HC to compare plasma results over time; the median time elapsed between these paired samples was 27 months (range 14-63 months; Data Supplement).

Sixty-six of the 127 HC (52%) had no abnormality detected on their repeat mammogram and 61 (48%) had a core biopsy where the benign findings were predominantly of a nonproliferative nature (Data Supplement). The median plasma cfDNA concentration in HC was 9 ng/mL plasma (range 1.2-41 ng/mL) compared to 32.4 ng/mL (range 2.83-6820 ng/mL) in patients with MBC (Fig 2, Data Supplement). The HC, DCIS, SD PBC, and PS PBC groups all had cfDNA levels that were significantly lower than in MBC; the SD PBC group also had significantly lower levels than PBC FU (all adjusted *P* values < .001, Dunn's multiple comparison test, Data Supplement).

Mutation Profiling Detects ctDNA Through the Natural History of Breast Cancer

Using a semiautomated standardized workflow and working to good clinical laboratory practice, all 404 plasma cfDNA samples and 121 gDNA samples were sequenced successfully and passed QC metrics (Data Supplement). Applying stringent thresholds, 128 of 373 (34%) first plasma cfDNA samples had one or more single nucleotide variants (SNVs) detected, and no INDELs were identified. Twelve individuals had identical variants detected in gDNA, potentially because of CHIP, and were subsequently removed from analysis. The remaining 116 plasma samples had a total of 197 SNVs detected and were classed as ctDNA-positive (Fig 3A, Data Supplement). The median VAF was 0.33% (range 0.06%-52.9%), and 140 (71%) and 86 (44%) SNVs were detected at a VAF of ≤ 1% and ≤ 0.25%, respectively (Fig 3B). Variants were detected

in eight of 10 genes, notably *TP53* (31.5%), *ESR1* (30.5%), and *PIK3CA* (29.3%). No mutations were found in *EGFR* and *FBXW7*. The median VAF was *TP53* (0.12%; range 0.06%-22.03%), *ESR1* (0.62%; 0.06%-30.16%), and *PIK3CA* (0.41%; 0.06%-33.11%).

The Number of SNVs and VAF Increase From Primary to Metastatic Disease

The frequency of ctDNA-positive samples increased from 15.7% in HC to 55% in MBC (*P* = < .001, Mann-Whitney U test; Fig 3A). Within the MBC cohort, there were 78 ER-positive and HER2-neg, 17 HER2 3+, and eight TNBC patients. Five of 28 HC with fibroadenomas and three of 22 HC with

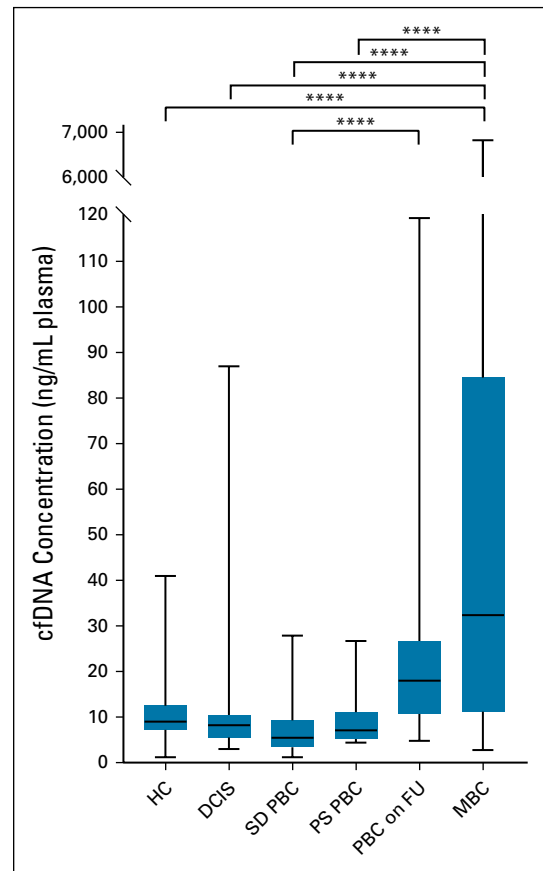


FIG 2. Plasma cfDNA concentration by cohort. Box and whisker plot showing plasma cfDNA concentration (ng/mL). The range, 25th and 75th percentiles, and median for each cohort (total, N = 373) are shown. HC (n = 127); DCIS (n = 28); SD PBC (n = 32); PS PBC (n = 28); PBC on FU (n = 47); MBC (n = 111). Adjusted *P* values were calculated by one-way ANOVA and Dunn's multiple comparison test. *P* values < .0001 are shown with ****; other *P* values are given in the Data Supplement. ANOVA, analysis of variance; cfDNA, cell-free DNA; DCIS, ductal carcinoma in situ; HC, healthy controls; MBC, metastatic breast cancer; PBC on FU, primary breast carcinoma on follow-up; PS PBC, presurgical primary breast carcinoma; SD PBC, screen-detected primary breast carcinoma.

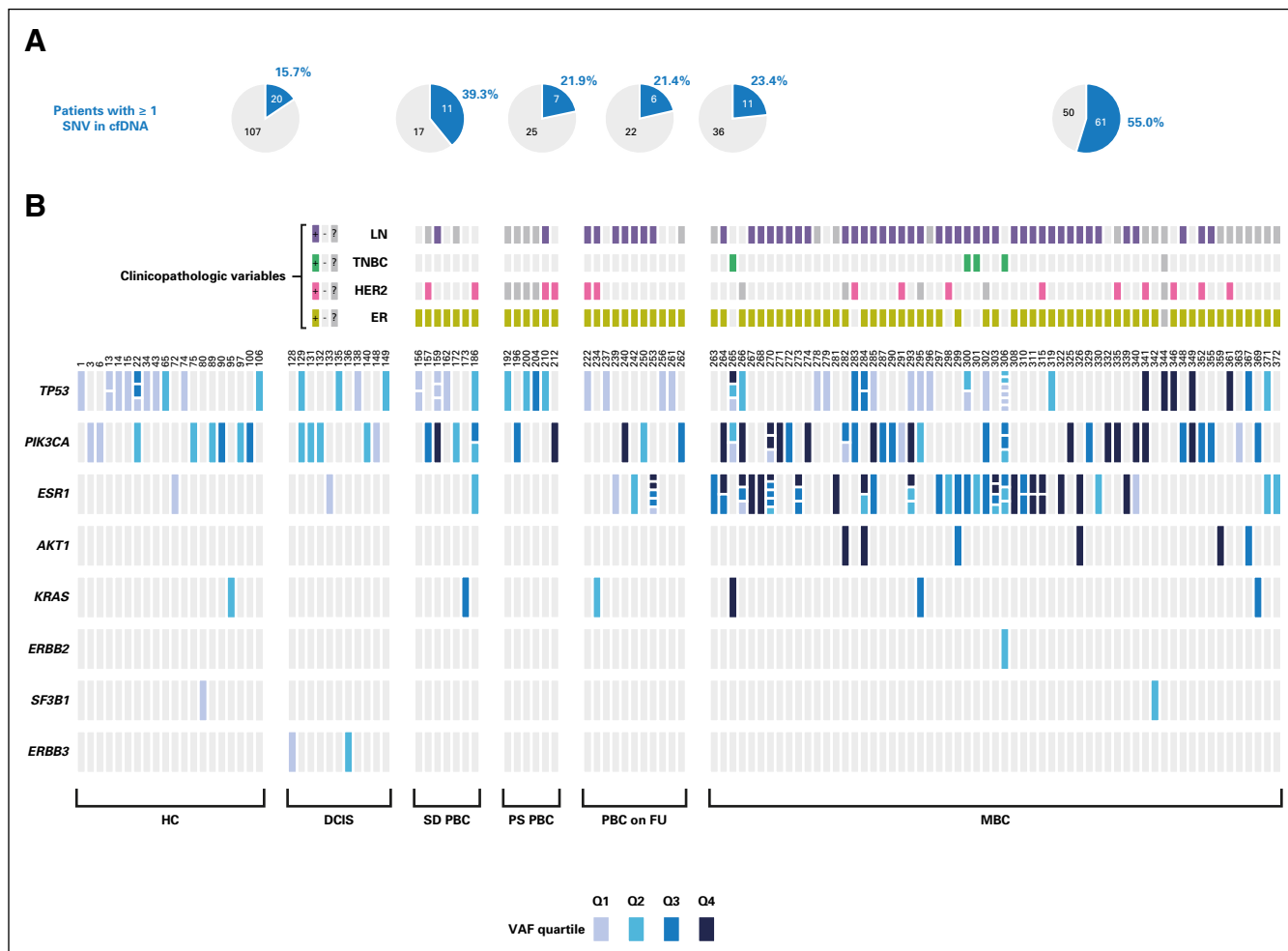


FIG 3. Summary of variants detected in plasma with the OncoPrint Breast cfDNA Assay. (A) Pie charts showing the number of ctDNA-positive (blue) and -negative (gray) participants by cohort. (B) OncoPrint showing all ctDNA-positive samples ($n = 116$) based on detection of ≥ 1 SNV; blue shading indicates the VAF range of each respective SNV. SNVs were ordered according to ascending VAF and placed into uneven quartiles (Q1, $n = 49$, VAF range 0.06%-0.135%; Q2, $n = 49$, VAF range 0.142%-0.325%; Q3, $n = 50$, VAF range 0.33%-1.409%; and Q4, $n = 49$, VAF range 1.44%-52.9%) to avoid multiple variants detected at the same VAF being placed into different quartiles. In those samples where multiple SNVs were detected within the same gene, the boxes were segmented accordingly. Clinicopathologic characteristics including hormone receptor status, HER2 status, TNBC status, and LN status are indicated. ctDNA, circulating tumor DNA; DCIS, ductal carcinoma in situ; ER, estrogen receptor; HC, healthy controls; HER2, human epidermal growth factor receptor 2; LN, lymph node; MBC, metastatic breast cancer; PBC on FU, primary breast carcinoma on follow-up; PS PBC, presurgical primary breast carcinoma; Q, quartile; SD PBC, screen-detected primary breast carcinoma; SNV, single nucleotide variant; TNBC, triple-negative breast cancer; VAF, variant allele frequency.

fibrocystic changes were ctDNA-positive, compared with one ctDNA-positive HC with a proliferative lesion (fibrocystic change with papillary apocrine metaplasia). In 31 of the HC, we also sought a second blood sample to compare the variant profile over time. The median time elapsed between the paired samples was 27 months (range 14-63 months). Four first and 2 second samples had one or more SNVs detected; there was no overlap, and all had low VAF ($< 0.5\%$; Data Supplement).

Overall, 85 of the 218 (39%) patients with breast cancer were ctDNA-positive (Data Supplement) comprising 54 of 143 (37.8%) ER-pos. HER2-neg. patients, 13 of 30 (43.3%) ER pos. HER2 3+ patients, two of six ER neg.

HER2 3+ patients, and four of 12 patients with TNBC. Of interest, three of the TNBC had SNVs detected in *ESR1*. A similar proportion of patients with DCIS (11 of 28 [39.3%]) were ctDNA-positive, seven of which were high grade (64%). Excluding DCIS, the number of ctDNA-positive samples was significantly lower in all groups compared with MBC (all $P < .005$; Data Supplement). Considering the 47 PBC FU patients, at the census date of November 30, 2020, two of 11 (18%) ctDNA-positive patients have relapsed, whereas only three of 36 ctDNA-negative patients (8%) have relapsed after a median follow-up of 64 months (range 50-81 months); there was no significant difference in VAF between relapsed and nonrelapsed patients. Finally,

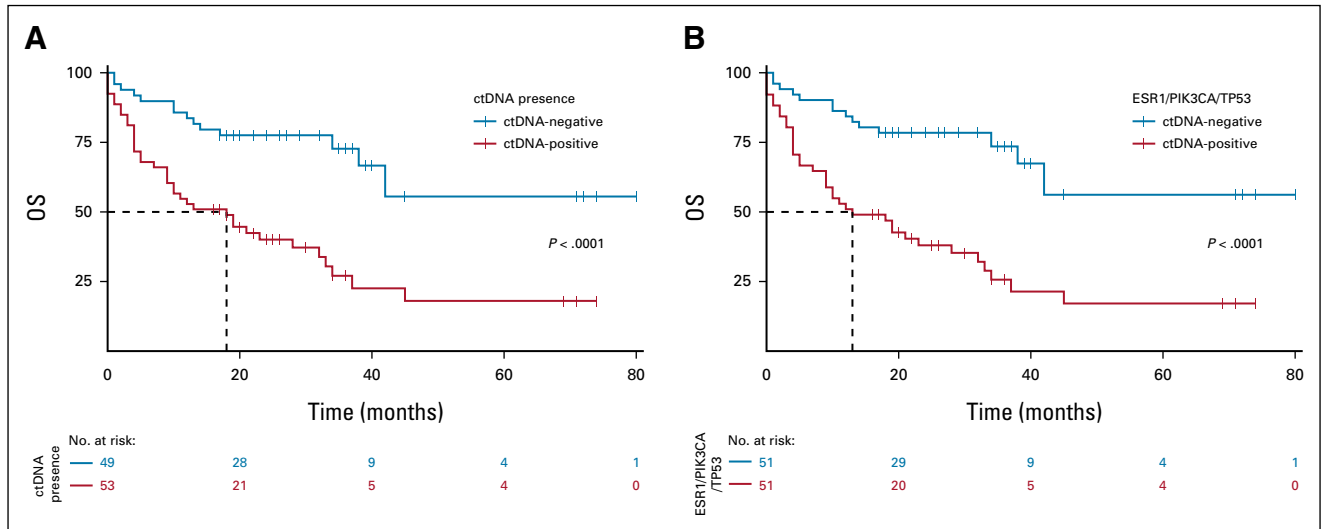


FIG 4. CtDNA predicts poor OS in MBC. OS analysis for MBC to explore the difference in survival outcome for ctDNA-positive patients across all genes or ctDNA-positive only in *ESR1*, *PIK3CA*, and *TP53*. Data for 100 patients with MBC; nine were removed from analysis because of incomplete information (unknown dates or state) and two were excluded with variants in other genes. (A) Median survival for ctDNA-positive patients was 18 months, whereas that for ctDNA-negative was > 80. Log-rank test P value < .001. Cox proportional hazards model analysis (P < .001) showed a hazard ratio value of 3.31 (95% CI, 1.784 to 6.142). (B) Median survival for ctDNA-positive patients (based on variants in *ESR1*, *PIK3CA*, and *TP53*) was 13 months, whereas for ctDNA-negative patients, it is > 80. Log-rank test P value < .001. Cox proportional hazards model analysis (P value < .001) showed a hazard ratio value of 3.461 (95% CI, 1.866 to 6.42). ctDNA, circulating tumor DNA; MBC, metastatic breast cancer; OS, overall survival.

correlation analysis of cfDNA quantity and the VAF showed no significant relationship within any group (Spearman's rank correlation). When applying a higher threshold of two or more SNVs detected to define ctDNA-positive samples,⁸ there was only one ctDNA-positive sample in each of the HC, DCIS, SD-PBC, and PBC FU groups, whereas 25 (2.5%) MBCs were ctDNA-positive.

ESR1, TP53, and PIK3CA Mutations Account for 93% of All Observed Alterations and Predict Poor Overall Survival in MBC

SNVs within *TP53*, *ESR1*, and *PIK3CA* genes accounted for 93.4% of all variants detected. *PIK3CA* and *ESR1* SNVs were also most common in MBC and with high VAFs (Fig 3; Data Supplement). The VAF of the *TP53* variants did not differ significantly between groups; however, the frequency of *PIK3CA* variants was significantly higher in MBC than in HC (P = .0013; exact P value, Mann-Whitney U test). OS was significantly worse for patients with detectable ctDNA (hazard ratio = 3.31; 95% CI, 1.78 to 6.14; P < .001) and for detection of variants in *TP53*, *ESR1*, and *PIK3CA* alone (hazard ratio = 3.461; 95% CI, 1.866 to 6.42; P = .001; Fig 4).

Oncomine Breast cfDNA Panel Identifies Evidence of CHIP

In 121 ctDNA-positive patients in whom we compared matched gDNA, 18 participants had an identical variant detected in their gDNA sample. Fifteen of these had variants in *TP53* and *KRAS* that were excluded as potential CHIP-derived mutations (Table 2, Data Supplement) and three of these also had other variants unique to plasma.

There were three other patients with variants detected in either *PIK3CA* or *ESR1*, not previously associated with CHIP.

DISCUSSION

To our knowledge, this is the most comprehensive targeted next-generation sequencing analysis to date of ctDNA in healthy women. In 127 HC recruited through BSMS who were recalled for further investigation following suspicious mammogram, about half had no abnormality detected on their repeat mammogram and half had a core biopsy but no abnormality was detected. Overall, the majority of samples from the screening population and symptomatic PBCs were ctDNA-negative, whereas 55% of MBC were ctDNA-positive based on detection of one or more SNVs. Of interest, seven of the 11 patients with DCIS (39.3%) who were ctDNA-positive had high-grade DCIS compared with only four of 17 (24%) with lower grades; however, the number of patients with DCIS within the study is limited.

Although larger gene or mutation panels are available, as more than 93% of mutations were detected in *TP53*, *PIK3CA*, or *ESR1* genes, and also predicted poor OS in MBC, we suggest that this assay can accurately detect mutations of clinical importance, such as in *ESR1*, where emergence of mutations heralds resistance to aromatase inhibitors.^{22,23} Of interest, three patients with TNBC had *ESR1* mutations detected, suggesting the presence of a small ER-positive clone; comparison with circulating tumor cells and/or metastatic tissue biopsy would be helpful in

TABLE 2. Summary of Potential CHIP Variants Detected Using the OncoPrint cfDNA Breast Assay

Sample ID	Age, years	Cohort	Tumor Subtype	cfDNA VAF (%)	BC VAF (%)	Gene	AA
22	48	HC	NA	0.33	0.095	<i>KRAS</i>	p.G12S
				0.31	0.00	<i>PIK3CA</i>	p.C420R*
				0.35	0.00	<i>TP53</i>	p.H179L*
				0.12	0.00	<i>TP53</i>	p.H179R*
				0.34	0.00	<i>TP53</i>	p.R175H*
37	53	HC	NA	7.94	1.579	<i>KRAS</i>	p.G12R
47	69	HC	NA	0.205	0.27	<i>TP53</i>	p.R248Q
79	68	HC	NA	0.25	0.37	<i>TP53</i>	p.R273H
119	53	HC	NA	0.593	0.46	<i>TP53</i>	p.V216M
121	67	HC	NA	0.317	0.34	<i>TP53</i>	p.V216M
				0.12	0.15	<i>TP53</i>	p.R175H
238	64	PBC FU	IDC, grade 1	0.09	0.06	<i>TP53</i>	p.R248W
241	71	PBC FU	IDC, grade 2	0.25	0.29	<i>TP53</i>	p.R248W
244	70	PBC FU	IDC, grade 3	13.62	6.84	<i>TP53</i>	p.R175H
300	61	MBC	IDC, grade 3	0.11	0.32	<i>TP53</i>	p.R282W
				0.08	0.00	<i>TP53</i>	p.Y220C*
				0.16	0.00	<i>TP53</i>	p.Y220H*
				0.53	0.00	<i>ESR1</i>	p.E380Q*
313	60	MBC	IDC, grade 2	0.16	0.12	<i>TP53</i>	p.K132E
334	66	MBC	IDC, grade 2	0.59	0.34	<i>TP53</i>	p.R248Q
344	67	MBC	IDC, grade unknown	0.266	0.06	<i>TP53</i>	p.C238Y
				2.64	0.00	<i>TP53</i>	p.G266E*
350	54	MBC	IDC, grade 2	48.43	0.13	<i>TP53</i>	p.G244D
370	75	MBC	Unknown	0.99	0.06	<i>TP53</i>	p.R248Q
157	54	SD PBC	IDC, grade 3	0.52	0.06	<i>PIK3CA</i>	p.H1047R
264	43	MBC	Unknown	19.71	0.42	<i>PIK3CA</i>	p.E545K
				25.86	0.25	<i>ESR1</i>	p.D538G
303	48	MBC	IDC, grade 2	6.216	0.06	<i>ESR1</i>	p.Y537N

NOTE. Fifteen individuals had an identical variant detected in their gDNA sample in *TP53* and/or *KRAS*, known to harbor CHIP. Three of these also had plasma exclusive variants (*). Three patients with BC also had an identical variant in gDNA in *PIK3CA* and *ESR1* genes with no previous reported evidence of CHIP (in bold).

Abbreviations: AA, amino acid; cfDNA, cell-free DNA; CHIP, clonal hematopoiesis of indeterminate potential; HC, healthy controls; gDNA, germline DNA; MBC, metastatic breast cancer; PBC, primary breast cancer; PBC-FU, primary breast cancer on follow-up; VAF, variant allele frequency.

these patients to explore tumor evolution. The prevalence of *TP53* and *PIK3CA* gene mutations were similar to other studies,²⁴⁻²⁶ indicating that this panel is effective in identifying actionable mutations including *PIK3CA* mutations that indicate those patients who might benefit from treatment with PI3K inhibitors such as alpelisib.²⁷ Our data show that variants in *ESR1* and *PIK3CA* are identified in only a small proportion of HC and become much more prevalent in MBC, indicating the presence of these mutations arise because of the evolving cancer and not because of the benign pathology of a core biopsy. In patients with MBC, as expected, the median VAF was significantly higher compared with HC, DCIS, and SD PBC, and a greater range of SNVs were detected, indicating the presence of different

subclones. A small number of studies have explored the OncoPrint cfDNA Assay in breast cancer; one was limited to advanced disease,²⁸ whereas another was used to determine whether mutations could be identified in cfDNA from serum stored for 30 years.²⁹

We also investigated the prevalence of CHIP-derived mutations in ctDNA, normal hemopoietic cells accumulating somatic mutations during the aging process in the absence of cancer.¹⁴ Such mutations can potentially arise in highly sensitive next-generation sequencing data because of inaccurate variant calling, sequencing artifacts, and somatic tumor-relevant SNPs. Swanton et al³⁰ demonstrated that CHIP variants occur at a VAF of < 0.1% and are more common than previously documented. In 15 ctDNA-positive

patients, identical variants were seen in the matched gDNA; 13 were missense, nonfunctional *TP53* mutations and two were in *KRAS*. This is in agreement with Chen and Liu²⁸ who identified similar *TP53* mutations, one of the five most commonly mutated genes in CHIP. We agree with others that matched gDNA should be analyzed in parallel to exclude CHIP-derived mutations, to facilitate appropriate and accurate variant calling.¹⁴

We hoped that in patients who have abnormal or indeterminate mammography results, ctDNA analysis at the time of breast screening could be used to help clinicians decide whether surgery is needed. Our results suggest that this test might be useful for stratifying biopsy-confirmed screen-detected cancers to neoadjuvant therapies. However, caution is needed in interpreting ctDNA results from a single blood test at this time as we found a small proportion of HC who had detectable ctDNA, but on retesting, no individual was found to have a persistent variant profile.

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R.C.C. and J.A.S. are joint senior authors.

PRIOR PRESENTATION

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Since the inception of this study, several groups have reported different assays potentially capable of helping in the differential diagnosis of breast diseases, and thus are candidate blood tests for use as a part of a screening strategy. These include DNA methylation analysis,²⁹ ctDNA fragmentation assays,²² and a multianalyte platform.²³ Our results show that screening with the assay could be used in combination with other such strategies, and future studies could focus on developing technologies to improve sensitivity, determining the added value of measuring other analytes,²³ and the application of machine learning²² in improving diagnostic accuracy.

In conclusion, the assay successfully detected key breast cancer mutations through ctDNA and predicted poor OS in MBC. Further investigation is needed regarding the prevalence of mutations in healthy women in a true cancer screening setting to attempt to define a ctDNA baseline in the healthy population.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, et al: Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer* 144:1941-1953, 2019
2. Pan H, Gray R, Braybrooke J, et al: 20-Year risks of breast-cancer recurrence after stopping endocrine therapy at 5 years. *N Engl J Med* 377:1836-1846, 2017
3. Fenton JJ, Taplin SH, Carney PA, et al: Influence of computer-aided detection on performance of screening mammography. *N Engl J Med* 356:1399-1409, 2007
4. Garcia-Murillas I, Schiavon G, Weigelt B, et al: Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Sci Transl Med* 7:302ra133, 2015
5. Olsson E, Winter C, George A, et al: Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. *EMBO Mol Med* 7:1034-1047, 2015
6. Shaw JA, Page K, Blighe K, et al: Genomic analysis of circulating cell-free DNA infers breast cancer dormancy. *Genome Res* 22:220-231, 2012
7. Diehl F, Schmidt K, Choti MA, et al: Circulating mutant DNA to assess tumor dynamics. *Nat Med* 14:985-990, 2008
8. Coombes RC, Page K, Salari R, et al: Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence. *Clin Cancer Res* 25:4255-4263, 2019
9. Johnston SR, Kilburn LS, Ellis P, et al: Fulvestrant plus anastrozole or placebo versus exemestane alone after progression on non-steroidal aromatase inhibitors in postmenopausal patients with hormone-receptor-positive locally advanced or metastatic breast cancer (SoFEA): A composite, multicentre, phase 3 randomised trial. *Lancet Oncol* 14:989-998, 2013
10. Allouchery V, Beaussire L, Perdrix A, et al: Circulating ESR1 mutations at the end of aromatase inhibitor adjuvant treatment and after relapse in breast cancer patients. *Breast Cancer Res* 20:40, 2018
11. Toy W, Shen Y, Won H, et al: ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* 45:1439-1445, 2013
12. Palmieri C, Cleator S, Kilburn LS, et al: NEOCENT: A randomised feasibility and translational study comparing neoadjuvant endocrine therapy with chemotherapy in ER-rich postmenopausal primary breast cancer. *Breast Cancer Res Treat* 148:581-590, 2014
13. Page K, Shaw JA, Guttery DS: The liquid biopsy: Towards standardisation in preparation for prime time. *Lancet Oncol* 20:758-760, 2019
14. Genovese G, Kahler AK, Handsaker RE, et al: Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* 371:2477-2487, 2014
15. Razavi P, Li BT, Brown DN, et al: High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. *Nat Med* 25:1928-1937, 2019
16. Dhingra D, Chien R, Gu J, et al: An NGS workflow to detect down to 0.1% allelic frequency in cfDNA for breast and colon cancers. *Cancer Res* 77, 2017 (abst 5396)
17. Page K, Guttery DS, Zahra N, et al: Influence of plasma processing on recovery and analysis of circulating nucleic acids. *PLoS One* 8:e77963, 2013
18. Guttery DS, Page K, Hills A, et al: Noninvasive detection of activating estrogen receptor 1 (ESR1) mutations in estrogen receptor-positive metastatic breast cancer. *Clin Chem* 61:974-982, 2015
19. Robinson JT, Thorvaldsdottir H, Winckler W, et al: Integrative genomics viewer. *Nat Biotechnol* 29:24-26, 2011
20. Andersen PK, Gill RD: Cox regression-model for counting-processes—A large sample study. *Ann Stat* 10:1100-1120, 1982
21. Fernandez-Garcia D, Hills A, Page K, et al: Plasma cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast cancer. *Breast Cancer Res* 21:149, 2019
22. Cristiano S, Leal A, Phallen J, et al: Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* 570:385-389, 2019
23. Cohen JD, Li L, Wang Y, et al: Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* 359:926-930, 2018
24. Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. *Nature* 490:61-70, 2012
25. Curtis C, Shah SP, Chin SF, et al: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 486:346-352, 2012
26. Martinez-Saez O, Chic N, Pascual T, et al: Frequency and spectrum of *PIK3CA* somatic mutations in breast cancer. *Breast Cancer Res* 22:45, 2020
27. Andre F, Ciruelos E, Rubovszky G, et al: Alpelisib for *PIK3CA*-mutated, hormone receptor-positive advanced breast cancer. *N Engl J Med* 380:1929-1940, 2019
28. Chen S, Liu Y: p53 involvement in clonal hematopoiesis of indeterminate potential. *Curr Opin Hematol* 26:235-240, 2019
29. Delmonico L, Silva Magalhães Costa MA, Gomes RJ, et al: Methylation profiling in promoter sequences of *ATM* and *CDKN2A* (*p14^{ARF}/p16^{INK4a}*) genes in blood and cfDNA from women with impalpable breast lesions. *Oncol Lett* 19:3003-3010, 2020
30. Swanton C, Venn O, Aravanis A, et al: Prevalence of clonal hematopoiesis of indeterminate potential (CHIP) measured by an ultra-sensitive sequencing assay: Exploratory analysis of the Circulating Cancer Genome Atlas (CCGA) study. *J Clin Oncol* 36, 2018 (suppl; abstr 12003)

