The Journal of Molecular Diagnostics, Vol. 21, No. 1, January 2019

the Journal of Molecular Diagnostics
jmd.amjpathol.org

An Optimized Workflow to Evaluate Estrogen Receptor Gene Mutations in Small Amounts of Cell-Free DNA

Silvia R. Vitale,[*†‡] Anieta M. Sieuwerts,[*†] Nick Beije,[*†] Jaco Kraan,[*†] Lindsay Angus,[*†] Bianca Mostert,[*†] Esther A. Reijm,[*†] Ngoc M. Van,[*†] Ronald van Marion,[§] Luc Y. Dirix,[¶] Paul Hamberg,[‖] Felix E. de Jongh,[‖**] Agnes Jager,[*†] John A. Foekens,[*†] Paolo Vigneri,[‡] Stefan Sleijfer,[*†] Maurice P.H.M. Jansen,[*†] and John W.M. Martens[*†]

From the Department of Medical Oncology and Cancer Genomics Netherlands,[†] Erasmus Medical Center Cancer Institute, and the Department of Pathology,[§] Erasmus Medical Center, Rotterdam, the Netherlands; the Department of Clinical and Experimental Medicine—Center for Experimental Oncology and Hematology,[‡] University of Catania, Catania, Italy; the Translational Cancer Research Unit,[¶] Department of Medical Oncology, Oncology Center Gasthuis Zusters Antwerp Hospital Sint Augustinus, Antwerp, Belgium; the Department of Internal Medicine,[‖] Franciscus Gasthuis and Vlietland, Rotterdam, the Netherlands; and the Department of Internal Medicine,** Ikazia Hospital, Rotterdam, the Netherlands*

Accepted for publication August 16, 2018.

Address correspondence to Anieta M. Sieuwerts, Ph.D., Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus MC Cancer Institute, Wytemaweg 80, 3015 CN Rotterdam, the Netherlands. E-mail: a.sieuwerts@erasmusmc.nl.

The detection of mutated genes in cell-free DNA (cfDNA) in plasma has emerged as an important minimally invasive way to obtain detailed information regarding tumor biology. Reliable determination of circulating tumor—derived DNA, often present at a low quantity amidst an excess of normal DNA in plasma, would be of added value for screening and monitoring of cancer patients and for hypothesis-generating studies in valuable retrospective cohorts. Our aim was to establish a workflow to simultaneously assess four hotspot estrogen receptor mutations (m*ESR1*) in cfDNA isolated from only 200 μL of plasma by means of uniplex or multiplex pre-amplification combined with digital PCR. This workflow was then applied in metastatic breast cancer (MBC) patients receiving systemic therapies for MBC. In accordance with previous studies, estrogen receptor mutations were more frequently detected in endocrine-treated MBC patients at progressive disease [34.1% (15/44)] than before the start of endocrine therapy [3.9% (2/51); $P = 0.001$]. For a subset of samples, results were compared with analysis of these mutations by Oncomine-targeted next-generation sequencing, which, although requiring a higher cfDNA input, yielded concordant results. The data establish development and validation of a digital PCR workflow for the simultaneous detection of several tumor-derived mutations in minute amounts of cfDNA and show the potential of this workflow for use on archived volume-limited blood samples. *(J Mol Diagn 2019, 21: 123—137; https://doi.org/10.1016/j.jmoldx.2018.08.010)*

Breast cancer (BC) is the most common cancer among women. Approximately 70% of BC cases are positive for the estrogen receptor (ER).[1—3] For patients with ER-positive metastatic BC (MBC), endocrine therapy is the preferred treatment modality. Unfortunately, approximately 40% of ER-positive MBC patients encounter swift progression after initiation of endocrine therapy and eventually virtually all MBC patients acquire resistance during treatment.[1]

Mutations in the gene coding for ER (*ESR1*) have been linked to endocrine resistance and are observed in 14% to 39% of MBC patients.[4—8] Although these mutations rarely occur in primary tumors,[2,9] they accumulate frequently during treatment,[2,10,11] usually in patients who received prior treatment for MBC with aromatase inhibitors.[6,12,13] The current consensus describes a hotspot region within the ligand-binding domain of *ESR1*, which, if mutated, results in

Supported by the Cancer Genomics Netherlands (CGC.nl)/Netherlands Organisation for Scientific Research (A.M.S. and J.W.M.), European Research Council advanced grant 322737 (S.R.V.), and a Pink Ribbon Netherlands grant (project WO 61). The next-generation sequencing experiments were supported by Merck KGaA.

S.R.V. and A.M.S contributed equally to this work.

Disclosures: None declared.

The authors are fully responsible for the content of this article, and the views and opinions described in the publication reflect solely those of the authors. Merck KGaA reviewed the manuscript for medical accuracy only before journal submission.

https://doi.org/10.1016/j.jmoldx.2018.08.010

an amino acid substitution at positions 537 and 538 in helix 12 of the ER, closing the pocket that otherwise captures estrogen and resulting in a constitutively active ER.[6,14] Depending on the type of endocrine therapy administered, this can result in endocrine resistance, which may be overcome by switching to agents such as fulvestrant.[2,10]

The detection of mutated *ESR1* (m*ESR1*) in MBC may play an important role in future clinical treatment decision making. However, because of the heterogeneous nature of metastases[15,16] and because repeated tissue biopsies to observe patients in time are costly, a burden to the patient, and often difficult, if not impossible, to perform, alternative ways to assess the genetic status of MBC, including the use of liquid biopsies, are being investigated.[3] Easily accessible body fluids, such as blood plasma and serum, contain cell-free DNA (cfDNA), in which circulating tumor DNA can be detected. Because cfDNA is also released by normal cells during physiological processes (eg, apoptosis and necrosis), the discrimination between scant circulating tumor DNA derived from tumor cells among that from normal cells can be challenging.[17]

Currently, research is focused on optimizing molecular methods to investigate and monitor the mutational status measured in cfDNA, and digital PCR (dPCR) has shown to be a highly sensitive approach, yielding encouraging results for the detection of m*ESR1* in MBC.[3,5,6,8,11,12,18–25] However, most of these studies do not take the—sometimes limited—amount of available starting material into account and typically start with at least 1 mL of plasma. To exploit plasma available from valuable retrospective studies, how to make optimal use of limited available amounts of plasma and, hence, circulating tumor DNA needs to be addressed.

Our aim was to optimize a reliable and reproducible workflow to investigate the m*ESR1* status for four hotspot mutations in minute amounts of cfDNA by dPCR and to improve cfDNA analyses from small volumes of plasma of MBC patients in general. To validate the workflow, dPCR data were compared with data obtained with a targeted next-generation sequencing (NGS) approach. Finally, to show the feasibility of using this workflow in a clinical setting with limited amounts of plasma available, our optimized workflow was used to evaluate the correlation between the presence of m*ESR1* and prior treatment within a retrospective cohort of MBC patients.

Materials and Methods

Study Design

Figure 1 shows a detailed overview of the study design.

Study Cohorts

A retrospective study was performed in MBC patients included in two multicenter studies primarily focusing on

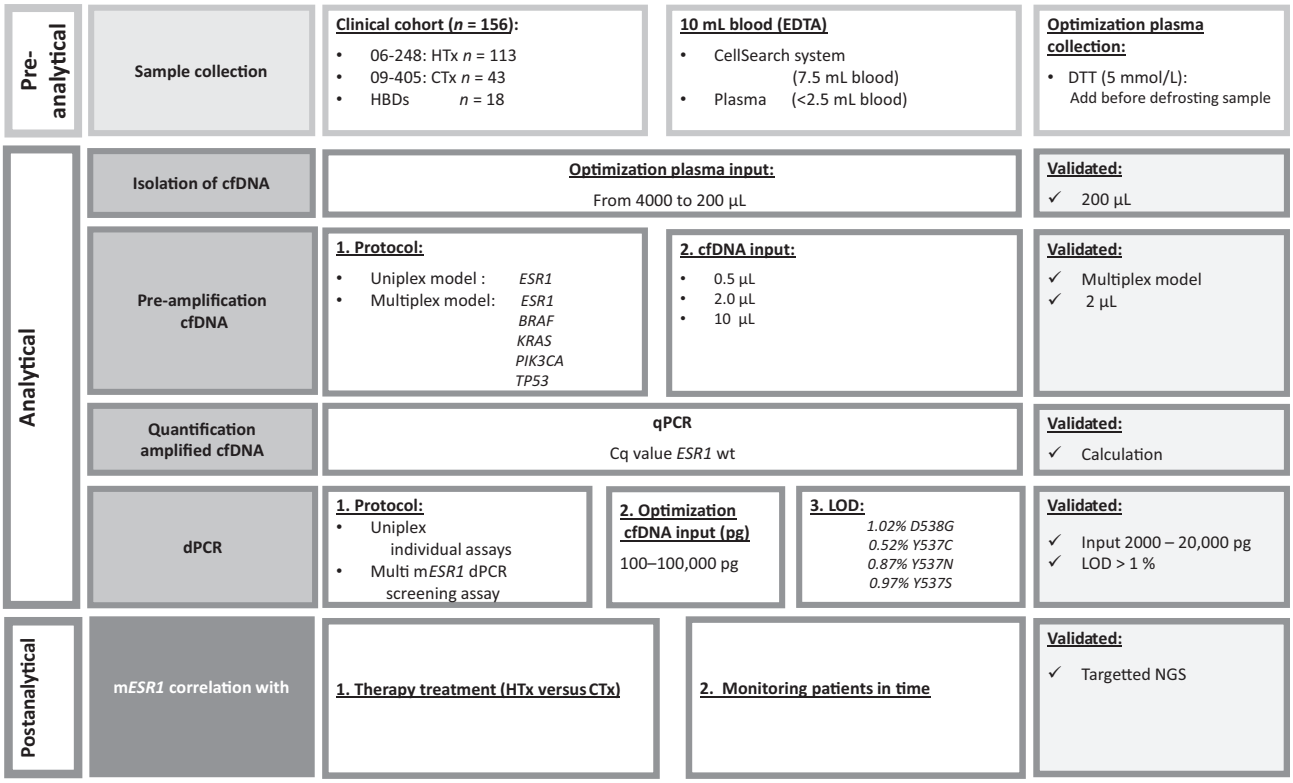

Figure 1 Workflow of processing samples. Cq, quantification cycle; cfDNA, cell-free DNA; CTx, chemotherapy; dPCR, digital PCR; DTT, dithiothreitol; HBD, healthy blood donor; HTx, endocrine therapy; LOD, limit of detection; NGS, next-generation sequencing; qPCR, quantitative PCR.

circulating tumor cell detection (study 06-248[26,27] and study 09-405[28]). Patients were recruited between February 2008 and March 2015 in several hospitals in the Netherlands and Belgium. The study protocols were approved by the local research ethics boards (Erasmus MC identifiers MEC-06-248 and MEC-09-405) and conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent before participation.

A total cohort of 156 MBC patients with at least 200 μL plasma available was evaluated; 113 patients were treated with endocrine therapy, and 43 patients were treated with chemotherapy, for MBC. More detailed information has been summarized in Table 1 for the 95 clinically evaluable endocrine-treated patients and in Supplemental Table S1 for all patients whose plasma was used in this study. In addition, plasma samples from 18 healthy blood donors (HBDs) were assessed.

Table 1 Treatment Characteristics, Endocrine Clinical Cohort

Parameter	BL subset (n = 51)	PD subset (n = 44)
Age at sample draw, median (range), years	67 (36–89)	67 (39–87)
Adjuvant endocrine therapy		
No	33 (65)	27 (61)
Yes, tamoxifen only	12 (23)	10 (23)
Yes, tamoxifen + AI	2 (4)	6 (14)
Yes, AI only	4 (8)	1 (2)
Adjuvant chemotherapy		
No	38 (75)	32 (73)
Yes	13 (25)	12 (27)
Neoadjuvant therapies		
No	51 (100)	42 (95)
Yes, chemotherapy		2 (5)
Previous endocrine therapy lines for MBC, n		
0	51 (100)	
1		28 (64)
2		13 (29)
≥3		3 (7)
Endocrine therapy after start (BL subset) or before PD (PD subset)		
AI	37 (73)	22 (50)
Tamoxifen	13 (25)	11 (25)
Fulvestrant	1 (2)	10 (23)
Fulvestrant + AI		1 (2)
Previous endocrine therapy lines for MBC (in case of inclusion at PD on second-line endocrine therapy or more)		
No	51 (100)	28 (64)
Yes, AI only		10 (23)
Yes, AI + tamoxifen		3 (7)
Yes, tamoxifen only		3 (7)

Data are given as n (%) of each group, unless otherwise indicated.
AI, aromatase inhibitor; BL, baseline; MBC, metastatic breast cancer; PD, progressive disease.

Plasma Collection and cfDNA Isolation

Blood (10 mL) was collected in Vacutainer EDTA tubes (BD, Franklin Lakes, NJ) and transported to the coordinating laboratory at ambient temperature for circulating tumor cell profiling and plasma preparation, both processed within 24 hours after blood draw. Of this blood, 7.5 mL was used for circulating tumor cell enrichment and characterization by quantitative RT-PCR (RT-qPCR), as described before,[26,29] and the remaining up to 2.5 mL of whole blood was used to prepare plasma. The plasma was collected after two sequential centrifugation steps (1711 × g for 10 minutes at room temperature and immediately stored at −80°C and a second time at 12,000 × g for 10 minutes at 4°C before cfDNA isolation).

cfDNA extraction was performed using the QIAamp Circulating Nucleic Acid kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's protocol, with some modifications: i) dithiothreitol at a 5 mmol/L final concentration was added to the plasma before thawing to prevent nucleic acid degradation; ii) the input volume of plasma was downscaled to 200 μL; and iii) the cfDNA was eluted in 20 μL AVE-Buffer (Qiagen, Venlo, the Netherlands) and applied to the column three times to increase the final cfDNA concentration. The cfDNA concentration was quantified with the Qubit dsDNA HS Assay kit (Thermo Fisher, Landsmeer, the Netherlands) and stored for use within 1 week at −30°C and long-term at −80°C.

Pre-Amplification and Quantitative PCR

cfDNA (0.1 to 1 ng/μL) was pre-amplified during 15 cycles using a single locus (specific for *ESR1* containing the four hotspot mutations at codon positions 537 and 538) or a multiple loci target-specific amplification for *ESR1*, *BRAF*, *KRAS*, *PIK3CA*, and *TP53* with TaqMan PreAmp Master Mix (Thermo Fisher), as recommended by the manufacturer. All primer pairs present in the two different pre-amplification combinations, as well as the TaqMan qPCR and digital PCR assays used to quantify the wild-type and mutated genes, are given in Table 2; details regarding the pre-amplification and digital PCR protocols are given in Supplemental Table S2. Before downstream processing, the pre-amplified product was diluted 10-fold in LoTE buffer (3 mmol/L Tris-HCl/0.2 mmol/L EDTA, pH 8.0).

Next, a quantitative PCR (qPCR) for wild-type (WT) *ESR1* was performed to quantify the number of WT copies present in the samples. To ensure equal loading of WT copies of all samples, the resulting quantification cycle value was used to empirically establish the correct dilution factor to apply to each sample before loading onto the chips for the dPCR analysis (Supplemental Table S3).

Table 2 Assay Details Pre-Amplification and Digital PCR

Description	Mutation	Assay identification*	Forward primer	Reverse primer	FAM TaqMan probe†	VIC TaqMan probe†	Size, bp
ESR1 uniplex model							
ESR1 pre-amplification[6]	*ESR1*		5′-AGGCATGGAG-CATCTGTACA-3′	5′-TTGGTCCGT-CTCCTCCA-3′			136
ESR1 mutation	*ESR1_D538G*		5′-CAGCATGAAG-TGCAAGAACGT-3′	5′-TGGGCGTCC-AGCATCTC-3′	5′-CCCTCTATG-GCCTGCT-3′	5′-CCCCT-CTATGAC-CTGCT-3′	63
ESR1 mutation	*ESR1_Y537C*[6]		5′-AGGCATGGAG-CATCTGTACA-3′	5′-TTGGTCCGT-CTCCTCCA-3′	5′-TGCCCCT-CTGTGA-CCTGC-3′	5′-TGGTGC-CCCTCT-ATGACC-TG-3′	136
ESR1 mutation	*ESR1_Y537N*[6]		5′-AGGCATGGAG-CATCTGTACA-3′	5′-TTGGTCCGT-CTCCTCCA-3′	5′-TGCCCCT-CAATGAC-CTGC-3′	5′-TGGTGC-CCCTCTA-TGACC-TG-3′	136
ESR1 mutation and qPCR screening	*ESR1_Y537S*		5′-CAGCATGAAGT-GCAAGAACGT-3′	5′-TGGGCGTCC-AGCATCTC-3′	5′-CCCTCTC-TGACCTGC-3′	5′-CCCCT-CTATGA-CCTGC-3′	63
Additional assays included in the multiplex model							
KRAS pre-amplification	*KRAS_codon 12/13*		5′-ACTGGTGGAGT-ATTTGATAGT-GTAT-3′	5′-CTCTATTGT-TGGATCATA-TTCGTCC-3′			204
BRAF pre-amplification	*BRAF_V600E*	AH6R5PH					149
PIK3CA pre-amplification	*PIK3CA_ E542K_760*	AHD2BSD					
PIK3CA pre-amplification	*PIK3CA_ H1047L_776*	AHLJ0TP					
TP53 pre-amplification	*TP53_Exon 5*		5′-CCCCTGCCCTC-AACAAGATG-3′	5′-GACCATCGC-TATCTGAG-CAG-3′			183
TP53 pre-amplification	*TP53_Exon 7*		5′-TGGCTCTGACT-GTACCACCA-3′	5′-CTGGAGT-CTTCCAGT-GTGATG-3′			108
TP53 pre-amplification	*TP53_Exon 8*		5′-ACTGGGACGG-AACAGCTTTG-3′	5′-CTGGGGGCA-GCTCGTG-3′			113
TP53 pre-amplification	*TP53_Exon 10*		5′-GTGAGCGCTTC-GAGATGTTC-3′	5′-TCCCCCCTG-GCTCCTTC-3′			81
Reference: synthetic *ESR1_D538G*	5′-GTCTTCCCACCTACAGTAACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGTGGTGCCCC-TCTATGGCCTGCTGCTGGAGATGCTGGACGCCCACCGCCTACATGCGCCCACTAGCCGTGGAGGGGCA-TCCGTGGAGGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTC-3′						181
Reference: synthetic *ESR1_Y537C*[6]	5′-GTCTTCCCACCTACAGTAACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGTGGTG-CCCCTCTGTGACCTGCTGCTGGAGATGCTGGACGCCCACCGCCTACATGCGCCCACTAGCCGTGGA-GGGGCATCCGTGGAGGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTC-3′						181
Reference: synthetic *ESR1_Y537N*[6]	5′-GTCTTCCCACCTACAGTAACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGTGGTGCC-CCTCAATGACCTGCTGCTGGAGATGCTGGACGCCCACCGCCTACATGCGCCCACTAGCCGTGGAGG-GGCATCCGTGGAGGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTC-3′						181
Reference: synthetic *ESR1_Y537S*[6]	5′-GTCTTCCCACCTACAGTAACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGTGGTGCCC-CTCTCTGACCTGCTGCTGGAGATGCTGGACGCCCACCGCCTACATGCGCCCACTAGCCGTGGAG-GGGCATCCGTGGAGGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTC-3′						181

*Thermo Fisher.
†MGB NFQ probe.
FAM, 6-carboxyfluorescein; MGB NFQ, Minor Groove Binder NonFluorescent Quencher; qPCR, quantitative PCR.

Chip-Based Digital PCR

dPCRs were performed with the QuantStudio 3D Digital PCR System (Thermo Fisher), according to the manufacturer's protocol. All four hotspot mutations for *ESR1* (D538G, Y537S, Y537C, and Y537N) were separately, or together by multiplexing the assays, analyzed with mutation-specific TaqMan assays (Table 2 and Supplemental Table S2); and

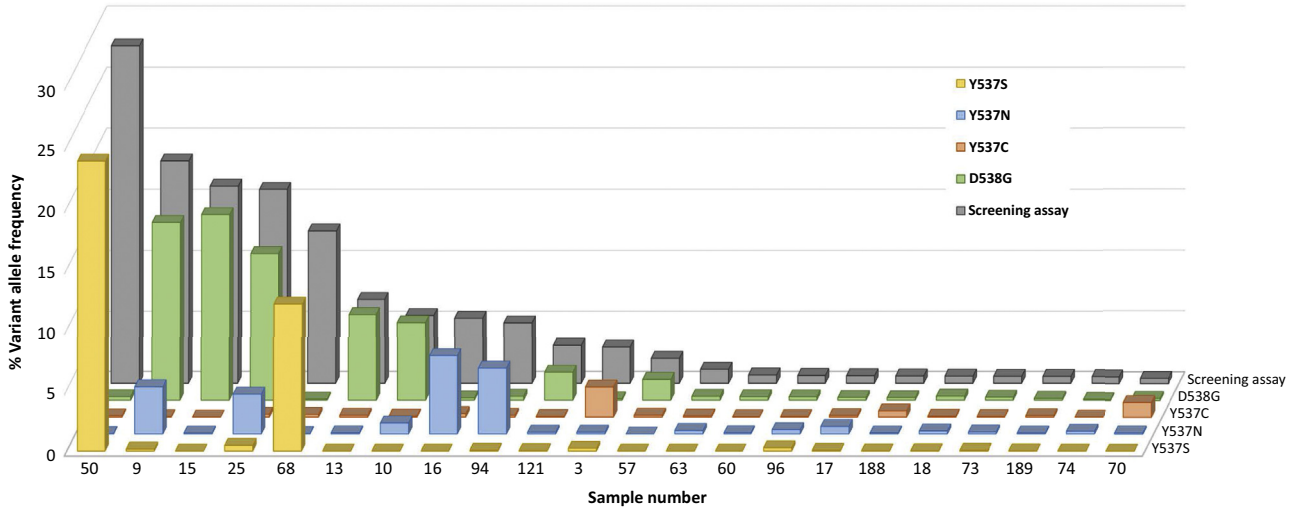

Figure 2 Performance of m*ESR1* digital PCR (dPCR) screening assay. To validate the performance of the m*ESR1* multiplex screening assay covering all four *ESR1* hotspot mutations, 36 different plasma samples were analyzed with the m*ESR1* dPCR screening assay. Variant allele frequency data were compared with the results of the individual m*ESR1* assays.

the variant allele frequencies (VAFs) were calculated for each of these mutations or for the combined mutations, respectively. For this, each pre-amplified cfDNA sample was partitioned into 20,000 wells of a QuantStudio 3D Digital PCR v2 Chip and run on a ProFlex 2x Flat PCR System (Thermo Fisher). The target-specific optimized PCR program was as follows: 10 minutes at 96°C, followed by 40 cycles of 30 seconds' incubation at 98°C and 2 minutes at 55°C, and a final pause for up to 16 hours at 10°C. Chips were read in a QuantStudio 3D Digital PCR Instrument and analyzed with the web-based Quantstudio 3D dPCR Analysis Software version 3.01 (Thermo Fisher). At least one positive and one negative control sample were included in every run, and all chips were read in duplicate (immediately after the dPCR run and again between 4 and 16 hours later).

Ion Torrent Next-Generation Sequencing

In addition to dPCR, cfDNA of 10 HBDs as controls for accuracy and specificity and serial samples of seven MBC patients were analyzed using the Ion Torrent Oncomine Breast cfDNA Assay (catalog number A31183; Thermo Fisher) in combination with the Ion Torrent S5XL NGS system (Life Technologies, Carlsbad, CA) to simultaneously evaluate multiple hotspot mutations. The Oncomine Breast Assay is designed to sequence 26 amplicons to detect 152 hotspots and insertions/deletions for a panel of 10 BC-relevant genes (*AKT1*, *EGFR*, *ERBB2*, *ERBB3*, *ESR1*, *FBXW7*, *KRAS*, *PIK3CA*, *SF3B1*, and *TP53*). This NGS assay applies unique molecule identifiers to improve the sensitivity by decreasing the amount of sequencing artifacts. With the recommended input of 20 ng cfDNA, the use of unique molecule identifiers enables a limit of detection (LOD) as low as 0.1%.[30–33]

Because of our retrospective analysis, suboptimal input amounts compared with the recommended input of 20 ng DNA were used for cfDNA from HBDs (range, 4.86 to 10.41 ng) and MBC patients (range, <1 to 6.1 ng) to generate targeted libraries following the manufacturer's protocol. First, concentrations of each Oncomine cfDNA library were determined by qPCR using the Ion Library

Table 3 Sensitivity and Specificity of Measuring D538G *ESR1* Variant Allele Frequencies

Sample		Without pre-amplification			With pre-amplification		
HBD plasma, mL	D538G spiked in	dPCR, pg	VAF, %	VAF, means ± SD, %	dPCR, pg*	VAF, %	VAF, means ± SD, %
4.0	Yes	11,955	18.4		9444	16.1	
2.0	Yes	7601	14.6		7261	17.4	
1.0	Yes	4543	20.2		8806	15.3	
0.5	Yes	2119	28.3		7489	15.7	
0.2	Yes	2798	20.6	20.4 ± 5.0	6891	25.8	18.0 ± 4.4
2.0	No	1999*	0.0		11,965	0.5	
1.0	No	1182*	0.0		7968	0.2	
0.2	No	412*	0.0	0.0 ± 0.0	12,076	0.2	0.3 ± 0.2

*Less than 2000 pg DNA input in the dPCR is considered a too low input for a reliable analysis of low mutated samples.
dPCR, digital PCR; HBD, healthy blood donor; VAF, variant allele frequency.

Table 4 dPCR Input Evaluation for Measuring D538G *ESR1* Variant Allele Frequencies

Without pre-amplification		With pre-amplification		
dPCR, pg*	VAF, %	dPCR, pg	VAF, %	VAF, means ± SD, %
116*	14.8	641*	18.4	
		1227*	25.5	
		1642*	14.8	
		1722*	15.9	
		1770*	16.6	
		1786*	13.3	17.4 ± 4.3
		2756	21.9	
		7777	24.1	
		12,254	23.3	
		12,371	21.6	
		17,216	22.7	
		18,147	22.4	
		20,118	21.1	
		20,125	21.1	
		24,859	22.2	
		309,706	21.5	22.2 ± 1.0

*Less than 2000 pg DNA input in the dPCR is considered too low an input for a reliable analysis of samples mutated at low frequency.

dPCR, digital PCR; VAF, variant allele frequency.

TaqMan Quantitation Kit (Life Technologies) and then diluted to a final concentration of 50 pmol/L. Next, sample barcoded libraries were pooled together for template preparation on the Ion Chef Instrument using the Ion 520/530 Kit−Chef (catalog number A30010) and loaded onto an Ion 530 chip (all from Life Technologies). The chip was sequenced on an Ion S5 XL Sequencer System, and the data were analyzed using the Ion Torrent Suite software version 5.2.2 (both from Life Technologies). For patient and HBD samples, general NGS quality measures (eg, median read depth, median molecular coverage, and mean read lengths) are presented in Supplemental Table S4.

Statistical Analysis

All data were entered in SPSS version 23 (IBM Corp., Armonk, NY) to generate the tables and perform the statistical analyses. For 2 × 3 and 2 × 4 tables, the online Freeman-Halton extension of the Fisher's exact probability test was used (*http://vassarstats.net/newcs.html*, last accessed October 1, 2018). All P values are two sided, and $P < 0.05$ was considered statistically significant.

Results

Sensitivity and Specificity of a Multiplex Mutant *ESR1* Hotspot dPCR Screening Assay

We designed a multiplex dPCR m*ESR1* screening assay (m*ESR1*_G_C_N_S) covering the four most common *ESR1* mutations (D538G, Y537C, Y537N, and Y537S) (Table 2 and Supplemental Table S2). To validate the performance of the m*ESR1*_G_C_N_S screening assay, 36 plasma samples were first analyzed with the m*ESR1* dPCR screening assay and subsequently compared with the four individual *ESR1* mutation assays (Supplemental Table S5). Using a VAF threshold of 2% for the m*ESR1* screening assay, 12 plasma samples were higher than the threshold for the multiplex assay (VAF range, 2.04% to 27.73%). Digital PCR analysis using individual mutation assays demonstrated that at this cutoff all individual hotspot mutations using a VAF threshold of 1% for the uniplex assays were efficiently captured with the m*ESR1* hotspot dPCR screening assay. Only one sample (Sample 70) with an *ESR1* Y537C mutation at 1.22% was missed with the dPCR m*ESR1* screening assay, giving a VAF of 0.43% (Supplemental Table S5). Figure 2 shows that the sum of the frequencies of the individual TaqMan assays correlated well with the VAF assessed with the m*ESR1* dPCR screening assay (Pearson's $r = 0.99$, $P < 0.0001$). In addition, as shown for Samples 9 and 25, the multiplex screening assay was also able to capture the possible presence of multiple mutations in the same sample, suggesting a different clonal background. The m*ESR1*_G_C_N_S dPCR screening approach was, therefore, applied to screen our samples before performing the individual dPCR *ESR1* mutation assays.

Sensitivity and Specificity of Single versus Multilocus Pre-Amplification

Because limited amounts of plasma were available, pre-amplification was required to assay multiple mutations in the same sample. Because whole-genome amplification using, for example, the REPLI-g Whole Genome Amplification method (Qiagen) is not suitable for fragmented (cf)DNA and, therefore, failed for our targets (data not shown), a target-specific pre-amplification method was designed to allow measurement of *ESR1* mutations next to *KRAS*, *BRAF*, *PIK3CA*, and *TP53* in cfDNA isolated from down to 200 μL plasma. These analyses showed that, using a 1% VAF threshold for the four individual mutations, no information was lost if the *ESR1* locus was pre-amplified in a multiplex protocol together with *KRAS*, *BRAF*, *PIK3CA*, and *TP53* (Supplemental Table S5). All samples identified as mutated at a 1% VAF threshold with the *ESR1* target-specific pre-amplification protocol were also identified as such with the multiplex pre-amplification protocol (Supplemental Table S5).

Range of Plasma Volume Input for Extraction, DNA Input for Digital PCR, and cfDNA Input for Pre-Amplification

To determine the minimum volume of plasma required for our analyses, cfDNA was isolated from different volumes of plasma from the same HBD with and without spiked-in

DNA containing D538G *ESR1* (isolated from a D538G-positive formalin-fixed, paraffin-embedded tissue) and then analyzed by dPCR both with and without *ESR1* locus-specific pre-amplification. An average VAF of 20.4% (range, 14.6% to 28.3%) and 18.0% (range, 15.3% to 25.8%) for D538G *ESR1* was measured in unprocessed and pre-amplified samples, respectively (Table 3), indicating that similar allele frequencies were detected in cfDNA isolated from 4 mL down to 200 μL of plasma with and without pre-amplification. Similar starting volumes of HBD plasma without spiked-in cfDNA were used as negative control. Although no D538G *ESR1* copies were detected in non−pre-amplified HBD samples, an average VAF of 0.3% (range, 0.2% to 0.5%) was measured in pre-amplified samples, which is, however, well below the 1% cutoff we used to assign a sample mutated (see also below). In conclusion, an input of 200 μL plasma was considered sufficient as input to detect variant molecules in cfDNA after pre-amplification.

Because the estimated VAF in dPCR at a final input of <2000 pg DNA [17.4% ± 4.3% (25% CV)] was lower and less reliable compared with a VAF assessed in at least 2000 pg DNA [22.2% ± 1.0% (4.5% CV)] (Table 4), it was decided that at least 2000 pg cfDNA input was required for dPCR. On the other hand, to prevent overloading of the dPCR chip, the most optimal maximum input for the dPCR needed to be determined. The VIC signal of the qPCR for Y537S *ESR1* (with the same TaqMan assay used for the dPCR) was used to assess the number of WT *ESR1* copies present in the pre-amplified cfDNA, irrespective of the mutation present. Using the resulting quantification cycle value as an estimate for the number of WT copies, the input range for the m*ESR1* dPCR was empirically established after pre-amplification to load the chip with between 2 and 100 ng WT *ESR1* DNA (Supplemental Table S3). When using this approach, the quantification cycle values versus input range of pre-amplified material need to be optimized first for any other dPCR assay.

To determine if the amount of cfDNA input in the pre-amplification biased the resulting VAF, cfDNA isolated from plasma of 26 patients was pre-amplified using 0.5 μL cfDNA [2.5% of the total isolated volume; median (range), 0.28 (0.17 to 3.9) ng] in a uniplex pre-amplification assay and 2 μL [10%; median (range), 1.12 (0.68 to 15.6) ng] in a

Table 5 Sensitivity and Specificity of Measuring *ESR1* Variant Allele Frequencies in Pre-Amplified cfDNA

Sample number	Uniplex pre-amplification (0.5 μL cfDNA)				Multiplex pre-amplification (2 μL cfDNA)				Multiplex pre-amplification (10 μL vacuum-dried cfDNA)				
	D538G	Y537C	Y537N	Y537S	D538G	Y537C	Y537N	Y537S	G_C_N_S	D538G	Y537C	Y537N	Y537S
1	0.2	0.1	0.0	0.0	0.0	0.2	0.1	0.0	0.5	0.3	0.0	0.0	0.0
2	0.1	0.1	0.1	0.0	0.3	0.2	0.2	0.0					
3	0.2	0.0	0.1	0.1	0.1	**2.5**	0.2	0.2					
4	0.1	0.3	0.0	0.0	0.1	0.1	0.1	0.2					
5	0.4	0.4	0.2	0.0	0.2	0.4	0.2	0.0					
6	0.1	0.1	0.4	0.0	0.2	0.4	0.2	0.1	0.4				
7	0.3	0.2	0.1	0.0	0.2	0.1	0.1	0.0	0.2				
8	0.5	0.2	0.2	0.1	0.3	0.1	0.1	0.0	0.2				
9	**27.1**	**12.1**	0.1	0.0	**14.6**	**3.9**	0.0	0.2					
10	**2.9**	0.2	0.3	0.0	**6.0**	0.4	0.7	0.1	**5.6**	**6.4**	0.1	0.9	0.0
11	**11.3**	0.4	0.2	0.0	**11.0**	0.5	0.7	0.0					
12	0.2	0.2	0.1	0.1	0.5	0.1	0.1	0.0	0.1				
13	**5.1**	0.1	0.0	0.1	**5.1**	0.2	0.4	0.0	**6.9**	**7.1**	0.2	0.1	0.0
14	0.7	0.2	0.1	0.1	0.3	0.1	0.3	0.5	0.4				
15	**10.2**	0.1	0.3	0.1	**17.8**	0.3	0.2	0.0	**16.2**	**15.3**	0.1	0.0	0.0
16	0.2	0.1	**6.3**	0.0	0.2	0.3	**6.4**	0.0					
17	0.5	0.1	0.4	0.1	0.2	0.1	0.5	0.6	0.6	0.2	0.6	0.1	0.1
18	0.2	0.2	0.1	0.0	0.4	0.6	0.3	0.0	0.6	0.3	0.3	0.1	0.0
19	0.3	0.4	0.1	0.1	0.2	0.2	0.1	0.0	0.3				
20	0.1	0.3	0.1	0.0	0.5	0.2	0.1	0.0	0.1				
21	0.3	0.2	0.0	0.1	0.3	0.2	0.0	0.0	0.1				
22	0.1	0.1	0.5	0.1	0.9	0.1	0.1	0.0	0.1				
23	0.1	0.0	0.2	0.1	0.0	0.2	0.0	0.0	0.1				
24	**20.6**	0.2	0.2	0.1	**20.5**	0.0	0.1	0.0					
25	**16.0**	0.1	0.2	0.0	**8.7**	0.1	**6.3**	0.0	**16.0**	**12.1**	0.2	**3.2**	0.5
26	0.4	0.2	0.1	0.5	0.6	0.1	0.1	0.0	0.2				

Date are given as variant allele frequency. Values >1% threshold cutoff are in bold.
cfDNA, cell-free DNA.

Table 6 Reproducibility of VAF Estimates after Pre-Amplification of a Multipool Sample

Pre-amplification protocol	*ESR1* mutation	dPCR, pg	VAF, %	VAF, means ± SD, %
ESR1 uniplex	D538G	23,742	0.30	
ESR1 uniplex	D538G	23,881	0.30	
ESR1 uniplex	D538G	31,284	0.30	0.32 ± 0.00
Multiplex	D538G	3206	0.20	
Multiplex	D538G	3826	0.40	
Multiplex	D538G	3851	0.50	
Multiplex	D538G	35,892	0.25	0.34 ± 0.14
ESR1 uniplex	Y537C	25,759	38.05	
ESR1 uniplex	Y537C	34,931	40.25	
ESR1 uniplex	Y537C	58,852	35.20	37.83 ± 2.53
Multiplex	Y537C	6540	32.10	
Multiplex	Y537C	6559	32.15	
Multiplex	Y537C	28,689	36.10	33.45 ± 2.30
ESR1 uniplex	Y537N	17,949	14.65	
ESR1 uniplex	Y537N	26,306	13.80	
ESR1 uniplex	Y537N	45,277	13.80	14.08 ± 0.49
Multiplex	Y537N	4806	12.60	
Multiplex	Y537N	4908	12.35	
Multiplex	Y537N	20,837	12.30	12.42 ± 0.16
ESR1 uniplex	Y537S	27,800	4.95	
ESR1 uniplex	Y537S	27,801	4.10	
ESR1 uniplex	Y537S	29,441	4.95	4.67 ± 0.49
Multiplex	Y537S	3951	2.90	
Multiplex	Y537S	4496	5.90	
Multiplex	Y537S	4663	6.00	
Multiplex	Y537S	34,605	3.25	5.05 ± 1.56
ESR1 uniplex	*ESR1*_G_S_C_N	59,887	50.70	
ESR1 uniplex	*ESR1*_G_S_C_N	56,253	49.55	50.13 ± 0.81

dPCR, digital PCR; VAF, variant allele frequency.

multiplex pre-amplification assay. Similar VAFs were observed for 24 of the 26 patients (Table 5). *ESR1* mutations were detected in eight samples pre-amplified using 0.5 μL cfDNA input (7× D538G, 1× Y537C, and 1× Y537N) and in nine samples using 2 μL input (7× D538G, 2× Y537C, and 2× Y537N). According to both pre-amplification protocols, one sample (Sample 9) showed two different mutations (D538G and Y537C). However, after using the multiplex pre-amplification protocol with 2 μL of cfDNA, the presence of an additional mutation in Y537N (6.3% VAF) was identified in Sample 25, which was not detected using the uniplex pre-amplification protocol with 0.5 μL cfDNA input (Table 5). Thus, increasing the cfDNA input for the pre-amplification to 2 μL led to the detection of one additional mutated sample. Because the volume of cfDNA in the pre-amplification can affect downstream analyses, additional tests were performed using higher cfDNA input volumes of 8 to 10 μL plasma (40% to 50% of the cfDNA isolated from 200 μL plasma). The data obtained in these analyses were similar to the data obtained with 2 μL cfDNA (Table 5). More important, no additional mutations were identified at an input of 10 μL, indicating that 2 μL cfDNA input in the pre-amplification was sufficient to identify all four *ESR1* mutations higher than the cutoff level of 1%.

Reproducibility of VAF Estimates after Pre-Amplification

To evaluate the reproducibility of our method to analyze mutations in minute amounts of cfDNA, the four hotspot *ESR1* mutations were measured by performing multiple independent technical replicates using a pool of cfDNA (multisample pool) containing mutated copies of three of our four *ESR1* hotspot mutations. D538G *ESR1* was not included and, thus, served as a negative control. The VAFs for the individual mutations present in the pooled sample were analyzed after independent pre-amplification sessions with both the uniplex and multiplex pre-amplification protocol. The independent replicates resulted in similar VAFs for both the protocols (Table 6). In addition, a frequency of 50.1% ± 0.8% was detected when the *ESR1* dPCR prescreening assay containing all four individual TaqMan assays was used. As observed before, this frequency equals the sum of the VAFs (56.9% after uniplex and 51.2% after multiplex pre-amplification) of the four individual *ESR1* mutations.

Genomic DNA isolated from the m*ESR1*-negative MDA-MB-435s cell line was also fragmented by sonication to obtain a negative control sample with the same characteristics of cfDNA (which is generally fragmented). Measuring

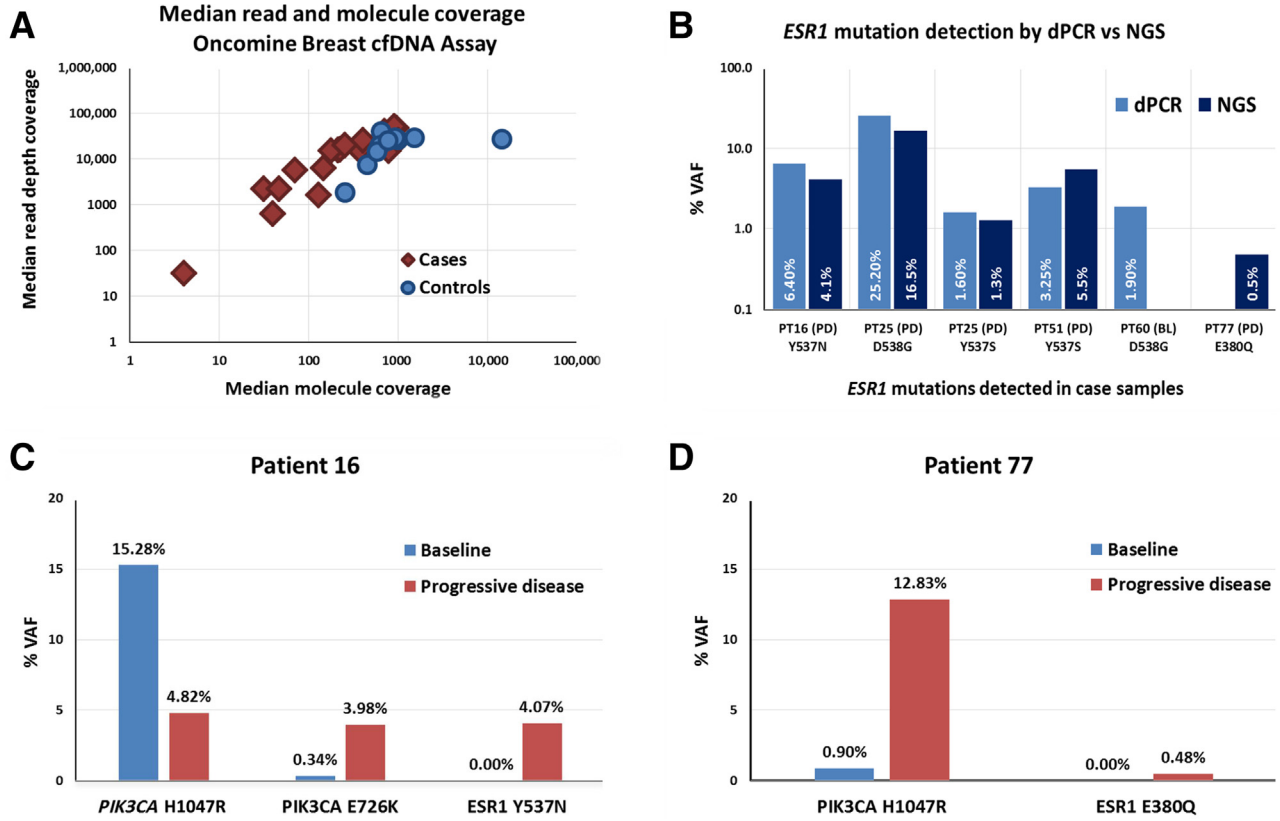

Figure 3 Digital PCR (dPCR) versus targeted next-generation sequencing (NGS) with the Oncomine Breast cfDNA Assay. **A:** The median molecule coverage versus the median read depth coverage of the investigated cases and controls by NGS. Five cases did not pass the cutoff of >100 independent molecules sequenced. **B:** Comparison of *ESR1* mutation status evaluated by dPCR and NGS for five samples for which both dPCR and NGS data were available. Only the *ESR1* D538G, measured in a sample of Case 60 by dPCR [variant allele frequency (VAF), 1.93%], was missed by the NGS approach. The low-frequency E380Q detected by NGS was not tested by dPCR. **C** and **D:** Different *ESR1* and *PIK3CA* mutations detected by NGS in matched baseline (BL) and progressive disease (PD) samples of two cases (Cases 16 and 77).

the *ESR1* mutational status after eight independent pre-amplification experiments with our multiplex pre-amplification protocol showed that, also for this negative control sample, the results were reproducible and compared well with the nonamplified parental cell line (Supplemental Table S5). In summary, the following are results for the parental cells before pre-amplification versus the data obtained after the eight independent multiplex pre-amplifications: D538G, 0.00% versus 0.24% ± 0.15%; Y537C, 0.45% versus 0.17% ± 0.19%; Y537N, 0.47% versus 0.22% ± 0.24%; Y537S, 0.00% versus 0.02% ± 0.03%; and for the screening multiplex dPCR assay, 0.20% versus 0.51% ± 0.22%.

Lower LOD

Now that we optimized the cfDNA input volume for pre-amplification at 2 μL, a cutoff could be set up for assays to assign a patient sample mutated for *ESR1*. The lowest VAF that could distinguish a negative VAF at three times the baseline noise was first established. For D538G, the LOD was calculated to be 0.89% in the multiplex pre-amplification model (Supplemental Table S6). In addition, the four *ESR1* mutations were measured in 18 individual HBDs and the highest VAF + 2.58 SDs was calculated to achieve an LOD with 99% confidence. These analyses resulted in the following cutoffs: 1.02% for D538G, 0.52% for Y537C, 0.87% for Y537N, and 0.97% for Y537S (Supplemental Table S7). For our patient samples, it was, therefore, decided to use a cutoff of at least 1% mutated for any of the hotspot mutations before a patient could be called positively mutated for *ESR1*.

Comparison with NGS

To explore whether targeted mutation analysis by NGS would confirm and/or improve sensitivity to detect circulating tumor DNA by m*ESR1* compared with the digital PCR m*ESR1* workflow, cfDNA samples of seven MBC patients at baseline, while receiving treatment, and/or at progressive disease were evaluated by both techniques.

A total of 17 cfDNA samples from seven MBC patients and 10 cfDNA samples from 10 HBDs as controls were analyzed by means of the Ion Torrent Oncomine Breast cfDNA Assay. The NGS results were defined as successful

in this study when the following occurred: i) samples had a median number of >100 independent molecules sequenced (Figure 3A), ii) variants were detected in at least two independent molecules, and iii) the VAF was higher than the calculated LOD of the NGS assay. No variants were detected in any of the control samples and in 7 of 17 patient samples in the 10 genes analyzed by the targeted Oncomine NGS panel. This failure to detect variants and the low-molecule coverage were explained by cfDNA input amounts of <5 ng in eight case samples (Supplemental Table S4). The remaining nine case samples had a total of 22 variants, with 10 Catalogue Of Somatic Mutations In Cancer (COSMIC)−reported hotspot missense mutations in four patients (44%) and detected in the genes *ESR1* (D538G, Y537N, Y537S, and E380Q), *PIK3CA* (H1047R and E726K), *KRAS* (G12C), and *TP53* (R213L, V274F, and R280T). All variants detected in cases were not present in the controls, although the positions were sequenced at similar or even higher median molecule coverage.

For all the seven MBC patients, the *ESR1* mutational status was investigated by both NGS and dPCR. Although the measured VAFs differed slightly by the two methods, four of the five *ESR1* mutations detected by dPCR were retrieved by NGS (Figure 3B). Only the *ESR1* D538G, measured in a sample of Case 60 by dPCR (VAF, 1.90%), was missed by the NGS approach. The latter may be explained by the differences in cfDNA input (ie, 18 ng of pre-amplified cfDNA as input for dPCR and 4.3 ng of cfDNA as input for NGS). NGS discovered the *ESR1* E380Q mutation, albeit at a low frequency (VAF, 0.48%), in a progressive disease sample of Case 77. However, this mutation was not included in the *ESR1* pre-amplification mix. Finally, Cases 16 (Figure 3C) and 77 (Figure 3D) had, next to the *ESR1* mutation found at progressive disease, also *PIK3CA* mutations detected at baseline and at progressive disease.

ESR1 Mutations in Clinical Cohorts

The optimized workflow was applied on a total of 156 samples of 132 individual patients who received first (67 patients; 81 samples) and/or later lines of endocrine treatment (35 patients; 35 samples) or first-line chemotherapy (37 patients; 40 samples) (Supplemental Table S1).

Table 7 *ESR1* Mutational Status in Endocrine-Treated Patients according to Time of Plasma Sampling

ESR1 mutated at 1% cutoff	Baseline		Progressive disease		*P* value (Fisher's exact test)
	Count	%	Count	%	
No	49	96.1	29	65.9	0.0001
Yes	2	3.9	15	34.1	

The *ESR1* mutational status of patients receiving first-line endocrine therapy for MBC (baseline subset, $n = 51$) was first compared with that of patients who had progressed on endocrine therapy (progressing subset, $n = 44$). The patient characteristics, including endocrine therapies received before the plasma sampling, are summarized in Table 1. In the progressive subset, a higher frequency of cases with *ESR1* mutations (34.1%) was observed compared with the baseline subset (3.9%) (Fisher's exact test $P = 0.0001$) (Table 7).

Next, the *ESR1* mutational status for the chemotherapy and endocrine cohorts was analyzed separately, subdivided in baseline samples taken before start of first-line therapy, samples taken at progressive disease, and samples taken in between these two time points (on-treatment phase; eg, 1 week up to 6 months from the start of any line of therapy). These analyses revealed that, although numbers for the chemotherapy cohort at the on-treatment and progressive disease stage were small, similar percentages of *ESR1* mutated samples were found at baseline in the chemotherapy and endocrine therapy cohorts (Table 8).

To further evaluate the prognostic and predictive value of liquid biopsies, m*ESR1* was analyzed in longitudinally collected cfDNA of 17 endocrine-treated MBC patients (Supplemental Table S8). A minimum of two and up to a maximum of five follow-up samples were available for each patient. Thirteen patients lacked m*ESR1* during follow-up. One patient (Patient 60) had an m*ESR1* (1.90% for D538G) at baseline that was no longer detected at 1 and 9 months after tamoxifen, although an increase (from 0.5 to 1.3 ng/μL) in cfDNA concentration was observed in the first follow-up sample, followed by a decrease down to baseline level 8 months later (Figure 4A). The patient was still alive without progression 2 years later at the last recorded follow-up data. In three additional patients (Patients 16, 25, and 51), an acquired m*ESR1* reflected the putative presence of progressive disease. Patient 16 acquired a Y537N mutation (from 0.02% to 6.39%) after letrozole, and Patient 51 acquired a Y537S mutation (from 0.00% to 3.25%) after tamoxifen, treatment (Supplemental Table S8). Patient 25 received exemestane as first-line therapy. At baseline and in two follow-up samples during the aromatase inhibitor treatment, no m*ESR1* was detected. However, at progression, a high frequency of the D538G mutation (25.16%) and a lower frequency of the Y537N mutation (1.61%) were detected in cfDNA, reflecting disease progression with a likely polyclonal mutation status. Fulvestrant plus dovitinib in the next line after an initial response seemed to induce *ESR1* Y537N (6.26%) and reduce D538G (8.73%), which were detected at disease progression (Figure 4B).

Discussion

cfDNA offers several important advantages for real-time monitoring of a tumor response to therapy, and as a result,

Table 8 *ESR1* Mutational Status according to the Inclusion Cohort

Therapy started after inclusion	*ESR1* mutated at 1% cutoff	Chemotherapy (n = 43)		Endocrine therapy (n = 113)	
		n	%	n	%
Baseline	No	15	34.9	49	43.4
	Yes	1	2.3	2	1.8
On treatment	No	21	48.8	20	17.7
	Yes	1	2.3	0	0.0
Progressive disease	No	4	9.3	27	23.9
	Yes	1	2.3	15	13.3

an increasing number of studies accessing cfDNA through a minimally invasive blood draw have been reported in the past few years.[17,34,35] However, published studies often give remarkable importance to clinical data without providing details about the conditions under which the clinical samples were analyzed. The main purpose of this study was to set up a reliable, easy-to-implement workflow for uniplex and multiplex mutation analyses in cfDNA derived from minute amounts of plasma.

In general, three phases are relevant to establish an assay: the preanalytical, analytical, and postanalytical phases. It is important to realize that all these three phases are prone to errors.[36–38] Although several studies reported a higher time-dependent increase of degenerated lysed white blood cells in EDTA tubes during the preanalytical phase,[39,40] our group previously showed that this increase is not significant up to 24 hours from blood draw.[41] Therefore, to reduce the risk of contamination with DNA derived from white blood cells, the input material was restricted to plasma collected within 24 hours from blood draw. Regarding the analytical phase, several challenges relate to the robust and reproducible analysis of m*ESR1* by dPCR in small amounts of cfDNA derived from cancer patients. To date, several studies have reported that dPCR is a highly sensitive method to detect *ESR1* mutations in cfDNA.[3,5–7,12,18–20] However, cfDNA concentrations and/or total input may affect the mutation detection rate at a low VAF and the possibility to perform multiple analyses on the same sample.[42,43] It is known that cfDNA is highly fragmented (approximately 150 bp) and usually constitutes only a small percentage of total cfDNA (often <1%).[44,45] As a consequence, optimal recovery efficiency and quality of cfDNA are usually only obtained from a sufficient amount of blood plasma (>1 mL).[46–48] For retrospective cohorts, the volume of an available sample is often limited (in this study, sometimes only 200 μL plasma samples were available), yielding a relatively lower amount of cfDNA (in this study, ranging between 2 and 200 ng). The generation of reliable data for multiple variants requires an unbiased pre-amplification step. Recent studies have tested the amplification of small amounts of DNA before dPCR using whole-genome amplification.[49,50] However, the application notes of the manufacturer and our own data suggested that some methods, such as REPLI-g, are not suitable for cfDNA because of their fragmented nature. For this reason, a target-specific pre-amplification step was used to increase the DNA quantity before dPCR. The experiments with different starting volumes of plasma from the same HBD, with or without pre-amplification, clearly confirmed that an input of 200 μL plasma was sufficient for the analyses (Table 3). Nevertheless, despite the claim of the manufacturer (Thermo Fisher) that TaqMan PreAmp Master Mix pre-amplifies small amounts of DNA without introducing amplification bias to the sample, the possibility that the pre-amplification method used may have introduced a bias cannot be ruled out. Data have been reported regarding errors introduced by Taq polymerase (up to 1 to 20 $\times$ 10^{-5} errors/bp per duplication)[51] that may be the source of false-positive wells in the dPCR chip.[50] This might explain the presence of some mutant copies detected in cfDNA from pre-amplified healthy blood donor samples, which were not detected in the matched not pre-amplified sample. Therefore, to prevent false positives, a stringent cutoff value at 1% was used for all of the four m*ESR1*, which was well above the average VAF + 2.58 $\times$ SD measured in 18 individual healthy blood donors.

Besides optimizing the target-specific pre-amplification step to enable mutation-specific analyses in small volumes of plasma, a custom multiplex m*ESR1* screening dPCR approach was also introduced in the workflow. The possibility to detect multiple mutations in parallel has already been demonstrated with droplet digital PCR in combination with, for example, the *KRAS* Screening Multiplex Kit (Biorad, Veenendaal, the Netherlands), which is able to screen for seven *KRAS* mutations.[52–54] However, no such kits are available yet for measuring m*ESR1*. For this study, an *ESR1* multiplex dPCR screening assay for detecting and quantifying the four most common mutations in the ligand-binding domain of the *ESR1* gene was, therefore, successfully developed and validated.

Finally, the outcome of the m*ESR1* workflow was compared with results obtained by targeted NGS covering hotspot mutations in 10 genes. Although the calculated variant allele frequencies were slightly different, four of the five *ESR1* mutations detected by dPCR were also identified by targeted NGS. One *ESR1* D538G mutation with a VAF of 1.9% was, probably because of the suboptimal amount of

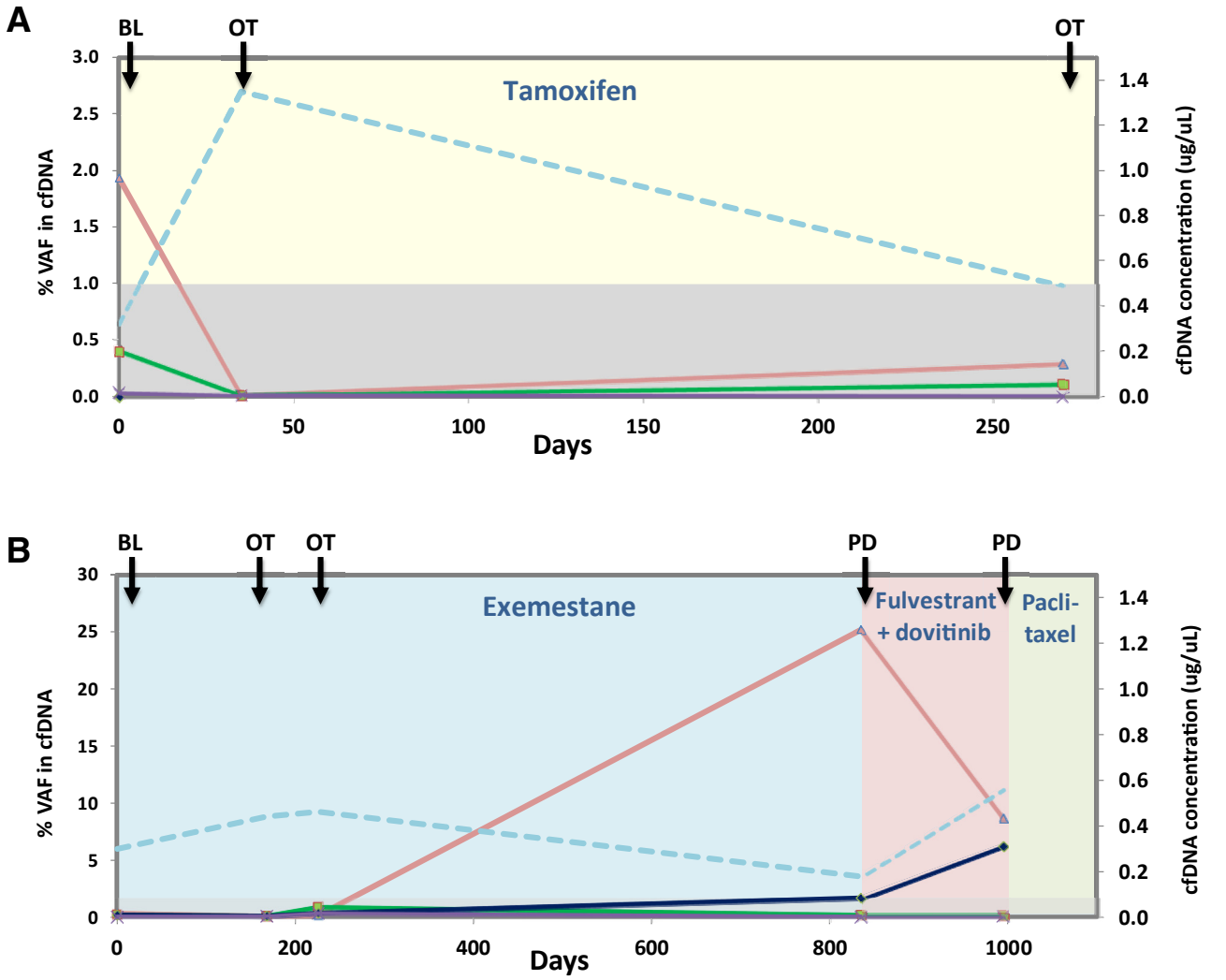

Figure 4 Longitudinal monitoring changes in cell-free DNA (cfDNA) concentration and *ESR1* mutational load. **A** and **B:** Longitudinal monitoring of cfDNA with respect to total concentration and variant allele frequency (VAF) of the four m*ESR1* for two metastatic breast cancer patients (**A**, Patient 60; **B**, Patient 25) monitored in time by digital PCR. Time is expressed as days from diagnosis of metastatic disease to plasma sampling. Details of therapies are indicated by colored shading. The dashed light blue line indicates the total concentration of cfDNA. The colored lines indicate the different m*ESR1*: pink, D538G; green, Y537C; dark blue, Y537N; and purple, Y537S. The gray area indicates the m*ESR1* cutoff at 1% VAF. BL, baseline; OT, on treatment; PD, progressive disease.

DNA available for NGS, only identified by our m*ESR1* dPCR workflow. The advantage of this m*ESR1* dPCR workflow is that far less starting material is required (down to 0.1 ng cfDNA versus at least 5 ng cfDNA for the targeted NGS).

Next, the optimized workflow was used to investigate whether a correlation between the presence of m*ESR1* and treatment existed within two retrospectively obtained patient cohorts that received chemotherapy or endocrine therapy for MBC. Although *ESR1* mutations are rarely detected in primary BC,[10,55] they are known to be enriched in metastatic lesions of patients receiving endocrine therapy.[3,6] These data are in line with these studies showing an enrichment of m*ESR1* in MBC patients treated with endocrine therapy for MBC. Although, to date, no direct evidence exists for the presence of these mutations in patients receiving chemotherapy, m*ESR1* was detected in three different patients who received chemotherapy and who had never received endocrine therapy, including one patient with an ER-positive primary tumor that expressed 8.2% VAF for *ESR1* Y537S in the cfDNA collected at progressive disease. Perhaps unexpected, the primary tumor of only two of these three chemotherapy-treated patients with mutated m*ERS1* copies was ER positive, according to the pathology reports. Besides tumor heterogeneity and sampling bias, this finding might be explained by intrinsic tumor genetic instability that allows cancer cells to develop mutations under treatment pressure or by the presence of the mutations already in the primary tumor, as previously reported by Wang et al.[5] Furthermore, and as we already reported before,[8] although most of the m*ESR1*-positive patients who progressed on endocrine therapy received aromatase

inhibitor, fulvestrant, or a combination of the two, four of the patients with mutated *ESR1* copies at progressive disease received tamoxifen single-agent therapy. These data, thus, indicate that the emergence of m*ESR1* copies is not necessarily restricted to one specific type of therapy. Nevertheless, knowing the m*ESR1* status may help patients by offering m*ESR1*-positive patients a different line of endocrine therapy to lengthen the progression-free survival time. Whether this should be an ER-degrading compound in combination with, for example, a cyclin-dependent kinase 4/6 inhibitor, in line with the PALOMA-3 study,[12] and if this results in a decrease in m*ESR1* remain to be seen in confirmatory studies.

In conclusion, we established a dPCR workflow to assess m*ESR1* in a limited amount of cfDNA, which was compared with targeted NGS. This workflow was successfully used to investigate the *ESR1* mutational status in a retrospectively collected cohort of MBC patients, treated with either endocrine therapy or chemotherapy, and showed a clear enrichment of m*ESR1* in samples of patients who progressed on endocrine treatment.

Acknowledgments

S.R.V., A.M.S., N.B., and M.P.H.M.J. designed the study; S.R.V., A.M.S., J.K., N.M.V., and R.v.M. performed experiments; L.Y.D., P.H., F.E.d.J., and A.J. included patients in the clinical study for this analysis; N.B., L.A., B.M., and E.A.R. collected the clinical data; S.R.V. and A.M.S. analyzed the data and compiled statistics; A.M.S., J.A.F., S.S., M.P.H.M.J., P.V., and J.W.M.M. supervised and approved the study; S.R.V. and A.M.S. wrote the manuscript; all authors reviewed, edited, and approved the manuscript.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2018.08.010*.

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