

# ***ESR1* Methylation: A Liquid Biopsy–Based Epigenetic Assay for the Follow-up of Patients with Metastatic Breast Cancer Receiving Endocrine Treatment**

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## **Abstract**

**Purpose:** Liquid biopsy provides real-time monitoring of tumor evolution and response to therapy through analysis of circulating tumor cells (CTCs) and plasma-circulating tumor DNA (ctDNA). *ESR1* epigenetic silencing potentially affects response to endocrine treatment. We evaluated *ESR1* methylation in CTCs and paired plasma ctDNA. We evaluated *ESR1* methylation in CTCs and paired plasma ctDNA as a potential biomarker for response to everolimus/exemestane treatment.

**Experimental Design:** A highly sensitive and specific real-time MSP assay for *ESR1* methylation was developed and validated in (i) 65 primary breast tumors formalin-fixed paraffin-embedded (FFPE), (ii) EpCAM<sup>+</sup> CTC fractions (122 patients and 30 healthy donors; HD), (iii) plasma ctDNA (108 patients and 30HD), and (iv) in CTCs (CellSearch) and in paired plasma ctDNA for 58 patients with breast cancer. *ESR1* methylation status was investigated in CTCs isolated from serial peripheral blood samples of

19 patients with ER<sup>+</sup>/HER2<sup>-</sup> advanced breast cancer receiving everolimus/exemestane.

**Results:** *ESR1* methylation was detected in: (i) 25/65 (38.5%) FFPEs, (ii) EpCAM<sup>+</sup> CTC fractions: 26/112 (23.3%) patients and 1/30 (3.3%) HD, and (iii) plasma ctDNA: 8/108 (7.4%) patients and 1/30 (3.3%) HD. *ESR1* methylation was highly concordant in 58 paired DNA samples, isolated from CTCs (CellSearch) and corresponding plasma. In serial peripheral blood samples of patients treated with everolimus/exemestane, *ESR1* methylation was observed in 10/36 (27.8%) CTC-positive samples, and was associated with lack of response to treatment ( $P = 0.023$ , Fisher exact test).

**Conclusions:** We report for the first time the detection of *ESR1* methylation in CTCs and a high concordance with paired plasma ctDNA. *ESR1* methylation in CTCs was associated with lack of response to everolimus/exemestane regimen. *ESR1* methylation should be further evaluated as a potential liquid biopsy-based biomarker. *Clin Cancer Res*; 24(6); 1500–10. ©2017 AACR.

## **Introduction**

Targeted therapies have remarkably changed the treatment of cancer over the last decade. However, almost all tumors acquire resistance to systemic treatment as a result of tumor heterogeneity, clonal evolution, and selection. Especially in breast cancer, our understanding of the molecular underpinnings of hormone-receptor-positive (HR<sup>+</sup>) breast cancer has led to new therapies that have substantially improved patient outcomes. However, endocrine-resistant disease still remains a leading cause of breast cancer mortality. Novel findings based on an integrated analysis of primary tumors and metastatic sites have revealed the tremen-

dous tumor heterogeneity in breast cancer, and moreover, the evolution of tumor that can occur during acquired resistance to systemic therapies (1).

"Liquid biopsy," based on the analysis of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in peripheral blood of cancer patients, provides noninvasive real-time monitoring of tumor evolution and therapeutic efficacy, with the potential to improve cancer diagnosis and treatment and has received enormous attention because of its obvious clinical implications for personalized medicine (2).

CTC analysis presents nowadays a promising field for both advanced- and early-stage patients, and their molecular characterization offers the unique potential to understand better the biology of metastasis and resistance to established therapies (3). In parallel, ctDNA analysis in plasma of patients with breast cancer enables the identification of actionable genomic alterations, monitoring of treatment responses, unraveling therapeutic resistance, and potentially detecting disease progression before clinical confirmation (4). CTCs are heterogeneous and rare, and the amount of available sample for their analysis is very limited. The clinical significance of CTCs has been evaluated in many tumor types (5, 6). CellSearch is the only system cleared by the FDA for breast cancer (7), because using this system, the number of CTCs has been associated with progression-free survival (PFS) and overall survival (OS) in metastatic (7, 8) and early (9, 10) disease.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

Epigenetic silencing of estrogen receptor could affect endocrine treatment efficacy in patients with breast cancer. "Liquid biopsy," based on the analysis of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in peripheral blood of patients with cancer, provides noninvasive real-time monitoring of tumor evolution and therapeutic efficacy, with the potential to improve cancer diagnosis and treatment. In this study, we evaluated for the first time *ESR1* methylation in CTCs, paired ctDNA, and primary tumors of patients with breast cancer. We report that *ESR1* methylation in CTCs and paired plasma ctDNA is highly concordant. *ESR1* methylation in CTCs was associated with lack of response to everolimus/exemestane. *ESR1* methylation should be further evaluated as a potential liquid biopsy-based biomarker for the follow-up of patients with breast cancer treated with the everolimus/exemestane combination.

Our group using highly sensitive RNA-based molecular assays was among the first to show the clinical significance of CTC detection in early breast cancer (11, 12). Moreover, nowadays qualitative and quantitative analysis of ctDNA is now successfully utilized to assess tumor progression and evaluate prognosis, diagnosis, and response to treatment in many types of cancer (13, 14).

Epigenetic changes are very important in cancer development, because tumor and metastasis suppressor genes can be silenced through DNA methylation of their promoters (15, 16) and affect virtually every step in tumor progression (17). Epigenetic modifications precede genetic changes and usually occur at an early stage in cancer development. Recent technological advances offer a better understanding of the underlying epigenetic alterations during carcinogenesis and provide insight into the discovery of putative epigenetic biomarkers for diagnosis, prognosis, risk assessment, and disease monitoring (18).

Analysis of DNA methylation in CTCs can give important information on the molecular and biological nature of these cells. Epigenetic silencing of tumor and metastasis suppressor genes plays a key role in the survival and regulation of their metastatic potential (19, 20). Our group was the first to demonstrate epigenetic alterations in CTCs (20) and corresponding ctDNA (21, 22). The methylation status of tumor and metastasis suppressor genes in CTCs and ctDNA is a very promising approach, because the starting material is accessed noninvasively, and very low percentages of DNA methylation are detected in healthy donors (20–22).

Adjuvant endocrine therapy, which aims to inhibit estrogen receptor (ER) signaling, represents an effective treatment for an important percentage of patients with ER  $\alpha$ -positive breast cancers. The unique transcriptional response to estrogens in each tissue-specific cell subtype is, in part, regulated by the epigenome. Differential DNA methylation and chromatin remodeling serve to dictate accessibility to functional, estrogen responsive regions of the genome, and thus define endocrine response. Inappropriate activation of the *ESR1* signaling network in mammary epithelial cells initiates oncogenic transformation and drives *ESR1*-positive breast cancer (23). However, resistance to hormone therapy occurs in some cases and often reflects a change in ER status, in

these patients. Recent data implicate that in ER-positive tumors, CpG methylation levels are inversely correlated with ER expression status, suggesting that single CpG site plays an important role in the regulation of ER transcription (24). Many studies are focused on *ESR1* methylation in various cancer types (25–29), but only a limited number proves a clinical relevance in terms of diagnosis (30), prognosis (31), and response to therapy (32).

In breast cancer, silencing of the *ESR1* gene due to *ESR1* methylation has an important role on protein expression, while *ESR1* methylation in peripheral blood is significantly correlated with lack of ER expression in excised tumor tissue (33). As such, evaluation of *ESR1* methylation may add prognostic value in identifying luminal phenotypes with poor prognosis and patients with potentially greater resistance to hormonal treatment (33).

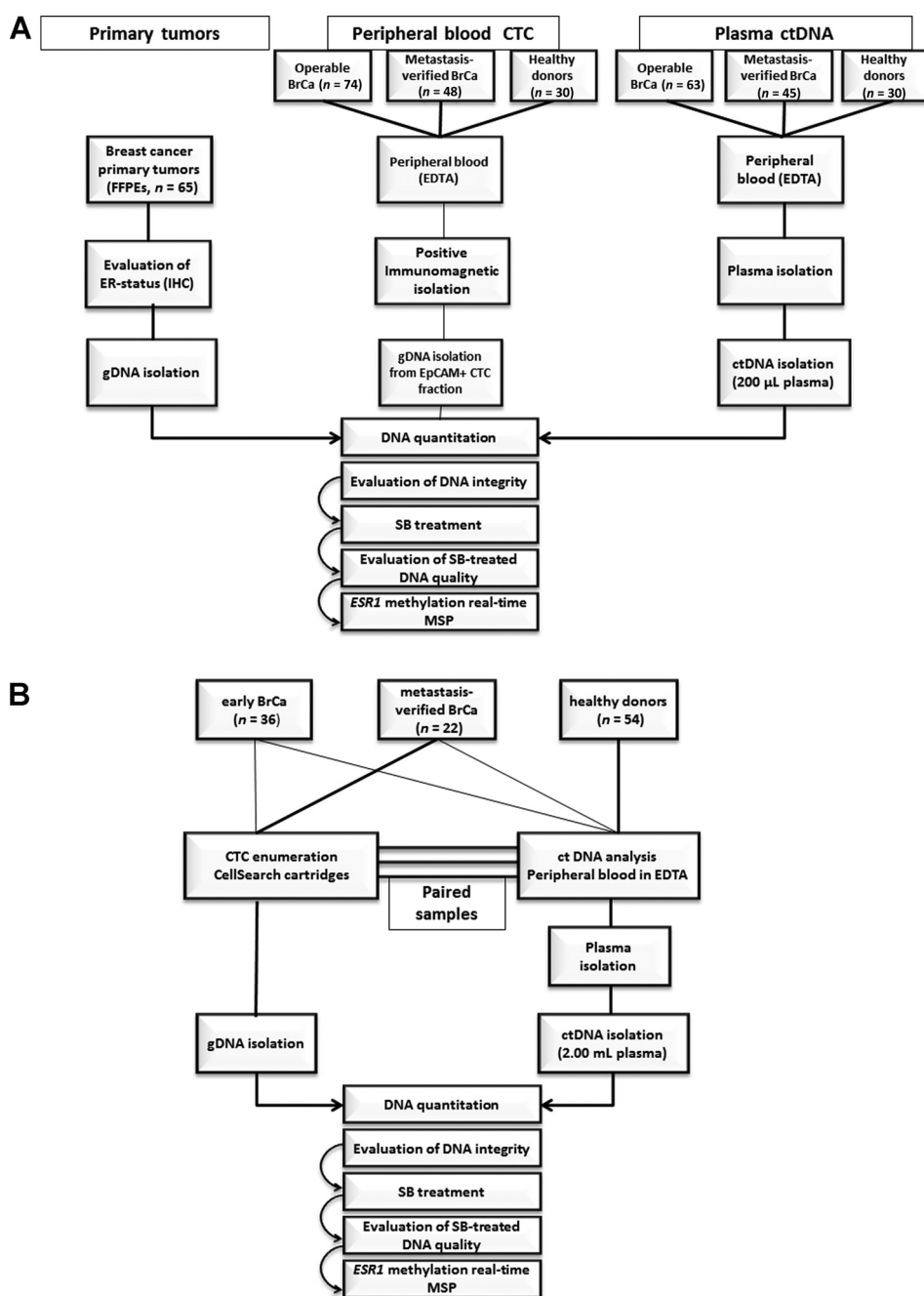
In this study, we report for the first time the presence of *ESR1* methylation in CTCs and paired ctDNA in patients with early and metastatic breast cancer. ER expression and *ESR1* methylation were found to be inversely correlated in primary breast tumors and *ESR1* methylation status in plasma ctDNA was highly concordant with *ESR1* methylation in CTCs. We further evaluated the clinical utility of *ESR1* methylation in CTCs isolated from serial peripheral blood samples of 19 patients with ER<sup>+</sup>/HER2<sup>-</sup> advanced breast cancer treated with the combination everolimus/exemestane. We report for the first time that *ESR1* methylation in CTCs is strongly associated with lack of response to everolimus/exemestane regimen.

## Materials and Methods

### Clinical samples

We used two different patient sample groups for the validation of the developed *ESR1* methylation assay: a training group, and an independent group. Formalin-fixed paraffin-embedded (FFPE) tissues from 65 patients with breast cancer were obtained from the Departments of Medical Oncology, University General Hospital of Heraklion, Crete and the "Attikon" University General Hospital of Athens. Peripheral blood (20 mL in EDTA) was obtained from 122 patients with breast cancer (74 with early and 48 with metastatic disease) and 30 HD (control group), and EpCAM<sup>+</sup> CTC fractions were isolated as previously described (20). ctDNA was isolated from plasma (200  $\mu$ L) of 63 and 45 patients with early and metastatic disease, respectively, and 30 HD. These samples were used as a training group (Fig. 1A). CTC enumeration using the CellSearch system (Menarini) was performed for 36 and 22 patients with early and metastatic disease before treatment initiation, and for all these patients, ctDNA was isolated from paired plasma using the same blood draw (2 mL); 54 HD were used as control group for ctDNA analysis. These samples were used as an independent group (Fig. 1B).

The group used for the clinical relevance of *ESR1* methylation in liquid biopsy samples comprised 19 metastatic patients with ER<sup>+</sup>/HER2<sup>-</sup> tumors; in this group, all patients had detectable CTCs, according to the CellSearch assay and received treatment with the everolimus/exemestane combination in the context of standard treatment. All these patients had received Tamoxifen as adjuvant therapy and/or letrozole as first-line therapy. Patients who achieved a partial response (PR) or stable disease (SD) as best response were classified as responders, and patients who experienced progressive disease (PD) were characterized as nonresponders. Serial peripheral blood samples were collected from patients prior to each cycle of treatment, and CTCs were isolated and

**Figure 1.**

Experimental flowchart of the study: **A**, Training group: *ESR1* methylation in primary breast tumors, CTCs, and ctDNA; **B**, Independent group: *ESR1* methylation in paired CTCs and ctDNA.

enumerated with the CellSearch system. Genomic DNA (gDNA) was extracted from the isolated CTCs from CellSearch cartridges, processed with sodium bisulfite (SB) treatment and samples were subsequently analyzed for *ESR1* methylation status using real-time MSP. On average, three samples were collected from each patient at three different time points (before treatment initiation, after one treatment cycle, and at relapse; a total of 58 samples were analyzed).

The study was conducted in accordance with the 1964 Declaration of Helsinki and was approved by the ethics and scientific committees of the participating institutions. All participating

patients gave their signed informed consent in order to participate in the study.

#### Sample preparation

To avoid contamination, different rooms, dedicated labware, and dedicated areas were used for all procedures. All DNA preparation and handling steps took place in specific laminar-flow hoods under DNase-free conditions. DNA concentration in all cases was measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific); isolated gDNA and SB-converted DNA samples were stored at  $-70^{\circ}\text{C}$  until further use. The analytical

performance of the developed *ESR1* real-time MSP assay was evaluated using the breast cancer cell lines MDA-MB-231, MCF7, and SKBR3.

**gDNA isolation from FFPEs.** FFPE tissue sections of 10 mm containing >80% of tumor cells were used for DNA extraction. gDNA was isolated with the QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's protocol.

**gDNA isolation from EpCAM<sup>±</sup> CTCs and cell lines.** gDNA was extracted from the EpCAM<sup>+</sup> CTC fractions and cell lines using the Trizol reagent as previously described (20, 22). Isolated gDNA was dissolved in 50  $\mu$ L of 8 mmol/L NaOH.

**ctDNA isolation from plasma.** Whole blood samples were collected into venous blood collection tubes using EDTA. Samples were mixed thoroughly and plasma was isolated within 2 to 4 hours from sample collection by centrifugation at  $530 \times g$  for 10 minutes at room temperature. Once isolated, plasma samples were centrifuged again at  $2,000 \times g$  for 10 minutes, before transferring into clean 2-mL tubes and freezing at  $-70^\circ\text{C}$  until time of processing. The High Pure Viral Nucleic Acid Kit (Roche Diagnostics) was used to extract ctDNA from 200  $\mu$ L of plasma, and the QIAamp Circulating Nucleic Acid Kit (Qiagen) was used to isolate ctDNA from 2 mL of plasma.

**Isolation of gDNA from CellSearch cartridges.** CellSearch analysis was performed according to the manufacturer's instructions; in the group of patients with metastatic disease, 7.5 mL of PB were used for each patient, while in the early breast cancer group, three CellSave tubes were used for each patient (22.5 mL of PB). Following CTC analysis, CellSearch cartridges were stored in a dark place at  $4^\circ\text{C}$  until gDNA isolation. CTCs and WBCs (prestained with antibody to CD45, pan-CK, and DAPI) were aspirated from the CellSearch cartridge and underwent downstream gDNA extraction using the QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's instructions.

#### SB treatment

Quality control checks were performed in all steps prior to sample analysis (Supplementary Fig. S1). Before proceeding to the SB treatment and real-time MSP, we assessed the gDNA integrity of all samples by amplifying the *PIK3CA* exon 20 (34). Only samples that were positive for *PIK3CA* exon 20 amplification were further processed to SB treatment (Supplementary Fig. S1A). gDNA samples were modified with SB, to convert all nonmethylated cytosines to uracil, while methylated cytosines were not converted, using the EZ DNA Methylation Gold Kit (ZYMO Research). Converted DNA was stored at  $-70^\circ\text{C}$  until use. In each SB reaction, dH<sub>2</sub>O and 100% methylated DNA were included as negative and positive control, respectively. Converted DNA from each cancer cell line was used for the evaluation of assay performance, prior to the analysis of clinical samples. The quality of SB-treated DNA was checked by a real-time PCR assay for  $\beta$ -actin (*ACTB*). The human placental gDNA (Sigma-Aldrich) was used as a negative real-time MSP control after SB treatment. The Universal Methylated Human DNA Standard (ZYMO Research) was used as fully methylated (100%) positive control (Supplementary Fig. S1B).

#### *In silico* design of primers for *ESR1* real-time MSP

We designed *in silico* primers for *ESR1* MSP using Primer Premier 5.00 software (Premier Biosoft) avoiding the formation of stable hairpin structures, primer dimers, cross dimers, and false priming sites. For maximal discrimination between methylated and nonmethylated alleles, both primers contained several CpGs. Additionally, both primers contained T bases derived from modified nonmethylated C regions to allow discrimination and amplification of the converted from the unconverted DNA. To verify that we could specifically detect only the methylated sequence, we used six different controls: gDNA not submitted to SB conversion (unconverted DNA), placental DNA submitted to SB conversion (placental converted DNA, 0% methylated), DNA derived from MDA-MB-231, MCF7, and SKBR3 cell lines and the Universal Methylated Human DNA Standard (ZYMO Research). Primers were synthesized by Integrated DNA Technologies (IDT). Primer sequences are given upon request.

#### Real-time MSP

Each reaction was performed in the LightCycler 2.0 (Roche) in glass capillaries in a total volume of 10  $\mu$ L. One microliter of SB-converted DNA was added to 9- $\mu$ L reaction mixture containing 0.05 U/ $\mu$ L GoTaq Hot Start Polymerase (Promega),  $0.1 \times$  of the supplied PCR buffer, 2 mmol/L of MgCl<sub>2</sub>, 0.15 mmol/L of each dNTP (Fermentas), 0.3  $\mu$ g/ $\mu$ L BSA, 0.2  $\mu$ mol/L of the forward and reverse primers, and  $1 \times$  LC-Green Plus Dye (Idaho Technology). Finally, deionized water was added to a final volume of 10  $\mu$ L. Real-time MSP protocol began with one cycle at  $95^\circ\text{C}$  for 2 minutes followed by 45 cycles of:  $95^\circ\text{C}$  for 10 seconds,  $63^\circ\text{C}$  for 20 seconds, and  $72^\circ\text{C}$  for 20 seconds. Immediately after amplification, a rapid cooling cycle to  $40^\circ\text{C}$  for 30 seconds was introduced in order to prepare the melting curve acquisition step. Real-time fluorescence acquisition was set at the elongation step ( $72^\circ\text{C}$ ). The following melting curve analysis included the steps of  $55^\circ\text{C}$  for 10 seconds,  $92^\circ\text{C}$  for 0 seconds with a ramp rate of  $0.2^\circ\text{C}/\text{s}$  (acquisition mode: continuous),  $92^\circ\text{C}$  for 1 minutes and  $40^\circ\text{C}$  for 1 minutes.

The developed MSP assay is not quantitative, so we do not use a cutoff. We report the sample as methylation positive, when we detect an MSP amplification signal and we report as methylation-negative samples only samples that are not amplified.

The analytical performance of the developed *ESR1* real-time MSP assay was evaluated using the breast cancer cell lines MDA-MB-231, MCF7, and SKBR3.

#### Statistical analysis

We used the  $\chi^2$  test of independence for data analysis and for the evaluation of the significance of differences between groups. The evaluation of agreement between ER expression (IHC) and *ESR1* methylation in primary breast tumors, CTCs, ctDNA and in paired samples of ctDNA and CTCs was assessed by using the Fisher exact test. The same statistical test was used in order to correlate *ESR1* methylation status in CTCs of patients with HR<sup>+</sup> advanced breast cancer with response to everolimus/exemestane treatment. PFS and OS curves were calculated by using the Kaplan–Meier method and comparisons were performed using the log-rank test. Finally, univariate Cox regression analysis was performed to identify the risk of progression and death in CTC-positive patients. *P* values < 0.05 were considered statistically significant. Statistical analysis was performed by using the SPSS Statistics, version 23.0 (SPSS Inc.).

## Results

### Development and analytical validation of the *ESR1* real-time MSP assay

**Optimization of experimental conditions.** The experimental conditions of real-time MSP were first optimized in detail for the annealing temperature and time, then for the optimum concentrations for the primer pair and finally for buffer, MgCl<sub>2</sub>, dNTPs, and BSA concentrations (data not shown).

**Analytical specificity.** To evaluate the analytical specificity of the *ESR1* real-time MSP, the primers were, initially, tested *in silico* and then in PCR, using gDNA (unconverted DNA) and SB-modified human placental gDNA samples that were not methylated; no amplification of the *ESR1* was observed. In contrast, amplification was observed only when SB-treated DNA from the MDA-MB-231 cell line and 100% methylated standard were used. The developed assay is highly specific because it can detect only SB-treated methylated sequences. No amplification was observed when DNA isolated from MCF7 and SKBR3 cell lines was used (Supplementary Fig. S2A).

**Analytical sensitivity.** The analytical sensitivity of the developed real-time MSP was evaluated by using synthetic mixtures based on serial dilutions of SB-converted DNA control samples (0% and 100% methylated) at various percentages of methylation (0.1%, 1%, 10%, and 50%). The developed real-time MSP assay for *ESR1* methylation could specifically and reliably detect the presence of 1% methylated *ESR1* sequences in the presence of 99% non-methylated *ESR1* sequences (Supplementary Fig. S2B).

### Training group: evaluation of the *ESR1* real-time MSP assay performance in primary tumors, EpCAM<sup>+</sup> CTC fractions and ctDNA

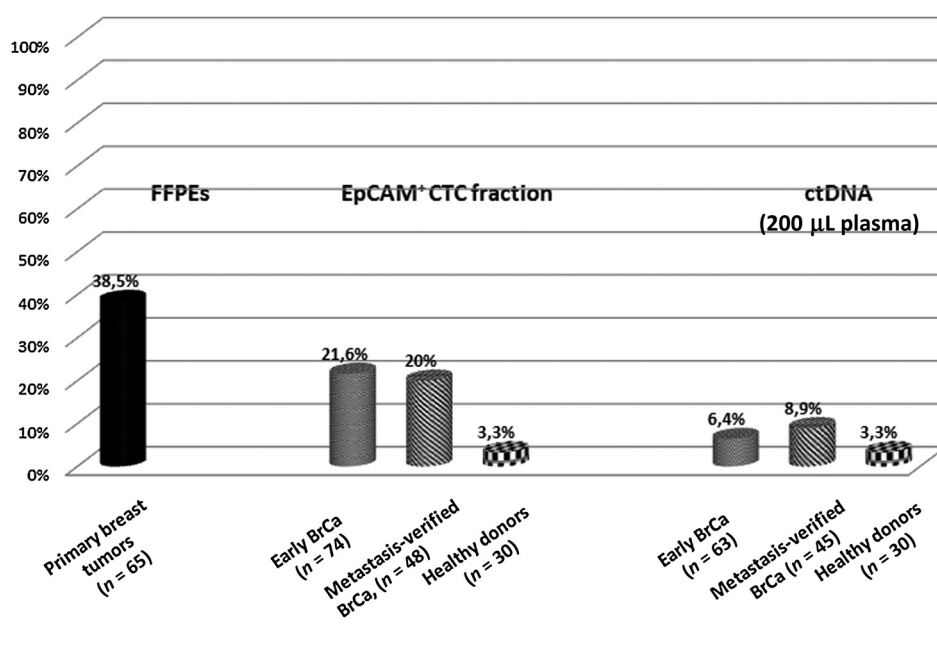
***ESR1* methylation in primary breast tumors (FFPEs).** Initially, we evaluated the DNA methylation status of *ESR1* in primary breast tumors (FFPEs). Methylation of *ESR1* was observed in 25/65 (38.5%) samples (Fig. 2). The *ESR1* methylation status was

further compared with the tumoral ER expression as assessed by IHC. There was a significantly negative correlation between *ESR1* methylation and ER expression in the primary tumor ( $P < 0.001$ , Fisher exact test) in 56/65 (86%) samples (Table 1).

***ESR1* methylation in EpCAM<sup>+</sup> CTC fractions.** The *ESR1* methylation status was subsequently evaluated in 122 EpCAM<sup>+</sup> CTC fractions isolated from peripheral blood of patients with breast cancer. Methylation of *ESR1* was observed in 26/112 (23.3%) patients with breast cancer [in 16/74 (21.6%) and in 10/48 (20%) patients with early and metastatic breast cancer, respectively, but only in 1/30 (3.3%) HD (Fig. 2)].

***ESR1* methylation in plasma ctDNA.** The DNA methylation status of *ESR1* was further examined in 108 plasma ctDNA samples (63 patients with early breast cancer and 45 patients with metastatic disease). *ESR1* methylation was detected in 8/108 (7.4%) patients [in 4/63 (6.3%) and in 4/45 (8.9%) early and metastatic breast cancer samples, respectively, and in 1/30 (3.3%) HD (Fig. 2)].

**Independent group: *ESR1* methylation in CTCs and paired plasma ctDNA.** The independent group consisted of 58 paired samples from CTC and plasma ctDNA. These samples were all isolated on the same day, using the same blood draw and were all processed in the same way minimizing all differences due to potential pre-analytical variations (Fig. 1B). CTC analysis for all these samples was performed using the CellSearch platform. For this reason, the *ESR1* methylation status was evaluated in all these 58 SB-treated gDNA samples isolated from CellSearch cartridges, irrespective of the detected CTC count (Fig. 3A). The *ESR1* methylation status was evaluated in all these 58 SB-treated gDNA samples isolated from CellSearch cartridges (Fig. 3A). This group consisted of: (i) 36 samples of patients with early breast cancer for which more than one CTC was detected in 13/36 (36.1%) and (ii) 22 samples from patients with metastatic disease for which more than five CTCs were detected in 7/22 (31.8%) of them. Interestingly, *ESR1* methylation was detected only in the CTC-positive samples, but in none of the CTC-negative samples. More specifically, *ESR1*



**Figure 2.** Training group: *ESR1* methylation in primary tumors, EpCAM<sup>+</sup> CTC fractions, plasma ctDNA, and healthy donors.

**Table 1.** Comparison of ER expression (IHC) and *ESR1* methylation in primary breast tumors, CTCs, ctDNA and in paired samples of ctDNA and gDNA isolated directly from CellSearch cartridges in patients with early and metastatic breast cancer

<b>Training group: Comparison of ER expression (IHC) and <i>ESR1</i> methylation in primary breast tumors</b>				
		<i>ESR1</i> methylation		Total
		METH	UNMETH	
ER expression (IHC)	ER <sup>-</sup>	18	2	20
	ER <sup>+</sup>	7	38	45
Total		25	40	65
Concordance: 56/65 (86.2%; $P < 0.001$ , Fisher exact test)				
<b>Comparison of ER expression (IHC) in the primary tumors and <i>ESR1</i> methylation in CTCs (gDNA isolated directly from CellSearch cartridges)</b>				
		<i>ESR1</i> methylation		Total
		METH	UNMETH	
ER expression (IHC)	ER <sup>-</sup>	1	7	8
	ER <sup>+</sup>	14	34	48
Total		15	41	56
Concordance: 35/56 (62.5%; $P = 0.428$ , Fisher exact test)				
<b>Comparison of ER expression (IHC) in the primary tumors and <i>ESR1</i> methylation in matched ctDNA</b>				
		<i>ESR1</i> methylation		Total
		METH	UNMETH	
ER expression (IHC)	ER <sup>-</sup>	1	18	19
	ER <sup>+</sup>	5	34	39
		6	52	58
Concordance: 35/58 (60.3%; $P = 0.653$ , Fisher exact test)				
<b>Independent group: Comparison of <i>ESR1</i> methylation in paired samples of ctDNA and gDNA isolated directly from CellSearch cartridges in early breast cancer (<math>n = 36</math>)</b>				
		<i>ESR1</i> methylation (CTCs)		Total
		-	+	
<i>ESR1</i> methylation (ctDNA)	-	33	0	33
	+	0	3	3
Total		33	3	36
Concordance: 36/36 (100%; $P < 0.001$ , $\chi^2$ test)				
<b>Independent group: Comparison of <i>ESR1</i> methylation in paired samples of ctDNA and gDNA isolated directly from CellSearch cartridges in metastatic breast cancer (<math>n = 22</math>)</b>				
		<i>ESR1</i> methylation (CTCs)		Total
		-	+	
<i>ESR1</i> methylation (ctDNA)	-	19	0	19
	+	1	2	3
Total		20	2	22
Concordance: 21/22 (95.4%; $P < 0.001$ , $\chi^2$ test)				

methylation was detected in 3/13 (23.1%) CTC-positive samples from patients with early breast cancer (Fig. 3B) and in 2/7 (28.6%) CTC-positive samples from patients with metastatic disease (Fig. 3C).

Subsequently, the *ESR1* methylation status was evaluated in corresponding plasma ctDNA samples of the same patients and 54 HD. *ESR1* methylation was observed in 3/36 (8.3%) early breast cancer samples, in 3/22 (13.6%) samples of patients with metastatic disease and in 2/54 (3.7%) HD (Fig. 3B and C).

*ESR1* methylation was highly correlated in ctDNA and CTCs samples (Table 1). There was an almost perfect agreement in findings for 57/58 (98.3%) patients between CTCs and ctDNA. In samples positive for CTCs, *ESR1* was found methylated in 3/13 (23.1%) and in 2/7 (28.6%) patients with early and metastatic breast cancer, respectively; the assessment of agreement between *ESR1* methylation in CTCs and matched ctDNA samples revealed that in 36/36 (100%) patients with early breast cancer, and in 21/22 (95.5%) patients with metastatic breast cancer, *ESR1* methylation status was concordant between CTCs and ctDNA ( $P < 0.001$  in both cases). These data are consistent with our previous findings suggesting a common origin between ctDNA and CTCs (22).

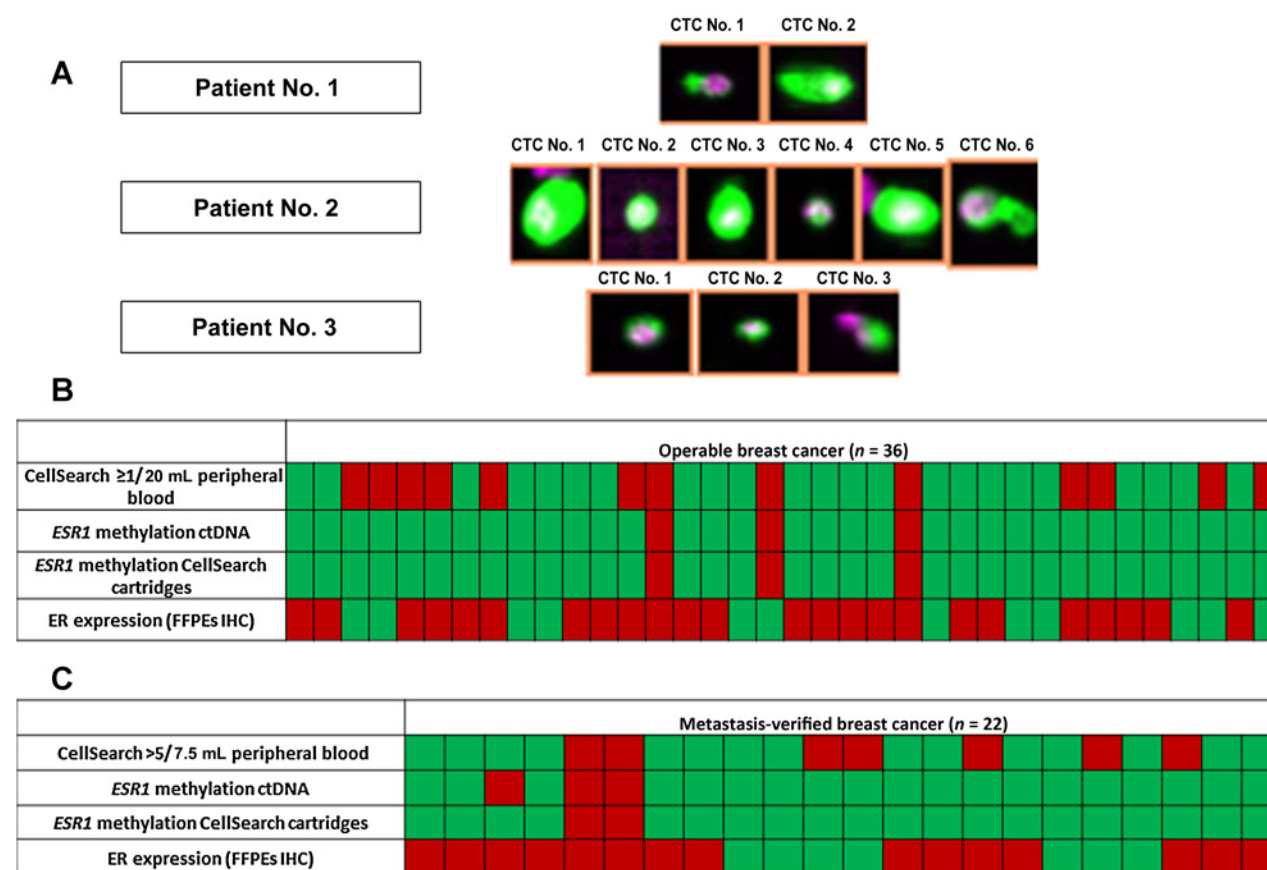
We further compared *ESR1* methylation in CTCs and ctDNA with the ER status of the primary cancers in these patients (Table 1). ER status in the primary tumor was negatively corre-

lated with *ESR1* methylation in CTCs in 35/56 (62.5%) cases while for ctDNA in 35/58 (60.3%) cases.

#### Clinical evaluation of *ESR1* methylation in CTCs of patients with ER<sup>+</sup>/HER2<sup>-</sup> advanced breast cancer under endocrine treatment

The clinical relevance of *ESR1* methylation in CTCs was further evaluated in a selected group of 19 patients with breast cancer with detectable CTCs before treatment initiation, as defined using the CellSearch assay treated with the everolimus/exemestane combination. gDNA was extracted from the isolated CTCs from CellSearch cartridges at the predefined time points and *ESR1* methylation status was evaluated. In average, at least three samples were collected from each patient. More than 5 CTCs/7.5 mL PB were identified in 36/58 (62.1%) of the analyzed samples; *ESR1* methylation was observed in 10/36 (27.8%) CTC-positive blood samples whereas in all CTC-negative samples *ESR1* methylation was not detected (Fig. 4A).

The presence of *ESR1* methylation in CTCs was subsequently correlated with the response to everolimus/exemestane regimen. Ten out of 19 (52.6%) patients achieved a PR or SD as best response and were classified as responders, and 9/19 (47.4%) patients experienced PD and were characterized as nonresponders. Nine patients harbored *ESR1* methylation in CTCs, and two of them (22.2%) achieved a PR or SD, whereas seven (77.8%)

**Figure 3.**

Independent group: **A**, CellSearch images for CTCs found in three different patients; **B**, heatmap of *ESR1* methylation in CTCs matched to ctDNA samples and ER expression in primary tumors of patients with early breast cancer, and **C**, heatmap of *ESR1* methylation in CTCs matched to ctDNA samples and ER expression in primary tumors of patients with metastasis.

experienced PD. Respectively, among the 10 patients with CTCs negative for *ESR1* methylation, eight (80%) were considered as responders, whereas two (20%) were nonresponders (Fig. 4B). According to these results, *ESR1* methylation status in CTCs of patients with HR+ advanced breast cancer is associated with response to everolimus/exemestane treatment ( $P = 0.023$ , Fisher exact test).

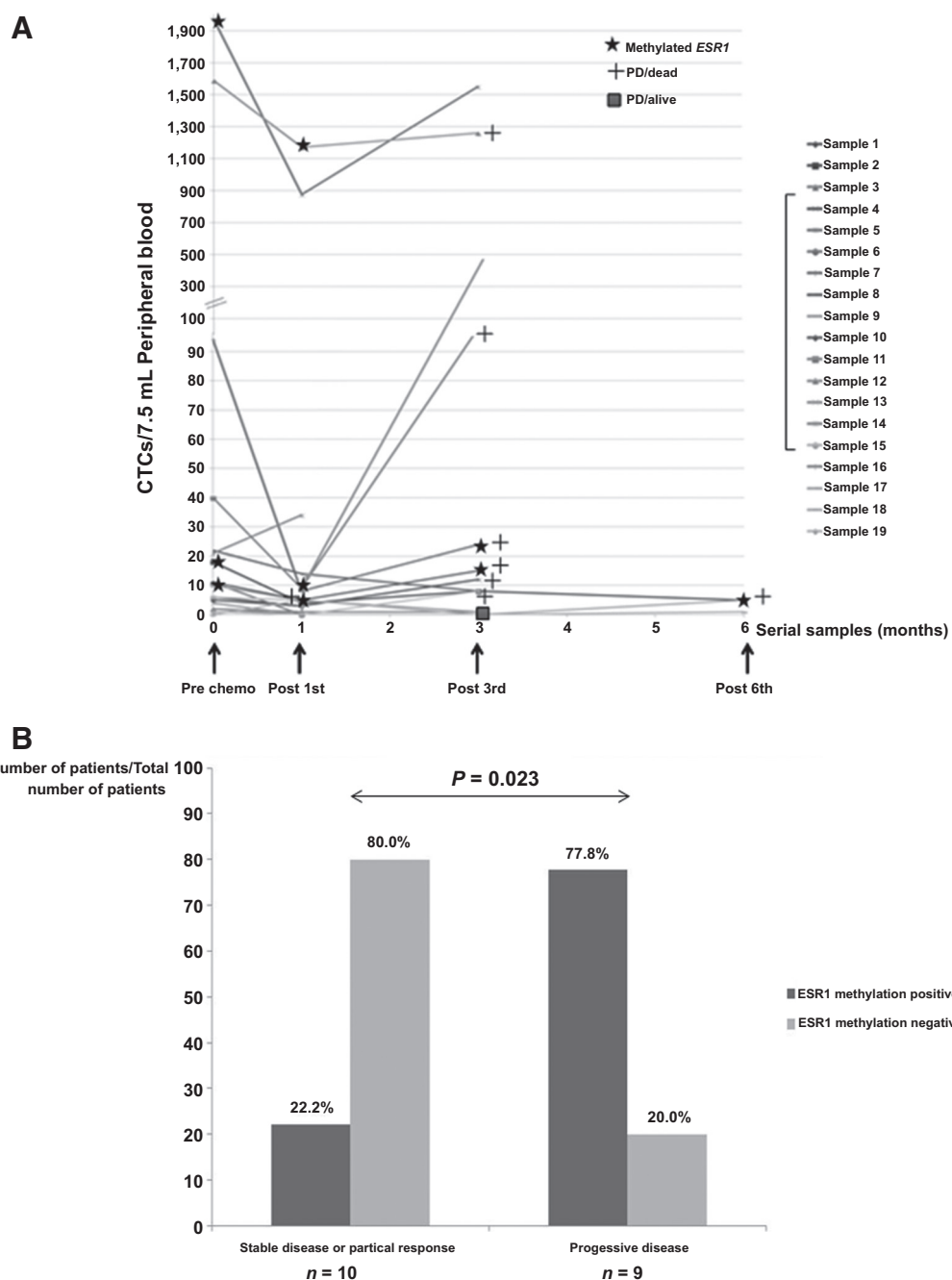
After a median follow-up of 20 months (range, 2–55), 14/19 (73.7%) patients developed PD, and all of them died. Patients with *ESR1* methylation–positive CTCs had significantly different PFS ( $P = 0.009$ ; Fig. 5A) and OS ( $P = 0.028$ ; Fig. 5B) compared with those with nonmethylated *ESR1*. Univariate analysis showed a significantly higher risk of progression (HR: 4.022; 95% CI, 1.277–12.670;  $P = 0.017$ ) and death (HR: 3.199; 95% CI, 1.063–9.632;  $P = 0.039$ ) in the *ESR1* methylation–positive CTC compared with *ESR1* methylation–negative CTC patients.

## Discussion

Our knowledge on the molecular evolution of cancer has been limited by the lack of access to tumor tissue throughout disease progression. Liquid biopsy based on the analysis of CTCs and plasma ctDNA offers the possibility of noninvasive real-time

monitoring of tumor progression (2–6, 35). It is likely that ctDNA and CTCs will have complementary roles as cancer biomarkers, although separate approaches may have distinct advantages in specific clinical contexts (2, 35). Up to now, however, there are a very limited number of studies where CTCs are directly compared with ctDNA, using the same blood draws and the same biomarkers (22).

Plasma ctDNA analysis is appealing due to the ease with which plasma can be collected and analyzed without the prior need to enrich and isolate a rare population of cells (36). For this reason, ctDNA analysis is likely to be the preferred option for genotyping and monitoring treatment response. The analysis of ctDNA can be applied as a high-throughput strategy for the assessment of clinical samples, but is limited to the analysis of point mutations, structural rearrangements, copy number aberrations, and changes in DNA methylation. EGFR mutations in cfDNA isolated from plasma of patients with non-small cell lung cancer (NSCLC) can stratify patients who may benefit from specific therapies (37, 38) and EGFR mutations in ctDNA analysis has been recently cleared by the FDA. On the other side, the analysis of CTCs provides the unique opportunity to study the whole cell, allowing DNA, RNA, and protein-based molecular profiling, and the opportunity for functional studies to guide personalized treatment selection (2–6, 35). However,



**Figure 4.**

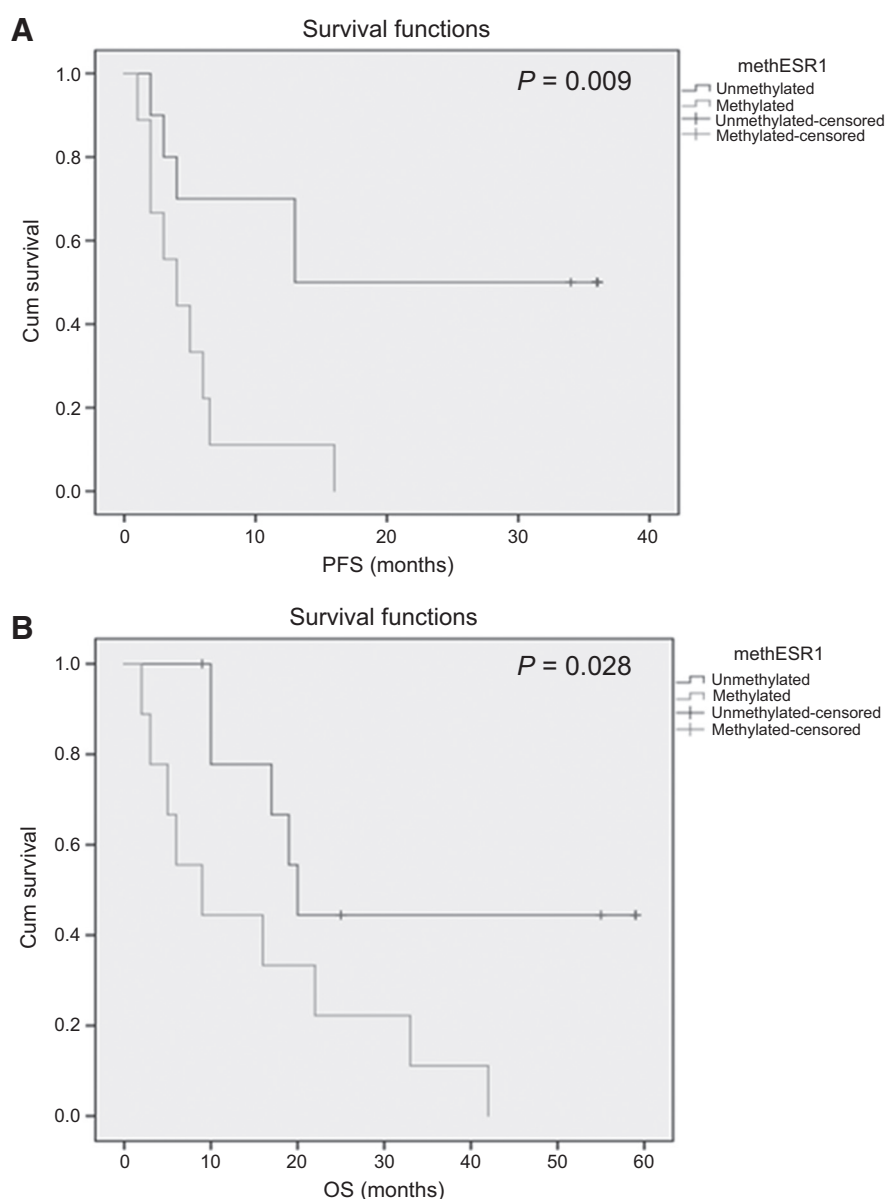
**A**, *ESR1* methylation status for each patient in serial CTC samples of BrCa patients receiving everolimus plus exemestane treatment at different time points during treatment, in relation to the number of detected CTCs. **B**, *ESR1* methylation status in CTCs of patients with HR<sup>+</sup> advanced breast cancer in association with response to everolimus plus exemestane treatment.

many open questions still remain in the fast-evolving field of liquid biopsy research (36, 39).

Within the last few years, DNA methylation is gaining ground as a potential biomarker for diagnosis, staging, prognosis, and monitoring of response to therapy (15–18). DNA methylation is an early event in carcinogenesis, thus tumor-specific methylation has a great potential to be used as a screening and/or diagnostic

tool in a noninvasive and cost-effective way. The first blood test that interrogates ctDNA methylation for cancer screening was approved by the FDA in April 2016. This assay, marketed as Epi proColon (Epigenomics, Inc.), is based on *SEPT9* promoter methylation in EDTA plasma derived from patients' whole blood samples and has been approved for screening and diagnosis of colorectal cancer (40).



**Figure 5.**

Kaplan-Meier estimates of patients with breast cancer receiving endocrine treatment with everolimus in relation to *ESR1* methylation in CTCs: **A**, *ESR1* methylation in relation to PFS; **B**, *ESR1* methylation in relation to OS.

In breast cancer, approximately 70% of cases are ER alpha positive ( $ER\alpha^+$ ) and, therefore, these patients are candidates for endocrine treatment. However, many of these patients relapse despite the administration of adjuvant endocrine therapy indicating that ER+ cancers may have primary or secondary resistance to endocrine therapy (1). Recent improvements in our understanding of how tumors evolve during treatment with endocrine agents have identified both changes in gene expression and mutational profiles, in the primary tumors (1, 41). Silencing, by methylation, of the promoter region of the *ESR1* affects the expression of the ER protein in patients with breast cancer; *ESR1* methylation is associated with ER-negative status which, in turn, may be implicated in the patient's resistance to hormonal treatment in breast cancer (24). Ung and colleagues used ENCODE CHIP-seq and DNase I hypersensitivity data, along with large-scale breast cancer genomic data from The Cancer Genome Atlas (TCGA) to computationally dissect the intricacies of DNA meth-

ylation in regulation of cancer transcriptomes. They report that they identified a relationship between  $ER\alpha$  activity and DNA methylation patterning in breast cancer and present evidence that methylation status of DNA sequences at  $ER\alpha$  binding sites is tightly coupled with  $ER\alpha$  activity (42).

Our group was the first to report on epigenetic changes in CTCs (20, 22) and demonstrated a close correlation between *SOX17* methylation in CTC and paired plasma ctDNA in patients with breast cancer (22). We have also shown that the breast cancer tumor suppressor (*BRMS1*) gene is highly methylated in CTCs isolated from patients with breast cancer that show a very low expression of this protein, as this was certified by immunofluorescence (43). Recently, we have performed a detailed comparative study of epigenetic markers in paired primary tumors, CTCs, and plasma ctDNA samples from breast cancer patients (44).

In the present study, we evaluated for the first time *ESR1* methylation status in  $EpCAM^+$  CTCs of patients with both early

and metastatic breast cancer and their corresponding plasma samples. We further evaluated the clinical utility of this assay in CTCs isolated from serial peripheral blood samples from 19 patients with ER<sup>+</sup>/HER2<sup>-</sup> advanced breast cancer under treatment with everolimus/exemestane.

To achieve this, we first developed a highly sensitive and specific real-time MSP assay for *ESR1* methylation. Using this assay, we found that ER expression and *ESR1* methylation in primary breast tumors are 100% inversely correlated. We, then, detected *ESR1* methylation in the EpCAM<sup>+</sup> CTC fraction of about 20% of patients with breast cancer and in ctDNA plasma samples of breast cancer patients in a lower percentage. This is the first time that *ESR1* methylation is studied in CTCs. We report that a significant percentage of patients with early-stage disease have detectable *ESR1* methylation. We know from numerous studies that methylation is an early event in breast cancer carcinogenesis. Moreover, we have seen similar results in our previous studies on methylation of tumor suppressor and metastasis suppressor genes in CTCs of patients with early breast cancer (20–22, 43, 44).

We further compared *ESR1* methylation in CTCs and plasma ctDNA isolated from peripheral blood samples taken at the same day, using the same blood draw and processed in the same way, thus minimizing all differences due to potential preanalytical variations. In these paired samples, *ESR1* methylation in plasma ctDNA showed a high concordance with *ESR1* methylation in CTCs, suggesting a possible connection between CTCs and the origin of ctDNA.

In a recently reported study, performed in a limited number of patients, a heterogeneous ER expression in CTCs was observed, suggesting diverse mechanisms of fulvestrant resistance such as insufficient dosage, *ESR1* mutations, or conversion to dependence on non-ER pathways (45). Our results in serial peripheral blood samples of patients with breast cancer receiving everolimus/exemestane indicate that *ESR1* methylation in CTCs is strongly associated with lack of response to this combination and should, thus, be further evaluated in a larger cohort of patients with breast cancer as a potential liquid biopsy-based biomarker for endocrine

treatment efficacy. A limitation of our study is the lack of access to ER status in the corresponding metastatic tissues.

In conclusion, we report for the first time that *ESR1* can be epigenetically silenced in CTCs through methylation, and that this reflects in paired plasma ctDNA samples. We have also shown that *ESR1* methylation is inversely correlated with ER protein expression by IHC in primary tumors. This is an interesting observation despite the limited number of serial patient samples, because *ESR1* methylation in CTCs was strongly associated with lack of response to everolimus/exemestane regimen. Therefore, the *ESR1* methylation status in CTCs should be further evaluated in prospective studies as a potential liquid biopsy-based biomarker for the selection of patients with endocrine-sensitive breast cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** S. Mastoraki, A. Strati, E. Lianidou  
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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Mastoraki, E. Politaki, A. Psyrri, V. Georgoulas, E. Lianidou

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Mastoraki, E. Politaki, E. Lianidou

**Writing, review, and/or revision of the manuscript:** S. Mastoraki, A. Psyrri, V. Georgoulas, E. Lianidou

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E. Tzanikou, M. Chimonidou, E. Lianidou  
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