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The prevalence of estrogen receptor-1 mutation in advanced breast cancer: The estrogen receptor one study (EROS1)

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ABSTRACT

Background: Breast cancer has, due its high incidence, the highest mortality of cancer in women. The most common molecular variety of breast cancer is luminal subtype that expresses estrogen and progesterone receptors. Estrogen receptor alpha (ERα), encoded by the estrogen receptor1 (ESR1) gene, is expressed in approximately 70% of all breast cancers, and hormonal therapy represents a major treatment modality in all stages of ER positive breast cancers. Acquired mutations in the ligand-binding domain (LBD) of ERα, referred as ESR1 mutation, result in resistance to different endocrine therapies leading to disease progression or recurrence. Recent studies reviled that these ESR1 mutations lead to constitutive activity of the estrogen receptor ER, meaning that the receptor is active in absence of its ligand conferring resistance against endocrine therapy and tumor growth. Published studies have not yet been able to determine the exact prevalence rate of ESR1 mutations, but set the outer boundaries between 11–55%.

Purpose: The goal of the present study is to determine the frequency rate of ESR1 mutations in ER positive recurrent breast cancer by using digital droplet PCR (ddPCR) technique.

Materials and methods: This retrospective study was conducted in the Multidisciplinary Breast Clinic of Antwerp University Hospital. The seven most common ESR1mutations (c.1138G>C (p. (E380Q)), c.1610A>G (p. (Y537C)), c.1613A>G (p.(p.D538G)), c.1607T>G (p.(L536R)), c.1387T>C (p.S463R)), c.16410A>C (p. (Y537S)), c.609T>A (p.(Y537N)) were assessed in available baseline plasma samples of 21 patients with ER positive recurrent breast cancer. Inclusion criteria for study participation were: female, age above 18 years, ER positive breast cancer, 5years adjuvant hormonal therapy of primary disease, and disease recurrence or metastasis during or after stop of endocrine therapy. ESR1 mutations were analyzed in cell-free DNA (cfDNA) by using digital droplet PCR (ddPCR).

Results: cfDNA was obtained from 21 patients with recurrent breast cancer. ESR1 mutations were found in 4/21 (19%; 95% CI, 5%–42%). The test sensitivity was lower than the targeted value <0.1% in 29% of patients (6/21). No significant statistical difference in baseline clinical characteristics was observed in patients with wild-type and mutant ER ($p > 0.05$). Adjuvant endocrine therapy for primary disease was Tamoxifen (TAM) for 57% of patients (12 of 21) of whom 8 patients had received aromatase inhibitor (AI) after two years, while 43% of patients (9 of 21) had received AI as first line adjuvant hormonal therapy. All the patients had received aromatase inhibitor AI therapy in first or second line therapy with initially a variable period of good response.

Conclusion: ESR1 mutation analysis could be determined in archived plasma samples using simple non-invasive methods. In the future, screening for mutation status could improve the therapeutic strategies in controlling ER signaling before the occurrence of wide spread disease metastasis.

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Introduction

Breast cancer is the most common cancer in women and one of the leading causes of death worldwide. Estrogen receptor alpha (ERα, "wild-type" estrogen receptor), encoded by the estrogen receptor1 (ESR1) gene, is expressed in approximately 70% of all breast cancers. Hormonal therapy has become the mainstay in treatment of ER+ breast cancer and prevention of disease recurrence [1,2]. The most commonly used anti-estrogen therapies inhibit ER activity by either targeting the ER protein itself or depriving the receptor of its ligand. The different classes of endocrine treatments include (1) direct inhibition of ER by selective estrogen modulators (SERMs) with mixed agonistic/antagonistic activities, such as Tamoxifen (TAM), (2) selective ER degraders (SERDs) such as Fulvestrant that are more potent anti-estrogens, and treatment strategies that deplete systemic estrogen levels by either (3) aromatase inhibitors or (4) ovarian suppression [3,4]. While endocrine therapies have proven to be very effective in both the early and the metastatic settings; both de novo and acquired resistance to endocrine treatments remain a key clinical challenge [5–7].

TAM is the first endocrine therapy in clinical use against breast cancer [8]. TAM binds to the receptor and can exhibit both agonist and antagonist properties, with agonistic effects on endometrium and antagonist effects on mammary tissue. Thus, the selectivity of responses led to it being described as a selective estrogen receptor modulator (SREM). TAM is considered as first line hormonal therapy in premenopausal women with ER+ breast cancer. Other anti-estrogens affect the stability of the ER and down regulate the receptor protein; these drugs often referred as pure anti-estrogens [9]. Currently, Fulvestrant is the most widely studied of this anti-estrogen class; which act by inhibition of ER protein dimerization leading to degradation of the receptor protein [10]. Given the mechanism of action of these drugs, they are described as a selective estrogen receptor downregulator/degraders (SERDs). Another category of anti-estrogens are Aromatase Inhibitors (AIs), which act to decrease circulating estrogen levels by interfering with estrogen production in the peripheral tissues. They are now considered as standard first line hormone therapy in postmenopausal women with ER+ breast cancer [11]. Other classes of drugs (CDK4/6 inhibitors, PI3K inhibitors, new ER antagonists) are being developed for adjuvant endocrine therapy in second or third regimens in patients with recurrent breast cancer, some of which were administered to the subjects in our study population.

Estrogens are synthesized from puberty until menopause within the ovaries. After menopause, ovarian estrogen ceases but other tissues in the body including the brain, adipose tissue and muscles continue to produce estrogens from androgens by the action of the aromatase enzyme (CYP 19) [12]. Normal ERα, a nuclear protein, is ligand dependent. Estrogen-ER complex will activate the receptor and induce conformational changes, allowing the complex to bind to particular DNA sequences. Meanwhile, co-repressors and co-activators could influence the process of gene transcription [13]. Therefore, anti-estrogens act primarily to block ER or deplete endogenous estrogen preventing ER activation and tumor growth.

Several mechanisms have been proposed to the development of resistance to endocrine therapies including: 1. Deregulation of ER pathway including loss of expression of ERα, post-translational modifications and altered activity of co-activators and co-repressors in tumor cells. 2. Alterations in cell cycle and cell survival signaling molecules in the tumor cells. 3. Activation of signaling pathways that can provide alternative ER independent proliferation and survival stimuli to the tumor cells such as EGF, the insulin/IGF-1 and the PI3K/Akt/mTOR pathways [14]. Recently, mutations in the gene encoding ERα have attracted particular interest as a mechanism for endocrine resistance in recurrent breast cancer.

Functional studies revealed that these ESR1 mutations lead to constitutive activity of the ER, meaning that the receptor is active in absence of estrogen, conferring resistance against several endocrine agents. Recent studies reported that the occurrence of ESR1 mutations is rare in ER+ primary breast cancers, however these mutations are frequently reported in ER+ recurrent breast cancers pretreated with endocrine therapy [15]. Darwinian theory could potentially explain this disease-related event; ESR1 mutations are selected and enriched during longstanding endocrine therapy. As a result, the subpopulation of resistant clones will grow and account over time for a larger fraction of the tumor mass [24,25].

Based on ongoing preclinical trials, ESR1 mutation has an effect on the response to endocrine therapy, [22,23]. Therefore, screening for ESR1 mutations may in the future allow for more individualized treatment controlling ER signaling before the occurrence of wide spread disease. In this study, we investigated the application of ddPCR analysis for detection of ESR1 mutations using archived plasma samples. EROS1 designed to determine the frequency rate of ESR1 mutation with ER+ recurrent breast cancer refractory to hormonal therapy.

Materials and methods

Patients' selection

The current study is a single institutional study that enrolled patients with disease recurrence in the period between September 2010 and July 2017. There are archived plasma samples from 250 patients with breast cancer available in the biobank-Antwerp University Hospital. Inclusion criteria were (1) female patients; (2) age more than 18 years; (3) positive ER expression; (4) 5 years endocrine therapy of primary disease; and (5) disease recurrence/progression refractory to endocrine therapy. Recurrence was defined as identification of positive spots by imaging diagnosis and/or tissue biopsy during follow-up period. All included patients had demonstrated prior good response to adjuvant endocrine therapy of the primary disease. Only 21 patients were eligible for ESR1 analysis. The patients were recruited from Breast Clinic of Antwerp University Hospital (study number) by using the electronic medical dossiers and multidisciplinary oncology consult. Selection was performed depending on the clinical course of the disease and response to endocrine therapy. Informed consent was obtained from all participants before using their plasma samples. The Ethics Committee of Antwerp University Hospital approved the study and conformd to relevant ethical guidelines for human research.

Plasma samples collection and extraction of circulating DNA

Baseline plasma samples were collected in EDTA tubes in outpatient clinic. The storage of samples was performed at −80 °C for a period of 1–12 months before start of study. All samples were collected, stored and analyzed in the same laboratory of pathological department in Antwerp University Hospital, to avoid interlaboratory bias. 2 mL of plasma was used to extract cfDNA by Maxwell RSC cfDNA isolation kit for large volumes (Promega) on a Maxwell 16 instrument (Promega) according the manufacturer's instructions.

Analysis of ESR1 mutations by ddPCR

For ESR1 mutation analysis, we used commercially available QX200 Droplet Digital Polymerase Chain Reaction system (ddPCR, Bio-Rad) assays for the most common ESR1 mutations (c.1138G>C (p.(E380Q)), c.1610A>G (p.(Y537C)), c.1613A>G (p.(p.D538G)), c.1607T>G (p.(L536R)), c.1387T>C (p.S463R)), c.16410A>C (p.(Y537S)), c.609T>A (p.(Y537N)). All samples were screened with two multiplex assays of Bio-Rad. The first multiplex assay detects E380Q, L536R, Y537C, D538G mutations (Biorad, assay ID dHsaMDXE91450042), whereas the second multiplex assay detects S463P, Y537S and Y537N (Biorad assay ID dHsaMDXE65719815).

TaqMan PCR mixtures were assembled by 10 μL 2x ddPCR Supermix for Probes (Bio-Rad), 1 μL ddPCR ESR1 Screening Multiplex Kit, and

9 μL of isolated sample.

Final volume reactions of 20 μL were loaded into sample wells of a DG8 cartridge (Bio-Rad) with 70 μL of Droplet Generation Oil for Probes (Bio-Rad). Droplets were generated by the QX200 Droplet Generator (Bio-Rad); 40 μL of the generated droplets was manually transferred with a multichannel pipette into a 96-well PCR plate and amplified in a Veriti thermal cycler (Thermo Fisher Scientific). The thermal cycling conditions were as follow:

95 °C for 10 min, 40 cycles of 95 °C for 15 s, then 52 °C for 1 min, followed by 98 °C for 10 min and cooling to 4 °C. Droplets were analyzed using the QX200 Droplet Reader (Bio-Rad). Data analysis was performed with the QuantaSoft version 1.7.4.0917 (Bio-Rad), which uses the number of positive and negative droplets to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% Cis.

Validation of analysis of ESR1 mutations in archived plasma samples

We validated the analysis of ESR1 mutations using archived plasma samples in context of research.

Statistical analysis

Continuous data (age at diagnosis and age at recurrence) were tested for normality by Shapiro-Wilk W test. As the normality assumption was appropriate we compared age based on t-testing. Categorical data were tested by Fisher's exact test. We considered $p < 0.05$ (two-sided) as statistically significant. We conducted a survival analysis (Kaplan Meier) defining time to recurrence as survival time and ESR1 mutation as failure. All statistical analyses were conducted in IBM SPSS Statistics version 24.0.0.0

Results

Patients' characteristics

A total of 21 (8.4%) of 250 patients were eligible for ESR1 analysis. Patients with ER- breast cancer or adjuvant endocrine therapy less than 5 years were excluded. The selection procedure was also limited to patients who show disease recurrence or progression refractory to endocrine therapy after a period of good response in primary disease. The rationale behind the inclusion criteria is to increase theoretical possibility of identifying ESR1 mutation [20]. patients had disease recurrence after stopping endocrine therapy, while 1 patient had disease progression with peritoneal metastasis at end of 5-years adjuvant therapy. The median duration of follow-up was 96 months (range 12–180 months). The four patients with mutant ESR1 were relatively younger at diagnosis and at recurrence with mean age 41.2 and 53.5 years respectively versus 55.8 and 63.2 years respectively for those with wild-type ER. No other pretreatment clinicopathological characteristics differed between the two groups. This suggests that the study population was well balanced in normal versus mutant ER groups, see Table 1.

ESR1 mutation with QX200 droplet digital PCR

ESR1 mutation status was successfully analyzed in baseline plasma samples using from 21 patients. To confirm the presence of ESR1 mutation, all samples were screened with two multiplex assays of Bio-Rad. The first multiplex assay detects E380Q, L536R, Y537C, D538G mutations, whereas the second multiplex assay detects S463P, Y537S and Y537N. Positive ESR1 mutations were found in 4 patients (19%; 95% CI, 5% − 42%) recently described with recurrent breast cancer. Additionally, another 6/21 patients had ambiguous positive test results below the detection threshold of 0.1% mutant cfDNA.

Table 1
Baseline clinical characteristics in patients with mutant versus wild-type ER.

	Mutant ER $n = 4$ (19.1%)	Wild-type ER $n = 17$ (80.9%)	*P value*
Age (y)			
Diagnosis	*41,2*	*55,8*	*ns*
Recurrence	*53,5*	*63,2*	*ns*
PR positivity	*4/4 (100%)*	*15/17 (88%)*	*ns*
Menopause	*3/4 (75%)*	*13/17 (76.4)*	*ns*
BRCA mutation status	*0/4 (0%)*	*0/17 (0%)*	*ns*
Her2 receptor positivity	*0/4 (0%)*	*3/17 (17.6)*	*ns*
Histology			
Ductal	*3/4 (75%)*	*15/17 (88%)*	*ns?*
Lobular	*1/4 (25%)*	*2/17 (12%)*	*ns?*

Table 2
Sites of metastasis at time of recurrence.

	Mutant ER ($N = 4$)	Wild-type ER ($N = 17$)
Metastasis		
Bone	3/4	12/17
Lung	2/4	2/17
Peritoneum	1/4	1/17
Supraclavicular LN	0/4	3/17
Liver	0/4	2/17
Adrenal Gland	0/4	2/17
Brain	0/4	1/17

ESR1 mutations and site of metastasis

The correlation of the presence of ESR1 mutations with site of metastasis was summarized in Table 2. Bone metastasis was the most common site of metastasis in 15 patients. However, ESR1 mutations were present in only 3 (20%) patients. Distant metastasis is associated with transfer of tumor cells through blood vessels to other organs, i.e. enriched tumor material could be demonstrated in plasma samples theoretically increasing test sensitivity. Other sites of metastases were: lung, peritoneum, supraclavicular LN, liver, adrenal gland and brain. However, in one study, analysis of samples from cfDNA, primary and metastasis tissues showed more ER1 mutation frequency in cfDNA in comparison to the primary and metastasis tissue analysis [20]. Further analysis was not performed because of small sample size.

Association of ESR1 mutations and type endocrine therapy

In primary disease, TAM was the first line adjuvant hormonal therapy for 12 patients of whom 8 patients had received AI after two years and 4 patients had received TAM for further 3 years, while 9 patients were treated with AI for 5 years as first line adjuvant hormonal therapy. The patients showed good response for a variable period during endocrine therapy of the primary disease. At time of relapse, AI was started as first line hormonal therapy in 16 patients, TAM was prescribed for 3 patient and Fulvestrant in combination with CD4/6 for 1 patient, see Table 3. All the patients received multiple endocrine therapy mainly TAM and AIs. The four ESR1 mutations were identified in patients previously treated with TAM and AIs, Fig. 1. Our results are

Table 3
Type of first endocrine therapy at time of recurrence.

	Mutant ER ($N = 4$)	Wild-type ER ($N = 17$)
Tamoxifen	0/4	3/17
Letrozole	2/4	9/17
Anastrozole	1/4	1/17
Exemestane	1/4	3/17
Fulvestrant	0/4	1/17

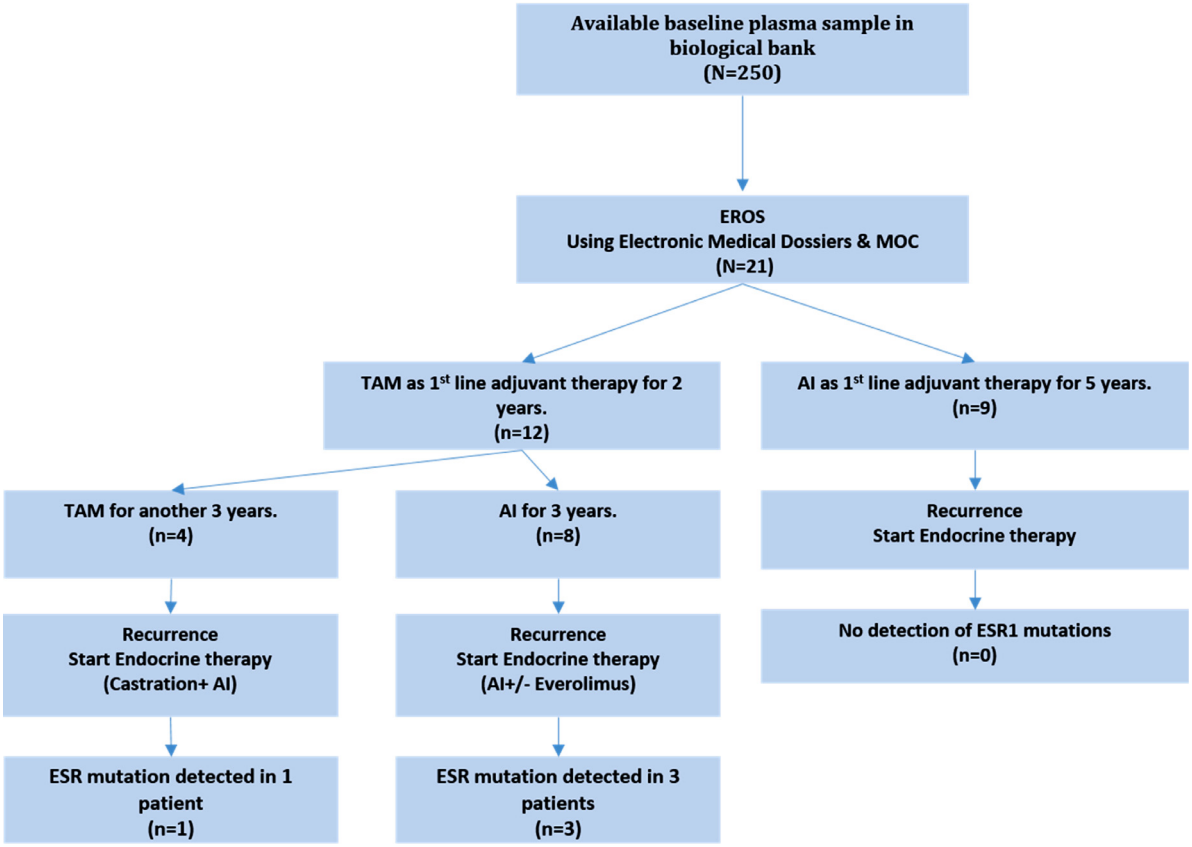


Fig. 1. Flow diagram of ESR mutation. Flow chart showed that the patients with ESR1 mutation received AIs as adjuvant endocrine therapy of the primary disease or as first line therapy at time of relapse.

consistent with other preclinical studies that showed a possible correlation between longstanding AIs therapy and existence of ESR1 mutations.

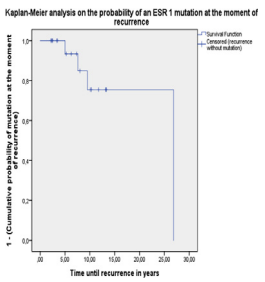
Discussion

Breast tumors are characteristically heterogeneous tumors at the cellular, molecular, and genetic/epigenetic levels. The potential for both spatial and temporal heterogeneity in ER expression is still not well understood. ESR1 mutation expression can fluctuate over time; some cells that stain negative at a point of time may express detectable ESR1 mutations at another tome. Resistance in cancer cells to endocrine therapy can be divided into two basic patterns, de novo and acquired resitance [16]. ESR1 mutations are rarely a cause of primary resistance; but breast cancers that show a good initial response to endocrine therapy but thereafter regrow or recur which reflect acquired resistance to endocrine therapy, 15 consistent with our findings.

The incidence of ESR1 mutations in breast cancers has been reported to be as low as less than 1% in primary cancers, but as high as 11%–55% in ER+ metastatic tumors [17]. More recent studies using ddPCR techniques show that ESR1 mutations could be found in approximately 2.5%−7% of primary breast cancers, 1 and ultrasensitive detection of rare ESR1 mutations may represent an important biomarker for early detection of endocrine-resistance disease. The 19% resistance mutation rate among recurrent cancers found in our study probably represents an underestimation, given the fluctuating ER expression and ambiguous DNA findings from 29% of our sample population. It should be noted that the analysis was retrospective and the storage-time factor might have an effect on ER analysis. Dynamic ER assessment through longitudinal analysis of plasma samples would be expected to display an even higher sensitivity. It should be noted that we assessed seven different ESR1 mutations. There may be other mutations or aberrations that could also contribute to hormone-resistance in recurrent breast cancer.

Association between ESR1 mutation status and type of endocrine therapy is an interesting subject. Longstanding endocrine therapy could provoke the occurrence of ER mutation according to Darwinian theory. One of the key findings of this work is the high prevalence of ER mutation in patients treated with TAM and AIs. Despite the interesting finding, the analysis of ER-medication interaction is limited by the small sample size ($n = 21$). Our study was not designed to address the impact of ER mutation on clinical outcome and retrospective response to endocrine therapy.

Exposure time to endocrine therapy and absolute time are important cofactors in development of acquired ER mutation in hormone sensitive breast cancer. Until now, no study has examined the time needed for the development of acquired resistance. In the current study, 19% patients with recurrent breast cancer developed ER mutation after 5–10 years of the diagnosis of the primary disease (Graphic 1). The correlation between time factor and risk for development ER mutation has



Graphic 1. The correlation between ESR1 mutation development and time factor.

significant impact on clinical outcome especially in young age patients who may develop disease relapse within short period after the primary disease.

Results from EROS1 on archived plasma samples demonstrate that cfDNA analysis has potential clinical utility in patients with ER + recurrent breast cancer. In patients with no detection of ER mutation, serial analysis of plasma samples is needed. Endocrine therapy resistance could be attributed to not well-known mutations and mechanisms leading to disease progression. Given the assumed impact that the presence of ESR1 mutations has an effect on endocrine therapy, assessing ESR1 mutations in recurrent breast cancer patients is likely to be of significant interest to further individualize treatment in patients with endocrine resistance.

In ER + group, Histopathological examination followed by Fluorescence in situ hybridization (FISH) had revealed the absence of HER2 receptors. Co-targeting of ER and HER2 appears to provide benefit without a significant increase in toxicity although formal trials have not been carried out [18]. Adopting routine ER mutation testing would depend not only on a demonstration of its clinical value in guiding choice of chemotherapy, but on cost and logistical issues as well, including identifying the optimal frequency of testing, taking patient age into account.

The current study has a number of important limitations. The biological analysis was retrospective using baseline plasma samples that were collected from patients within a period of 1–12months before the start of the study, and degradation of the samples prior to testing is a possibility. Furthermore, the study is designed to calculate the incidence rate of ESR1 mutation in recurrent breast cancer not the impact of ER status on clinical outcome and endocrine therapy. Our center is currently enrolling subjects in a prospective study with larger population and with longitudinal serial analysis of plasma samples to assess the frequency rate of ESR1 mutation in patients with progressive/ recurrent breast cancer.

Liquid biopsy is a highly sensitive, less invasive method of detecting activating ESR1 mutations via circulating cfDNA and tumor cells, without the spatial and temporal limitations of core biopsies [19]. Early identification of ESR1 mutations by liquid biopsy might allow for timely switching or intensification of chemotherapy through addition of other agents to a compromised endocrine therapy, without need for tissue biopsy and before the emergence of metastatic disease. ddPCR technique is robust for mutation detection in liquid biopsy despite the contamination from white blood cell breakdown leading to suboptimal analysis of cfDNA [21].

In general, ESR1 mutations may be indicative of progressive disease and could be associated with poor clinical outcome. Therefore, it will be of particular interest to see whether certain medications are able to overcome endocrine therapy resistance. With this information, clinicians may avoid the occurrence of metastasis in patients with hormone sensitive breast cancer in young age women.

Conclusion

In our retrospective study ESR1 mutations are found in 19% of patients with recurrent breast cancer previously treated with adjuvant TAMs and AIs. These mutations can be detected relatively simply and cheaply with ddPCR, the sensitivity of which is increased by repeated testing over time. Their implications for therapy are potentially significant, while questions remain as to the optimal frequency of testing, taking patient age into account, and mutation-specific therapeutic susceptibilities.

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Authors' contribution

Study design, analysis, and interpretation of data: ON.
Acquisition of data, technical laboratory support: SG, PP and KZ.
Statistical analysis: JW.
Study supervision: WT.
ON wrote the manuscript, which was edited, reviewed and approved the final manuscript by MH, KP, XBT, SA, PVD and WT.

Conflicts of interest

There are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2019.100123.

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