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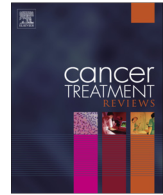

Hot Topic

ESR1 mutations: Moving towards guiding treatment decision-making in metastatic breast cancer patients

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ABSTRACT

Mutations in the gene coding for the estrogen receptor (ER), *ESR1*, have been associated with acquired endocrine resistance in patients with ER-positive metastatic breast cancer (MBC). Functional studies revealed that these *ESR1* mutations lead to constitutive activity of the ER, meaning that the receptor is active in absence of its ligand estrogen, conferring resistance against several endocrine agents. While recent clinical studies reported that the occurrence of *ESR1* mutations is rare in primary breast cancer tumors, these mutations are more frequently observed in metastatic tissue and circulating cell-free DNA of MBC patients pretreated with endocrine therapy. Given the assumed impact that the presence of *ESR1* mutations has on outcome to endocrine therapy, assessing *ESR1* mutations in MBC patients is likely to be of significant interest to further individualize treatment for MBC patients. Here, *ESR1* mutation detection methods and the most relevant pre-clinical and clinical studies on *ESR1* mutations regarding endocrine resistance are reviewed, with particular interest in the ultimate goal of guiding treatment decision-making based on *ESR1* mutations.

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Introduction

Endocrine therapy with selective estrogen receptor modulators/downregulators (SERMs/SERDs) or by estrogen deprivation using aromatase inhibitors (AIs), is the most important treatment modality for estrogen receptor (ER)-positive metastatic breast cancer (MBC) patients [1]. Unfortunately, 40% of patients do not benefit from first-line endocrine therapy due to intrinsic resistance, and the remainder of patients initially responding will eventually develop resistance during therapy [1]. Several mechanisms have been linked to endocrine resistance, however, no marker for resistance has reached wide clinical use yet [2–4]. Recently, mutations in the gene encoding ERα, *ESR1*, have attracted particular interest as a mechanism for endocrine resistance in MBC. Large-scale next-generation sequencing (NGS) efforts on MBC tissues revealed that these mutations are enriched in MBC patients treated with endocrine agents while these variants are not or only at very low frequencies present in primary tumor tissue [5,6]. Importantly, this implies that their presence has to be assessed in metastatic lesions, or in "liquid biopsies" such as circulating cell-free DNA (cfDNA) as a representative of metastatic tumor cells. Here we review the pros and cons of current detection methods for *ESR1* mutations, the pre-clinical and clinical studies investigating *ESR1* mutations and highlight its potential role in treatment decision-making in MBC patients.

Functional studies on ESR1 mutations

The ER belongs to the nuclear hormone receptor superfamily [7] and consists of two activation function (AF)-1/2 domains, DNA binding and hinge domains, and a ligand binding domain (LBD) (Fig. 1). The ER functions as a ligand-dependent transcription factor. Binding of estradiol to the LBD leads to a conformational change of helix 12, resulting in recruitment of coregulatory proteins [8]. This eventually yields transcription of genes important in normal physiological processes but also for breast tumorigenesis and breast cancer (BC) progression [9].

Recent NGS efforts revealed that somatic *ESR1* mutations in the LBD were more frequently present in metastatic lesions than previously thought. In preclinical models to evaluate the role of *ESR1* mutations in endocrine resistance, it was demonstrated that cell lines transfected with a D538G, Y537S, L536Q, Y537N, Y537C, S463P or E380Q *ESR1* mutation exert activity in the absence of estrogen [6,10–15] (Fig. 1). This constitutive activity suggests that estrogen-depriving therapies such as AIs are not or less effec-

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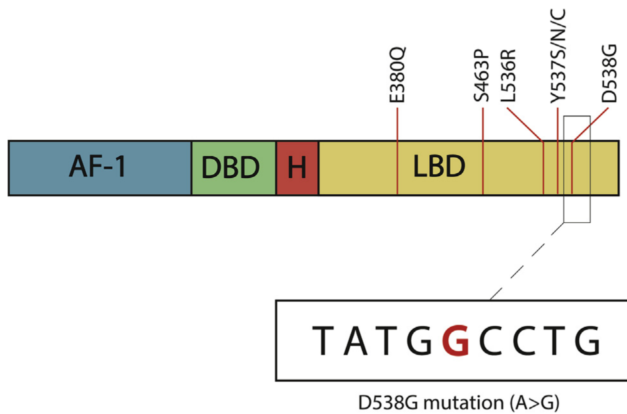

Fig. 1. Schematic overview of the different domains of the ER. Activation function (AF) domain-1 present at the N-terminus acts in a ligand-independent manner, whereas, the AF-2 within the ligand binding domain (LBD) is dependent on estradiol for its activation [52]. The DNA binding domain encodes two zinc finger molecules, playing an important role in receptor dimerization and binding of the ER to specific DNA sequences: the estrogen response element (ERE) [53]. H = hinge region. *ESR1* mutations, some hotspot mutations shown as vertical red lines, mainly occur in the C-terminal domain of the receptor encoding for the LBD of the ER.

tive in patients with activating *ESR1* mutations. Cell lines transfected with mutant *ESR1* variants were however still responsive to treatment with tamoxifen and fulvestrant, though sensitivity to these drugs was relatively impaired compared to *ESR1* wildtype transfected cell lines [5,6,12,13]. Similar observations were recently made for novel SERM/SERD hybrid endocrine therapies pipendoxifene and bazedoxifene [16].

Techniques to detect ESR1 mutations

Several techniques can be used to assess *ESR1* mutations in tissue or cfDNA (Fig. 2), all having their own advantages and disadvantages. Importantly, these techniques widely vary in their sensitivity. NGS can be performed either in the context of whole genome sequencing, as part of a whole exome panel, or as part of a targeted *ESR1* panel. While NGS is an established and widely used approach for mutation detection in tumor tissue, mutation detection in cfDNA is more challenging, as the relative number of mutant to wildtype DNA alleles has to be taken into account. Frequencies of circulating tumor DNA (ctDNA) vary largely between patients, frequently being below 1% of the total cfDNA [17], which is beyond the sensitivity of conventional NGS. Therefore, techniques based on digital PCR (dPCR) have been introduced enabling the detection of ctDNA in frequencies as low as 0.001% [18,19]. In dPCR-based techniques, each individual DNA molecule, within its own partition, is able to react with a specific probe for wildtype *ESR1* and another probe for a specific *ESR1* mutant. There are several commercially available dPCR-based assays (e.g. digital PCR, droplet digital PCR (ddPCR), BEAMing), differing in used reagents and sample readouts, but generally having similar sensitivity [17,20]. In a study comparing conventional targeted NGS with dPCR to detect mutations in cfDNA, threefold more D538G *ESR1* mutations in cfDNA were observed using dPCR than with NGS [21]. One disadvantage of dPCR assays is however that only a subset of hotspot mutations can be evaluated. Other assays, using some sort of target-enrichment prior to analysis, can be used to detect multiple hotspot mutations (OnTarget assay [22,23]) or multiple frequently mutated genes (e.g. SafeSeqS [24], CAPP-Seq [25]), however to date these assays have not yet been reported to be used to detect *ESR1* mutations.

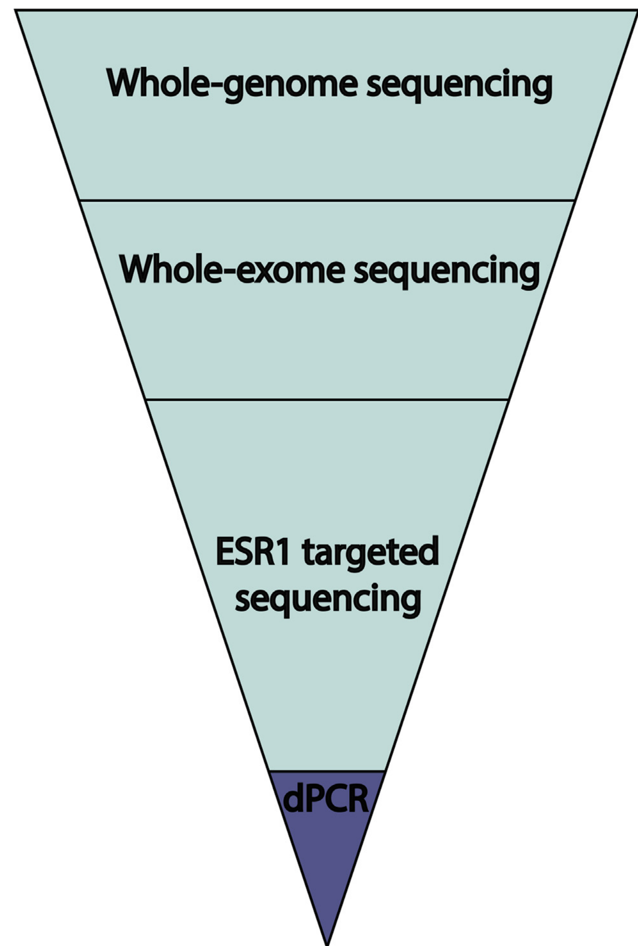

Fig. 2. Various techniques for *ESR1* mutation detection. The pyramid represents the range in which the genome is investigated. *ESR1* mutations can be detected by large-scale NGS efforts such as whole-genome sequencing or whole-exome sequencing, or by more targeted methods as targeted sequencing of the *ESR1* gene only, or by the interrogation of individual mutations in *ESR1* by digital PCR.

Clinical studies on the significance of *ESR1* mutations

ESR1 mutations in primary and metastatic tumor tissue

Although already described anecdotally in the the ’90s [11,26,27], *ESR1* mutations were thought to be rare in BC. They occur only in up to 3% of primary tumors using NGS (Supplementary Table 1) [5,6,12,13]. Using more sensitive dPCR-based techniques, the *ESR1* mutation rate in primary BC tumors may mildly increase [28,29], however, only at very low variant allele frequencies (VAF; 0.07–0.2%) [29].

In contrast to mutation rates in primary BC, the landmark papers of Toy et al. [6] and Robinson et al. [13] showed much higher *ESR1* mutation rates in metastatic lesions (Supplementary Table 2). Toy and colleagues [6] found *ESR1* mutations (predominantly D538G and Y537S) in metastatic tissues in 9/36 ER-positive MBC patients who had received at least 3 months of endocrine therapy. All patients with an *ESR1* mutation were at least treated with two lines of endocrine therapy; all containing an AI. In an independent cohort of 44 metastatic tumors from patients pretreated with endocrine therapy, 5 metastases (11%) harbored an *ESR1* mutation.

Likewise, Robinson et al. [13] demonstrated *ESR1* mutations in 6/11 (55%) evaluated metastatic biopsies of ER-positive MBC patients. All patients with an *ESR1* mutation were pretreated with

AIs and SERMs or SERDs. None of three available matched primary tumors of patients with a metastatic *ESR1* mutation harbored an *ESR1* mutation. Based on these findings and the accompanied functional studies, both groups hypothesized that *ESR1* mutations are a common mechanism underlying endocrine resistance, developing during estrogen deprivation, especially in the context of AI treatment.

Prompted by these findings, several studies investigated *ESR1* mutations in metastatic tissue of MBC patients. In 5/13 (38%) ER-positive MBC patients, who failed on multiple lines of endocrine treatment, a D538G *ESR1* mutation was reported [12]. Furthermore, Jeselsohn et al. [5] detected in 9/76 (12%) ER-positive metastatic tumors *ESR1* mutations (Y537N/C/S and D538G) using NGS, whereas none of the 115 ER-negative tumors they assessed had such mutations. In both studies no *ESR1* mutations in matched primary tumors were detected [5,12].

In a study using dPCR, an *ESR1* mutation was revealed in metastatic tissue of 11/55 ER-positive MBC patients [28]. Notably, polyclonal *ESR1* mutations (multiple *ESR1* mutations in one sample) were observed in 4/11 (36%) patients. Also of particular interest was that two patients with *ESR1* mutations were not pretreated with any therapies at all and 4/11 only received prior treatment with tamoxifen, supporting a previous observation [5] that *ESR1* mutations are not exclusively found following AI treatment. In another study [29] applying dPCR, *ESR1* mutations were found in 3/43 primary tumors, 1/12 bone metastasis tissues and 3/38 brain metastasis tissues in ER-positive MBC patients. The prevalence of *ESR1* mutations and their VAF were higher in bone (1.4% VAF) and especially in brain metastases (34.3–44.9% VAF) compared to primary tumors (0.07–0.2% VAF), suggesting an enrichment of *ESR1*-mutant subclones in metastatic tissue.

All these tissue-based studies provided important insights into the prevalence of *ESR1* mutations and the population of patients in which they occur. However, the biggest disadvantage of these studies is that they concerned mostly small, heterogeneously treated, and retrospectively selected patient cohorts. Furthermore, of note is that biopsies were usually taken at various time points and therefore the evidence at which moment *ESR1* mutations emerge, which is suggested to be mainly after AI treatment, is indirect. The majority of the above mentioned drawbacks are mainly driven by the fact that taking metastatic biopsies is a cumbersome procedure and even impossible in some patients, not easily allowing the assessment of *ESR1* mutations over time. In addition, taking metastatic biopsies may lead to sample bias due to tumor heterogeneity [30]. Therefore, recent studies have focused on *ESR1* mutation detection in "liquid biopsies" as a patient-friendly alternative to taking biopsies from metastatic lesions.

ESR1 mutations in 'liquid biopsies'

Circulating blood biomarkers such as circulating tumor cells (CTCs) and cfDNA are increasingly used as non-invasive surrogate "liquid biopsies", and are thought to represent the most important metastatic tumor sites [31,32]. Both these types of liquid biopsies can be measured in peripheral blood, with CTCs being intact tumor cells and cfDNA being DNA mainly derived from apoptotic tumor cells. Recently, several studies investigating the presence of *ESR1* mutations in liquid biopsies, particularly in cfDNA, have been published (Table 1).

To evaluate NGS and dPCR techniques to detect *ESR1* mutations in plasma, Guttery et al. examined cfDNA of 48 ER-positive MBC patients [21]. In 3/48 patients (6%), they observed an *ESR1* mutation in cfDNA using NGS. In one patient with a D538G mutation also CTCs, isolated by the CellSearch system, were sequenced, and the same mutation was detected in CTCs. When dPCR was performed in the same cohort for the D538G mutation only, the D538G mutation was found in 6 additional patients (15%) at VAF below 1%, underlining the limited sensitivity of NGS to detect low frequent mutations. In eleven patients, serial plasma samples were available. Interestingly, in one patient an *ESR1* mutation was present at baseline and was further enriched (0.4% VAF to 13.6% 3 months later) while treated with chemotherapy (docetaxel/vinorelbine).

To further explore whether *ESR1* mutations present in metastases are also represented in the cfDNA, Chu et al. [33] assessed *ESR1* mutations in plasma cfDNA in 11 ER-positive MBC patients in whom the *ESR1* mutation status in a metastatic lesion was assessed by NGS. All *ESR1* mutations (8/8) observed in the metastatic lesions were also observed in the cfDNA using dPCR. In one patient with an *ESR1* wildtype metastatic lesion, a low frequency *ESR1* mutation was observed in the cfDNA. It should however be noted that the cfDNA was obtained two months after the biopsy, meaning that changes in *ESR1* mutation status could also be due to therapy-related effects emerging after the initial biopsy. In an independent cohort consisting of 8 ER-positive patients, dPCR was once more demonstrated to be able to detect *ESR1* mutations in cfDNA, and in two more patients an *ESR1* mutation was observed in the cfDNA but not in the metastatic lesion. This study further underscored that dPCR is able to readily detect *ESR1* mutations in the cfDNA and that cfDNA seems to represent *ESR1* mutations in the metastatic lesions. Also, strikingly, *ESR1* mutations were detected in cfDNA but not in metastatic lesions, which may be indicative of heterogeneity within the metastatic lesion or between multiple metastases.

Another study only used dPCR to detect *ESR1* mutations [29], and *ESR1* mutations were detected in 7/29 MBC patients (24%), with one patient having polyclonal *ESR1* mutations. All patients with an *ESR1* mutation in cfDNA received at least one line of endocrine treatment, mainly AIs or tamoxifen. In this series, also an *ESR1* mutation was seen in a patient who had only received prior treatment with fulvestrant. Of particular interest were the serial blood draws in the patient with the polyclonal *ESR1* mutations, which revealed that two mutations were enriched during AI treatment and chemotherapy, while one mutation was absent after treatment. This may suggest that different mutations react differently to different treatments.

Schiavon and colleagues [34] were the first to present a study in which *ESR1* mutations were assessed in a relatively large cohort of MBC patients. With dPCR to examine cfDNA from MBC patients at the time of progression under endocrine therapy, *ESR1* mutations were observed in 18/128 patients (14%), with D538G mutations comprising 56% of all observed *ESR1* mutations. Polyclonality of *ESR1* mutations was observed in 21% of the patients. All patients in whom *ESR1* mutations were observed had received prior AI treatment, while no *ESR1* mutations were observed in a subset of 22 patients who had only received tamoxifen treatment. Interestingly, *ESR1* mutations were mainly detected in patients who received AIs only in the metastatic setting (36%), and not in patients who received AIs only in the adjuvant setting (4%) or in the adjuvant and metastatic setting (8%). In accordance were observations in two relatively small independent cohorts, in which no *ESR1* mutations were observed in 32 BC biopsies taken at recurrence after adjuvant AI treatment or in 7 cfDNA samples of MBC patients who received adjuvant AI treatment only. Regarding the outcome of patients with *ESR1* mutations, subgroup analyses in *ESR1* mutant versus wildtype patients revealed a significantly poorer progression-free survival (PFS) on subsequent AI treatment in patients harboring an *ESR1* mutation, although these analyses should be seen as exploratory given the small number of patients eligible for such analyses.

The observations by Schiavon et al. suggest that AI treatment in the metastatic setting, but not in adjuvant setting, causes *ESR1*

Table 1
Overview of *ESR1* mutation analysis in "liquid biopsies" of metastatic breast cancer patients.

Patients	Method	*ESR1* mutation	Substrate	Number of patients with *ESR1* mutations	D538G	Y537S	Y537N	Y537C	Other	Refs.
6 pts with ER-positive MBC either off or progressing on therapy	RNA sequencing	whole transcriptome	Cultured CTCs	3/6 (50%)	1/6 (17%)	1/6 (17%)	–	–	1/6 (17%) L536P	[43]
48 pts with ER-positive MBC receiving endocrine therapy	NGS	E380Q, V392I, P535H, Y537C/N/S, D538G	cfDNA	3/48 (6%)	1/48 (2%)	1/48 (2%)	–	–	1/48 (2%) E380Q	[21]
48 pts with ER-positive MBC receiving endocrine therapy	ddPCR	D538G	cfDNA	9/48 (19%)	9/48 (19%)	NP	NP	NP	NP	
3 pts with *ESR1*mutation detected in cfDNA by NGS	NGS	E380Q, V392I, P535H, Y537C/N/S, D538G	CTCs	1/3 (33%)	1/3 (33%)	–	–	–	–	
128 pts with ER-positive MBC, progression on therapy	ddPCR	D538G, Y537C/N/S, L536R	cfDNA	18/128 (14%)	14/128 (11%)	3/128 (2%)	4/128 (3%)	2/128 (2%)	2/128 (2%) L536R	[34]
11 pts with ER-positive MBC (8 with known *ESR1* mutation in metastatic biopsy by NGS)	ddPCR	D538G, Y537N/S	cfDNA	9/11 (82%)	6/11 (55%)	3/11 (27%)	1/11 (9%)	NP	NP	[33]
8 pts with ER-positive MBC	ddPCR	D538G, Y537N/S	cfDNA	6/8 (75%)	4/8 (50%)	2/8 (25%)	1/8 (13%)	NP	NP	
29 pts with MBC	ddPCR	K303R, S463P, Y537C/N/S, D538G	cfDNA	7/29 (24%)	6/29 (21%)	2/29 (7%)	–	1/29 (3%)	–	[29]
161 pts ER-positive MBC with prior sensitivity to nonsteroidal AI (SoFEA)	ddPCR	E380Q, L536R, Y537C/N/S, D538G, S463P	cfDNA	63/161 (39%)	29/161 (18%)	16/161 (10%)	23/161 (14%)	3/161 (2%)	6/161 (4%) E380Q, 6/161 (4%) S463P, 2/161(1%) L536R	[38]
360 pts with ER-positive MBC with progression on endocrine therapy (PALOMA3)	ddPCR	E380Q, L536R, Y537C/N/S, D538G, S463P	cfDNA	91/360 (25%)	51/360 (14%)	23/360 (6%)	14/360 (4%)	5/360 (1%)	22/360 (6%) E380Q, 4/360 (1%) S463P, 1/360 (1%) L536R	
153 pts with ER-positive MBC pre-treated with AI (FERGI)	BEAMing	E380Q, S463P, V524E, P535H, L536H/P/Q/R, Y537C/N/S, D538G	cfDNA	57/153 (37%)	31/153 (20%)	19/153 (12%)	10/143 (7%)	6/143 (4%)	15/153 (26%) E380Q, 5/143 (3%) S463P, 7/143 (5%) L536P	[36]
5 pts with MBC (4 ER+, 1 TN), with ⩾100 CTCs	NGS	*ESR1* exome	40 single CTCs	10/40 (25%)	7/40 (18%)	–	–	–	3/40 (8%) E380Q	[44]
5 pts with MBC (4 ER+, 1 TN), with ⩾100 CTCs	NGS	*ESR1* exome	cfDNA	3/5 (60%)	2/5 (40%)	–	–	1/5 (20%)	1/5 (20%) E380Q	
541 pts with ER-positive MBC with progression after nonsteroidal AI (BOLERO-2)	ddPCR	D538G, Y537S	cfDNA	156/541 (29%)	113/541 (21%)	72/541 (13%)	–	–	–	[40]

ddPCR = droplet digital PCR, NP = Not performed. Number of patients with a *ESR1* mutation in different study cohorts are listed. The specific mutations observed in these patients are also shown; in case of polyclonality, these numbers may exceed the total number of patients with a *ESR1* mutation.

mutations. This may suggest selection of subclones already present in the primary tumor, or in the metastases when the tumor load is increased and the probability of acquiring mutations increases [35]. This first observation could be in line with the previously mentioned findings by Wang et al. whom found *ESR1* mutations at extremely low VAF in primary tumors of MBC patients with *ESR1* mutations. While the study by Schiavon and colleagues also provided evidence for an impaired response to AI treatment, larger studies were needed to confirm these findings and to examine whether MBC patients with *ESR1* mutations will have improved responses on alternative therapies.

ESR1 mutations and outcome on endocrine therapies

In the randomized phase II FERGI trial, baseline plasma samples of patients failing to AI treatment randomized either to fulvestrant combined with the pan-PI3K inhibitor pictilisib or to the combination of fulvestrant and placebo, were examined for *ESR1* and *PIK3CA* mutations in tissue and cfDNA using BEAMing [36]. They detected *ESR1* mutations in cfDNA in 57/153 (37%) of patients at baseline; 13 patients (23%) harbored polyclonal mutations. Surprisingly, the prevalence of the E380Q mutation was rather high (26%), while this mutation was previously not often observed. No *ESR1* mutations were detected in 42 matched primary tumors of patients with *ESR1* mutations in cfDNA. *PIK3CA* mutations were observed in the cfDNA of 40% of the patients and were generally concordant with findings in matched metastatic tissue. For the *ESR1* mutations, discordances between the cfDNA and metastatic biopsies occurred more frequently and cfDNA sometimes harbored more *ESR1* mutations than the metastatic biopsies. These analyses were however limited by the fact that metastatic tissue and cfDNA were generally not collected on the same day. Of note was that the median VAF of *PIK3CA* mutations was markedly higher than for *ESR1* mutations (3.6% versus 0.45%). The higher VAFs and concordance with tissue probably reflect that *PIK3CA* mutations usually occur in earlier stages of BC [37], in contrast to *ESR1* mutations. Similar to Wang and colleagues [29], it was observed in multiple longitudinal samples in patients with polyclonal *ESR1* mutations that different *ESR1* mutations reacted differently under treatment.

The clinical analyses in the fulvestrant/placebo arm of the FERGI study revealed that patients with an *ESR1* mutation in ctDNA had no impaired PFS on fulvestrant compared to *ESR1* wildtype. When the analyses were further restricted to those patients with polyclonal *ESR1* mutations or *ESR1* mutation with VAF above the median, also no effect on PFS was observed. Also no differences in PFS were observed in patients with and without *ESR1* mutations receiving fulvestrant and pictilisib.

The data from the FERGI study suggested that fulvestrant does not have reduced activity in patients with *ESR1* mutations. However, data on the impact of *ESR1* mutations on outcome to fulvestrant versus AI treatment and the addition of other agents to fulvestrant treatment were still missing. These gaps were filled by data from two phase III randomized trials, reported by Fribbens et al. whom assessed *ESR1* mutations in cfDNA by dPCR [38]. In the SoFEA study, patients who had previously benefited from a non-steroidal AI were randomly assigned to fulvestrant combined with anastrozole, fulvestrant with placebo, or exemestane alone. Mutations were detected at baseline in 63/161 (39%) patients; 27/55 (49%) patients evaluable for polyclonal mutations had such mutations. Patients with an *ESR1* mutation had an improved PFS after taking a fulvestrant-containing regimen versus exemestane (median PFS fulvestrant-containing 5.7 versus exemestane 2.6 months, $P = 0.02$), in contrast to *ESR1* wildtype patients in whom a similar PFS was found (5.4 months versus 8.0 months, $P = 0.77$). Within the exemestane-treated patients, patients with *ESR1* mutations ($n = 18$) had a worse PFS compared to patients having an *ESR1* wildtype ($n = 39$), (median PFS 2.6 versus 8.0 months $P = 0.01$).

In the PALOMA3 study, patients who failed on prior endocrine therapy were randomized to fulvestrant in combination with the CDK4/6-inhibitor palbociclib or to fulvestrant and placebo. In 91/360 patients (25%), *ESR1* mutations were detected with polyclonal mutations observed in 26/91 (29%). The main study revealed a significant PFS benefit in patients receiving fulvestrant/palbociclib versus patients receiving fulvestrant alone (median 9.5 versus 4.6 months, $P = 0.0001$) [39]. This PFS benefit was maintained in patients with *ESR1* mutations (median 9.4 versus 3.6 months, $P = 0.002$), while no PFS difference was observed between *ESR1* mutants and wildtype in patients treated with fulvestrant/palbociclib (median 9.4 versus 9.5 months, respectively). Although median PFS seemed to be slightly worse in the *ESR1* mutated patients treated with fulvestrant alone (3.6 months 95% CI, 2.0–5.5) compared to *ESR1* wildtype (5.4 months 95% CI 3.5–7.4), this was not statistically significant, which is in line with the results of the FERGI study [36].

So far, the only large study providing overall survival (OS) data with respect to *ESR1* mutations is the phase III BOLERO-2 study [40]. In this study, postmenopausal women who progressed on an AI were randomized to the AI exemestane combined with everolimus, or exemestane and placebo. Overall, 156/541 (29%) of evaluable patients had either a D583G and/or Y537S *ESR1* mutation detected in their cfDNA, with double-mutations detected in 30/541 (6%) patients. *ESR1* mutations were more prevalent in patients who had previously received AI treatment for metastatic disease (33%) than in patients who had received AIs as adjuvant therapy (11%), supporting previous data from Schiavon et al. [34]. The results of the main study revealed that median PFS was significantly improved in patients treated with everolimus and exemestane compared to exemestane and placebo (7.8 months versus 3.2 months), though the combination therapy did not result in improved OS [41,42]. In the *ESR1* mutation-driven subgroup analyses for PFS in the exemestane arm, patients with a mutation in D538G had a shorter PFS than *ESR1* wildtype patients (2.7 versus 3.9 months), which is in accordance with the findings of the SoFEA study [40,38]. When the analysis was restricted to patients with a Y537S mutation only, this association was not observed, which may be related to the limited sample size for these subgroup analyses. Of note is that the PFS of *ESR1* wildtype patients was 3.9 months in this study, while in the SoFEA study this was 8 months. This discrepancy in PFS might be due to differences in selection criteria of both studies. In the SoFEA trial only patients who received a non-steroidal AI as adjuvant therapy or as first-line therapy for MBC were included whereas patients in the BOLERO-2 trial were also included after receiving more lines of therapy for MBC representing a more advanced disease stage. When everolimus was added to exemestane this resulted in an improved median PFS in both D538G mutated (5.8 months; HR 0.34, 95% CI 0.02–0.6) and wildtype patients (8.5 months; HR 0.4, 95% CI 0.3–0.5), suggesting that *ESR1* mutated patients could still benefit from the addition of everolimus. Of note is that benefit of the addition of everolimus was not demonstrated for patients with a Y537S mutation alone (4.2 months; HR 0.98, 95% CI 0.5–1.9), or with both a Y537S and D538G mutation (5.4 months; HR 0.53, 95% CI 0.2–1.3). Again, one should keep in mind that these analyses may have suffered from the limited sample size of patients with only a Y537S mutation or a polyclonal *ESR1* mutation. If larger future studies confirm that patients with a Y537S indeed do not benefit from the addition of everolimus, this mutation might be used to select for patients who should be treated with other treatment modalities. Overall, the absolute median PFS interval seemed to be shorter in patients with an *ESR1* mutation than in *ESR1* wildtype patients, however, no formal analyses on these potential

differences were observed. In this context, it was intriguing that OS analyses according to *ESR1* mutation status showed that patients with an *ESR1* mutation had a worse OS compared to wildtype patients (median OS 22 versus 32 months). Noteworthy, the type of individual mutations was also suggested to influence OS, with a median OS of 26 months for patients with a D538G mutation only and 20 months for the Y537S mutation alone. In patients harboring both mutations the OS was even worse with a median OS of 15 months. Overall, these results may indicate that *ESR1* mutations are associated with more aggressive disease biology.

Discussion

The putative role of *ESR1* mutations in endocrine resistance has sparked a wide interest in techniques enabling their detection, the conditions under which they appear, and whether their detection can ultimately assist treatment decision-making in MBC patients.

Regarding the best substrate for *ESR1* mutation detection, data from multiple studies suggests that the cfDNA compartment sometimes provides additional mutations compared to matched metastatic tumor material. This may indicate that cfDNA is more representative of the whole somatic tumor landscape. Another obvious advantage of cfDNA over metastatic biopsies is that it can easily be obtained repeatedly during treatment. Therefore, future studies on the clinical relevance of *ESR1* mutations should preferably be performed using cfDNA, measuring mutations not only at baseline but also sequentially during treatment. Of note, *ESR1* mutations can also be detected in CTCs [21,43,44], but at this point it is unclear how *ESR1* mutation detection in CTCs relates to *ESR1* mutation detection in cfDNA, and if this adds anything to *ESR1* mutation analyses in cfDNA.

Assessing *ESR1* mutations in tissue and cfDNA provided clues as to how these *ESR1* mutations are enriched in MBC patients. Very strong indirect evidence exists for the enrichment of these *ESR1* mutations during treatment with AIs in the metastatic setting. However, to date no direct evidence for the enrichment of *ESR1* mutations under AI treatment has been presented yet. In this context it is also of note that several studies observed *ESR1* mutations in metastases or cfDNA from patients treated with SERMs or SERDs only, or from patients not treated with endocrine therapy at all [5,21,28,29]. This further underlines that the understanding on how *ESR1* mutations exactly occur is still limited. *ESR1* mutations are present at very low frequencies in primary BC tumors using dPCR [29], supporting the hypothesis that *ESR1* mutations may already be subclonally present in the primary tumor, and because of growth advantages, become the more prominent clone under treatment pressure [34]. *ESR1* mutations might also occur as a result of mutational processes such as initiated by the APOBEC enzymes, however the mutational pattern of the hotspot *ESR1* mutations (T > A/C/G) does not follow an APOBEC pattern or the pattern of any other mutational signature known to date [45,46].

While the exact mechanism behind the enrichment of *ESR1* mutations in MBC is still unknown, the clinical relevance of *ESR1* mutations being present in cfDNA becomes evident. PFS after treatment with the AI exemestane was impaired in the patients harboring an *ESR1* mutation [38,40], while fulvestrant had similar efficacy in patients with an *ESR1* mutation versus patients without an *ESR1* mutation. Given these results with fulvestrant, efficacy of tamoxifen may also be unaffected in patients harboring *ESR1* mutations, however, no clinical data on this is present as of yet. For the addition of other agents to endocrine treatment, for example palbociclib or everolimus, the question remains whether the presence of *ESR1* mutations is of any predictive significance for the efficacy of these agents. While the *ESR1* mutation status did not seem to impact median PFS in patients receiving fulvestrant and palbociclib, the presence of an *ESR1* mutation in patients treated with exemestane and everolimus might be associated with decreased PFS compared to *ESR1* wildtype patients.

Since a raise in *ESR1* mutation ratio during the course of treatment may be indicative of progressive disease [47] and *ESR1* mutations in general are associated with poor outcome [40], it will be of particular interest to see whether certain treatments (for example fulvestrant combined with palbociclib or specific chemotherapeutic regimen) are able to select against *ESR1* mutant subclones. Recently, it was shown that upon the discontinuation of anti-epidermal growth factor receptor (EGFR) antibodies, resistant KRAS mutant clones decay, allowing re-challenges with anti-EGFR antibodies in particular patients [48]. If *ESR1* mutations are lost with certain treatment regimen, this could potentially allow re-challenges with AIs in a subset of patients.

Also currently unknown is whether the different *ESR1* mutations result in distinctive phenotypes. Functional studies on *ESR1* mutations did not specifically focus on differences between various *ESR1* mutations, and for some *ESR1* mutations that have been measured in clinical studies (e.g. K303R, V524E, P535H, L536H/P/R), very little functional evaluation of its constitutive activity and potential role in endocrine resistance has been performed at all. In addition, clinical studies to date have generally been underpowered for subgroup analyses evaluating differential effects of different *ESR1* mutations. Even further complicating such analyses is the described polyclonality of *ESR1* mutations. Multiple studies with anecdotal longitudinal sampling data suggested that in patients with polyclonal *ESR1* mutations there are differential effects of therapy on different *ESR1* mutations. This suggests that *ESR1* mutations are present in different subclones, and not in the same cell. Theoretically, this may mean that patients with polyclonal mutations are more difficult to treat given the wider repertoire of resistance mutations. However, in rather small analyzed groups of patients with polyclonal *ESR1* mutations treated with fulvestrant such effects were not observed. Given that some *ESR1* mutations are rarer than others, the most pragmatic way to evaluate the prognostic value of these rare *ESR1* mutations will likely be in the form of a meta-analysis in due time, as it is virtually impossible to evaluate the prognostic value of these mutations in single studies. In addition, functional studies evaluating all LBD *ESR1* mutations described in patients to date, validating their constitutive activity and exploring potential differential effects of different *ESR1* mutations are of interest.

The current evidence on *ESR1* mutations warrants prospective studies in which patients are randomized and treated according to the *ESR1* mutation status in cfDNA. Therefore, standardized methods to process plasma, to isolate cfDNA and to prepare and analyze the dPCR chips are needed. A lot of the recent *ESR1* mutation research was performed on cfDNA samples that were suboptimally collected. For example, in the SoFEA trial, plasma was collected in EDTA tubes and processed up to 9 days after sample collection which may have consequences for the sensitivity to detect *ESR1* mutations, especially in the context of longitudinal sampling [49,50]. Recently, it was demonstrated that blood collected in CellSave or BCT blood tubes assures optimal quality of cfDNA for dPCR or NGS analyses for up to 96 hours after the blood draw [49–51], providing opportunities to send blood samples to remote locations for plasma isolation. In addition, it is of utmost importance to assess variables such as intra-assay, inter-assay, inter-lab and inter-observer variability when using dPCR, which are currently not only poorly studied for *ESR1* mutations, but also for cfDNA analyses in general.

In conclusion, the presence of *ESR1* mutations in patients with ER-positive MBC has high potential for clinical validity and utility. Prospective studies in which the exact role of how *ESR1* mutations can be used to guide treatment decision-making have to be initi-

ated, but firstly standardization of protocols to assess these mutations will be necessary to eventually allow clinical implementation.

Contributors

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Declaration of interest

Conflicts of interest: none

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ctrv.2016.11.001.

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