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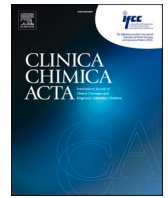
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## Development of sensitive and robust multiplex digital PCR assays for the detection of *ESR1* mutations in the plasma of metastatic breast cancer patients

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### ABSTRACT

**Background:** Early detection of *ESR1* mutations is a key element for better personalization of the management of patients with HR+/HER2- Metastatic Breast Cancer (MBC). Analysis of circulating tumor DNA from liquid biopsies is a particularly well-suited strategy for longitudinal monitoring of such patients.

**Materials and methods:** Using the naica® three-color digital PCR platform, we developed a screening assay allowing the detection of 11 *ESR1* mutations and designed a sequential strategy for precise mutation identification. We then applied this strategy in the analysis of plasma circulating cell-free DNA from 109 HR+/HER2-MBC patients and performed a double-blind comparison study on a subset of patients with the multiplex assay used at the Institut Curie (IC) for the PADA-1 study.

**Results:** Thirty-one patients (28.4%) harboured at least one *ESR1* mutation, with the following frequencies: D538G (41.03%), Y537S (25.64%), E380Q (10.26%), Y537N (10.26%), “(536–540)” (7.69%), Y537C (2.56%), and L536R (2.56%). The presence of *ESR1* mutation(s) was significantly associated with liver metastases ( $p = 0.0091$ ). A very good agreement (91%) was observed with the IC assay.

**Conclusion:** Our assays have proven to be robust and highly sensitive and are very well-suited for monitoring *ESR1* mutations in the plasma of MBC patients.

### 1. Introduction

Estrogen receptor alpha (ER) is encoded by the *ESR1* gene. About 70% of primary breast cancers are characterized by ER expression, and targeting ER with endocrine therapy (ET) is the current standard of care

at the initial or metastatic stages of the disease [1,2]. In 1997, it was discovered that mutations could occur in the *ESR1* gene, resulting in constitutive receptor activity with reduced sensitivity to ET [3].

Such *ESR1* mutations are rarely detected in primary tumors. In a large series of 3,217 primary non-metastatic breast cancers, the presence

**Abbreviations:** AI, Aromatase Inhibitor; CEM, Centre Eugène Marquis; cfDNA, circulating cell-free DNA; COSMIC, Catalogue of Somatic Mutations in Cancer; ctDNA, circulating tumor DNA; CV, Coefficient of Variation; DO, Drop-Off; dPCR, digital PCR; ER, Estrogen Receptor alpha; *ESR1*, Estrogen Receptor 1; ET, Endocrine Therapy; HER2, Human Epidermal growth factor Receptor 2; HR, Hormone Receptor; IC, Institut Curie; LBD, Ligand-Binding Domain; LOB, Limit of Blank; LOD, Limit of Detection; MBC, Metastatic Breast Cancer; MUT, Mutated; NGS, Next-Generation Sequencing; OS, Overall Survival; PBMC, Peripheral Blood Mononuclear Cells; PFS, Progression Free Survival; REF, Reference; SERD, Selective Estrogen Receptor Degradator; SD, Standard Deviation; WT, Wild-Type.

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of *ESR1* mutation was detected in 0.9% of cases using RNA sequencing [4]. Interestingly, for ET-treated patients, *ESR1* mutation was associated with worse progression free survival (PFS) and overall survival (OS) [4]. In another study, *ESR1* mutation was detected by digital PCR (dPCR) in 9 of 121 (7.4%) ER + breast cancer patients who had relapsed after surgery and was associated with primary endocrine resistance in an adjuvant setting [5]. This indicates that, even though *ESR1* mutations are rare events in primary breast cancer, screening for them may be applicable in identifying patients likely to develop early ET resistance.

*ESR1* mutations are usually acquired during ET treatment (particularly after treatment with Aromatase Inhibitor (AI)), which leads to an enrichment at the metastatic stage of the disease compared to the initial stage. *ESR1* mutations are identified in about 20% to 40% of ER+ Metastatic Breast Cancer (MBC) cases after ET therapy, and their detection could guide clinicians in their choice of treatment [6]. AI treatment alone may not be advocated in patients displaying *ESR1* mutations, but it has been shown that fulvestrant (a selective estrogen receptor degrader (SERD)) is still effective in MBC harbouring these mutations [7,8]. In addition to being associated with ET resistance, the presence of *ESR1* mutation is associated with the acquisition of enhanced proliferative, stem-cell, epithelial-to-mesenchymal transition and metastatic phenotypes [6]. This may be connected to the fact that *ESR1* mutation is associated with decreased PFS and OS in ER+/HER2-MBC [9–11].

Given that *ESR1* mutations appear under the pressure of treatment, identification of their emergence requires longitudinal analysis. Blood-based liquid biopsy is therefore particularly well suited for *ESR1* mutation detection, especially because it has been shown that different metastatic sites can harbour different mechanisms of endocrine resistance, such as *ESR1* mutations [12]. Circulating tumor DNA (ctDNA) analysis has the double advantage of being adapted to longitudinal monitoring and able to capture potential genetic heterogeneity of metastatic lesions. Recently, the randomized phase III PADA-1 trial (NCT03079011) has shown the usefulness of periodic monitoring for the emergence or rise of *ESR1* mutations in ctDNA in order to trigger the shift from AI to fulvestrant in hormone receptor (HR) positive/HER2-MBC patients treated by first line AI-palbociclib without radiologic progressive disease. After a median follow-up of 26 months, the median PFS of patients who switched to fulvestrant was over twice as long as that of those who remained on an AI treatment (11.9 months, compared with 5.7 months, respectively) after *ESR1* mutation detection [13]. Another phase III randomised study (SERENA-6, NCT04964934) is currently underway to assess switching to AZD9833 (a next-generation oral SERD) plus CDK4/6 inhibitor versus continuing AI plus CDK4/6 inhibitor treatment in HR+/HER2- MBC with a detectable *ESR1* mutation in plasma.

*ESR1* mutations are currently regarded as an interesting emerging clinical biomarker for HR+ MBC, and testing has begun to be integrated into patient care. The two most frequently used techniques for mutation detections in ctDNA are next-generation sequencing (NGS) and digital PCR (dPCR). In the PADA-1 study, *ESR1* mutations in ctDNA were analysed by dPCR using a QX200 system (Bio-Rad) [14]. The intrinsic qualities of this technique are high sensitivity and robustness at a low cost; with the limitation of having to know a priori the mutations to target. Using the three-color Crystal dPCR™ naica® platform (Stilla Technologies), we designed a screening assay that allows the simultaneous detection of 11 pathogenic *ESR1* mutations in a single test. This assay combines a drop-off system targeting the mutations on codons 536–540 with the specific detection of the E380Q mutation. The drop-off system is a two-color detection strategy that involves using two differently labelled probes simultaneously on the same amplicon, with a “reference” probe targeting an invariant region and a “drop-off” probe targeting a mutational hotspot. Both probes target the wild-type (WT) sequence. Thus, a double-positive fluorescence signal indicates the presence of WT sequences, while a lower fluorescence intensity in the color assigned to the drop-off probe indicates the presence of any given

mutation on the targeted mutational hotspot. This is induced by the complete or partial loss of drop-off probe binding, which makes the mutation(s) clearly distinguishable from the WT signal. This particular strategy is perfectly adapted to multiplexed screening assays, as it allows for the detection of multiple potential mutations using only two probes. Here, we present the analytical performances of this assay, our strategy for precisely identifying the *ESR1* mutation(s) in case of a positive screening assay, and the results obtained from a series of plasmas taken from HR+/HER2- MBC, with a cross-validation with the dPCR assay used for the PADA-1 study.

## 2. Materials and methods

### 2.1. Patients

A total of 138 plasma samples from 109 HR+/HER2- MBC female patients treated at the Department of Medical Oncology of the Centre Eugène Marquis (CEM) in Rennes were included in this study (Supplementary Dataset). A blood sample was prospectively collected from each patient at the time of disease progression for circulating cell-free DNA (cfDNA) extraction. The results obtained from the cfDNA samples were compared to the results obtained at the Institut Curie (IC) using their own multiplex assay for a subset of 33 samples from 30 patients. The research protocol was conducted under French legal guidelines and was approved by the medical ethics committee, CREDO, in the CEM. Written informed consent was obtained from all patients.

### 2.2. Sample collection and processing

For cfDNA samples, 20 ml of blood were collected using two 10 ml K<sub>2</sub>EDTA blood collection tubes (BD Vacutainer®, Beckton, Dickinson) and processed within four hours of collection. Plasmas were obtained through double centrifugation at 1,600g for 15 min and 4,500g for 10 min, and were stored at -80 °C prior to cfDNA extraction.

### 2.3. Nucleic acid extractions, quality and quantity assessments

cfDNA samples were extracted from 4 to 5 ml of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) and were resuspended in a final volume of 50 µl of AVE buffer. The quality of the extracted nucleic acids was assessed using the High Sensitivity DNA kit on a 2100 Bioanalyzer Instrument (Agilent Technologies) and the quantity was assessed using the Qubit™ dsDNA HS Assay kit on a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific) (Supplementary Table S1).

### 2.4. In silico design and verifications of the *ESR1* assays

All primers and hydrolysis probes (Supplementary Table S2) were designed as previously described [15], using the sequences of the *ESR1* gene (NG\_008493.2) for the studied mutations (Supplementary Table S3). All oligonucleotides were synthesized by Eurogentec.

### 2.5. Design of *ESR1*-mutated cfDNA-like positive controls

We designed gBlock® Gene Fragments (gBlock) (Integrated DNA Technologies) of 166 bp to serve as positive controls (Supplementary Table S4). For the Drop-Off<sub>536–540</sub> system, we selected the seven most frequent mutations occurring on codons 536–540, representing 98% of all of the pathogenic mutations identified on this hotspot for breast carcinoma tumor samples in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Supplementary Figures S1 and S2).

### 2.6. Other sources of DNA used during validation experiments

Wild-type gDNA were extracted from the peripheral blood mononuclear cells (PBMC) of three healthy donors.

## 2.7. Digital PCR workflow and Crystal dPCR™ data analysis

All dPCR experiments were performed and analysed following the same workflow detailed in [15]. Briefly, each PCR was performed in a final volume of 25  $\mu$ l, containing 5  $\mu$ l of 5X PerfeCTa Multiplex qPCR ToughMix (Quanta BioSciences), 2.5  $\mu$ l of 1  $\mu$ M Fluorescein (VWR), 2.5  $\mu$ l of homemade 10X *ESR1* assay (Supplementary Table S2) and 15  $\mu$ l of input cfDNA sample. Highly concentrated samples were diluted in DNase/RNase Free UltraPure™ distilled water (Invitrogen) to reach a maximum theoretical concentration of 10,000 copies/PCR (33 ng/PCR) in order to limit background noise. Additionally, samples with low concentrations were assayed in two or three replicates to increase sensitivity by investigating at least 10 ng/PCR, while a negative H<sub>2</sub>O control and a positive control containing a mixture of WT gDNA and mutated gBlocks were included in each run. PCR programs included an initial “partition” step allowing for the formation of 15,000 to 30,000 droplets of  $0.59 \pm 0.03$  nl, self-arranged into a crystal-like pattern, followed by PCR amplification cycles (Supplementary Table S5). The chips were imaged with the naica® Prism3 scanner using Crystal Reader™ software v2.4.0.3 with optimized parameters (Supplementary Table S6), and the analyses were performed using Crystal Miner™ software v2.4.0.3. Notably, thresholds for the classification of WT and mutated (MUT) droplet clusters for patient samples were set manually using the polygon gates on 2D dot plots based on the positions of the positive control clusters. These results were then “LOB-corrected” to account for the potential presence of false-positive droplets using the equation presented in [15].

## 2.8. Determination of the limits of blank (LOB<sub>95%</sub>) and the theoretical limits of detection (LOD<sub>95%</sub>)

Determination of the limits of blank (LOB<sub>95%</sub>), defined as the maximum number of false-positive droplets expected in a chamber at a probability of 95% (i.e., an  $\alpha$  risk equal to 5%) in a sample containing no target sequence, and the theoretical limits of detection (LOD<sub>95%</sub>), defined as the minimum concentration that can be considered non-zero and statistically higher than the LOB at a probability of 95%, were

performed as previously described [15]. A total of 34 replicates of WT-only samples (gDNA from healthy donors), with theoretical concentrations (based on the Qubit quantifications) of 10,000 copies/PCR, were tested, and the means of the numbers of false-positive droplets were calculated for each detection. The corrected means were then calculated using the following equation:  $\mu_{\text{corr}} = \mu + 1.645\sigma\sqrt{N}$ , where  $\mu$  is the mean,  $\sigma$  is the standard deviation (SD) of false-positive events, and  $N$  is the number of experiments performed. Notably, the LOB<sub>95%</sub> were determined by fitting the  $\mu_{\text{corr}}$  on Normal Law approximation and Chernoff's inequality, and the theoretical LOD<sub>95%</sub> were calculated following the instructions that Stilla Technologies provided (Supplementary Table S7).

## 2.9. Statistical analyses

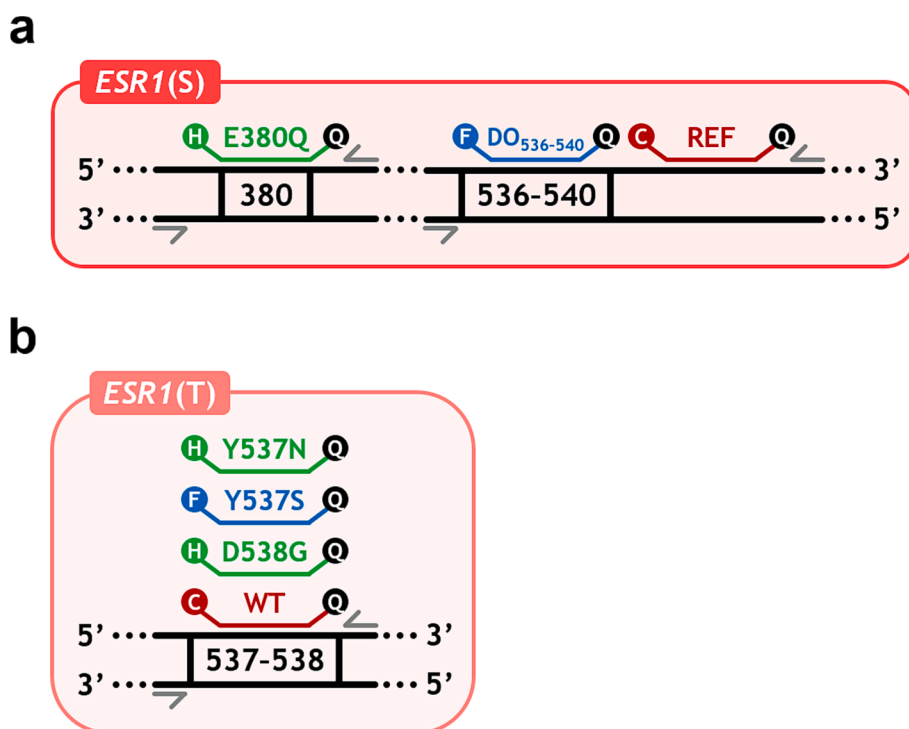
All statistical analyses were carried out using GraphPad Prism v8.0.0 (GraphPad Software). Coefficients of determination ( $R^2$ ) were calculated using linear regression analyses, while coefficients of variation (CV) were calculated using the following equation:  $CV (\%) = \frac{\sigma}{\mu} \times 100$ , where  $\sigma$  is the SD and  $\mu$  is the mean of the replicate results.

## 3. Results

Following the *in silico* design and verification methods previously described [15], we designed two multiplex assays. The *ESR1* screening assay (*ESR1*(S) assay) (Fig. 1a) associates a drop-off system for the detection of the 536–540 hotspot mutations (Drop-Off<sub>536-540</sub>) using a FAM-labelled drop-off probe covering the 536–540 hotspot and a Cy5-labelled reference probe located on the same amplicon, and the individual detection of the E380Q mutation. The *ESR1* targeted assay (*ESR1*(T) assay) (Fig. 1b), includes the detection of three of the most frequent *ESR1* mutations (Y537N, Y537S and D538G) using FAM and HEX-labelled probes together with a Cy5-labelled WT probe.

### 3.1. *ESR1* assays optimisation

We first checked the quality of the signals obtained in simplex



**Fig. 1. Design diagrams of the two multiplex assays for *ESR1* mutations detection.** (a) Design diagram of the *ESR1* screening (*ESR1*(S)) assay: two amplicons are simultaneously amplified using two couples of primers (grey arrows) to permit the detection of the E380Q mutation with a HEX-labelled probe (green) and the mutations on codons 536–540 using a FAM-labelled drop-off (DO) probe (blue) combined with a Cy5-labelled reference (REF) probe (red). (b) Design diagram of the *ESR1* targeted (*ESR1*(T)) assay: the detection of WT sequences is performed using a Cy5-labelled probe in competition with a FAM-labelled probe in order to detect Y537S mutations and HEX-labelled probes for the detection of Y537N and D538G mutations. (C: Cy5; DO: drop-off; F: FAM; H: HEX; MUT: mutation; REF: reference).



reactions (Fig. 2, “1D” left panels). The positive signals generated showed great separability from negative signals and very low amounts of “rain” droplets (of intermediate fluorescence). We then performed optimisation experiments using mixtures of WT gDNA and MUT gBlocks to identify the optimal oligonucleotide concentrations (Supplementary Table S2), annealing/elongation temperatures and scanning parameters (Supplementary Table S5). We defined the quantification strategies using the polygon gates for droplets classification on 2D dot plots (Fig. 2, “2D” centre panels) with the help of the 3D visualization for cluster identification (Fig. 2, “3D” right panels).

### 3.2. *ESR1* assays validation

The  $LOB_{95\%}$  and  $LOD_{95\%}$  were determined for one-, two- or three-replicate assays (Supplementary Table S7). The  $LOB_{95\%}$  for the one-replicate *ESR1*(S) assay was three droplets for the E380Q detection and four droplets for the 536–540<sub>MUT</sub> detection. For the *ESR1*(T) assay, we obtained four, six and three droplets for the Y537N, Y537S and D538G detections, respectively. Samples with numbers of positive droplets between the  $LOB_{95\%}$  and the  $LOD_{95\%}$  were systematically investigated using the *ESR1*(T) assay by performing two replicates, or three when needed, to increase the sensitivity.

For the sensitivity analyses, DNA mixtures were prepared using serial dilutions of MUT gBlocks in a constant WT gDNA background of 10,000 copies/PCR (Fig. 3). DNA mixtures were assayed in triplicate, except for the dilutions at Mutant Allelic Frequency (MAF) = 0.05%, which were performed in quadruplicate. We considered any detection with at least two replicates that had equal or higher numbers of positive droplets than the corresponding  $LOD_{95\%}$  to be positive, and calculated the means of the measured MAFs (%) obtained for all replicates. Thus, we obtained sensitivities of 0.16% for E380Q and 0.12% for 536–540<sub>MUT</sub> for the *ESR1*(S) assay, and 0.11% for Y537N, 0.09% for Y537S, and 0.07% for D538G for the *ESR1*(T) assay.

We performed a linearity study over a dynamic range from 5 to 10,000 copies/PCR (Supplementary Figure S3). The coefficients of determination calculated for the linear regressions performed between expected and measured concentrations of each detection ranged from  $R^2 = 0.9944$  to  $R^2 = 0.9999$ .

The coefficients of variation (CV) for the repeatability studies ranged from 4.6% to 8.2% (Supplementary Table S9).

For the *ESR1*(S) assay, inter-assay CV (reproducibility) was 15.2%, 12.9% and 12.4% for the WT, E380Q and 536–540<sub>MUT</sub> detections, respectively; for the *ESR1*(T) assay, it was 10.9%, 9.6%, 8.0% and 7.8% for the WT, Y537N, Y537S and D538G detections, respectively (Supplementary Figure S4).

We also performed cross-reactivity experiments to validate the specificity of detection for mutations involving competing probes, and no impact on quantifications was found (Supplementary Figure S5).

### 3.3. Diagnostic strategy for *ESR1* mutations identification

For the detection and identification of *ESR1* mutations, we developed a three-step diagnostic strategy (Fig. 4). We performed the screening assay first. If there was no evidence of *ESR1* mutation, samples were considered negative (Supplementary Figure S6). Otherwise, we performed the *ESR1* targeted assay (Fig. 5a). Finally, we performed individual WT-MUT Duplex assays as a third step to account for the less frequent *ESR1* mutations, such as L536R and Y537C (Fig. 5b).

### 3.4. *ESR1* assays results in patients

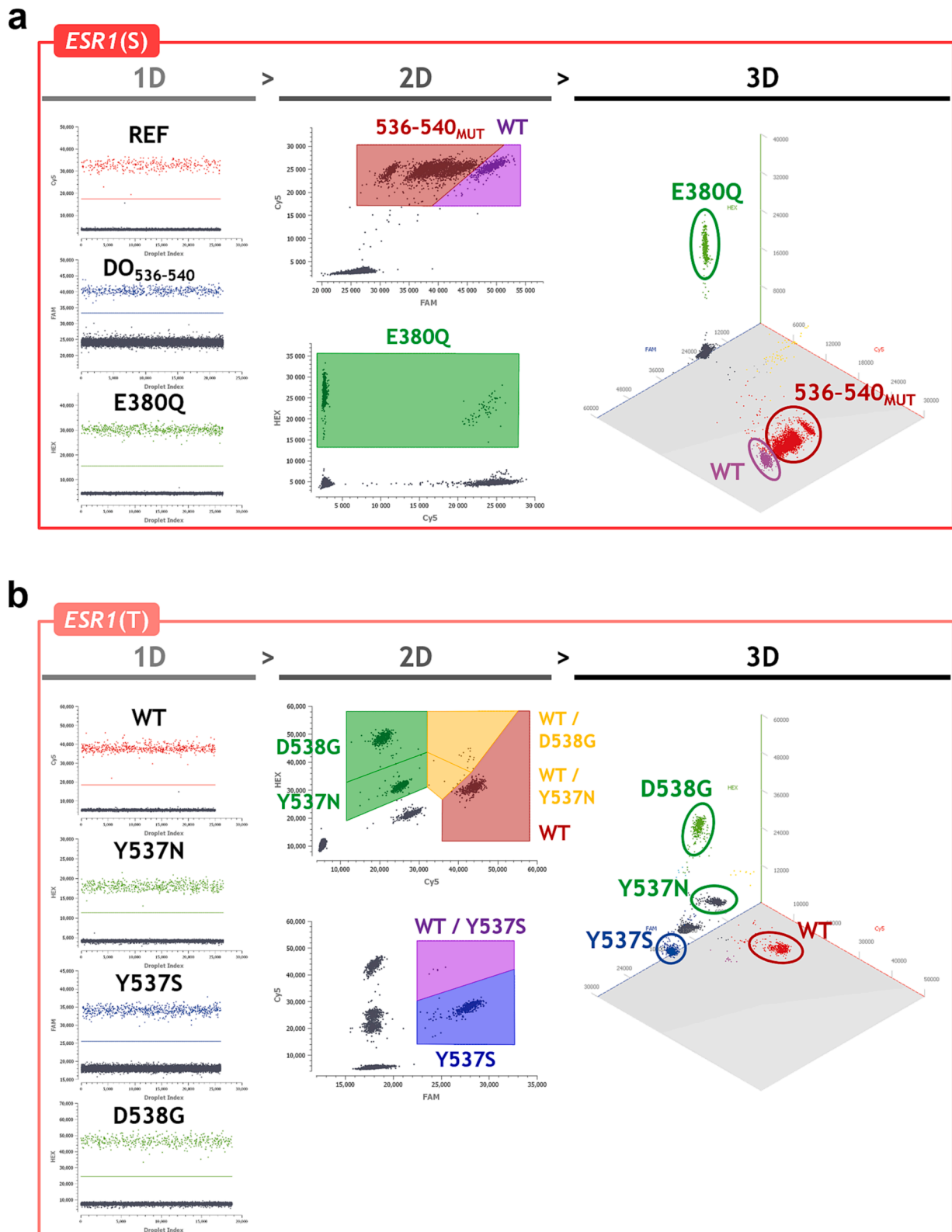
The median cfDNA concentration for the 138 plasma samples from the 109 patients was 10.1 ng/ml of plasma (range: 2.4–568 ng/ml of plasma; mean: 23.1 ng/ml of plasma) (Supplementary Dataset). Thirty-one patients (28.4%) harboured at least one mutation. A total of 39 mutations were found, with the simultaneous detection of two

mutations per sample occurring in five patients and the detection of four mutations occurring in one patient (Supplementary Figure S7). Among the 11 pathogenic mutations potentially detected, six were detected in our series of plasma samples with the following results: D538G (16/39, 41.03%), Y537S (10/39, 25.64%), E380Q (4/39, 10.26%), Y537N (4/39, 10.26%), Y537C (1/39, 2.56%), and L536R (1/39, 2.56%). Three mutations on codons 536–540 were revealed in the *ESR1*(S) assay but could not be identified: “(536–540)” (3/39, 7.69%) (Fig. 6a and 6b). Moreover, the relative frequencies obtained from our series of patients were rather consistent with the frequencies listed in the COSMIC database (Supplementary Table S8). The distribution of the quantifications obtained was quite broad in regards to copies/ml of plasma with the following results (median (min–max)): D538G (59 (3–712)), Y537S (38 (7–1,727)), E380Q (16 (3–246)), Y537N (10 (7–12)), Y537C (11), L536R (13,821), and “(536–540)” (41 (15–64)). The distribution was similarly broad for MAFs (%), with the following values (median (min–max)): D538G (1.10 (0.13–11.07)), Y537S (1.22 (0.22–14.07)), E380Q (0.25 (0.09–5.55)), Y537N (0.25 (0.16–0.33)), Y537C (0.14), L536R (28.68), and “(536–540)” (0.59 (0.18–1.87)) (Fig. 6c).

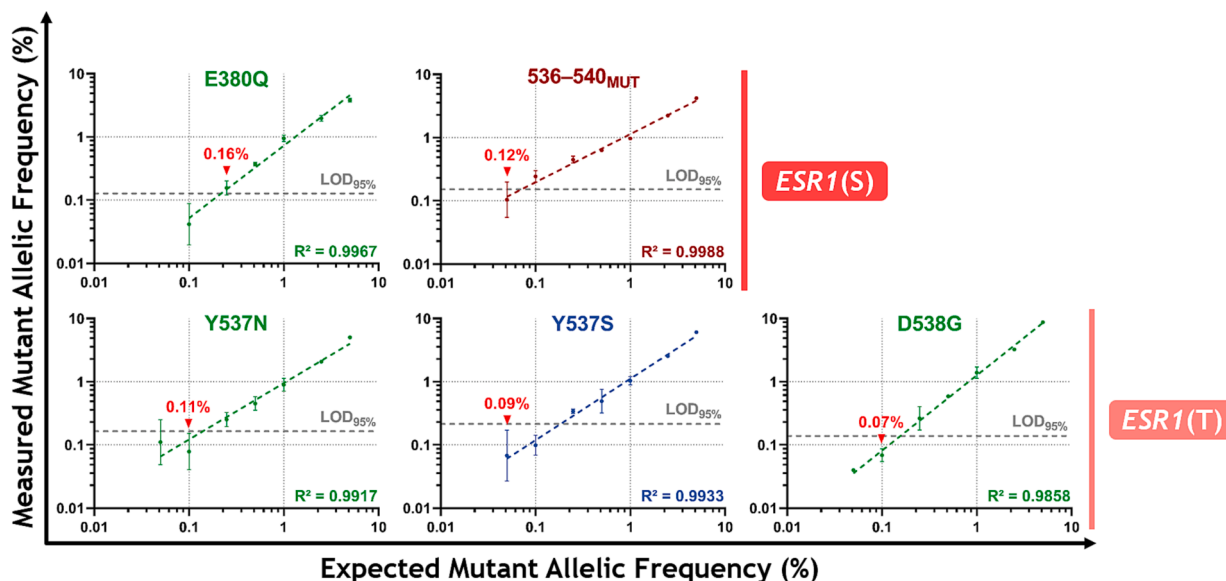
In a double-blind comparative study, we used a subset of 33 samples from 30 patients of this study to compare the results obtained from the *ESR1* assays to those obtained with the IC assay [16] (Fig. 7a, Supplementary Dataset). Out of the 20 mutated samples, 17 were also found to be mutated in the IC assay, while the three remaining samples had “indeterminate” results (i.e., few mutant droplets detected at first pass, a setting in which more cfDNA must be analysed to reach a conclusion about sample positivity status). These three samples had relatively low *ESR1* MAFs (0.89%, 0.22% and 0.13%) and the remaining 13 samples were detected to be non-mutated by both assays. Thus, we obtained 91% (30/33) concordance in this double-blind cross-validation experiment. Overall, the results obtained by the two assays showed a good correlation with a coefficient of determination, calculated for the linear regression performed between the MAFs obtained in the CEM and in the IC, of  $R^2 = 0.9822$  (Fig. 7b).

We also investigated whether there was an association between the presence of *ESR1* mutation and the following different clinical factors: age, de novo advanced or MBC status, type of metastases (bone-only, liver, visceral), duration of treatment (AI and ET) in the adjuvant and advanced settings. *ESR1* mutation was only significantly associated with liver metastases ( $p = 0.0091$ ) (Table 1).

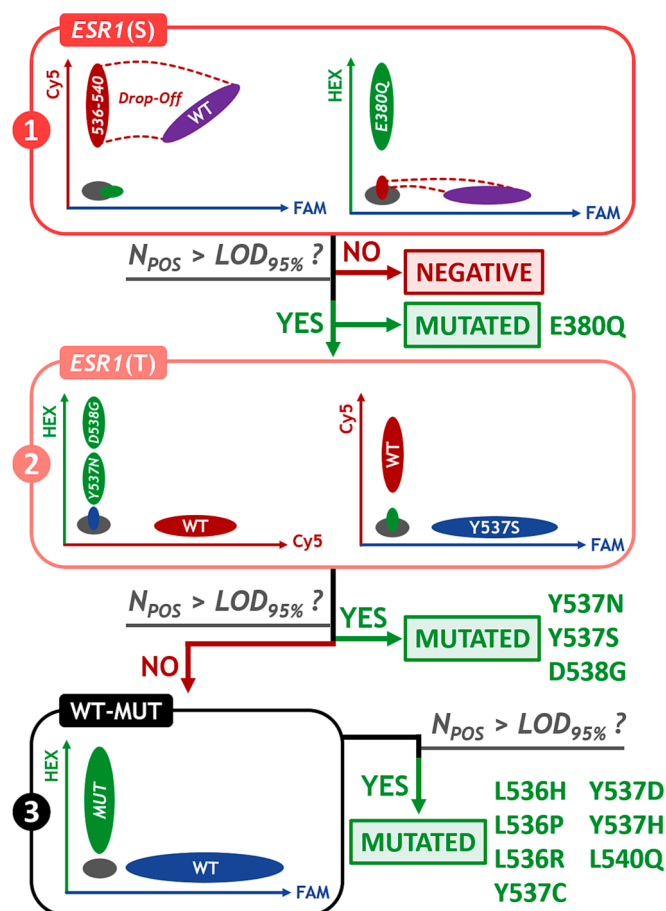
Longitudinal follow-ups were also performed for five patients (Fig. 8). After treatment with AI and fulvestrant, patient #5 harboured a low concentration of Y537S mutation (indeterminate result). Six months later, the Y537S mutation was present in high concentration, with the emergence of a D538G-mutated subclone. For patient #22, an increase in D538G concentration was observed over three consecutive years (11, 267, and 712 copies/ml of plasma, respectively), with a therapeutic failure that led to the patient’s death six months after the last sampling. Patient #27 received chemotherapy for bone metastases progression, with an initial significant decrease in Y537S concentration associated with a therapeutic response. However, a Y537S-D538G double-mutation was later detected and was concomitant with the appearance of new metastatic bone locations. Since both mutations were present at the same level, a new double-mutated tumor subclone may be suspected. In patient #73, after nine months of AI and palbociclib treatment, we observed the emergence of a D538G mutation, the concentration of which increased in parallel with clinical progression under the same treatment. The follow-up with patient #74 was particularly interesting: at progression, after 33 months on AI and palbociclib, during which several samplings had been done, no mutation was detected. One month after the end of HT and the start of chemotherapy, two mutations were identified. One explanation could be the emergence of mutated *ESR1* resistance clones, while another could be the lysis of pre-existing mutated clones under chemotherapy.



**Fig. 2. Optimisation and quantification strategies for the ESR1 assays.** (a) *ESR1* screening (*ESR1(S)*) assay: “1D” left panel show optimised fluorescence signals produced by each probe in a simplex reaction; “2D” centre panel show the polygon gating quantification strategy defined with a mixture of WT gDNA and MUT gBlocks of the E380Q, L536H/P/R, Y537C/N/S and D538G mutations; “3D” right panel show the cluster positions according to their relative FAM-HEX-Cy5 fluorescence signals. (b) *ESR1* targeted (*ESR1(T)*) assay: “1D” left panel show optimised fluorescence signals produced by each probe in a simplex reaction; “2D” centre panel show the polygon gating quantification strategy defined using a mixture of WT gDNA and MUT gBlocks for Y537N, Y537S and D538G mutations; “3D” right panel show the cluster positions according to their relative FAM-HEX-Cy5 fluorescence signals.



**Fig. 3. Evaluation of the sensitivity of the *ESR1* assays.** DNA mixtures were prepared through serial dilutions of MUT gBlocks in a constant WT gDNA background of 10,000 copies/PCR to reach theoretical MAFs of 5%, 2.5%, 1%, 0.5%, 0.25%, 0.1% and 0.05%. The *ESR1*(S) assay mixtures included MUT gBlocks for E380Q and the seven most frequent mutations identified on codons 536–540. Any detection with at least two replicates equal to or higher than the theoretical LOD<sub>95%</sub> (represented as grey, dotted lines) were considered positive, and the sensitivities of each detection (displayed in red) were defined as the means of the measured MAFs (%) obtained for all replicates. (MAF: Mutant Allelic Frequency).

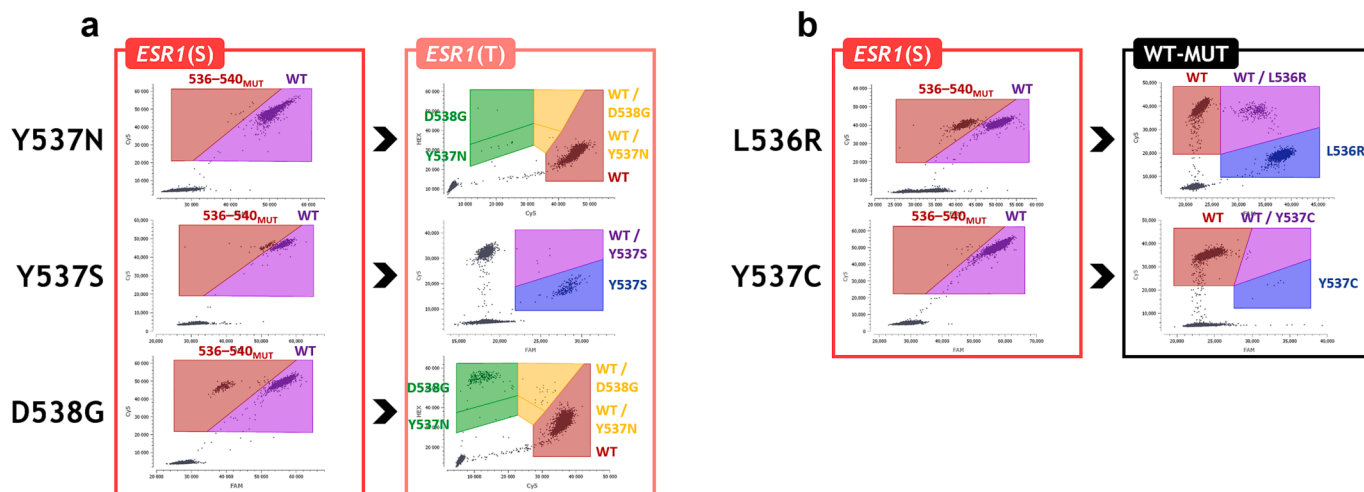


**Fig. 4. Diagnostic strategy for *ESR1* mutations identification.** The *ESR1*(S) assay, was performed as a first-line screening assay and was followed, in case of a positive result, by the *ESR1*(T) assay focusing on three of the most frequent *ESR1* mutations. Lastly, WT-MUT Duplex assays were performed to identify the less frequent mutations.

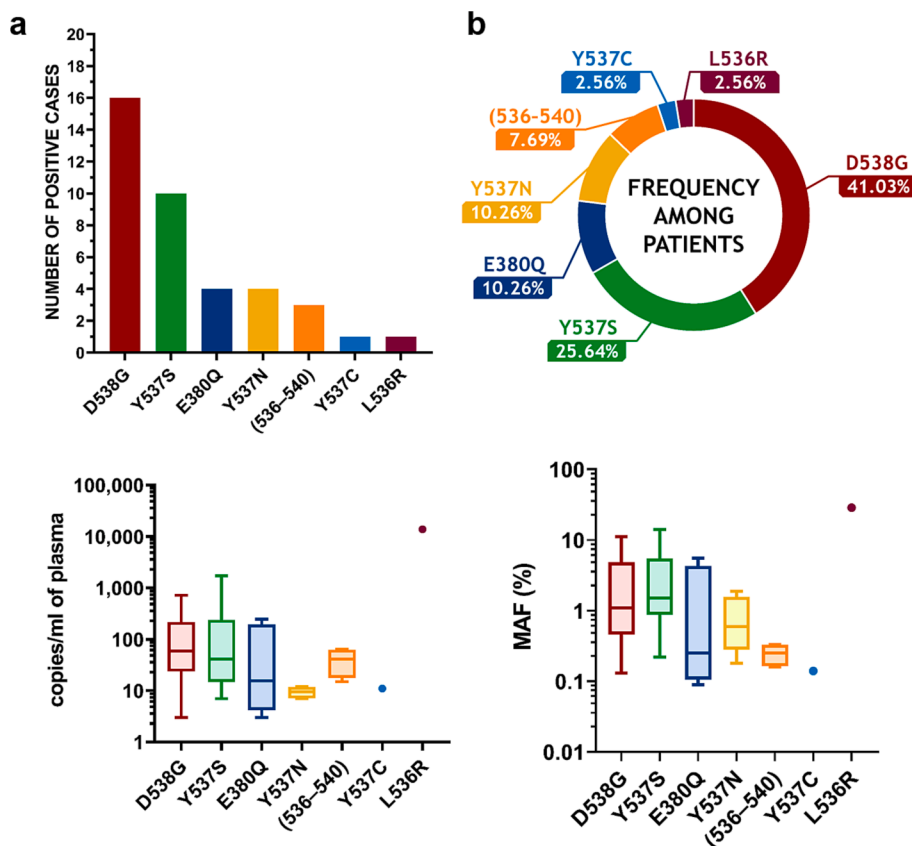
#### 4. Discussion

We developed multiplex dPCR assays on the three-color naica® platform to detect *ESR1* mutations in the plasma of HR+/HER2- MBC patients. Our screening test allowed the detection of the four most frequently searched mutations Y537C, Y537N, Y537S and D538G as well as other minority mutations found in codons 536–540 that affects the ligand-binding domain (LBD), thus potentially pathogenic. We also included the E380Q mutation, as it also affects LBD-inducing ER ligand independence [17] and because it is one of the most frequently reported *ESR1* mutations, representing between 10 and 19% of *ESR1* mutations [7,8,12,14,17].

Our three-step strategy allows for a single test to be performed in the case of a negative result and for the mutation(s) (other than E380Q) to be identified through further testing in the case of a positive screening test. As not all mutations have the same effect on resistance to hormone therapy and/or the same prognostic impact, it is important to identify them precisely. Depending on the type of mutation, Toy et al. [17] observed varying levels of *in vitro* receptor activity in the absence of estradiol. The Y537S mutation conferred the most potent estrogen-dependant activity and was the only type of ER mutant that could not be further activated by the addition of estradiol. Certain *ESR1* mutants altered fulvestrant sensitivity, with the Y537S mutant, again, requiring the highest dose for full inhibition [17]. The clinical translation of this has been reported in the PALOMA-3 phase III trial (NCT01942135) of palbociclib plus fulvestrant versus placebo plus fulvestrant in pre-treated ER+/HER2- MBC patients: in both arms of treatment a positive selection of *ESR1* Y537S (and not for the other mutants) was observed, which is consistent with the possibility that *ESR1* Y537S promotes resistance to fulvestrant [18]. Partly corroborating this, in the MONARCH2 phase III trial (NCT02107703) of abemaciclib plus fulvestrant versus placebo plus fulvestrant in pre-treated ER+/HER2- MBC patients, in the placebo arm, PFS was similar for D538G-mutant and wild-type tumors but shorter in patients harbouring Y537C/N/S mutations. However, OS was longer in patients with an *ESR1* mutation, and this was particularly so for the subgroup of the D538G mutant. In this study, only four mutations (Y537C, Y537N, Y537S and D538G) were searched for [19]. In the plasmaMATCH study (NCT03182634), patients



**Fig. 5.** Examples of *ESR1* mutations identified on patient cfDNA samples with the *ESR1* assays and WT-MUT duplexes. (a) 2D dot plot results of three major *ESR1* mutations (Y537N, Y537S and D538G), first detected using the *ESR1*(S) assay with the respective MAFs of 0.39%, 13.60% and 8.46%, and confirmed using the *ESR1*(T) assay with the respective MAFs of 0.33%, 14.07% and 7.68%. (b) 2D dot plot results of two other mutations (L536R and Y537C), first detected using the *ESR1*(S) assay, with the respective MAFs of 23.66% and 2.85%, and confirmed using the WT-MUT duplexes with the respective MAFs of 28.68% and 2.67%. (MAF: Mutant Allelic Frequency).



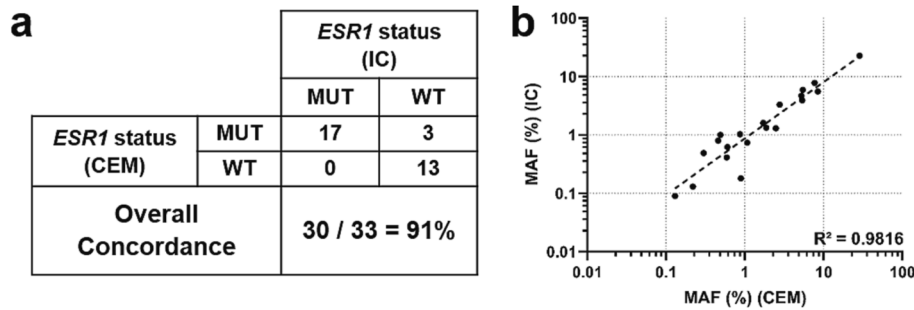
**Fig. 6.** *ESR1* mutations detection in the plasma of 109 HR+/HER2- metastatic breast cancer patients. (a) Number of positive cases per mutation for the *ESR1* mutations found among the 109 HR+/HER2- metastatic breast cancer patients tested. (b) Relative frequencies of the *ESR1* mutations identified. (c) Distributions of the mutations in copies/ml of plasma and in MAF (%) (mutations with more than three instances are presented as box plots, whereas mutations with less than three instances are presented as points, with median lines for more than two instances). (MAF: Mutant Allelic Frequency).

with Y537S mutations were not less sensitive to fulvestrant than those with other *ESR1* mutations. It should be noted that, in this trial, patients were heavily pre-treated and fulvestrant was not very effective (median PFS of two months), regardless of the presence of *ESR1* mutation [20]. Other differential effects by the type of *ESR1* mutations have been reported. In the BOLERO-2 phase III trial (NCT00863655) of exemestane plus everolimus versus placebo plus exemestane after a first line of ET, D538G and Y537S were analysed with ctDNA in a subgroup of patients.

The *ESR1* D538G mutant and wild-type derived a PFS benefit from addition of everolimus, unlike the Y537S mutant [11]. These results therefore further evidence the necessity for precise identification of *ESR1* mutations.

Using our test for our study population, we found that 28.4% (31/109) of patients had one or more *ESR1* mutations detected in their ctDNA. This is comparable to the data found in the literature, although it is interesting to note that there were significant differences in





**Fig. 7. Clinical data on *ESR1* mutations detection.** (a) Comparison of *ESR1* status performed in the Centre Eugène Marquis (CEM) or in the Institut Curie (IC) in a subset of 33 samples from 30 patients. (b) Linear regression of the results comparing MAFs obtained in the CEM and in the IC. (*MAF*: Mutant Allelic Frequency).

**Table 1**

**Baseline characteristics of patients with *ESR1* mutation (*ESR1*<sub>MUT</sub>) compared to *ESR1* wild-type(*ESR1*<sub>WT</sub>)patients.** Statistical comparison of baseline characteristics of patients with *ESR1* mutation (*ESR1*<sub>MUT</sub>) versus *ESR1* wild-type (*ESR1*<sub>WT</sub>) patients. Mann-Whitney tests were performed to compare the age distribution between the two groups and the different durations of treatment. Fisher’s exact tests were performed to compare the “de novo advanced or metastatic breast cancer” status and the location of metastatic sites. The results were considered not significant (ns) when p-values (p) were superior to 0.05.

	<i>ESR1</i> <sub>MUT</sub> (N = 31)	<i>ESR1</i> <sub>WT</sub> (N = 78)	p
Age (years), median (min–max):	64 (31–86)	66 (41–89)	ns
De novo advanced or metastatic breast cancer, n (%):	13 (41.9)18 (58.1)	25 (32.1)53 (67.9)	ns
Yes			
No			
Bone only metastasis, n (%):	11 (35.5)20 (64.5)	26 (33.3)52 (66.7)	ns
Yes			
No			
Liver metastasis, n (%):	9 (29.0)22 (71.0)	8 (10.3)70 (89.7)	<b>0.0091</b>
Yes			
No			
Visceral metastasis, n (%):	10 (32.3)21 (67.7)	29 (37.2)49 (62.8)	ns
Yes			
No			
Duration of treatment (months) in the adjuvant setting, median (min–max) [n (%):	46 (16–110) [9 (29.0)]	38 (3–112) [36 (46.2)]	ns
AI treatment	149 (13–120)	160 (8–145)	ns
ET treatment	14 (45.2)	51 (65.4)	
	1	1	
Duration of treatment (months) in the advanced setting, median (min–max) [n (%):	22 (6–43) [28 (90.3)]	26 (1–93) [54 (69.2)]	ns
AI treatment	130 (6–62) [29 (93.5)]	126 (1–129) [66 (84.6)]	ns
ET treatment	1	1	
Total duration of treatment (months), median (min–max) [n (%):	25 (6–148) [29 (93.5)]	32 (1–112) [75 (96.2)]	ns
AI treatment	142 (6–172)	170 (1–158)	ns
ET treatment	31 (100)]	77 (98.7)	
	1	1	

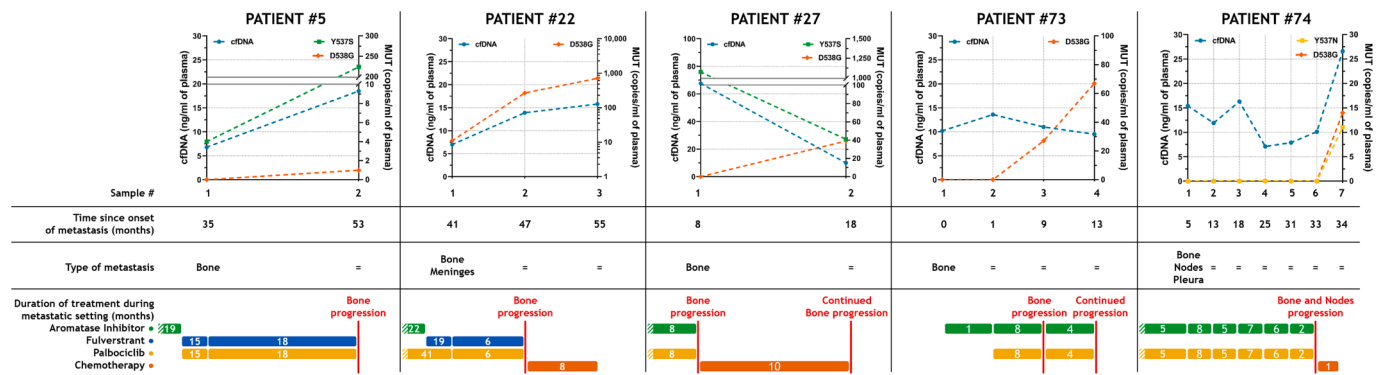
(AI: Aromatase Inhibitor, ET: Endocrine Therapy; n: number of patients; ns: not significant, p: p-value).

prevalence between studies: 25% (195 patients, PALOMA-3 study) [18], 26% (1,017 patient, PADA-1 study) [14], 29% (541 patients, BOLERO-2 study) [11], 30% (377 patients, SOFEA (NCT00253422) and EFECT (NCT00065325) studies) [21], 31% (144 MBC HR+ patients after first line AI failure [22], 37% (153 patients, FERGI study (NCT01437566)) [8], 38% (659 patients, plasmaMATCH study) [20], and 59% (248 patients, MONARCH2 study) [19]. In all cases, except one that used OncoBEAMing Digital PCR with a DNA pre-amplification, the analysis of at least Y537S and D538G mutations was done with a classical dPCR.

The difference in the percentages of positivity cannot, therefore, be explained by a difference in technique. Rather, the reason for this is likely to be a difference in the populations tested and/or in the time of collection. In our series, the two most frequently found mutations were D538G and Y537S (41% and 26% of all mutations, found in 14.7% and 9.2% of patients, respectively). E380Q and Y537N were each found in 3.7% of patients, while 4.6% of patients had other minority mutations. This is consistent with data from other studies in which D538G is always the most frequent mutation, followed by Y537S, then E380Q and/or Y537N [7,8,12]. Our assays, developed on the Stilla Technologies’ naica® platform, showed an excellent agreement of 91% with the technique used in the PADA-1 assay with a Bio-Rad platform, and a very strong correlation in MAF for positive cases. The three discordant results were mutated samples with low *ESR1* MAFs (0.89%, 0.22% and 0.13%), which were classified as indeterminate with the IC assay. This could indicate a slightly higher sensitivity for our test, given that the same amounts of cfDNA were tested. This could reasonably be explained by the significant difference in the numbers of droplets generated by the two different dPCR platforms, as well as by a probable impact of sampling for such low MAFs. A slight difference in sensitivity was also observed between our two assays, with the *ESR1*(T) assay being slightly more sensitive than the *ESR1*(S) assay. However, this had no clinical impact because the samples that the *ESR1*(S) assay classified as indeterminate were systematically checked with the *ESR1*(T) assay by performing two replicates (or three if necessary) to increase sensitivity. We also observed a good correlation between the MAFs obtained with the *ESR1*(S) and *ESR1*(T) assays (Supplementary Figure S8).

At present, ctDNA analysis via NGS is becoming increasingly popular because it allows for a wider range of mutations to be covered with multigene analyses. However, NGS only reaches the sensitivity of the dPCR when using specific designs and algorithms that are not always easy to implement, such as CAnCER Personalized Profiling via Deep Sequencing (CAPP-Seq), Safe Sequencing (Safe-Seq), Tagged-Amplicon Deep Sequencing (TAm-Seq), and Targeted Error Correction Sequencing (TEC-Seq) [23–24]. The main disadvantages of these techniques, however, are that they require significant bioinformatics resources; they lack flexibility, which can lead to relatively long turnaround times for results (with possible delays in decisions to change treatment); they are expensive. Therefore, the fast results, robustness, low cost, and relative ease of implementation of the dPCR make it very competitive with NGS in this particular case of *ESR1* mutation detection in liquid biopsies.

The occurrence of *ESR1* mutation is generally associated with exposure to an AI [7]. In our study, three patients received tamoxifen only in the adjuvant phase; in one of them, we found an L536R mutation with a very high MAF of 30% at the beginning of the metastatic stage. It is not possible to know whether this mutation occurred as a result of the pressure of hormone therapy. However, L536R is a pathogenic mutation that can influence treatment. It may therefore be prudent not to reserve the search for *ESR1* mutations to patients who have had an AI treatment. The presence of a circulating *ESR1* mutation was not related to the



**Fig. 8. Examples of longitudinal follow-ups allowing the identification of *ESR1* mutations.** Time course of total cfDNA (in ng/ml of plasma) and Y537N, Y537S and D538G mutated (MUT) ctDNA (in copies/ml of plasma) concentrations measured during follow-ups with five patients (Supplementary Dataset). For each patient, the time since onset of metastasis is represented in number of months at the time of sampling, as well as by the type of metastasis. The durations of treatment during metastatic setting are indicated, in number of months between each sample, by coloured histograms (green for aromatase inhibitor, blue for fulvestrant, yellow for palbociclib and orange for chemotherapy); the times of progression are identified by red lines.

median time of AI and/or ET treatment at the initial stage and/or at the advanced stage in this study. Conflicting data have been reported in the literature on this subject, with some reporting a significantly longer median time of AI exposure during the metastatic setting for patients with *ESR1* mutation [21,25] and others finding no link between the presence of *ESR1* mutation and the duration of AI treatment [10].

Several studies have reported an organotropism of mutations in advanced breast cancer, with *ESR1* mutations more frequently observed in the liver [26–29]. This may explain the high frequency of mutation detection in cases of liver metastasis; more than half of the patients in our cohort had one or more such *ESR1* mutation. For other metastatic locations, including, notably, bone-only metastasis, less than 30% of patients were found to be positive. Tolaney et al. [19] also reported high positivity rates (57%) of liver involvement, but, in contrast to our study, more than half of the patients with bone-only metastasis were also positive. This is certainly related to the selection of a heavily pre-treated population; in two other cohorts of patients, rates close to ours were reported for this category of patients [7,20].

**5. Conclusions**

Given the prevalence of *ESR1* mutations in MBC and the benefit of their early detection by a sensitive technique (as shown in the PADA-1 trial), the development of new endocrine treatments that will directly target and/or circumvent *ESR1* mutation, and the better understanding of the role played by these mutations (it has recently been shown that patients harbouring *ESR1* mutation could be good candidates for immune therapies [29]), it is likely that *ESR1* mutation testing will become a standard practice in follow-up with MBC patients in the coming years. We successfully developed highly sensitive and robust dPCR assays for the qualitative and quantitative clinical detection of *ESR1* mutations in plasma, which we have shown to be very suitable for this purpose.

**Data availability**

The data generated during the current study are available from the corresponding author on request.

**Authors contributions**

Conception and design of the study: TdLMR and VQ; Provision of biological material and clinical data from patients: CC, PP, FCB, EJ, FLD, LR, HB, AB, LC, CP, CLP, VD and TdLMR; Design of the dPCR assays: JC; Analysis of genomic results: JC, VQ and FG; Drawing of figure images: JC; Interpretation of the data: all authors; Drafting, revising and approval of the manuscript: all authors.

**Competing Interests**

An “ICMJE Conflict of Interest form” has been provided for each author.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2023.117366>.

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