Complementary information provided by simultaneous sequencing of CTC, cfDNA and metastatic tissue in endocrine-resistant metastatic breast cancer

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Abstract

**Background:** Endocrine resistance is a major cause of therapeutic failure in metastatic breast cancer (MBC). The information provided by DNA sequencing of plasma cell free DNA (cfDNA) and by the analysis of circulating tumor cells (CTC) may be useful to determine the occurrence and type of endocrine resistance. However, different levels of concordance between cfDNA and CTC, and also between liquid biopsy and tissue biopsy, have been reported. Thus, the best strategy for performing DNA sequencing in the setting of therapeutic decisions for luminal MBC patients is not well defined. The purpose of this study was to determine the concordance between DNA sequencing data from CTC, cfDNA, metastasis, and primary tumor in endocrine-resistant luminal MBC.

**Methods:** Using the same panel (10 genes) we performed simultaneous sequencing of cfDNA, CTC, metastases and primary tumor in 38 patients with luminal MBC. CTC isolation was performed with an Epcam-based immunomagnetic system. Concordance of DNA sequencing data of the different types of sample was analyzed and correlated with clinical data.

**Results:** CTC were detected in 21% of the patients. The rate of detection of genetic alterations was 87.5% for CTC, 61.5% for cfDNA, 63.2% for metastasis and 67.7% for primary tumor, and involved mainly TP53, PIK3CA, ESR1 and AKT1. A higher number of mutations was found in cfDNA of MBC patients with progressive disease (p=0.02) and the same trend was observed for endocrine resistance (p=0.08), especially for PIK3CA, ESR1 and AKT1 mutations. Global concordance was low to intermediate between CTC and cfDNA, but significantly higher between CTC and metastasis. Concordance also varied according to the specific genetic alteration, with no ESR1 mutations detected in CTC and with a low concordance for PIK3CA and AKT1 mutations between CTC and cfDNA samples.

**Conclusions:** DNA sequencing of different types of tumor samples (cfDNA, CTC, metastasis and primary tumor) may provide different rates of genomic alterations detection in luminal endocrine-resistant MBC. A moderate to low concordance, with discordances depending both on the clinical situation and on the sequenced gene, support the complementarity of the different samples, with cfDNA and metastatic tissue as the preferred sources of tumor DNA.

**Background**

Although new therapeutic options are now available, metastatic breast cancer (MBC) is still the first or second cause of death by cancer in women. Luminal MBC must be treated with endocrine therapy except in those cases presenting with a visceral crisis [1]. However, endocrine resistance invariably occurs, leading to disease progression and making chemotherapy the only option for most patients after two or three lines of hormonal treatment [2]. A better knowledge of endocrine resistance causes and of genomic alterations in patients with luminal MBC may offer additional therapeutic options for some patients.

In previous publications, enrichment of metastatic disease with respect to the primary tumor has been observed in certain genomic alterations related to endocrine resistance, such as ESR1, RAS, AKT1 and...
RB1 [3, 4]. The acquisition of at least part of these alterations, especially ESR1 and ERBB2, seems to be related to hormonal treatment [3]. Although better knowledge of these alterations could guide the treatment of luminal MBC, especially for guiding inclusion in clinical trials [5], until very recently most alterations have not modified the patient's treatment in clinical practice. However, the recent approval of alpelisib, a PIK3CA inhibitor, is linked to determination of PIK3CA mutations as predictors of response. There is also a growing conviction that the availability of new drugs, such as AKT inhibitors and new selective estrogen receptor degraders, among others, could increase the relevance of DNA sequencing in MBC.

Biopsies of metastatic tumor have been the main source of samples for next-generation DNA sequencing (NGS) in the metastatic disease setting. However, other alternatives, such as liquid biopsy, based either on cell-free circulating DNA (cfDNA) or on circulating tumor cells (CTC) obtention [6], may avoid invasive procedures and provide a dynamic strategy for analyzing resistance biomarkers in MBC [7, 8]. Thus, it is relevant to understand the contribution of each of the potential sources of information on endocrine resistance mechanisms in patients with luminal MBC. While cfDNA is able to identify resistance markers and therapeutic targets, CTC are usually preferred for prognostic evaluation and tumor monitoring [9]. However, some recent works have shown that CTC evaluation might be potentially informative about endocrine resistance [10] and resistance mechanisms heterogeneity [11]. In studies with ctDNA in luminal CM, up to 78% of patients have shown GA [12], with an average of 2-2.5 GA, mainly affecting TP53, PIK3CA, ESR1 and, less frequently, AKT1 [12, 13]. Differences in the degree of concordance of genomic alterations between CTC and cfDNA have been reported for ESR1 mutations [14, 15] and for NGS panels [11, 16]. These differences may be derived from technical sources of variation or from true differences in the cell compartments of origin. Since the level of concordance between cfDNA and CTC DNA is unclear, and discordant results have been communicated particularly for luminal MBC, the potential contribution of CTC DNA sequencing to therapeutic decisions in luminal MBC is also undetermined. A recent work has shown that CTC sequencing may provide additional information in certain patients [17], beyond the prognostic value of CTC levels [18]. Regarding primary tumor and metastatic tissue, the correlation of findings between ctDNA and both types of tissue is highly variable among the different publications, with only 10–15% concordance rates in some of them [12].

Our objective was to determine the mutational concordance between CTC, cfDNA and metastatic tissue in luminal metastatic breast cancer (MBC) and to explore the potential mechanisms of endocrine resistance in the different tumor compartments. The final aim was to determine which type of sample or sample combination might maximize the detection of targetable mutations in the setting of advanced disease.

**Methods**

**Patients and sample collection**

We included 40 patients with luminal (ER positive and/or PgR positive; HER2 non amplified) MBC at the Department of Hematology and Medical Oncology of Hospital Universitario Morales Meseguer, Murcia,
Spain. All patients had histologic confirmation of breast invasive carcinoma and clinical evidence of metastatic disease. Endocrine resistance was defined for the first recurrence or first progression according to established clinical criteria [1] as primary, secondary or no resistance. Response evaluation for the current treatment of the patient was performed according to RECIST 1.1 criteria.

We collected two 10 ml tubes of whole blood (WB) for each patient, EDTA and PaXGene ccfDNA. Plasma was obtained from blood collected in PaxGene tube. Primary and metastatic tumors were obtained as FFPE blocks.

**CTC enrichment and identification**

CTC isolation was performed in a whole blood (WB) sample of 7.5 mL by Epcam-based immuno-magnetic enrichment with anti-Epcam antibody conjugated microbeads and a column system (MCAS Miltenyi Biotec, Madrid, Spain) following manufacturer's instruction. Parallel confirmation of the presence of CTC in each case was obtained in a simultaneous blood sample of 5mL: after immuno-magnetic enrichment with ADNaTest Breast Cancer Select, (Qiagen, Hilden, Germany), multiplex RT-PCR was performed with AdnaTest Breast Cancer Detect (GA733-2, Muc1, HER2 and control actin) in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). ADNaTest RT-PCR was further confirmed by qPCR using specific primers for the three markers (GA733-2, Muc1, HER2); only confirmed results were considered as indicative of CTC presence. We used MCF-7 spike-n in human blood to determine the sensitivity of the test (Supplementary Figure S1). No CTC quantification was performed, and patients were classified either as CTC positive (CTC+) or CTC negative (CTC-).

**DNA isolation from CTC, plasma and tumor tissue**

cfDNA was extracted from plasma (5 ml) derived from the PaXGene ccfDNA tubes with QIAmp Circulating Nucleic Acid Kit. After purification, we quantified DNA concentration with a QUBIT dsDNA HS Assay (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Median DNA concentration was 24 ng/mL (range: 4.7–84 ng/mL).

tctDNA from CTCs was isolated using the “Cells and Tissue Genomic DNA Isolation Microkit” (Norgen). The efficiency of the isolation was confirmed by a technical validation with digital PCR (Supplementary Figure S2).

Tumor DNA was extracted from paraffin-embedded tissues (breast primary tumor and/or metastatic biopsies) using the AllPrep DNA/RNA FFPE kit (Qiagen) after marking of those areas with at least a 50% of tumor cellularity by a pathologist (ACB, FMD).

**DNA sequencing**

Sequencing of cfDNA and ctcDNA was performed in an Ion Torrent PGM System (Thermo Fisher Scientific, Waltham, MA, USA) using the Oncomine Breast cfDNA Assay, a panel of 152 hotspots and indels targeting 26 amplicons of 10 genes (AKT1, EGFR, ERBB2, ERBB3, ESR1, FBXW7, KRAS, PIK3CA, SF3B1, TP53). Libraries were constructed using multiplex PCR according with the manufacturer's
instructions and were purified with Agentcourt AMPurez XP (Beckman Coulter, Barcelona, Spain. Limit of detection range was 0.01–0.15%, with 92.1% cases equal or less than 0.1%.

Sequencing of tissue samples was performed with an Ampliseq custom panel designed to target just our sequences of interest (the same 152 hotspot mutations that the commercial panel Oncomine Breast cfDNA). This design was made using the algorithm Ion Ampliseq Designer 6.1.3.

For bioinformatic analysis, we used Torrent Suite software 5.8 (reference: hg19) and annotation with Ion Reporter 5.6

Statistical analysis

All statistical analyses were performed with SPSS 21.0 (IBM España, Madrid, Spain). The comparison of the mutational profile of CTC and cfDNA and its correlation with endocrine resistance and patient characteristics was performed with Chi-squared test for comparison of proportions and with Kendall’s kappa for concordance. Overall survival was calculated from the time of inclusion in the study; Kaplan-Meier curves were obtained, and univariate comparisons were done with the log-rank test. A p value of < 0.05 was considered as significant. The study report is in accordance to the REMARK guidelines [19].

Results

Patient characteristics and availability of CTC, cfDNA and primary and metastatic tissue.

We performed DNA sequencing of CTC and cfDNA samples in a cohort of 38 women with metastatic breast cancer patients treated in our center. A valid sample was available in 34 of them. In 20 of them there was available tissue of metastasis, and 32 also had tissue from primary tumor. Median age of the patients was 58 (range, 37–85). As shown in Table 1, most patients (81.6%) had endocrine resistant disease, either primary (21.1%) or secondary (60.5%). The plasma samples were taken in different response settings corresponding to different moments of the clinical evolution: 2 at time of metastatic recurrence or after metastasis surgery (5.3%), 21 with stable disease (55.3%), 5 with partial or complete response (13.2%), and 9 of them at time of disease progression (23.7%). Treatment at time of sample obtention consisted in endocrine therapy in 60.5% of the patients and was chemotherapy for the remaining 39.5%. Median overall survival for the whole group has not been reached, and 2-years survival is 60% (95%CI, 45–75%).

Among the 38 patients, only 8 (21%) had detectable CTC. All CTC+ patients showed endocrine resistance (37.5% primary and 62.5% secondary), although no significant association was found between endocrine resistance and CTC detection (Fisher’s exact test, p = 0.31). Detection of CTC was associated with progressive disease (45.5% vs. 10.3% of patients with SD/OR, p = 0.03) and showed a trend to worse overall survival (median: 13.2 months vs. not reached; p = 0.13).
Table 1
Patient characteristics (n = 38).

| Characteristicsa | N (%) |
|------------------|-------|
| **Age, median, range** | 58 (37–85) |
| **Metastasis location** | |
| Bone only | 23 (60.5%) |
| Visceral only | 6 (15.8%) |
| Visceral + Bone | 9 (23.7%) |
| **Metastatic disease** | |
| At diagnosis (M1) | 16 (42.1%) |
| Recurrence (M0) | 22 (57.9%) |
| **Type of endocrine resistance** | |
| No resistance | 7 (18.4%) |
| Primary resistance | 8 (21.1%) |
| PD < 6 months 1st line ET | 5 (13.2%) |
| Recurrence < 2yrs adjuvant ET | 3 (7.9%) |
| Secondary resistance | 23 (60.5%) |
| PD > 6 months 1st line ET | 15 (39.5%) |
| Recurrence > 2yrs adjuvant ET | 8 (21.1%) |
| **Drug related to endocrine resistance** | |
| Aromatase inhibitors | 28 (73.7%) |
| Tamoxifen | 9 (23.7%) |
| Fulvestrant | 1 (2.6%) |
| **Response setting** | |
| PD | 9 (23.7%) |
| SD | 21 (55.3%) |
| PC/CR | 5 (13.2%) |
| Pre-treatment/NED | 3 (7.9%) |
| **Endocrine therapy** | |
| 1st line | 17 (44.7%) |
| 2nd line | 4 (10.5%) |
| 3rd line | 2 (5.3%) |
| **Chemotherapy** | |
| 1st or 2nd line CT | 7 (18.4%) |
| 3rd line | 8 (21.1%) |

a CR: complete response; CT: chemotherapy; CTC: circulating tumor cell; ET: endocrine therapy; NE: non-evident disease; PD: progression of disease; PR: partial response; SD: stable disease;
### Characteristics

| Characteristics | N (%) |
|-----------------|-------|
| CTC detection   |       |
| CTC-            | 30 (79%) |
| CTC+            | 8 (21%)  |

* CR: complete response; CT: chemotherapy; CTC: circulating tumor cell; ET: endocrine therapy; NE: non-evident disease; PD: progression of disease; PR: partial response; SD: stable disease;

### Sequencing of cfDNA, CTC, metastases and primary tumor

DNA sequencing was based on a targeted panel comprising 10 genes and 152 hotspots. The same panel was used for all samples. The rate of detection of genetic alterations was 87.5% (7/8) for ctcDNA, 61.5% for cfDNA, 63.2% for metastatic tissue and 67.7% for the primary tumor biopsy. The type and number of mutations for each type of samples are summarized on Fig. 1 and the complete list is shown in Supplementary Table S1.

The median number of genetic alterations detected by DNA sequencing did not significantly differ among the different types of samples, with a range of 0 to 4 for all of them, except for primary tumor biopsies (range: 0–8).

The specific type of genetic alterations also differed among the different types of samples (Table 2). In particular, sequencing of CTC showed a high frequency of TP53 mutations (75% of cases) and a comparable number of PIK3CA (25%), but no ESR1 mutations were found. In contrast with CTC results, cfDNA was enriched in ESR1 mutations (6/34; 17.6%) while keeping a comparable frequency of PIK3CA (23.5%) and AKT1 (14.7%) mutations and an intermediate number of TP53 mutations (50%).
Table 2
Frequency of genomic alteration according to type of sample.

| Gene          | ctDNA (n = 34) | CTC (n = 8) | Metastasis (n = 20) | Primary tumor (n = 32) |
|--------------|---------------|------------|---------------------|------------------------|
| TP53         | 17 (50.0%)    | 7 (87.50%) | 4 (20.0%)           | 9 (28.0%)              |
| PIK3CA       | 9 (26.5%)     | 2 (25.0%)  | 7 (35.0%)           | 14 (43.8%)             |
| ESR1         | 6 (17.6%)     | 0 (0%)     | 2 (10.0%)           | 1 (3.1%)               |
| SF3B1        | 4 (11.7%)     | 1 (12.5%)  | 2 (10.0%)           | 3 (9.4%)               |
| AKT1         | 5 (14.7%)     | 2 (25.0%)  | 4 (20.0%)           | 2 (6.3%)               |
| FBXW7        | 0 (0%)        | 1 (12.5%)  | 0 (0%)              | 0 (0%)                 |
| ERBB3        | 0 (0%)        | 2 (25.0%)  | 0 (0%)              | 1 (3.1%)               |
| Others       | 0 (0%)        | 0 (0%)     | KRAS: 1 (5%);       | ERBB2: 1 (3.1%); KRAS: 2 (6.2%); EGFR: 1 (5%) |

Sequencing of metastatic tissues revealed a similar pattern with predominance of PIK3CA (35%), AKT1 (20%) and ESR1 (10%) mutations. Finally, the distribution of primary tumor genetic alterations was different, with a very high rate of PIK3CA mutations (43.7%), a lower occurrence of TP53 mutations (28%) and only one case with an ESR1 mutation (3.1%). Interestingly, a few mutations were only found in some type of samples. In particular, FBXW7 and ERBB3 mutations were detectable only in CTC, while ERBB2 mutations were only present in the primary tumor sample of one patient.

The number of different mutations of the same gene for a particular patient was also higher for TP53 and PIK3CA, especially in the primary tumor and cfDNA, thus yielding a larger total number of genomic alterations (Fig. 2).

**Matched assessment and concordance of somatic mutations on liquid biopsies (CTC, ctDNA) and metastasis tissue**

Globally, we found a low to intermediate concordance between CTC and cfDNA, for the detection of any genomic alterations (Table 3). Concordance was also low to moderate between primary and metastatic tissue. A higher overall concordance was found between CTC and primary tumor sequencing (k = 0.57; p = 0.12), which was significantly better between cfDNA and metastasis (k = 0.51, p = 0.03).

We performed a concordance analysis specifically focused on the three main groups of mutations related to endocrine resistance. Also, in order to determine the clinical utility of each type of sample to maximize the identification of targetable mutations, we calculated the percentage of positivity provided for each
type of sample over the total number of cases with mutations identified by sequencing every pair of matched samples. As shown in Table 3, our findings are consistent with different degrees of overall concordance according to the type of mutation.

For PIK3CA, the concordance between CTC and cfDNA was low, and the same occurred when CTC results were compared with tissue biopsy of either the primary tumor or metastases, which suggest some degree of complementarity between CTC and other type of samples. Our results also showed a low concordance of cfDNA positive results with those provided by sequencing of tumor (25%) or metastasis (50%) biopsy, which might provide a higher sensitivity for detection of mutations (70-87.5% rate of positive results in comparison with 25–40% for cfDNA).

Regarding AKT1, complete concordance was found for detection of mutations between CTC and primary tumor or metastasis biopsy, while only 33% of mutations identified in cfDNA sequencing were found in the matched CTC samples. The concordance between cfDNA and primary or metastatic biopsies was also low, with the identification of only 16–25% of the mutations found in cfDNA.

ESR1 mutations were not found in any of the CTC samples, which has limited the concordance analysis with other samples. However, the finding of ESR1 mutations in cfDNA again showed a low concordance (25%) with metastases biopsy detection, and null concordance with primary tumor, as expected. Finally, although not shown in Table 3, the concordance for TP53 mutations was very low in every pair of matched samples, with kappa values below 0.20 in all cases.

**Association of genomic alterations in metastatic samples (CTC, cfDNA, metastatic tissue) with progressive disease and endocrine resistance**

The average number of mutations found in cfDNA was significantly higher in those patients with disease progression (2.27 vs. 0.81; Mann-Whitney U, p = 0.002), but we did not find the same differences for CTC (p = 0.39) or metastatic tissue (p = 0.79) samples. Similarly, the number of patients with any mutation present in cfDNA showed a non-significant association with the response status (progression disease: 90.9%; stable disease or objective response: 60.9%) (Fisher’s exact test, p = 0.11). Endocrine resistant patients also showed a trend to a higher number of genetic alterations in cfDNA when compared with patients without resistance (1.42 vs. 0.43; p = 0.08), again without differences for the rest of samples (Fig. 1).

We also analyzed the association of finding specific genetic alterations in any of the metastatic samples (CTC, cfDNA, metastasis biopsy) with endocrine resistance. The association differed according to the specific gene mutation: the frequency of PIK3CA mutations trended to be higher (p = 0.22) in patients with endocrine resistance (primary: 50%, secondary: 34.7%) than in patients without resistance (14.3%). The same non-significant tendency was found for AKT1 mutations, which only occurred in endocrine resistant
cases (primary: 37.5%; secondary: 17.4%). Similarly, ESR1 mutations were only present in cases with endocrine resistance (22.5%), most of them (85.7%) corresponding to secondary resistance to aromatase inhibitors. Finally, only ESR1 mutations were associated with disease progression (45.5% mutated vs. 7.4% of patients with SD or OR; \( p = 0.01 \)). This association was observed neither for PIK3CA (\( p = 0.46 \)) nor for AKT1 mutations (\( p = 0.39 \)).
Table 3
Concordance of matched samples for endocrine resistance-related genomic alterations.

|                  | CTC-cfDNA | CTC-MTS | CTC-Tumor | cfDNA-MTS | cfDNA-Tumor | MTS-Tumor |
|------------------|-----------|---------|-----------|-----------|-------------|-----------|
| **N**            | N = 8     | N = 4   | N = 6     | N = 20    | N = 32      | N = 16    |
| **Any genomic alteration** | 62.5%     | 50%     | 83.3%     | 77.7%     | 60.7%       | 60%       |
| **Overall concordance** | CTC 87.5% | CTC 100% | CTC 83.3% | cfDNA 72.2% | cfDNA 67.9% | MTS 60%   |
| **Mutation detection rate** | cfDNA 75% | MTS 50% | 66.6%     | MTS 61.1% | Tumor 71.4% | Tumor 73.3% |
| **Positivity concordance** | 62.5%     | 50%     | 83.3%     | 77.7%     | 60.7%       | 60%       |
| **Statistics**   | K = 0.20, p = 0.54 | | K = 0.57, p = 0.12 | K = 0.51, p = 0.03 | K = 0.07, p = 0.70 | | K = 0.12, p = 0.63 |

| **PIK3CA**       | 62.5%     | 75% Moderate | 50% Low | 80% Moderate | 62.5% Low | 62.5% Low |
| **Overall concordance** | CTC 50% (2/4) | CTC 100% (1/1) | CTC 33.3% (1/3) | ctDNA 62.5% (5/8) | ctDNA 37.5% (6/16) | MTS 70% (7/10) |
| **Mutation detection rate** | ctDNA 75% (3/4) | Mts 50% (1/2) | Tumor 66.7% (2/3) | MTS 87.5% (7/8) | Tumor 87.5% (14/16) | Tumor 70% (7/10) |
| **Positivity concordance** | 25% (1/4) | 0% (0/3) | 50% (1/2) | 0% (0/3) | 50% (4/8) | 25% (4/16) |
| **Statistics**   | κ = 0.14, p = 0.67 | | κ = 0.53, p = 0.45 | κ = 0.53, p = 0.21 | | | κ = 0.24, p = 0.34 |

| **AKT1**         | 75% Low | 100% Very high | 100% Very high | 75% Low | 90.6% Low | 93.8% High |
| **Overall concordance** | CTC 33% (1/3) | CTC 100% (1/1) | CTC 100% (3/6) | ctDNA 50% (3/4) | ctDNA 75% (3/4) | MTS 100% (3/3) |
| **Mutation detection rate** | ctDNA 100% (1/1) | MTS 100% (2/3) | MTS 100% (2/4) | | | | |
| **Positivity concordance** | 33% (1/3) | 100% (1/1) | 100% (1/1) | 66.7% (16/24) | 66.7% (6/9) | | |
| **Statistics**   | κ = 0.38, p = 0.17 | | κ = 1.0, p = 0.01 | κ = 0.14, p = 0.04 | κ = 0.35, p = 0.04 | | κ = 0.76, p = 0.002 |
|                | CTC-cfDNA | CTC-MTS  | CTC-Tumor | cfDNA-MTS | cfDNA-Tumor | MTS-Tumor |
|----------------|-----------|----------|-----------|-----------|-------------|-----------|
| **ESR1**       | –         | –        | –         | 85% Low   | 84.4% Very low | –         |
| **Overall concordance** | CTC 0% (0/8) | CTC 0% (0/4) | CTC 0% (0/6) | ctDNA 75% (3/4) | ctDNA 80% (4/5) | MTS 100% (2/2) |
| **Mutation rate** | ctDNA 100% (1/1) | MTS 0% (0/6) | Tumor 0% (0/6) | MTS 50% (2/4) | Tumor 20% (1/5) | Tumor 0% (0/2) |
| **Positivity concordance** | 0% | 0% | 0% | 25% (1/4) | 0% (0/5) | 0% (0/2) |
| **Statistics** | – | – | – | κ = 0.32, p = 0.14 | κ = 0.05, p = 0.70 | – |

**Discussion**

The identification of genomic alterations associated with endocrine resistance and tumor progression may provide additional therapeutic opportunities for women with luminal MBC. In this work, we analyzed the concordance of genomic alterations, including those relevant for endocrine resistance, in matched samples of CTC, cfDNA, metastatic tissue and primary tumor in a series of 38 women with luminal advanced breast cancer and diverse degrees of resistance.

The finding of relevant genomic alterations may have implications for clinical trial inclusion and for decision-making concerning next treatments. Our analysis focused on concordance, which was low to moderate between the different types of samples. However, in the clinical setting, the maximization of mutation detection is the main goal to improve therapeutic options for patients. Consequently, positive concordance is the true measure of potential clinical utility, and our evaluation was directed to determine whether sequencing other type of samples might increase the detection rate. While CTC sequencing results were similar to primary tumor, a higher concordance was found between cfDNA and metastatic tumor, in agreement with previous whole-exome sequencing data [20]. The low identification of ESR1 mutations in CTC is also interesting, and previous data have shown similar results concerning the low concordance of ESR1 variants between CTC and cfDNA [17]. Although these data are limited by the small number of cases and by technical factors such as the extent of the gene panel, there are also plausible biological explanations related to space and time tumor heterogeneity. In particular, the low overall concordance between both forms of liquid biopsy, CTC and cfDNA, suggest relevant biological differences among the different tumor compartments.

The use of pooled CTC samples for sequencing deserves a comment. While isolated CTC sequencing may provide additional information on tumor heterogeneity of resistance mechanisms [21], pooled CTC sequencing may be a more accessible and practical clinical approach for liquid biopsy. Additionally, recent data on CTC clusters suggest a prominent role of CTC aggregates in metastasis generation and resistance to cancer therapy [22], thus supporting a less selective approach for studying CTCs in the
clinical setting. In any case, CTC sequencing provided additional data to cfDNA in order to identify genomic alterations in luminal MBC patients. When only potentially targetable mutations were analyzed, 25% patients with CTC+ had additional findings for PIK3CA mutations. Consequently, partly due to the low yield of CTC in this group of patients, less than 5% of the total group of luminal MBC patients obtained therapeutically relevant results after CTC isolation and sequencing. Together with the absence of ESR1 mutations in CTC pooled samples, our results suggest that, in most cases, cfDNA alone might provide sufficient information from a clinical perspective, and that a metastasis biopsy should be the second preferred option in order to maximize the identification of targetable genomic alterations. Alternatively, a sequential approach with CTC sequencing in those cases in which cfDNA or tissue sequencing do not yield any targetable genomic alteration or in which a metastasis biopsy is not feasible might be useful in some patients. CTC sequencing is still a valid research tool and may be a relevant source of information about tumor heterogeneity; prognostic complementarity of ctDNA and CTC dynamic evaluation has also been suggested [18, 23, 24] and new approaches for liquid biopsy are being developed that include both types of information [25].

A higher number of genomic alterations was found in patients with endocrine resistance and progressive disease. This finding is in agreement with previous reports [3, 26], and poses the question of the best moment for obtaining a liquid or tissue biopsy for DNA sequencing. Although our work is based on a one-time sample collection, our data support a dynamic evaluation of tumor genomics and a preference for obtention of tissue or plasma at time of progression or when endocrine resistance occurs [4, 8].

Our work has limitations, the first of them being the different moments in the evolution of the disease and the different response settings in which samples were obtained. However, it should be noted that blood obtention for cfDNA and CTC samples was simultaneous, making sequencing results directly comparable. Comparability was further assured with the utilization of the same gene panel for all samples of the same patient and our results of around 2 genomic alterations in each patient is in accordance with previous publications [13]. A second potential limitation is the utilization of an Epcam-based method for CTC isolation, with the potential loss of undifferentiated or mesenchymal CTC [27]. However, Epcam-positive CTC seem to be the most relevant population in terms of metastasis generation [28] and prognosis [9] of MBC. Also, although our CTC isolation method was validated with spike-in experiments, its sensitivity is probably lower than that of other marketed systems such as CellSearch; in fact, the 21% detection rate suggest that positivity for CTC with this approach might be equivalent to finding > 5 CTC/10 mL with other methods. Finally, the utilization of a panel with a limited number of hotspots, although including the main genomic alterations previously linked to resistance, might have provided a partial estimation of the concordance between samples.

Conclusions

In conclusion, in luminal MBC the performance of sequencing different types of tumor samples (CTC, cfDNA, metastasis, primary tumor) is different, and may be variable for the different genetic mutations. Sequencing of CTC does not seem to be the best approach due to the low yield of CTC and the low rate of
identification of targetable mutations. The obtention of liquid biopsy at time of progression or when endocrine resistance develops is associated with a higher rate of detection genomic alterations. This information may be valuable for developing sequential algorithms of sample sequencing to maximize the rate of detection of potentially targetable genetic mutations in luminal metastatic breast cancer.

**Declarations**

**Ethics approval and consent to participate**

The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants and the protocol was approved by the institutional Ethics and Clinical Research Committee (CEIC Hospital Universitario Morales Meseguer; EST24/15).

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

ENM participated in the conception and design of the study, performed laboratory procedures, participated in data retrieval and analysis, performed statistical analysis and drafted the manuscript; MPFP participated in the design of the study and performed laboratory procedures and data retrieval; EGM, PMB, AIR and EGT participated in the obtention of clinical data, data analysis and critically revised the manuscript; ACB and FMD performed the pathologic studies, selected the samples for DNA sequencing, participated in data retrieval and critically revised the manuscript; RTM participated in the design of the study, performed laboratory procedures and critically revised the manuscript; FAP conceived
of the study, participated in its design, obtained clinical data, participated in data analysis and revised the manuscript. All authors read and approved the final manuscript.

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Abbreviations

cfDNA: cell-free DNA; CTC: circulating tumor cell; ctcDNA: circulating tumor cell DNA; MBC: metastatic breast cancer

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Figures
Figure 1

Sequencing results for each type of sample.
Figure 2

Total number of genomic alterations found in each type of sample for each gene.

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