Development of Circulating Tumor Cell-Endocrine Therapy Index in Patients with Hormone Receptor–Positive Breast Cancer

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Abstract

Background: Endocrine therapy (ET) fails to induce a response in one half of patients with hormone receptor (HR)–positive metastatic breast cancer (MBC), and almost all will eventually become refractory to ET. Circulating tumor cells (CTC) are associated with worse prognosis in patients with MBC, but enumeration alone is insufficient to predict the absolute odds of benefit from any therapy, including ET. We developed a multiparameter CTC-Endocrine Therapy Index (CTC-ETI), which we hypothesize may predict resistance to ET in patients with HR-positive MBC.

Methods: The CTC-ETI combines enumeration and CTC expression of four markers: estrogen receptor (ER), B-cell lymphoma 2 (BCL-2), Human Epidermal Growth Factor Receptor 2 (HER2), and Ki67. The CellSearch System and reagents were used to capture CTC and measure protein expression by immunofluorescent staining on CTC.

Results: The feasibility of determining CTC-ETI was initially established in vitro and then in a prospective single-institution pilot study in patients with MBC. CTC-ETI was successfully determined in 44 of 50 (88%) patients. Eighteen (41%), 9 (20%), and 17 (39%) patients had low, intermediate, and high CTC-ETI scores, respectively. Interobserver concordance of CTC-ETI determination was from 94% to 95% (Kappa statistic, 0.90–0.91). Inter- and cell-to-cell intrapatient heterogeneity of expression of each of the CTC markers was observed. CTC biomarker expression was discordant from both primary and metastatic tissues.

Conclusions: CTC expression of ER, BCL-2, HER2, and Ki67 can be reproducibly measured with high analytical validity using the CellSearch System. The clinical implications of CTC-ETI, and of the heterogeneity of CTC biomarker expression, are being evaluated in an ongoing prospective trial. Clin Cancer Res; 21(11): 2487–98. ©2014 AACR.

See related commentary by Mathew et al., p. 2421

Introduction

Endocrine therapy (ET) is routinely recommended for patients with hormone receptor (HR)–positive metastatic breast cancer (MBC), while patients with HR-negative disease are typically offered more toxic chemotherapy as first-line treatment (1). However, approximately one half of patients with HR-positive MBC do not respond to ET, 15% to 30% of patients treated with ET experience progression within the first two to three months of therapy, and almost all patients with HR-positive MBC will eventually become refractory to ET (1).

Patients with HR-positive MBC who have ET-refractory disease are treated with chemotherapy, as is done for HR-negative patients. Unfortunately, only clinical judgment, or a failed trial of ET, is available to identify such patients. Moreover, such empiric decisions do not permit consideration of tumor heterogeneity, which has been implicated as one of the main barriers to effective anticancer therapy, but the molecular tools to quantify and monitor tumor heterogeneity have been lacking (2–4). Several studies have demonstrated that estrogen receptor (ER) and human epidermal growth factor receptor type 2 (HER2) differ from the primary cancer and metastatic biopsies in 5% to 30% of patients with MBC (5–7). Ideally, serial real-time analysis of expression of ER and other markers associated with response or resistance to ET could be determined before or during ET to guide therapeutic decisions. However, biopsies of metastatic tissue are difficult because they are invasive, inconvenient, and associated with potential morbidity and prohibitive costs.
Circulating biomarkers represent an appealing alternative to baseline or serial tissue biopsies to predict ET resistance and to monitor evolving tumor heterogeneity. Prior studies have demonstrated that, at least when performed by the CellSearch System (Janssen Diagnostics, LLC), enumeration of circulating tumor cells (CTC) is a specific, independent predictor of progression-free and overall survival in patients with MBC (8, 9). However, CTC enumeration alone is insufficient to predict the absolute odds of benefit from any therapy, including endocrine therapy (ET). CTCs represent an appealing alternative to baseline or serial tissue biopsies to predict ET resistance and to monitor evolving tumor heterogeneity. We developed a multiparameter CTC-Endocrine Therapy Index (CTC-ETI), which is based on CTC enumeration, as well as highly validated semiquantitative analyses of several important biomarkers (CTC-ER, BCL-2, HER2, and Ki67 expression). Our data demonstrate extensive CTC biomarker heterogeneity in patients with hormone receptor (HR)-positive MBC. This heterogeneity creates enormous challenges to treatments with ET and to development of new, targeted therapies. The CTC-ETI assay, which was analytically validated in this study, may serve as a real-time predictive factor for resistance to ET in patients with HR-positive MBC.

Translational Relevance

Circulating tumor cells (CTC) are associated with worse prognosis in patients with metastatic breast cancer (MBC), but enumeration alone is insufficient to predict the absolute odds of benefit from any therapy, including endocrine therapy (ET). CTCs represent an appealing alternative to baseline or serial tissue biopsies to predict ET resistance and to monitor evolving tumor heterogeneity. We developed a multiparameter CTC-Endocrine Therapy Index (CTC-ETI), which is based on CTC enumeration, as well as highly validated semiquantitative analyses of several important biomarkers (CTC-ER, BCL-2, HER2, and Ki67 expression). Our data demonstrate extensive CTC biomarker heterogeneity in patients with hormone receptor (HR)-positive MBC. This heterogeneity creates enormous challenges to treatments with ET and to development of new, targeted therapies. The CTC-ETI assay, which was analytically validated in this study, may serve as a real-time predictive factor for resistance to ET in patients with HR-positive MBC.

Materials and Methods

Human breast cancer cell lines

Human breast cancer MCF-7 cells were originally from Michigan Cancer Foundation, but directly obtained from the laboratory of Dr. Stephen Ethier and were cultured in RPMI medium 1640 (Invitrogen) with 10% FBS and 1 mmol/L sodium pyruvate (GIBCO; 100× Sodium Pyruvate 100 mmol/L lot 786714). BT-474, MDA-MB-231, and Sk-Br-3 cells were obtained from the laboratory of Dr. Stephen Ethier via ATCC, and Dr. James Rae's laboratory, respectively, and they were cultured in DMEM medium (Invitrogen) with 10% FBS. MDA-MB-231 cells were cultured in IMEM supplemented with 10% FBS. For ER-positive control cells, MCF-7 cells were cultured in hormone-depleted condition for 2 days using DMEM (Lonza) with 4.5 g/L glucose and without l-Glutamine or Phenol Red supplemented with 10% charcoal/dextran-stripped FBS (JR Scientific, Inc.) and fixed with 1× CellSave. All cell lines were confirmed by DNA fingerprinting in February 2011 and expanded and frozen for future use.

Reagents to phenotype CTC

The following antigen-specific fluorescent-labeled antibodies were used to characterize ER, HER2, BCL-2, and Ki67 expression on CTC: ERα monoclonal murine ER-119.3 Ab (Janssen Diagnostics, LLC), HER2 monoclonal murine Her81 Ab (Janssen Diagnostics, LLC), BCL-2 monoclonal murine Ab BCL-2/(100) (BD Pharmingen), Ki67 monoclonal murine B56 Ab (BD Pharmingen; Supplementary Table S1).

In vitro experiments and development of positive and negative control specimens

Twenty-four milliliter of whole blood (WB) was drawn into three 10-cc vacutainer tubes that contain a cellular fixative (CellSave Tubes; Janssen Diagnostics, LLC), from healthy volunteers after signed informed consent approved by the University of Michigan Institutional Review Board (IRB). Cultured breast cancer cells were spiked at different concentrations into 7.5 mL aliquots of the pooled WB, and the aliquots were processed for CTC enumeration and characterization using the CXC Kit (Janssen Diagnostics, LLC). The cultured breast cancer MCF-7 and Sk-Br-3 cell lines were used as positive and negative controls, respectively, for ER staining and as negative and positive controls, respectively, for HER2 staining. The cultured breast cancer cell lines BT-474 and Sk-Br-3 were used as positive and negative controls, respectively, for BCL-2 staining. All cultured cell lines express Ki67, but heterogeneously, so MCF-7 cells were used as a control cell line for both positive and negative staining.

CTC-ETI in vitro experiments

To test the CTC-Bio-Point assay for each marker and the Bioscore calculation for different cell lines, the following cultured human breast cancer cell lines were spiked into healthy WB and processed in the CellSearch system in a manner identical to that described below for patient samples: MCF-7 (ER+, BCL-2+, HER2+, Ki67+), Sk-Br-3 (ER+, BCL-2+, HER2+, Ki67+), and MDA-MB-231(ER+, BCL-2+, HER2+, and Ki67+).

Patient accrual, blood collection, and processing

Patients with progressive MBC scheduled to start a new therapeutic regimen of any type (ET or chemotherapy or other) were enrolled onto a prospective single-institution pilot study to establish the analytical validity of the CTC-ETI assay. All patients signed informed consent approved by the University of Michigan IRB (see full protocol in Supplementary Data). The trial was conducted in two stages, with futility stopping rules based on unacceptable analytical failure (Supplementary Fig. S1 and Supplementary Table S2). Following demonstration of an acceptable rate of successful CTC-ETI analyses in the first stage, accrual was restricted to patients with HR-positive MBC (stage 2). We enrolled patients until we had at least 5 patients in each group with elevated CTC (≥5 CTCs/7.5 mL of WB; Supplementary Fig. S1).
Before starting a new systemic treatment, all patients had approximately 40 mL WB drawn into 4 CellSave tubes. These tubes were pooled and divided into four different 7.5-mL aliquots for CTC enumeration and characterization using the CellSearch System.

**CTC enumeration and characterization**

CTCs were isolated and enumerated using the CXC CellSearch Kit and CellSearch System according to the manufacturer's instructions (Janssen Diagnostics LLC) and as previously described (8, 22). Three of the four fluorescent CellSearch channels were used to distinguish CTC from WBC (DAPI, anti-cytokeratin, anti-CD45). The fourth channel was used to measure ER, BCL-2, HER2, and Ki67 expression in separate aliquots of the pooled WB specimens, using antigen-specific phycoerythrin-labeled antibodies. Positive and negative control specimens for each marker were included in each batch, as previously stated above.

After CTC enumeration of each aliquot (23), the relative expression of each biomarker was determined to be 0, 1+, 2+, or 3+ on a scale developed from the expression of that marker in cultured human cell lines known to express each marker (0 = nondetectable; 1+ = low; 2+ = intermediate; 3+ = high; Supplementary Fig. S2), similar to HER2 staining scale reported in previous publications (24). CTC biomarker visual phenotyping was independently performed by two operators (C. Paoletti and M.C. Mutiu). Discordant results were reconciled by joint readings. All CTC biomarker results were determined without knowledge of tissue expression of the marker, except that all of the last 40 patients enrolled were known to have had ER-positive breast cancer (primary or metastatic).

**Calculation of CTC-ETI**

To generate CTC-ETI, assumptions were made about the relative prognostic and predictive effects of each of the biomarkers, including the use of a CTC cutpoint of ≥5/7.5 mL WB, based on published literature (8, 10–13, 15–19). CTC-ETI was considered low if a patient had <5 CTCs/7.5 mL of WB. For those patients with ≥5 CTCs/7.5 mL of WB, the percentage of cells that had each category (0, 1+, 2+, and 3+) of relative expression of ER, BCL-2, HER2, and Ki67 of the CTC was used to derive the CTC-ETI. The expression was evaluated visually with the semiquantitative scale described above. Staining of 2+ and 3+ was considered "positive", and staining of 0 and 1+ was considered "negative."

**CTC-Enumeration points.** CTC levels were enumerated in each of the four different aliquots of 7.5 mL of WB. The average CTC count of the four tubes was used to assign the CTC-Enumeration points for that blood draw as follows: 0 points = average <5 CTCs/7.5 mL WB (favorable outcome), 1 point = average 5–10 CTCs/7.5 mL WB (intermediate outcome), and 2 points = average >10 CTCs/7.5 mL WB (worst outcome, Supplementary Table S3A). If the average of CTC counts was <5 CTCs/7.5 mL of WB, but two aliquots had ≥5 CTCs/7.5 mL of WB, the average was rounded down to the closest integer.

**CTC-Bio-Points and Bio-Score.** If the average CTC/aliquot was ≥5/7.5 mL WB, CTC-Bio-Points were determined for each marker, based on the percentage of CTCs that were positive (2+ or 3+) for the respective marker. Arbitrarily, we established three categories of positive staining: 0%, 1% to 10%, and >10% of CTC staining for each marker (Supplementary Table S3B). Because ER has an absolute role in ET response, ER expression was weighted more heavily than the other three markers. Positive CTC-ER and BCL-2 readings were given low points (=sensitivity to ET), whereas positive CTC-HER2 and Ki67 readings were given high points (=resistance to ET; Supplementary Table S3B). The sum of assigned CTC-Bio-Points for each marker produces a final CTC-Bio-Score.

**CTC-ETI.** The CTC-Enumeration points were combined with the CTC-Bio-Score to derive the final CTC-ETI score (Supplementary Table S3C), according to the following equations:

\[
\text{CTC-ETI} = \left[ \text{CTC-Enumeration Points} \right] + \left[ \text{Bio-Points for ER} \right] + \left[ \text{Bio-Points for BCL-2} \right] + \left[ \text{Bio-Points for HER2} \right] + \left[ \text{Bio-Points for Ki-67} \right].
\]

Thus, CTC-ETI can range from 0 to 14 (Supplementary Table S3D). To make the CTC-ETI score clinically applicable, the scores were placed into three categories, much as histologic grading is calculated: low CTC-ETI score = 0 to 3, intermediate CTC-ETI score = 4 to 6, high CTC-ETI score = 7 to 14. No CTC enumeration, biomarker, or CTC-ETI results were returned to the patient or her caregiver.

**Central immunohistochemical staining of breast cancer tissue specimens**

Patient’s blocks were obtained from the University of Michigan Department of Pathology archives or from the referring institution. All pathologic analyses were performed by a pathologist (D.C. Thomas).

Immunohistochemical staining was performed on a DAKO Autostainer (DAKO) using diaminobenzadine (DAB) as the chromogen and the detection system as noted below. Deparaffinized sections of formalin-fixed tissue at 5-μm thickness were stained with ER, BCL-2, HER2, and Ki67 antibodies (Supplementary Table S1). Appropriate negative (no primary antibody) and positive controls (breast carcinoma) were stained in parallel with each set of tumors studied. ER, BCL-2, and HER2 were scored using the Allred system (25). Ki67 slides were scanned into an APERIO imaging system and the percent positive cells calculated using the positive nuclear algorithm. ER and BCL-2 were considered positive if Allred score was ≥3, and HER2 was considered positive if Allred score was ≥4. For Ki67, >10% cells staining was considered positive.

**Statistical analysis**

**Study design.** Feasibility and analytical validity of determination of CTC-ETI was designed by using two-step futility clinical trial design (see Supplementary Fig. S1 and Supplementary Data for full protocol, including planned statistical analysis). Success of the CTC-ETI assay was defined as the ability to measure each of the phenotypic markers of the ETI (ER, BCL-2, HER2, and Ki67) on ≥60% of CTC-informative patients (those patients with an average of ≥5 identified CTCs/7.5 mL of WB). Initial accrual goal was 20 to 40 patients, depending on successful determination of CTC-ETI in each stage. The trial was amended to accrue up to 50 patients for additional experience.

This study is reported according to the REMARK guidelines (26).
Reproducibility of CTC-ETI. CTC enumeration. The average number and coefficient of variation (CV; SD/mean) of CTC enumeration was calculated for each of four possible aliquots per patient.

Interreviewer reproducibility of CTC-ETI. Concordance of CTC-ETI between reviewers was assessed using the Kappa agreement statistic and 95% confidence interval. The statistic was calculated across all CTC with identified staining levels for each marker.

Concordance between CTC and tissue biomarker expression. Spearman rank correlation and Kappa coefficients were determined for agreement between tissue and CTC expression of each biomarker (25). To calculate the kappa coefficient, we dichotomized the Allred and visual scores between positive and negative by using the cutoff for each biomarker as described above. For CTC, ≥10% of cells staining of 2+ or 3+ was considered positive.

Results

Preclinical development of the CTC-ETI assay

The overall goal of this set of investigations was to develop a CTC-based biomarker index for patients with HR-positive MBC starting a new ET. Before initiation of any clinical studies, we performed preclinical studies to develop the technical aspects of the CTC-ETI assay.

Characterization of antibodies in cultured breast cancer cell lines. As illustrated in Supplementary Fig. S3A–S3D, each antibody appropriately stained positive or negative for the respective marker in the selected cell lines. To model what we anticipated in the subsequent clinical studies, we developed a semiquantitative immunofluorescent staining scale (0, 1+, 2+, and 3+) determined by visual readings for each biomarker against the respective cell lines (Supplementary Fig. S2). The location and degree of staining for each marker in these cell lines were then used in subsequent batches as internal positive and negative controls.

Calculation of CTC-Bio-Points and CTC-Bio-Score. As proof of principle, we calculated a CTC-Bio-Score (Supplementary Table S3) for the cultured human breast cancer cell lines known to represent various intrinsic subtypes (MCF-7 = luminal, Sk-BR-3 = HER2-like, and MDA-MB-231 = basal). As expected, the luminal-type, hormone-depleted MCF-7 cells were assigned 0 Bio-Points for CTC-ER and CTC-BCL-2, because in both cases >10% of the cells stained either 2+ or 3+ (Supplementary Fig. S4A). Further, MCF-7 cells were assigned a CTC-Her2 Bio-Point of 1, because 1% of the cells stained 2+ for this biomarker, and CTC-Ki67 Bio-Point of 2, because more than 10% of the cells stained 2+ to 3+ (Supplementary Fig. S4A). In contrast, the basal-like MDA-MB-231 cell line was assigned 6 CTC-ER Bio-Points, because 0% of the cells were 2+ or 3+ for this biomarker. This cell line was also assigned 1, 0, 2 Bio-Points for BCL-2, HER2, and Ki67, respectively (Supplementary Fig. S4B). The HER2-amplified Sk-BR-3 cell line was assigned 6, 2, 2, and 2 Bio-Points for ER, BCL-2, HER2, and Ki67, respectively (Supplementary Fig. S4C).

For each cell line, a CTC-Bio-Score was then calculated by summing the Bio-Points assigned to it for each of the biomarkers, as explained in Supplementary Table S3. As expected, the MCF-7 cell line had a relatively low CTC-Bio-Score (=3), whereas MDA-MB-231 and Sk-BR-3 cell lines had higher CTC-Bio-Scores (=12 and 9, respectively; Supplementary Fig. S4 and Supplementary Table S4).

Heterogeneity of cell line CTC biomarker expression

Even though these experiments were performed using cultured human breast cancer cell lines grown and harvested under optimal conditions, we observed remarkable intraline heterogeneity of biomarker staining as illustrated for ER staining of hormone-depleted MCF-7 cells (Supplementary Fig. S5). Two thirds of the cells stained 2+, whereas 18% and 15% stained 1+ or 0, respectively. Similar heterogeneity was seen for BCL-2, HER2, and even Ki67. Ki67 expression for each cell line was consistently >10%, as expected, but, not all cell lines were positive for this proliferative marker within any of the cell lines.

CTC-ETI in patients with MBC

CTC-ETI in pilot clinical trial. Following preclinical development of the CTC-Bio-Point/Bio-Score assays, we extended the CTC-ETI assay to patients with MBC (Supplementary Fig. S1 and Fig. 1). CTC-ETI was successfully determined in all five of informative patients (of eight total) enrolled onto the first stage of the protocol. One patient in stage 2 of group 1 was ineligible because she had ER-negative breast cancer and was replaced with a new patient. CTC-ETI was successfully determined for three of the five informative patients (≥5 CTCs/7.5 mL of WB) patients enrolled into Group 1, two of the five informative patients in Group 2, and four of five informative patients in Group 3. In summary, in Groups 1 to 3, CTC was determined successfully in 9 of 15 informative patients, exceeding our protocol-stipulated criterion for successful analytical validity (≥9 of 15 informative patients). Examples of CTC-ETI calculation from three selected patients who had low, intermediate, or high CTC-ETI are provided in Supplementary Table S5.

CTC-ETI could not be calculated due to technical failure of the machine for one or more of the four aliquots in 2 patients (#15 and #20) and due to analytical failure in 4 patients (#10, #17, #19, and #29). Analytical failure was defined in these cases if the average enumeration of all four aliquots was ≥5 CTCs/7.5 mL of WB, but one of four aliquots had <5 CTCs/7.5 mL of WB, while CTCs were ≥5/7.5 mL WB in the other three aliquots from the same blood draw.

To gain additional experience with clinical determination of CTC-ETI, the protocol was amended to enroll a total of 50 patients (those included in the original feasibility plus the expansion cohort; Fig. 1). In total, CTC-ETI was successfully determined in 44 of the 50 eligible patients, ranging from 0 to 14 (Fig. 1). CTC-ETI was considered low (score ranged 0–3) in 18 (41%) of the 44 patients due to either a patient having <5 CTCs/7.5 mL of WB (CTC-enumeration points = 0; n = 13) or ≥5 CTCs/7.5 mL of WB (CTC-enumeration points = 1 or 2), but with low Bio-Scores (n = 3; see Supplementary Table S3A–S3C for definitions). Nine (20%) and 17 (39%) patients had intermediate (score range, 4–6) and high CTC-ETI (score range, 7–14), respectively (Fig. 1). CTC-ETI details for all patients are provided in Supplementary Table S6.

Because this study was a feasibility study designed to determine analytical validity only, patients were treated with many different types of therapies (both endocrine and chemotherapy), did not necessarily have measurable disease, and outcomes were not uniformly determined. These multiple confounding issues...
precluded a valid exploration of associations between CTC-ETI and clinical outcomes.

**Performance characteristics of CTC-ETI**

*Coefficient of variation of CTC-enumeration.* CV for CTC-enumeration varied from 0% to 0.7% and did not vary as the mean increased (Supplementary Fig. S6). As expected, the variability was higher with low number of CTC and this generally lessened with higher numbers. However, an aberrant variation was observed around 50 cells.

*Interreader concordance of CTC-Bio-Scores.* There was strong agreement in assessment of CTC-Bio-Score and CTC-ETI between the two independent reviewers, with a Kappa statistic for interreader concordance of >0.9 for all of the markers (Table 1). In particular, discordances for each marker between readers that would have changed the CTC-ETI category occurred in <3% of patients, demonstrating that the assay has high analytical reproducibility.

Taken together, these data demonstrate the robust analytical validity and interviewer reproducibility of the CTC-ETI assay, as well as the broad distribution of CTC-ETI across the population of patients with HR-positive MBC.

**Heterogeneity of CTC-biomarkers**

As expected, CTC-enumeration varied widely among the patients, ranging from 0 to 837 CTCs/7.5 mL of WB, as did CTC-biomarker expression. Further, we observed enormous intrapatient heterogeneity of each CTC biomarker (Fig. 2A–D). Interestingly, even though all of these patients had ER-positive breast cancer as determined by their primary institutional pathology laboratory at some point before study entry, 13 of the 29 evaluable patients with ≥5 CTCs/7.5 mL (45%) had negative (0–1+) CTC-ER expression (Fig. 2A). Among patients who had elevated CTC (≥5 CTCs/7.5 mL of WB) and some level (1, 2, or 3+) of CTC-ER expression, the relative CTC-ER expression was quite heterogeneous. CTCs were not 100%

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**Table 1. Interreader concordance of CTC-ETI**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Concordance</th>
<th>Kappa (95% confidence intervals)</th>
<th>CTC-ETI important discordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>95%</td>
<td>0.91 (0.90–0.92)</td>
<td>3%</td>
</tr>
<tr>
<td>BCL-2</td>
<td>94%</td>
<td>0.91 (0.89–0.92)</td>
<td>2%</td>
</tr>
<tr>
<td>HER2</td>
<td>95%</td>
<td>0.90 (0.88–0.91)</td>
<td>2%</td>
</tr>
<tr>
<td>Ki67</td>
<td>95%</td>
<td>0.92 (0.91–0.93)</td>
<td>2%</td>
</tr>
</tbody>
</table>

*Important discordance* — would have changed CTC-ETI category for patient; final CTC-ETI was calculated after resolution of discordance between observers.
positive (2+, 3+) for ER in any patient (Fig. 2A). The results of patient #4 are particularly illustrative (Fig. 2A and Supplementary Fig. S7). Her primary cancer was originally ≥95% positive with strong staining for ER (Supplementary Fig. S7). She had 790 CTCs/7.5 mL of WB in the aliquot evaluated for ER expression (Fig. 2A). Of these, 36% were CTC-ER 0, 11% were 1+, 50% were 2+, and 3% were 3+.

Likewise, for most patients, intrapatient CTC-BCL-2, HER2, and Ki67 expression were highly heterogeneous (Fig. 2B–D). For example, patient #28 had metastatic tissue that was FISH negative.
for HER2, but CTC-HER2 staining was 0, 1+, 2+, and 3+ in 7%, 29%, 50%, and 14%, respectively (Fig. 2B).

Comparison of CTC-biomarker expression to cancer tissue biomarker expression

We investigated the correlation between CTC and cancer tissue biomarker expression. None of the available tissues was collected at the time of the CTC draw. Each was either the excised primary cancer or a biopsy of the first metastases. Therefore, we hypothesized that CTC and tissue biomarker expression would be discordant due to natural and therapeutic-induced genetic and phenotypic drift over time.

We included patients with CTC ≥1/7.5 mL WB and for whom archived breast cancer tissue was available. We excluded patients progressing on fulvestrant immediately before being enrolled in this pilot study because they were expected to have artificially induced CTC-ER–negative results (n = 5). Thus, 26 and 19 patients with available primary or metastatic tissues, respectively, met these criteria (Fig. 3A and B).

As expected, substantial discordance was found between CTC and cancer tissue biomarker expression, including both primary and metastatic sites (Fig. 4A–H, Table 2A–D, and Supplementary Table S7). Kappa scores for CTC and tissue concordance were quite low for all the markers (Supplementary Table S7). For example, of the 19 patients for whom primary cancer was available and was positive by central ER staining, 9 (47%) had negative CTC-ER (defined as <10% CTC with 2+ or 3+ ER staining; Table 2A). Likewise, of the 11 patients who were not taking fulvestrant and for whom metastatic tissue was available and ER was positive, 7 (60%) had negative CTC-ER (Fig. 4A, Table 2A). Two of the four patients (50%) who were found to have ER-negative metastatic tissue upon central staining had at least 10% of their CTC stain positively for ER (Fig. 4B, Table 2A).

Similar discordance between CTC and tissue expression was seen for the other biomarkers (Fig. 4C–H, Table 2B–D). To generate a more dynamic range of values than would be available using assays suggested by the American Society of Clinical Oncology and College of American Pathologists (ASCO-CAP) Practice Guidelines Committee, HER2 was determined by Alled score (25, 27). Of 10 patients with primary and 9 patients with metastatic HER2-positive Alled scores, five (50%) and six (66%), respectively, had negative CTC-HER2 expression (Fig. 4D and E, Table 2C). Perhaps more importantly, of 10 and 6 patients with negative HER2 primary or metastatic tissues, two (20%) and four (66%) had at least 10% of their CTC stain positively for HER2.

Discussion

In this study, we have established the analytical validity for determining CTC-ETI, based on immunomagnetic capture, enumeration, and biomarker characterization using the CellSearch system in patients with HR-positive MBC. We have demonstrated that CTC-ETI, and its individual components, can be reliably and reproducibly evaluated by two separate operators. The analytical failure rate (12%) was well within our protocol-stipulated acceptable limits for the assay (protocol in Supplementary Data).

Furthermore, we have observed an expected broad distribution of CTC-ETI scores across 44 patients whose primary and/or metastatic tumors were known to be HR positive. Roughly 2 of 3 of these patients with HR-positive MBC had low or intermediate CTC-ETI scores, whereas 1 of 3 of these patients had high CTC-ETI scores.

We hypothesize that those patients with high CTC-ETI score are likely to be refractory to, and unlikely to benefit from, ET. Therefore, they might be better palliated with chemotherapy, in spite of the higher side effect profile of the latter. However, this pilot study was designed to determine analytical, not clinical validity (28). Entry criteria, patient characteristics, and treatments were quite broad, and therefore we did not attempt to evaluate associations between CTC-ETI and patient outcomes. Moreover, the CTC-ETI was based on a series of informed, but relatively arbitrary assumptions. These included the selection of thresholds for CTC levels and expression of each biomarker to calculate CTC-Points and Bio-Scores. In our assay, we have used external cultured human breast cancer cells that are known not to express the relative marker as negative controls for each run. Other investigators have reported using leukocytes that are still present in the assay as an internal negative control for HER2 (29). At present, it is unclear which of these methods is preferable.

The intraindividual, cell-to-cell heterogeneity we observed in CTC biomarker expression underscores our belief that the CTC-ETI algorithm will need to be adjusted according to correlation to clinical outcomes of patients with HR-positive MBC. Taking these considerations together, our results are the basis of two currently accruing, multinational, multi-institutional prospective trials specifically designed to evaluate the clinical validity of CTC-ETI: (i) the characterization of CTCs from patients with metastatic breast cancer using the CTC-endocrine therapy index, COMETI P2, ClinicalTrials.gov identifier: NCT01710150; and (ii) SWOG S1222, ClinicalTrials.gov identifier: NCT02137837. The data generated in these trials will permit us to determine if CTC-ETI is correlated with outcomes in patients with HR-positive MBC starting a new second- or third-line ET. They will also permit us to retrain the multiparameter ETI algorithm to specifically identify the 15% to 30% of such patients who rapidly progress within the first two to three months after initiation of ET.

We and others have previously reported evaluation of various biomarkers on CTC, using CellSearch or other systems (30). However, few if any reports have examined combining several markers on CTC, using CellSearch or other systems (30). To our knowledge, our multiparameter ETI algorithm is the only one to use (in this case, resistance to ET) using rigorous analytical methodologies. The observed heterogeneity of each CTC marker highlights the complexity of such an assay. It is essential that such analytical validity be established before any tumor biomarker test can be evaluated for clinical utility (28). We have demonstrated that the CTC-ETI, based on the CellSearch system, is highly reproducible, with strong interobserver concordance. Indeed, our data further support a recently reported study that determined highly concordant interreader reproducibility of CTC analysis using the CellSearch system (31).

Our results also further highlight the long-recognized degree of tumor heterogeneity in MBC, which is presumably the major obstacle to cure of patients with metastatic cancer (2–4, 32). Ideally, one would like to measure tumor heterogeneity in all metastatic sites, at baseline and then serially during treatment, but such an approach is costly and logistically difficult. Serial evaluation of CTC biomarker expression might provide an opportunity to monitor evolving tumor heterogeneity, especially during treatment (33). Moreover, CTC biomarker...
expression, and in particular CTC-ETI, provides an integrated picture of tumor heterogeneity within the entire patient, as opposed to biopsies of individual sites (34), and serial CTC analysis may provide an opportunity to monitor the effects of treatment on CTC biomarker expression (35). However, in the current study, we did not address whether CTC-ETI, or CTC-individual biomarker scores, changes over time in individual patients, either independently or under selective pressure of
systemic therapies. This issue is also a key secondary objective of the ongoing COMETI and S1222 trials.

Several studies have demonstrated discordance in tissue biomarker expression, particularly ER and HER2, between primary and metastatic cancers (5–7, 36–40). These changes may have clinical importance in regard to ET and anti-HER2 therapy. In this regard, differences between CTC and primary tumor biomarker expression have been reported. For example, Babayan and colleagues (41) observed that over two thirds of women with ER-positive MBC exhibited heterogeneous CTC-ER expression, although 19% were homogeneously negative. Similarly, we also observed discordance between CTC and tissue expression for each biomarker. For example, one third of our patients, all of whom were considered to have HR-

Figure 4.
Correlation of CTC and tissue biomarker expression. A, B: ER; C, D: BCL-2; E, F: HER2; G, H: Ki67. A, C, E, and G: CTC biomarker compared with primary cancer tissue biomarker. B, D, F, and H: CTC biomarker compared with metastatic cancer tissue biomarker. For each biomarker, 1–4 CTCs/7.5 mL of WB; 5–10 CTCs/7.5 mL of WB; ≥10 CTCs/7.5 mL of WB; and relative biomarker staining score (0, 1, 2, and 3+). See Materials and Methods for details. Allred Score: see Materials and Methods; for Ki67, results are expressed as % positive cells. Dotted lines represent cutoff levels for CTC biomarker and for Allred score or % positive Ki67.
positive primary or metastatic cancer as determined at their local institutions, had ER-negative CTC.

The appearance of HER2-positive CTC in patients whose primary or metastatic tissues were previously considered HER2 negative has been reported previously (24, 42, 43); and could broaden the indication for anti-HER2 therapies. The heterogeneity we observed in CTC-HER2 is consistent with reports demonstrating changes in the same patient in biomarker expression between primary and metastatic tumor (44), between different metastatic lesions, and even within the same tumor (45, 46). These data are also supported by studies from several investigators demonstrating upregulation of HER2 when ER-positive breast cancer cells are placed in low-estrogen environments (45, 47).

In summary, we have developed a highly analytically validated assay to provide semiquantitative analyses of several important biomarkers combined in a single test: CTC enumeration, and CTC-ER, BCL-2, HER2, and Ki67 expression. The CTC-ETI assay may serve as a real-time predictive factor for resistance to ET in patients with HR-positive MBC. Furthermore, serial monitoring of CTC-ETI may provide insight into mechanisms of resistance to all, or specific types of ET. The CTC-ETI data demonstrate the extensive tumor heterogeneity that exists within patients with HR-positive MBC. This heterogeneity creates enormous challenges to treatments with ET and to development of new, targeted therapies. It is possible that serial CTC biomarker evaluation will provide a pharmacodynamic tool to monitor biomarker expression over time, and help guide personalized therapeutic management for patients with metastatic malignancies.

### Disclosure of Potential Conflicts of Interest

M.C. Miller was an employee of Veridex, LLC. D.A. Chianese has ownership interest (including patents) in Johnson & Johnson. D.F. Hayes reports receiving a commercial research grant from Janssen Diagnostics and other commercial research support from Janssen Therapeutics and Pfizer; is a consultant/advisory board member for Pfizer; has ownership with iBiomed and Oncimmune LLC; and is an inventor of a patent regarding use of the Circulating Tumor Cell Endocrine Therapy Index to manage patients with estrogen receptor positive metastatic breast cancer, which is owned by the University of Michigan and licensed to Janssen Diagnostics, LLC. No potential conflicts of interest were disclosed by the other authors.

### Authors’ Contributions

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** C. Paoletti, M.C. Muniz, D.G. Thomas, N. Tokudome, D.L. Blossom, A.F. Schott, N.L. Henry, D.F. Hayes

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C. Paoletti, M.C. Muniz, D.G. Thomas, K.A. Griffith, K.M. Kidwell, K. Aung, M.C. Miller, A.F. Schott, N.L. Henry, J.M. Rae, D.A. Chianese, D.F. Hayes

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### Table 2. Concordance between CTC and tissue biomarker expression

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<th>Metastatic tissue</th>
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</table>

*a For tissue ER and BCL-2, positive = Allred Score ≥ 3; for HER2, positive = Allred score ≥ 4; for Ki67, positive = ≥ 10% staining

*b For CTC-ER, BCL-2, HER2, and Ki67, positive = ≥10% staining 2-3* in sample with ≥1CTC/7.5 ml of WB.
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22. Yamauchi H, Stearns V, Hayes DF. When is a tumor marker ready for prime ahead of print?


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