Clinical relevance of circulating CK-19mRNA-positive tumour cells before front-line treatment in patients with metastatic breast cancer

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BACKGROUND: To investigate the clinical relevance of CK-19mRNA-positive circulating tumour cells (CTCs) detected before the initiation of front-line treatment in patients with metastatic breast cancer (MBC).

METHODS: The presence of CTCs was detected in 298 patients with MBC using a real-time PCR (RT-PCR) assay. In 44 patients, the detection of CTCs was evaluated by both the CellSearch and the RT-PCR assay. Interaction with known prognostic factors and association of CTCs with clinical outcome were investigated.

RESULTS: There was a strong correlation between the detection of CTCs by both assays. CK-19mRNA-positive CTCs were detected in 201 (67%) patients and their detection was independent of various patients’ clinico-pathological characteristics. The median progression-free survival (PFS; 9.2 vs 11.9 months (mo), \( P = 0.003 \)) and the overall survival (OS; 29.7 vs 38.9 mo, \( P = 0.016 \)) were significantly shorter in patients with detectable CK-19mRNA-positive CTCs compared with patients without detectable CTCs. Multivariate analysis demonstrated that oestrogen receptor status, performance status and detection of CTCs were emerged as independent prognostic factors associated with decreased PFS and OS.

CONCLUSION: The detection of CK-19mRNA-positive CTCs in patients with MBC before front-line therapy could define a subgroup of patients with dismal clinical outcome.

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The prognosis of patients with metastatic breast cancer (MBC) mainly depends on several patient and tumour characteristics such as age, hormone receptor and HER2 status, histological grade, site of metastasis and performance status, (PS; Chung and Carlson, 2004), which in the daily practice influence clinical decisions. Recent studies have shown that the detection of cytokeratin-expressing (CK\textsuperscript{+}) cancer cells in the blood (circulating tumour cells (CTCs)) or bone marrow (disseminated tumour cells) also holds significant prognostic information for patients with breast cancer. Indeed, the detection of peripheral blood CK-19mRNA-positive CTCs in patients with early disease has been proven as an independent prognostic factor for reduced disease-free survival (DFS) and overall survival (OS) (Stathopoulou et al, 2002, 2003; Xenidis et al, 2003, 2006, 2009; Ignatiadis et al, 2007). Moreover, a pooled analysis showed that bone marrow CK-positive disseminated tumour cells, detected in 31% of stage I–III breast cancer patients, have an independent prognostic impact (Braun et al, 2005). Similarly, in the metastatic setting, Cristiano\textit{n}i\textit{ll}i\textit{ et al} (2004), using the CellSearch platform, reported that the detection of \( \geq 5 \) CTCs/7.5 ml blood at baseline and before the administration of front-line or second-line treatment was associated with a poor clinical outcome both in terms of progression-free survival (PFS) and OS. These findings were further confirmed by several other investigators using either the CellSearch (Cristo\textit{f}o\textit{n}i\textit{n}i\textit{illi} et al, 2007; Dawood et al, 2008; Nole et al, 2008) or immunocytochemical assays (Bidard et al, 2008). In addition, the prognostic value of CTCs, as detected by the CellSearch system, was unrelated to hormonal receptor status, site of recurrence, previous treatments or the tumour burden (Cristo\textit{f}o\textit{n}i\textit{n}i\textit{illi} et al, 2007; Dawood et al, 2008; Nole et al, 2008).

Our group, using a molecular (real-time PCR (RT-PCR)) assay, based on the detection of CK-19mRNA transcripts in peripheral blood mononuclear cells (PBMCs) of patients with early-stage breast cancer (Stathopoulou et al, 2003), demonstrated in a cohort of 444 patients that their detection was an independent factor associated with an increased risk of relapse and disease-related death (Xenidis et al, 2003, 2006; Ignatiadis et al, 2007). In addition, it was shown that adjuvant chemotherapy does not always eliminate CK-19mRNA-positive CTCs, and that patients with chemotherapy-resistant CTCs have an extremely reduced median DFS and OS (Xenidis et al, 2009). As clinical relapses are believed to be originated from these chemotherapy- and hormone-resistant
for the detection of CTCs in whole blood has been reported previously (Cristofanilli et al, 2004). Briefly, CTCs expressing the epithelial cell adhesion molecule were immunomagnetically enriched and stained with 4,2-diamidino-2-phenylindole dihydrochloride, cytokeratins 8,18,19 and CD45. Circulating tumour cells morphology was confirmed in all cases. Quantitative results were expressed as number of CTCs per 7.5 ml blood.

RNA extraction and real-time RT-PCR assay for CK-19mRNA-positive cells
Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque gradient-density centrifugation within 2–4 h from vein puncture, and total RNA isolation was carried out in a laminar flow hood under RNase-free conditions by using Trizol (Invitrogen, Grand Island, NY, USA; Stathopoulou et al, 2002). The isolated RNA was dissolved in RNA storage buffer (Ambion, Grand Island, NY, USA) and stored at −80 °C until used. RNA concentration was determined by absorbance reading at 260 nm with the NanoDrop spectrophotometer (Wilmington, DE, USA) at the time of CK-19mRNA analysis. RNA integrity was tested by PCR amplification of the β-actin housekeeping gene. RNA samples prepared from the MCF-7 breast cancer and ARH-77 leukaemic cell lines were used as positive and negative controls, respectively (Stathopoulou et al, 2002).

Reverse transcription of RNA was carried out by synthesising cDNA using the Superscript III Platinum Two-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions; the real-time RT-PCR assay, performed in a total volume of 10 μl in the LightCycler glass capillaries, for the detection of CK-19mRNA-positive cells and its analytical methodological details (specificity, sensitivity, cut-off for positivity), the used primers and probes, as well as the cycling protocol have been previously described (Stathopoulou et al, 2003). The quality of cDNAs was always evaluated by real-time PCR using the β-actin housekeeping gene. The lower detection limit for positivity of the assay was determined to be ≥0.6 MCF-7 cell equivalents/5 μg RNA for the patients' PBMCs (Stathopoulou et al, 2003). Using this cut-off value, only 2 out of 89 healthy individuals tested were found positive (Stathopoulou et al, 2003).

Statistical analysis
This is a retrospective analysis aiming to investigate the clinical relevance of the detection of CK-19mRNA-positive CTCs, evaluated prospectively and blindly, at the time of diagnosis of metastatic disease before the initiation of any systemic treatment. Therefore, there was no predetermined sample size estimation in this study because of its observational nature. Progression-free survival was defined as the time elapsed between the date of the administration of the first chemotherapy cycle, and either the date of clinical or radiological progression, or death or the last follow-up. Overall survival was measured from the date of the administration of the first chemotherapy cycle until the date of death or last follow-up. To detect significant differences between survival curves, Log-rank or Breslow tests were used. Cox proportional hazards model for time to event data was used to evaluate the hazard of every important variable, as well as for the construction of a multivariate model, explaining the relative significant influence of the covariates on outcomes (conditional backwards procedure). The interaction between the prognostic value of the detection of CK-19mRNA and known clinico-pathological prognostic factors was tested using the heterogeneity tests for OS and PFS (Figures 1 and 2). Analysis included contingency tables and χ²-tests for categorical variables used to assess differences between groups. All statistical tests were carried out using the Statistical Package for Social Sciences (SPSS) version 16.
out at the 5% significance level using the statistical software SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patients’ demographics

During the study period, 298 consecutive patients with MBC at first presentation were evaluated for the presence of circulating CK-19mRNA-positive CTCs before the administration of any systemic treatment. The patients’ median age was 59 years (range, 27–83), 218 (73.2%) were post-menopausal and 166 (55.7%) had visceral disease. A total of 136 (45.6%) patients had hormone receptor (either ER or PR)-positive/HER2-negative tumours, 61 (20.5%) HER2-positive tumours (3+ or FISH +) and 66 (22.1%) triple-negative tumours. Another 129 (43.3%) patients had received prior adjuvant treatment, most of them in the context of research protocols of the Hellenic Oncology Research Group, whereas 169 (56.7%) patients were diagnosed with metastatic disease from the beginning. There was no difference in terms of the main clinicopathological characteristics in patients with detectable and undetectable CK-19mRNA-positive CTCs (Table 1). At the time of analysis, 16 (5.4%) patients are still in first remission and 58 (19.5%) are alive.

Detection of CTCs by CellSearch and RT-PCR

In 44 patients treated from January 2007 to May 2009, the detection of CTCs was performed in parallel using the two different assays. Circulating tumour cells were detected in 17 (38.6%) patients using the CellSearch assay (cut-off, \( \geq 5 \) CTCs/7.5 ml), and in 24 (54.6%) using the RT-PCR assay \((P = 0.001)\). The RT-PCR assay failed to detect CTCs in two (4.5%) patients in whom CellSearch could detect CTCs; conversely, CellSearch failed to detect CTCs in nine (20.5%) patients in whom RT-PCR could detect CTCs (Table 2). However, there was a statistically significant correlation of the detection of CTCs between the two assays \((\rho = 0.373, P = 0.0001)\). The correlation remained significant when the cut-offs for CTC detection with the CellSearch were set at \( \leq 1 \) and \( \leq 2 \) CTCs (Table 2).

Detection of CTCs

CK-19mRNA-positive cells were detected in 199 (66.8%) patients. There was no association between the detection of CK-19mRNA-positive CTCs and the menopausal status, the PS, the hormone receptor or HER2/neu status, the presence of visceral or bone metastases and the administered front-line treatment (Table 1). The median number of CK-19mRNA-positive CTCs was 1.0 MCF-7 cell equivalents/5\( \mu \)g RNA (range, 0.0–1221.0). Front-line chemotherapy resulted in an objective response (complete plus

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**Figure 1** Forest plot: overall survival for CK-19mRNA pre-chemotherapy.
partial response) in 95 (48.2%) out of 199 patients with detectable CK-19mRNA-positive CTCs and in 58 (58.6%) out of 99 patients without detectable CK-19mRNA-positive CTCs (P = 0.109). There was no significant correlation between the detection of CTCs and the presence of visceral (53.8% vs 59.6% in CK-19mRNA-positive and CK-19mRNA-negative patients, respectively; P = 0.441) or bone (36.2% and 36.4%, respectively; P = 0.999) disease.

Clinical outcome according to the detection of CK-19mRNA-positive cells

At the time of analysis, 240 (80.5%) patients had died. The median follow-up was 83.3 mo (range, 2.2–119.6). The median PFS was significantly lower in patients with detectable CK-19mRNA-positive CTCs compared with patients without detectable CTCs (9.2 vs 11.9 mo; P = 0.003). The median OS was significantly higher in patients without detectable CK-19mRNA-positive CTCs compared to patients with detectable CTCs (38.9 vs 29.7 mo; P = 0.016).

Univariate and multivariate analysis

The univariate analysis using the menopausal status, the hormone receptor status (ER and PR), the HER2/neu status, the PS, the disease localisation (bone or visceral), the adjuvant chemotherapy and the detection of CK-19mRNA-positive CTCs at baseline revealed that PS, ER, PR, HER2/neu, adjuvant chemotherapy and CTC status at baseline were significantly associated with PFS (Table 3). Multivariate analysis demonstrated that only the ER negativity (hazard ratio (HR) 1.627; 95% CI: 1.267–2.090) and the detection of CK-19mRNA-positive CTCs (HR 1.424; 95% CI: 1.092–1.857) could be emerged as independent factors associated with a decreased PFS (Table 4).

DISCUSSION

The aim of the current study was to evaluate the prognostic value of CK-19mRNA-positive CTCs in women with newly diagnosed metastatic breast cancer. The study included 240 patients who were assessed for CTCs using the epithelial membrane antigen 19 (EMA-19) at baseline and at the end of adjuvant chemotherapy. The detection of CK-19mRNA-positive CTCs was associated with a worse PFS and OS, even after adjusting for other prognostic factors. The results suggest that CK-19mRNA-positive CTCs may be a useful biomarker for prognosis in metastatic breast cancer.
recurrent or de novo MBC, using our previously described RT-PCR assay, which has been validated in patients with early breast cancer (Stathopoulou et al., 2003; Xenidis et al., 2006, 2009; Ignatiadis et al., 2007). The results of this retrospective study demonstrate that the detection of CK-19mRNA-positive CTCs at baseline and before the initiation of any systemic treatment is a poor prognostic factor, as it was independently associated with a significantly reduced median PFS and OS.

Recent studies demonstrated the prognostic value of baseline CTCs measurement in MBC. Indeed, the study by Cristofanilli et al.

### Table 1: Patients' demographics and clinico-pathological characteristics

|                | All patients | CK-19 (+) (66.8%) | CK-19 (-) (33.2%) | \(\chi^2\)-test |
|----------------|--------------|-------------------|-------------------|-----------------|
| **Age**        |              |                   |                   |                 |
| Median (min-max)| 59.0 (27–83) | 59.0 (27–83)      | 60.0 (29–81)      | 0.426           |
| **Menopausal status** |            |                   |                   |                 |
| Pre (%)        | 80 (26.8%)  | 55 (27.6%)        | 25 (25.3%)        |                 |
| Post (%)       | 218 (73.2%) | 144 (72.4%)       | 74 (74.7%)        | 0.680           |
| **Performance status** |         |                   |                   |                 |
| 0–1 (%)        | 265 (88.9%) | 176 (88.4%)       | 89 (89.9%)        |                 |
| 2–3 (%)        | 25 (8.4%)   | 18 (9.0%)         | 7 (7.1%)          |                 |
| Missing (%)    | 8 (2.7%)    | 5 (2.5%)          | 3 (3.0%)          | 0.661           |
| **Molecular profile** |          |                   |                   |                 |
| ER- and/or PR-positive/HER2-negative (%) | 136 (45.6%) | 84 (42.2%) | 52 (52.5%) |                 |
| ER- and/or PR-positive/HER2-positive (%) | 25 (8.4%) | 13 (6.5%) | 12 (12.1%) |                 |
| ER-PR-negative/HER2-positive (%) | 36 (12.1%) | 29 (14.6%) | 7 (7.1%) |                 |
| Triple-negative (%) | 66 (22.1%) | 49 (24.6%) | 17 (17.2%) |                 |
| Unknown (%)    | 35 (11.7%)  | 24 (12.1%)        | 11 (11.1%)        | 0.034           |
| **Disease location** |          |                   |                   |                 |
| (a) Visceral (%) | 166 (55.7%) | 107 (53.8%) | 59 (59.6%) |                 |
| Non-visceral (%) | 114 (38.3%) | 79 (39.7%) | 35 (35.4%) |                 |
| Missing (%)    | 18 (6.0%)   | 13 (6.5%)         | 5 (5.1%)          | 0.441           |
| (b) Bones (%)  | 108 (36.2%) | 72 (36.2%)        | 36 (36.4%)        |                 |
| No bones (%)   | 182 (57.7%) | 114 (57.3%)       | 58 (58.6%)        |                 |
| Missing (%)    | 18 (6.0%)   | 13 (6.5%)         | 5 (5.1%)          | 0.999           |
| **Adjuvant chemotherapy** |          |                   |                   |                 |
| Yes (%)        | 169 (56.7%) | 105 (52.8%)       | 64 (64.6%)        |                 |
| No (%)         | 129 (43.3%) | 94 (47.2%)        | 35 (35.4%)        | 0.063           |
| **Front-line chemotherapy** |          |                   |                   |                 |
| Taxane-based (%) | 125 (41.9%) | 78 (39.2%) | 47 (47.5%) |                 |
| Anthracycline-based (%) | 32 (10.7%) | 20 (10.1%) | 12 (12.1%) |                 |
| Taxane/anthracycline –based (%) | 104 (34.9%) | 72 (36.2%) | 32 (32.3%) |                 |
| Other (%)      | 37 (12.4%)  | 29 (14.6%)        | 8 (8.1%)          | 0.276           |
| **Survival status** |          |                   |                   |                 |
| Alive (%)      | 58 (19.5%)  | 29 (14.6%)        | 29 (29.3%)        |                 |
| Dead (%)       | 240 (80.5%) | 170 (85.4%)       | 70 (70.7%)        | 0.003           |

Abbreviations: ER = oestrogen receptor; PR = progesterone receptor.

### Table 2: Comparison of detection of CTCs by both CellSearch and RT-PCR assays

|                  | \(\geq 1\) CTC/7.5 ml | \(\geq 2\) CTCs/7.5 ml | \(\geq 5\) CTCs/7.5 ml |
|------------------|------------------------|------------------------|------------------------|
|                  | Negative (n = 17)     | Positive (n = 27)     | P-value                 |
| RT-PCR-negative  | 12 (27.3%)            | 8 (18.8%)             | 0.008                   |
| RT-PCR-positive  | 5 (11.4%)             | 19 (43.2%)            |                         |

Spearman’s test: \(r = 0.385, P = 0.01\)

|                  | Negative (n = 20)     | Positive (n = 24)     | P-value                 |
| RT-PCR-negative  | 14 (31.8%)            | 6 (13.6%)             | 0.003                   |
| RT-PCR-positive  | 6 (13.6%)             | 18 (40.9%)            |                         |

Spearman’s test: \(r = 0.373, P = 0.0001\)

Abbreviations: CTC = circulating tumour cell; RT = real-time PCR.
(2004) demonstrated that the detection of ≥5 CTCs per 7.5 ml of blood from MBC patients was associated with a shorter PFS and OS, and has superior and independent prognostic value than the tumour burden and disease phenotype. This finding was subsequently confirmed by the same investigators in untreated patients with MBC eliminating factors, which may influence the results (i.e., differences between prior therapies, stage of the disease, line of treatment, etc); in this particular study, CTC measurement performed at baseline and before initial therapy was found to be highly predictive for worse PFS and OS (Cristofanilli et al, 2005).

In a similar study, Dawood et al (2008) confirmed that the detection of <5 CTCs/7.5 ml of blood at baseline in patients with newly diagnosed MBC was associated with a significantly better OS compared with those with ≥5 CTCs/7.5 ml of blood, irrespectively of the hormone receptor and HER-2/neu status, site of first metastasis or whether the patient had recurrent or de novo metastatic disease. Similar results have been reported by other investigators who evaluated the presence of CTCs using either the CellSearch platform (Bidard et al, 2010; Pierga et al, 2012) or other techniques (Wong et al, 2006; Bidard et al, 2008; Nakamura et al, 2010).

### Table 3 Univariate analysis for PFS and OS

| Variables | PFS HR | 95% CI | P-values | OS HR | 95% CI | P-values |
|-----------|--------|--------|----------|--------|--------|----------|
| Menopausal status (pre vs post) | 1.110 | 0.853–1.446 | 0.437 | 1.197 | 0.903–1.587 | 0.210 |
| ER status (− vs +) | 1.677 | 1.308–2.150 | <0.001 | 1.433 | 1.098–1.869 | 0.008 |
| PR status (− vs +) | 1.442 | 1.118–1.859 | 0.005 | 1.334 | 1.019–1.747 | 0.036 |
| HR status (ER−/PR− vs other) | 1.641 | 1.272–2.117 | <0.001 | 1.385 | 1.058–1.815 | 0.018 |
| HER2 status (− vs +) | 1.420 | 1.061–1.900 | 0.019 | 1.140 | 0.831–1.563 | 0.418 |
| HER2/HER2 (triple-negative vs other) | 1.302 | 0.983–1.724 | 0.065 | 1.345 | 0.996–1.816 | 0.053 |
| Adjuvant chemotherapy (yes vs no) | 1.293 | 1.019–1.639 | 0.034 | 1.254 | 0.972–1.619 | 0.082 |
| Bone disease (yes vs no) | 0.985 | 0.769–1.262 | 0.908 | 1.210 | 0.927–1.579 | 0.161 |
| Visceral disease (yes vs no) | 1.093 | 0.855–1.396 | 0.478 | 1.049 | 0.806–1.365 | 0.724 |
| CK-19 pre chemo (+ vs −) | 1.456 | 1.132–1.875 | 0.003 | 1.406 | 1.064–1.858 | 0.016 |
| Performance status (2–3 vs 0–1) | 1.667 | 1.100–2.525 | 0.016 | 2.184 | 1.425–3.347 | <0.001 |

Abbreviations: CI = confidence interval; ER = oestrogen receptor; HR = hazard ratio; OS = overall survival; PFS = progression-free survival; PR = progesterone receptor.

### Table 4 Multivariate analysis for PFS and OS

| Variables | PFS HR | 95% CI | P-values | OS HR | 95% CI | P-values |
|-----------|--------|--------|----------|--------|--------|----------|
| ER status (− vs +) | 1.627 | 1.267–2.090 | <0.001 | 1.480 | 1.129–1.940 | 0.005 |
| Performance status (2–3 vs 0–1) | 1.424 | 1.092–1.857 | 0.009 | 1.957 | 1.199–3.193 | 0.007 |
| CK-19 pre chemo (+ vs −) | 1.424 | 1.092–1.857 | 0.009 | 1.371 | 1.019–1.845 | 0.037 |

Abbreviations: CI = confidence interval; ER = oestrogen receptor; HR = hazard ratio; OS = overall survival; PFS = progression-free survival.
2010). In a recent report, the MD Anderson group reported that the risk of death was linearly increased with increasing CTC count in all molecular tumour subtypes; however, it was higher in ER+ and triple-negative than in HER2+ tumours (Giordano et al, 2011).

The detection rate of CK-19mRNA transcripts in peripheral blood was 67% in this group of patients with metastatic disease. Using the same RT-PCR assay, CK-19mRNA-positive CTCs were detected in 181 (40.8%) of 444 patients with early breast cancer (Ignatiadis et al, 2007). Van der Auwera et al (2010), using a RT-PCR for CK-19mRNA showed that 26% of patients with MBC were CTC-positive, whereas Reinholz et al (2011) were able to detect CK19+ mRNA cells in 56–75% of 86 patients with metastatic disease. Using a multi-marker RT-PCR assay for four marker genes, including CK-19 (Bosma et al, 2002), tumour cells were detected in 31% of patients with advanced breast cancer (Weigelt et al, 2003), and using the AdnaTest BreastCancer, 52% of metastatic patients were tested positive for CTCs (Tewes et al, 2009). It should be mentioned here, however, that the use of various enrichment procedures, the variability in the millilitres of blood analysed, and the different methods used for the evaluation of the sensitivity and specificity of the assays, preclude firm conclusions to be drawn regarding the actual incidence of CTC detection. To further complicate the issue, in the above-mentioned reports, different numbers of patients have been evaluated in different stages of metastatic disease. In the present study, 20 ml of peripheral blood were used to detect CTCs in previously untreated patients with advanced breast cancer. Usually, smaller volumes of blood are used for gene expression studies in metastatic patients, which could explain, at least in part, the relatively high incidence of CTC detection reported here.

Nevertheless, in the present study, the detection of CK-19mRNA-positive CTCs in metastatic patients was significantly and independently associated with worse outcome. The independent prognostic value of CTC detection using this method was observed irrespectively of the quantitative burden of CTCs, as patients were classified according to the presence or the absence of the CK-19mRNA transcripts. The cut-off of \( \geq 0.6 \) MCF-7 cell equivalents/5 \( \mu \)g RNA of the patients’ PBMCs, which has been determined in a previous analytical study (Stathopoulou et al, 2003) and has been shown to be able to define patients with early breast cancer at high risk for relapse and disease-related death (Xenidis et al, 2006, 2009; Ignatiadis et al, 2007), was also appropriate for the selection of poor prognosis patients with newly diagnosed MBC. Despite the fact that no quantitative data have been reported, preliminary results seem to indicate that quantitative classification of patients according to the numerical burden of CTCs is not superior than the qualitative one (data not shown). This might be a difference with the results obtained with the CellSearch platform (Giordano et al, 2011). In a small cohort of patients, the detection of CTCs was performed, in parallel, using both the CellSearch platform and the RT-PCR assay; the findings indicate that there was a close correlation concerning the detection of CTCs between the two assays, suggesting that they, probably, recognise similar sub-populations of breast cancer cells. Additional small number of parallel lines with statistical power of these comparisons necessitating a comparison of the two assays in a larger number of patients. In any case, this correlation could, at least partly, explain the fact that our findings are in line with those of the literature concerning the independent prognostic value of CTC detection before the initiation of any systemic treatment.

The current study represents the cumulative experience of >10-year involvement of our group in the study of CK-19mRNA-positive CTCs in patients with early or/and MBC. Our study demonstrates, for the first time, that the detection of CK-19mRNA-positive CTCs in a large group of previously untreated patients with metastatic disease, using a well-validated RT-PCR assay, is clinically relevant, as it was significantly associated with an increased incidence of relapse and disease-related death. The prognostic value of the detection of CK-19mRNA-positive CTCs was independent of whether the patients presented with recurrent or de novo MBC or the different well-known clinico-pathological characteristics. Similarly, Reinholz et al (2011), showed in a smaller group of metastatic patients that the presence of CK19 + mRNA CTCs was associated with poor prognosis.

Our findings, taken together with those of the literature, strongly suggest that the detection of CTCs represent an interesting marker characterising the biology of the disease, and thus, might identify subgroups of patients with different prognosis. On the basis of these findings, we strongly support the Dawood et al’s (2008) proposition that CTC’s status could be used as a new stratification factor for women with newly diagnosed MBC. However, despite these data, CTCs measurement at baseline has not yet been incorporated into cancer-staging protocols or even considered as a ‘standard’ clinical tool; the American Society of Clinical Oncology (ASCO) 2007 guidelines (Harris et al, 2007) do not recommend its routine use in clinical practice, owing to insufficient clinical evidence. Nevertheless, Dawood et al (2008) proposed that the distinction of the two different prognostic groups of patients with MBC based on the detection of CTCs could provide a biological (and not merely anatomic) distinction of prognoses upfront, on the basis of a reliable and reproducible test (e.g., stage IV A vs IV B); in addition, the upfront use of CTCs would allow patients with more aggressive or stage IV B disease to be actively enrolled into clinical trials designed to address specific questions related to the selection of more specific and targeted therapies aimed at the elimination of CTCs, which might have an impact on patients’ survival (Cristofanilli et al, 2004; Bidard et al, 2010, 2012). However, limiting factors such as the variable expression of different epithelial antigens in poorly differentiated tumours, the different sensitivity and specificity of various detection assays, and the lack of their direct comparison support the need for cautious consideration of the use of CTC measurement in the daily clinical practice. We have to wait for the data of the large validation trial, which has been undertaken by the Southwestern Oncology Group to definitively decide on the integration of CTC measurement into clinical practice and to provide the rational for an individualised therapeutic approach. Additionally, the better biological analysis of CTCs may help us to better understand the mechanism of resistance of tumour cells to the different anti-tumour agents, as well as to define new molecular targets for their elimination (Bozionellou et al, 2004). This is most important, as experimental data seem to indicate that the population of CTCs is heterogeneous and an important number of them are apoptotic (Mehes et al, 2001), whereas another subpopulation may express stem cell or/and epithelial–mesenchymal transition markers (Fehm et al, 2008; Aktas et al, 2009; Theodoropoulos et al, 2010) facilitating the disease dissemination.

In conclusion, the results of the present study demonstrate that the detection of CK-19mRNA-positive CTCs in patients with MBC at baseline and before the initiation of first-line treatment is associated with significant diagnostic and prognostic impact. Further prospective studies are needed to investigate whether the genotypic or/and phenotypic characterisation of CTCs may allow a more effective and rational treatment of MBC, which could confer a survival benefit in these patients.

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