**Molecular
Cancer
Therapeutics**

Circulating Tumor Cells with Stemness and Epithelial-to-Mesenchymal Transition Features Are Chemoresistant and Predictive of Poor Outcome in Metastatic Breast Cancer

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Abstract

Circulating tumor cells (CTCs) bearing phenotypes related to cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT) have been identified in breast cancer; however, their clinical significance is not clear. In the current study, we investigated the prognostic relevance of single CSC$^+$/partial-EMT$^+$ CTCs in patients with metastatic breast cancer and the effect of first-line chemotherapy on their incidence. For this purpose, triple immunofluorescence against cytokeratin, ALDH1, and TWIST1 was performed in peripheral blood mononuclear cell (PBMC) cytospins from 130 patients before and after first-line chemotherapy. CSC$^+$/partial-EMT$^+$ CTCs were characterized as cells co-expressing cytokeratin, high levels of ALDH1, and nuclear TWIST1. CSC$^+$/partial-EMT$^+$ CTCs were evident in 27.7% of patients at baseline and were correlated to lung metastases ($P = 0.010$) and decreased progression-free survival [PFS; median 10.2 (8.9–11.6) vs. 13.5 (11.3–15.7) months; $P = 0.024$].

Their detection was an independent factor predicting for increased risk of relapse [multivariate analysis; HR (95% confidence interval (CI)): 1.785 (1.171–2.720); $P = 0.007$]. In HER-2–negative patients, CSC$^+$/partial-EMT$^+$ CTCs were additionally associated with reduced overall survival (OS) [median 39 (26.2–51.9) vs. 51 (15.7–86.4) months; $P = 0.020$] and increased risk of death [multivariate analysis; HR (95% CI): 2.228 (1.066–4.655); $P = 0.033$]. Chemotherapy resulted in a significant increase in the incidence of CSC$^+$/partial-EMT$^+$ CTCs (mean CTC% per patient: 59.4% post vs. 39.5% pre; $P = 0.018$), which was subsequently confirmed only in HER2-negative patients ($P = 0.040$) and in non-responders at the end of treatment ($P = 0.020$). In conclusion, CSC$^+$/partial-EMT$^+$ CTCs represent a chemoresistant subpopulation, which independently predicts for unfavorable outcome in metastatic breast cancer. Efficient targeting of these CTCs could potentially increase patient survival.

Introduction

The prognostic and predictive value of circulating tumor cells (CTC) identified in peripheral blood of patients with metastatic breast cancer has been clearly demonstrated by the use of different techniques (1–4). However, recent functional studies showed that only a minority of CTCs can successfully form metastases when injected in mice (5), which is attributed to the profound intra- and interpatient CTC heterogeneity (6, 7). Phenotypic analysis of CTCs could recognize the most aggressive CTC subpopulations, which could then serve for risk assessment, patient stratification, and monitoring (8, 9). Furthermore, efficient targeting of these highly metastatic CTCs could be a promising strategy to improve patients' outcome (10).

Epithelial-to-mesenchymal transition (EMT) and stemness have been extensively investigated in the field of CTC-related research (11). EMT, a dynamic process leading to a partial or complete loss of epithelial characteristics and acquisition of a mesenchymal phenotype, holds an important role in promoting cancer cell migration to distant sites (12). Recent evidence suggests that tumor cells co-expressing epithelial and mesenchymal phenotypes, thus being in a partial EMT state, have the highest metastatic potential (13). Cancer stem cells (CSC), tumor cells bearing stemness properties, display enhanced tumorigenicity, metastatic ability, and resistance to radiation and chemotherapy (14, 15). EMT has been strongly associated with the acquisition of stem cell properties (16, 17) and interestingly, differentiated non-CSCs were transformed to dedifferentiated breast CSCs via EMT (18). Tumor cells bearing both stemness and EMT features are empowered with increased metastatic capacity, combining enhanced motility and invasiveness (EMT traits), with increased self-renewal and proliferation potential (CSC properties; ref. 19). Conventional therapeutics are frequently inefficient to eradicate cancer cells that have entered a CSC state through the activation of EMT, thus leading to a CSC-driven disease relapse (20, 21). As a consequence, CTCs co-expressing these characteristics may

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constitute an aggressive, chemoresistant CTC population that could serve as a prognostic and/or predictive tool, as well as a promising therapeutic target.

TWIST1 is an important EMT-inducing transcription factor (EMT-TF), which represses E-cadherin, activates mesenchymal markers and induces cell motility, whereas its depletion completely blocked EMT in breast cancer cells (22, 23). High activity of the detoxifying enzyme aldehyde dehydrogenase (ALDH) is widely used for the isolation of breast CSCs with self-renewal and tumorigenic capacity (24). Moreover, ALDH1 expression has been correlated to ALDH activity and is predictive of poor outcome in breast cancer (24). To date, the expression of EMT and CSC markers has been demonstrated on CTCs of patients with different solid tumors by the use of different methodologies; however, controversy exists on the clinical relevance of these cells (25). We have previously demonstrated that the putative stemness phenotypes, $CD44^+CD24^{low/-}$ and $ALDH1^{high}$ (26), as well as the EMT markers, Vimentin and TWIST1, are frequently expressed on single CTCs from patients with breast cancer (27). Moreover, we developed a multi-immunofluorescence methodology to evaluate the co-expression of CSC (ALDH1) and EMT (TWIST1) markers on CTCs (28) and observed that the CSC^+/partial-EMT^+ CTC subpopulation prevailed in metastatic patients, whereas those with early disease mainly harbored a non-CSC/epithelial$^+$ CTC phenotype (28).

In the current study, we used the same methodology to detect CTCs co-expressing the CSC and partial-EMT phenotypes in a well-defined cohort of 130 patients with metastatic breast cancer receiving first-line chemotherapy. Our goals were to investigate the effect of standard chemotherapy regimens on the incidence of these cells and to evaluate their relevance in the prediction of patient outcome.

Materials and Methods

Study design

The current study included 130 patients with metastatic breast cancer, who received first-line chemotherapy at the Department of Medical Oncology of the University General Hospital of Heraklion (Crete, Greece) during 2005 to 2010. Clinical characteristics and follow-up information for each patient were prospectively collected. Peripheral blood samples were obtained at baseline and at the end of first-line chemotherapy and CTC detection and characterization was performed on peripheral blood mononuclear cells (PBMC) cytospin preparations. This study was conducted in accordance with the Declaration of Helsinki ethical guidelines and was approved by the Ethics and Scientific Committees of the University General Hospital of Heraklion, Crete, Greece. All patients gave their written informed consent to participate in this study.

PBMC cytospin preparation

Peripheral blood (10 mL) was obtained at the middle of vein puncture after the first 5 mL were discarded, to avoid contamination with epithelial cells derived from the skin. PBMCs were isolated by Ficoll–Hypaque density gradient (d = 1,077 g/mL) centrifugation at $650 \times g$ for 30 minutes. PBMCs were washed twice with PBS and aliquots of 250.000 cells were cyto-centrifuged at $500 \times g$ for 2 minutes on glass slides. Air-dried cytospins were stored at $-80°C$ until use.

Cell culture

The HepG2 cell line was obtained from ATCC in February of 2013 and stored at liquid nitrogen. The cell line was not authenticated by us. High glucose GlutaMAX DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin (GIBCO-BRL Co.) was used for cell culture and cells were maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. Subcultivation was performed using 0.25% trypsin and 5 mmol/L ethylenediaminetetraacetic acid (EDTA; GIBCO-BRL). Following *Mycoplasma* testing by the use of MycoAlert assay, cytospins of HepG2 cells (passage 4 after obtainment) were prepared as described above to be included as controls during the immunofluorescence stainings in patient samples.

Detection and characterization of CTCs according to CSC and EMT phenotypes

Triple immunofluorescence was performed on PBMC cytospin preparations, using antibodies against putative markers for epithelial cells (Cytokeratins, CK 8, 18, and 19), CSCs (ALDH1), and EMT (TWIST1) as described previously (28). Briefly, cytospins were fixed with 3% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100 at room temperature, and blocked overnight with PBS/1% BSA at 4°C. The primary antibodies used were pan-cytokeratin (A45-B/B3 anti-mouse; Micromet), ALDH1 (anti-mouse; Abcam), and TWIST1 (anti-rabbit; Abcam); secondary antibodies were Alexa 555- and Alexa 633–conjugated (Molecular Probes) and Zenon technology (FITC-conjugated; Molecular Probes, Invitrogen); the DAPI-antifade reagent (Invitrogen) was used for cell nuclei staining. Cells were further postfixed with 3% PFA.

A total of 500.000 PBMCs per patient sample were analyzed for the detection and further characterization of CK-positive CTCs, by the use of the ARIOL system CTCs software (Genetix), as described previously (7, 9, 29). Cytospins of spiked HepG2 cells were included in each separate immunofluorescence experiment as controls to evaluate CK, ALDH1, and TWIST1 co-expression in patient samples. Positive controls included all three primary and the corresponding secondary antibodies. Negative controls, one for each marker, prepared by adding the secondary IgG isotype antibody only and omitting the corresponding primary antibody, were also included in each separate experiment. A45-B/B3 pan-cytokeratin antibody exhibits a specific, filamentous, and nonfused CK expression pattern in tumor cells only. Its specificity has been demonstrated in previous studies from our group, showing that no $CK^+/CD45^+$ cells were observed in PBMCs from healthy donors, PBMCs spiked with different breast cancer cell lines (28), or in PBMC cytospins from patients with breast cancer (7, 27, 29–31).

ALDH1 expression was quantified using the ARIOL system and characterized as high, low, or absent, according to the methodology used in our previous study (28). Briefly, ALDH1 expression levels were first measured among HepG2 control cells included in each staining experiment, by quantifying 1.500 $ALDH1^{high}$, $ALDH1^{low}$, and $ALDH^{neg}$ cells in 50 randomly selected microscope vision fields. The resulting cutoffs were subsequently used for the characterization of ALDH1 expression in CTCs. TWIST1 expression was characterized as nuclear, cytoplasmic, or absent according to its subcellular localization.

The characterization of cells as CTCs was exclusively based on CK expression. CK^+ CTCs with high ALDH1 expression were considered as having the CSC phenotype, whereas cells with low or absent expression were characterized as non-CSCs-like. CK^+ CTCs with nuclear TWIST1 localization were of the partial-EMT

Table 1. Patient and disease characteristics

	Patients (*n*, %)			
Age, years (median, range) 59 (23–82)		Metastatic sites		
Performance status		Lymph nodes		42 (32.3)
0–1	111 (85.4)	Lung		46 (35.4)
2–3	17 (13.1)	Bones		58 (44.6)
Unknown	2 (1.5)	Liver		44 (33.8)
Menopausal status		Skin		10 (7.7)
Premenopausal	41 (31.5)	CNS		7 (5.4)
Postmenopausal	82 (63.1)	No. of affected organs		
Unknown	7 (5.4)	1–2		85 (65.4)
Histological grade		≥3		42 (32.3)
I–II	53 (40.8)	Unknown		3 (2.3)
III	61 (46.9)	Type of first-line chemotherapy		
Unknown	16 (12.3)	Tax or Anthr single agents		21 (16.2)
Adjuvant chemotherapy		Tax-based combinations		75 (57.7)
Yes	67 (51.5)	Combinations without Tax or Anthr		34 (26.2)
No	62 (47.7)	Response to treatment		
Unknown	1 (0.8)	Complete response		10 (7.7)
Histology subtype		Partial response		52 (40)
Ductal	99 (76.2)	Stable disease		46 (35.4)
Lobular	13 (10)	Progressive disease		17 (13.1)
Mixed	8 (6.2)	Unknown		5 (3.9)
Unknown	10 (7.7)	Disease status at the end of chemotherapy		
Molecular subtype		Complete response		12 (9.2)
ER-positive	89 (68.5)	Partial response		37 (28.5)
PgR-positive	83 (63.8)	Stable disease		44 (33.8)
HER2-positive	28 (21.5)	Progressive disease		32 (24.6)
Triple-negative	19 (14.6)	Unknown		5 (3.9)

NOTE: No of patients: *n* = 130.

Abbreviations: Anthr, anthracyclines; CNS, central nervous system; ER, estrogen receptor; PgR, progesterone receptor; Tax, Taxanes.

phenotype and cells with cytoplasmic or absent TWIST1 were characterized as epithelial-like. Consequently, four different CTC subpopulations could be recognized: CSC$^+$/partial-EMT$^+$, non-CSC/partial-EMT$^+$, CSC$^+$/epithelial-like or non-CSC/epithelial-like.

Statistical analysis

χ^2 and Mann–Whitney tests were used to correlate the different CTC subpopulations with patient and disease characteristics and to compare their incidence among individual patients. Wilcoxon signed rank test was performed to compare the frequency of distinct CTC subsets before and after treatment. Kaplan–Meier analysis was used to estimate survival curves; progression-free survival (PFS) was calculated from the day of the administration of the first cycle of chemotherapy until the day of documentation of disease progression or death from any cause; overall survival (OS) was calculated from the day of treatment initiation until the date of death from any cause. Univariate Cox regression analysis was performed to investigate the associations between different parameters and the risk for progression and death; the factors with statistical significance or a trend for significance were subsequently included in a multivariate Cox proportional hazards regression model to evaluate whether they were independently associated with the risk for progression and death. All statistical analyses were performed using IBM SPSS Statistics version 20. *P* values were calculated by two-sided tests and were considered statistically significant at the 0.05 level.

Results

Patient and disease characteristics

The median age of patients was 59 years and the median progression-free and overall survival was 12.5 (9.9–15.1) and 37.6 (31.4–43.7) months, respectively. First-line chemotherapy included taxanes or anthracyclines as single agents (16.2%), taxane-based regimens (57.7%), and combinations without taxanes or anthracyclines (26.2%). All HER2-positive patients also received trastuzumab. CTC analysis was performed before the initiation of first-line chemotherapy (*n* = 130 patients) as well as at the end of chemotherapy for patients who were found to be CTC-positive in the prechemotherapy sample and who had available post-treatment samples (*n* = 62 patients). Patient and disease characteristics are summarized in Table 1.

Detection and characterization of CTCs pre- and post-treatment

CTCs (CK$^+$ cells) were detected in 72 of 130 patients (55.4%) at baseline, with a total number of 884 CTCs identified [median CTC number per CTC-positive patient; 2 (range, 1–286)]. Further CTC characterization according to the co-expression of the CSC and EMT markers revealed the presence of four different CTC subpopulations (Fig. 1A). CSC$^+$/partial-EMT$^+$ CTCs, co-expressing both phenotypes, represented the most frequently encountered CTC subset; these cells were identified in 27.7% of patients and constituted 40.7% of the total number of CTCs detected. CTCs lacking the expression of both phenotypes, namely non-CSC/epithelial-like CTCs, were the second more frequent subpopulation, which was evident in 27.7% of patients and represented 28.8% of total CTCs. The intermediate phenotypes, CSC$^+$/epithelial-like and non-CSC/partial-EMT$^+$ CTCs, were identified in 12.3% and 13.8% of patients, respectively, representing 7.5% and 22.9% of total CTCs.

Post-treatment, CK$^+$ CTCs were identified in 38 (61.3%), with a total number of 312 CTCs identified [median CTC number per CTC-positive patient; 3.5 (range, 1–94)]. Chemotherapy resulted in reduction or elimination of CK$^+$ CTCs in 61.3% of patients.

Papadaki et al.
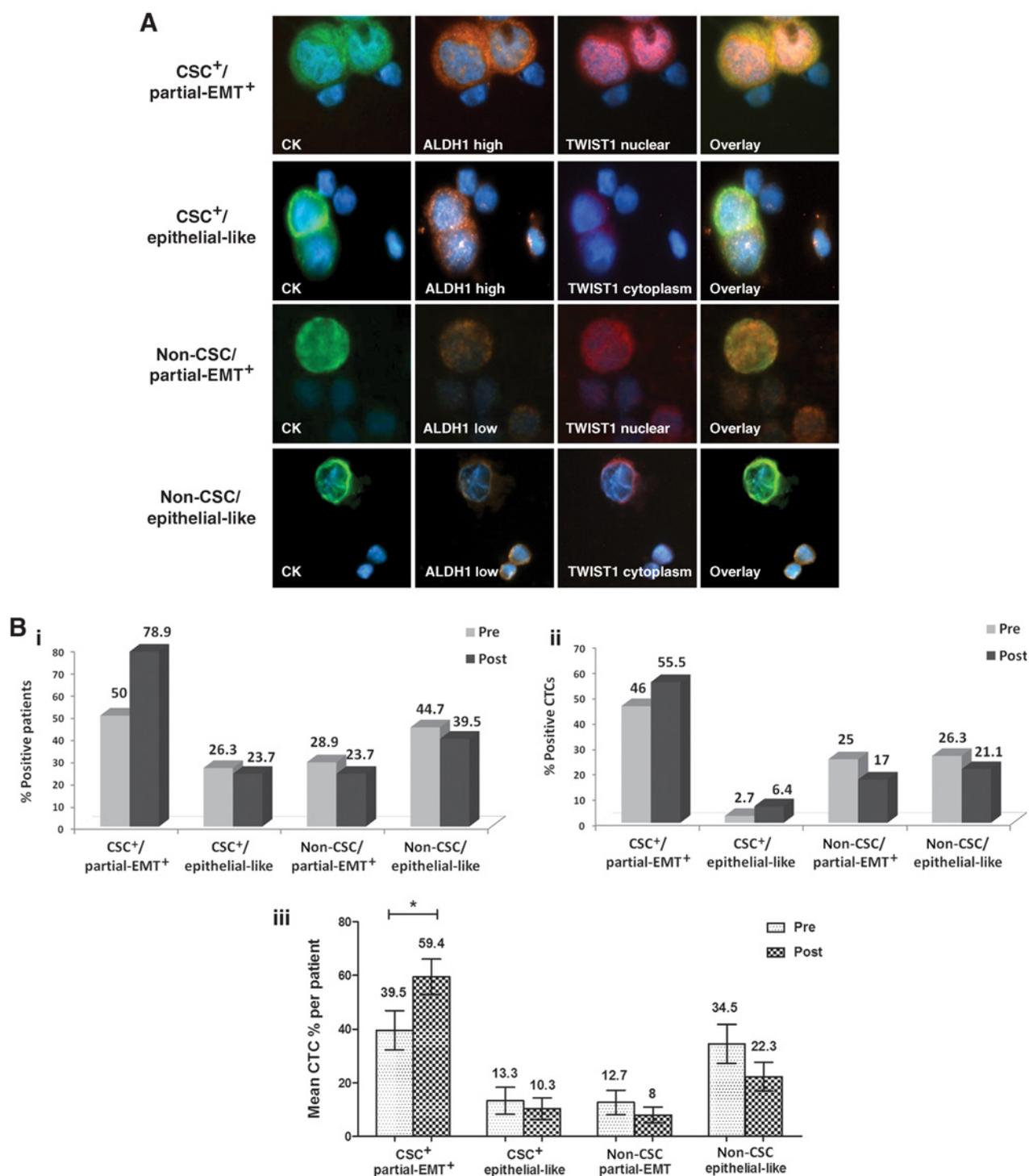
Figure 1.
Investigation of putative CSC and partial-EMT phenotypes on single CTCs of patients with metastatic breast cancer receiving first-line chemotherapy. **A,** Representative images of phenotypically different CTC subsets, ARIOL system (X400); high ALDH1 expression was used to define the CSC phenotype, whereas co-expression of CK with nuclear TWIST1 was considered as a partial-EMT phenotype; four different CTC subpopulations were identified: CSC$^+$/partial-EMT-like, non-CSC/partial-EMT-like, CSC$^+$/epithelial-like and non-CSC/epithelial-like CTCs. **B,** Incidence of the different CTC subpopulations pre- and post-chemotherapy; (**i**) percentage of patients bearing each CTC subset, (**ii**) percentage of CTC subsets per total CTCs, and (**iii**) mean percentage of CTCs per patient. An increased frequency of CSC$^+$/partial-EMT$^+$ CTCs was evident after treatment at the patient and the CTC level. Wilcoxon signed rank test was used.
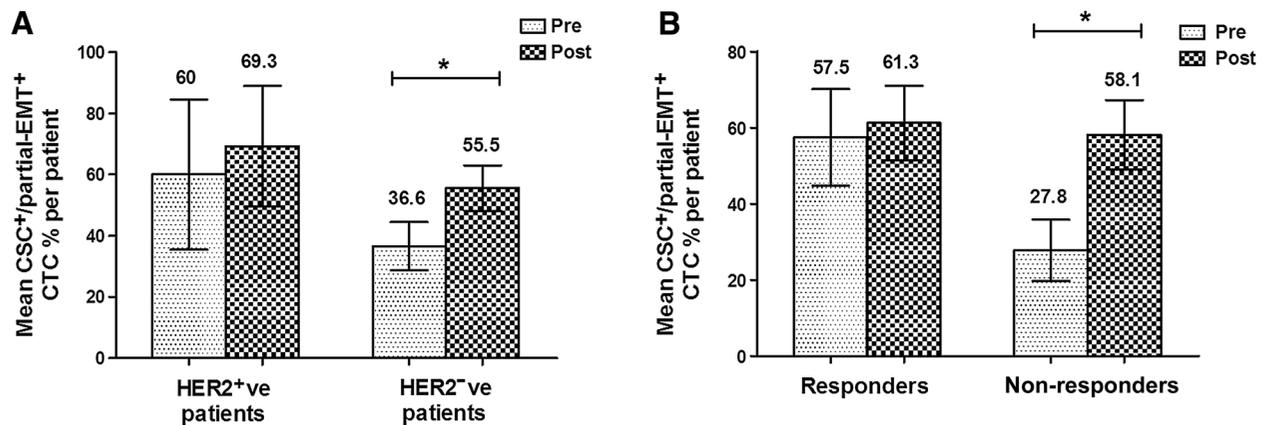
Figure 2.
Effect of first-line chemotherapy on the incidence of CSC$^+$/partial-EMT$^+$ CTCs according to HER2 disease status and response to treatment. Incidence of CSC$^+$/partial-EMT$^+$ CTCs pre- and post-chemotherapy among HER2-positive and HER2-negative patients (**A**) and responders and Non-responders (**B**). Wilcoxon signed rank test was used.

However, a significant enrichment of the CSC$^+$/partial-EMT$^+$ subpopulation was observed post-treatment in patients with detectable CTCs at both time points (Fig. 1B i–iii). Specifically, this CTC subset was detected in 30 (78.9%) patients postchemotherapy compared with 19 (50%) at baseline ($P = 1.000$; Fig. 1Bi) and represented 55.5% and 46% of total CTCs, respectively (Fig. 1Bii). The mean percentage of CSC$^+$/partial-EMT$^+$ CTCs per patient significantly increased from 39.5% to 59.4% postchemotherapy ($P = 0.018$; Fig. 1Biii). Instead, the frequency of the other CTC subpopulations was numerically decreased after chemotherapy, both at the patient and the CTC level (Fig. 1B i–iii). In addition, a significant increase in CSC$^+$/partial-EMT$^+$ CTC counts was confirmed among patients who increased their total CK$^+$CTCs after treatment (median number: 0 pre- vs. 2 postchemotherapy, $P = 0.001$, Wilcoxon signed rank test). Instead, in patients with unchanged or reduced total CTC numbers after chemotherapy, CSC$^+$/partial-EMT$^+$ CTC counts were significantly decreased (median number: 1.5 vs. 0, pre- and post-chemotherapy, respectively, $P = 0.000$).

Correlation of the detection of CTCs and of the different CTC subpopulations with patient and disease characteristics and with the type of treatment

The presence of CK$^+$ CTCs before treatment was associated with tumor positivity for estrogen receptors ($P = 0.046$) and progesterone receptors ($P = 0.036$) and with the presence of lung ($P = 0.001$) and lymph node metastases ($P = 0.024$). The detection of CSC$^+$/partial-EMT$^+$ CTCs at baseline was correlated with lung metastases ($P = 0.010$), whereas no associations were observed between the other CTC subpopulations and clinicopathologic characteristics before or after chemotherapy.

Separate analysis according to HER2 status of the primary tumor revealed that in HER2-positive patients, the detection of CK$^+$ CTCs was associated with grade 3 tumors ($P = 0.005$). In HER2-negative patients, the detection of CK$^+$ CTCs was associated with lymph node ($P = 0.041$) and lung ($P = 0.003$) metastases; CSC$^+$/partial-EMT$^+$ CTCs were more frequently evident among patients with lung metastases ($P = 0.035$), in contrast to those with lymph node metastases, who mainly harbored non- CSC$^+$/epithelial-like CTCs ($P = 0.039$; Supplementary Table S1).

The type of therapy was not correlated to the presence or the phenotype of CTCs at baseline, among patients with either HER2-positive or HER2-negative disease (Supplementary Table S2). However, in the HER2-negative setting, treatment with taxanes or anthracyclines as single agents was associated with negative CTC status post-treatment ($P = 0.000$) and with complete absence of CSC$^+$/partial-EMT$^+$ CTCs ($P = 0.002$), whereas combination regimens without taxanes or anthracyclines resulted in 100% CTC positivity and an increased incidence of CSC$^+$/partial-EMT$^+$ CTCs.

Moreover, the enrichment in the CSC$^+$/partial-EMT$^+$ subset post-treatment was observed in the HER2-negative patient cohort only (mean CTC% per patient: 36.6% pre- vs. 55.5% postchemotherapy, $P = 0.041$), whereas no change was shown among HER2-positive patients ($P = 1.000$; Fig. 2A). In the whole group of patients, CSC$^+$/partial-EMT$^+$ CTCs were significantly enriched only among patients presenting non-response (stable or progressive disease) at the end of treatment (mean CTC% per patient: 27.8% pre- vs. 58.1% post-chemotherapy, $P = 0.014$), whereas their frequency remained unchanged among responders ($P = 0.721$; Fig. 2B).

Correlation of the detection of CTCs and CTC subpopulations with patient outcome

Overall, no correlation was found between the detection of CK$^+$ CTCs before treatment initiation and PFS [median 12.6 (range, 9.4–15.8) vs. 12.2 (range, 8.9–15.5) months; $P = 0.063$] or OS [median 39.9 (range, 31.7–48.2) vs. 35.3 (range, 19.8–50.8) months; $P = 0.838$]. However, the presence of CSC$^+$/partial-EMT$^+$ CTCs at baseline was significantly correlated to decreased PFS [median 10.2 (range, 8.9–11.6) vs. 13.5 (range, 11.3–15.7) months; $P = 0.024$; Fig. 3A]. Multivariate Cox regression analysis confirmed that the detection of CSC$^+$/partial-EMT$^+$ CTCs [HR (95% confidence interval (CI)): 1.785 (1.171–2.720); $P = 0.007$], triple-negative tumor subtype [HR (95% CI): 1.826 (1.055–3.161), $P = 0.031$], presence of liver metastases [HR (95% CI): 1.945 (1.276–2.963); $P = 0.002$], and

Papadaki et al.
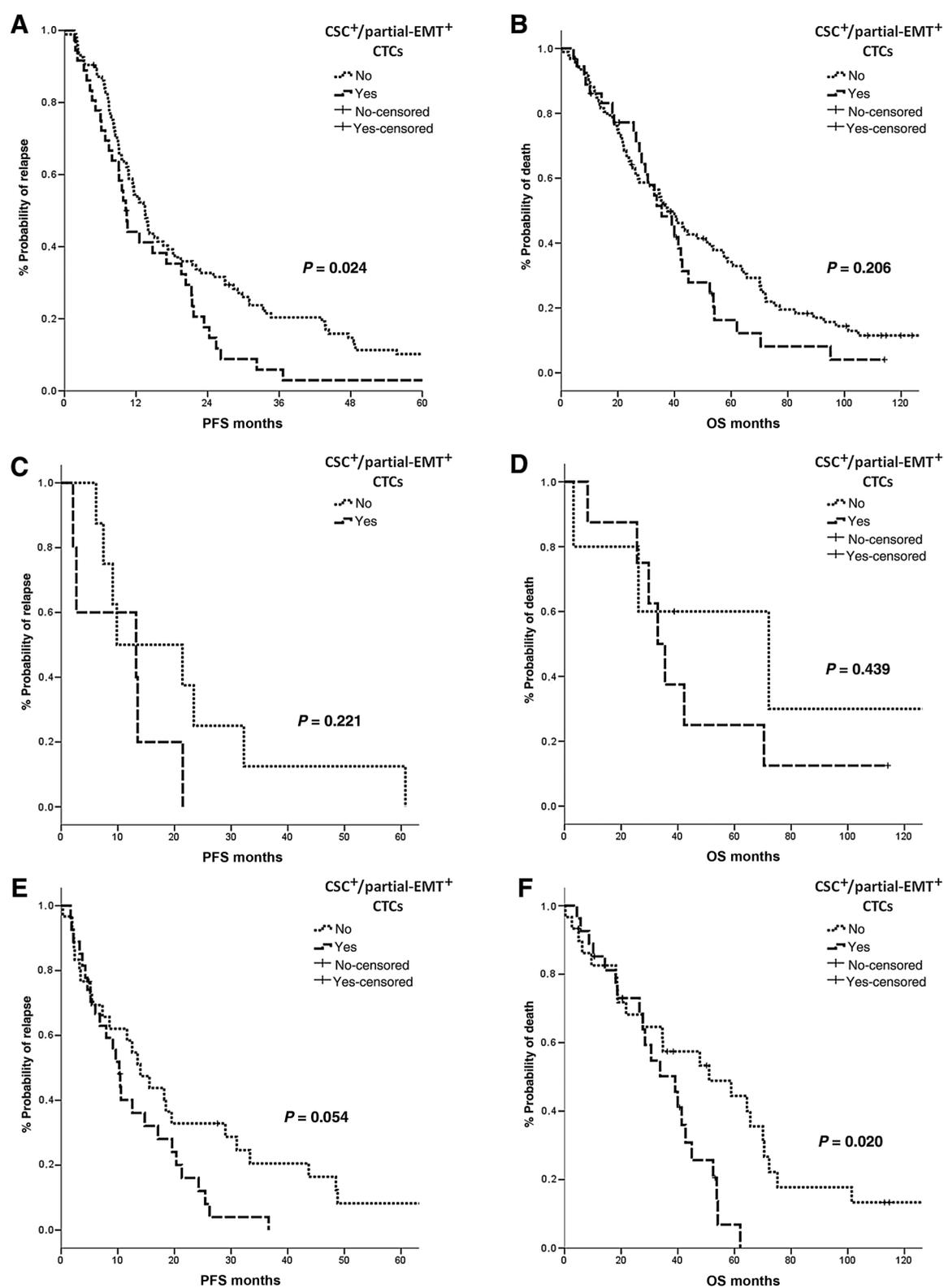
Figure 3.
Prognostic relevance of CSC^+/partial-EMT^+ CTCs at baseline of first-line chemotherapy. Kaplan–Meier survival curves for PFS and OS according to the detection of CSC^+/partial-EMT^+ CTCs among the total metastatic breast cancer patients (**A**), and separately for patients with HER2-positive (**C** and **D**) and HER2-negative disease (**E** and **F**). P values were obtained by log-rank test.

combination therapy without taxanes or anthracyclines [HR (95% CI): 2.086 (1.154–3.774); $P = 0.015$] could independently predict for high risk of disease progression (Table 2). No correlation was observed between the detection of the other three CTC subsets and outcome.

Separate analysis according to the HER2 status revealed that although the incidence of CSC$^+$/partial-EMT-like CTCs prechemotherapy was similar among HER2-negative and HER2-positive patients (29.3% vs. 28.6%), their detection at baseline was associated with decreased survival only in the HER2-negative cohort (Fig. 3C–F; Table 3). Specifically, Kaplan–Meier analysis revealed a significantly reduced OS among HER2-negative patients with CSC$^+$/partial-EMT$^+$ CTCs compared with those not harboring this CTC subset [median 39 (range, 26.2–51.9) vs. 51 (range, 15.7–86.4) months ($P = 0.020$; Fig. 3F]. Multivariate Cox regression analysis confirmed that, in the HER2-negative patient cohort, the detection of CSC$^+$/partial-EMT$^+$ CTCs [HR: 2.283 (1.211–4.305); $P = 0.011$], triple negative tumors [HR (95% CI): 3.660 (1.602–8.360); $P = 0.002$], liver metastases [HR (95% CI): 3.503 (1.734–7.078); $P = 0.000$], and combination therapy without taxanes or anthracyclines [HR (95% CI): 5.264 (1.814–15.264); $P = 0.002$] were independently associated with increased risk for relapse (Table 3). The detection of CSC$^+$/partial-EMT$^+$ CTCs [HR: 2.228 (1.066–4.655); $P = 0.033$], triple negative tumors [HR (95% CI): 6.748 (2.833–16.072), $P = 0.000$], and liver metastases [HR (95% CI): 2.892 (1.448–5.775); $P = 0.003$] were also independently associated with increased risk for death (Table 3). In contrast, no correlations were observed between the detection of CK$^+$ CTCs or of the other CTC phenotypes and outcome in either the HER2-positive or the HER2-negative cohorts.

Discussion

In the current study, we evaluated the incidence and the prognostic significance of different CTC subpopulations, characterized according to the detection of stemness and EMT markers, before and after the administration of first-line chemotherapy in patients with metastatic breast cancer. We showed that only the CSC$^+$/partial-EMT$^+$ CTCs, co-expressing stemness and partial-EMT phenotypes, were enriched post-chemotherapy and that their enrichment was associated with lack of response to treatment. Moreover, the detection of this CTC subpopulation at baseline was an independent factor predictive of reduced PFS, whereas in the HER2-negative cohort, it was also predictive for decreased OS.

In this report, we confirmed, in a different and larger patient cohort, our previous findings showing that the CSC$^+$/partial-EMT$^+$ CTC subset is the most prevalent among patients with metastatic breast cancer (28). The detection of CSC$^+$/partial-EMT-like CTCs at baseline was associated with the presence of lung metastases suggesting that CTC characterization could be suggestive of the pattern of metastatic spread. Importantly, CSC$^+$/partial-EMT$^+$ CTCs were highly enriched after treatment, in contrast to the other CTC subsets, namely CSC$^+$/epithelial-like, non-CSC/epithelial-like or non-CSC/partial-EMT$^+$ CTCs, which were numerically reduced post-chemotherapy. These findings suggest that CSC$^+$/partial-EMT$^+$ CTCs are resistant to conventional chemotherapeutic regimens. Conceivably, the enrichment of this CTC subset was associated with lack of objective response (stable or progressive disease) at the end of treatment. These observations

are consistent with a study by Creighton and colleagues, showing that residual breast tumor cell populations surviving after conventional treatment were enriched for subpopulations of cells co-expressing stemness and EMT characteristics (20). Our findings also corroborate previous evidence in metastatic breast cancer, demonstrating a higher frequency of stemness and/or EMT markers on CTCs among non-responders compared with responders (32, 33); however, these studies included small numbers of patients.

In the current study, we show for the first time that the detection of CSC$^+$/partial-EMT$^+$ CTCs before the initiation of first-line chemotherapy was significantly associated with reduced PFS in patients with metastatic breast cancer. Importantly, the detection of this CTC subpopulation emerged as an independent prognostic factor for increased risk of relapse. This is in line with preclinical data showing that the partial-EMT state is critical for the tumorigenic activity of CSCs (34, 35). Although it is suggested that MET is required for the efficient colonization of tumor cells (36), the CSC$^+$/epithelial-like subset did not show any prognostic significance. Because the liquid biopsy represents a "snapshot" of the CTC status in the circulation, it is unknown whether these cells initially lost and then regained their epithelial phenotype, or whether they passively entered the circulation without losing their epithelial character. Instead, CSC$^+$/partial-EMT$^+$ CTCs could maintain the ability for full epithelial transformation through MET after their extravasation at the metastatic site. Nevertheless, our findings are in accordance with a recent study in metastatic breast cancer showing that the detection of partial-EMT-like CTCs was predictive of poor outcome, in contrast to fully epithelial or fully mesenchymal CTCs, which lacked prognostic relevance (37).

An interesting finding of our study was that CSC$^+$/partial-EMT$^+$ CTCs were enriched post-chemotherapy only among HER2-negative patients, whereas no change in their frequency was evident in the HER2-positive cohort. The presence of these cells was also independently associated with decreased OS only in the HER2-negative cohort. These findings could be explained by a series of preclinical and clinical data, showing that HER2 expression drives the CSC population in breast cancer cell lines, mouse xenografts, and HER2-positive tumors and that HER2-targeted therapies effectively eliminate breast CSCs (38). In this study, we could not investigate HER2 expression on CSC$^+$/partial-EMT$^+$ CTCs due to the limitation of our technique in using up to three markers for combined analysis; nevertheless, a strong correlation has been previously shown between ALDH1 and HER2 overexpression (38, 39). We could thus hypothesize that CSC$^+$/partial-EMT$^+$ CTCs were neither enriched nor prognostic among HER2-positive patients because of the administration of HER2-targeting therapy, which can effectively eliminate these cells and is routinely applied as first-line treatment in these patients. Moreover, we could speculate that the improved clinical outcome observed for these patients is due to the increased effectiveness of anti-HER2 therapy on CSC$^+$/partial-EMT$^+$ CTCs.

The current study was performed in a large and well-defined cohort of patients with metastatic breast cancer who had samples available before and after first-line chemotherapy. This allowed the evaluation of the kinetics of phenotypically different CTC populations during treatment and consequently the detection of a chemoresistant subset. Thus, our results contribute and expand on the existing limited data regarding the phenotypic characterization of CTCs that persist post-chemotherapy in metastatic patients. In addition, EMT and stemness markers are frequently

Table 2. Univariate and multivariate analysis for PFS and OS in patients with metastatic breast cancer

Cox regression analysis	PFS				OS			
	Univariate		Multivariate		Univariate		Multivariate	
Covariates	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age (≤59)	**1.089** (0.759–1.562)	0.643			**1.021** (0.697–1.496)	0.915		
Performance status (2–3)	**1.451** (0.860–2.447)	0.163			**1.425** (0.833–2.439)	0.196		
Menopausal status (pre)	**1.201** (0.535–2.696)	0.658			**1.085** (0.481–2.450)	0.844		
Grade (III vs. I/II)	**1.128** (0.766–1.662)	0.542			**1.072** (0.712–1.613)	0.739		
Adjuvant chemotherapy (yes)	**1.206** (0.839–1.733)	0.312			**1.151** (0.785–1.687)	0.471		
Molecular subtype of tumor								
ER-positive	**0.737** (0.491–1.106)	0.141			**0.491** (0.321–0.753)	0.001*	**0.709** (0.400–1.256)	0.239
PgR-positive	**0.865** (0.587–1.274)	0.162			**0.743** (0.494–1.116)	0.152		
HER2-positive	**0.972** (0.630–1.502)	0.899			**1.055** (0.665–1.675)	0.819		
Triple-negative	**1.649** (0.982–2.770)	0.059	**1.826** (1.055–3.161)	0.031*	**2.738** (1.612–4.650)	0.000*	**2.591** (1.500–4.477)	0.001*
No of organs affected (≥3)	**1.196** (0.812–1.762)	0.366			**1.256** (0.838–1.882)	0.269		
Metastatic sites								
Liver	**1.709** (1.161–2.516)	0.007*	**1.945** (1.276–2.963)	0.002*	**1.746** (1.157–2.635)	0.008*	**1.639** (1.082–2.482)	0.020*
Lung	**1.014** (0.692–1.486)	0.941			**1.024** (0.681–1.539)	0.910		
Bones	**1.308** (0.905–1.890)	0.153			**1.222** (0.827–1.805)	0.314		
Lymph nodes	**1.088** (0.737–1.606)	0.672			**1.036** (0.686–1.566)	0.866		
CNS	**0.958** (0.419–2.186)	0.918			**0.771** (0.337–1.763)	0.538		
Skin	**0.869** (0.440–1.717)	0.686			**0.959** (0.484–1.903)	0.906		
Type of first-line chemotherapy								
Taxane- or anthracyclin- single agents (reference)								
Taxane-based	**1.317** (0.782–2.219)	0.301			**0.993** (0.585–1.687)	0.981		
Combination without taxanes or anthracyclines	**1.791** (1.002–3.201)	0.049*	**2.086** (1.154–3.774)	0.015*	**1.004** (0.553–1.823)	0.989		
CSC+/partial-EMT+ CTCs	**1.586** (1.060–2.374)	0.025*	**1.785** (1.171–2.720)	0.007*	**1.322** (0.856–2.042)	0.207		

NOTE: Univariate and multivariate Cox regression analysis was performed to estimate the risk for relapse and death of patients with metastatic breast cancer. Parameters showing statistical significance (*) or a trend for significance (Italics) in the univariate Cox regression analysis were subsequently included in a multivariate Cox proportional hazards regression model to evaluate whether they were independently associated with the risk for progression and death. The detection of CSC+/partial-EMT+ CTCs at baseline was an independent factor predicting for increased risk of relapse. Similarly, combination therapy not including taxanes or anthracyclines was associated with increased risk of relapse. Triple-negative status of the primary tumor and liver metastases independently predicted for increased risk of relapse and death.

Abbreviation: HR; hazard ratio (Bold).

Table 3. Univariate and multivariate analysis for PFS and OS in the HER2-negative cohort

Cox regression analysis	PFS				OS			
	Univariate		Multivariate		Univariate		Multivariate	
Covariates	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age (≤59)	**1.172** (0.674–2.037)	0.573			**1.107** (0.605–2.027)	0.741		
Performance status (2–3)	**1.047** (0.411–2.664)	0.924			**1.228** (0.430–3.507)	0.701		
Menopausal status (pre)	**1.196** (0.342–4.183)	0.779			**1.622** (0.458–5.736)	0.453		
Grade (III vs. I/II)	**1.647** (0.911–2.977)	0.098			**1.209** (0.642–2.278)	0.556		
Adjuvant chemotherapy (yes)	**1.732** (0.958–3.131)	0.069			**1.342** (0.731–2.465)	0.343		
Molecular subtype of tumor								
Triple-negative vs. ER- or PgR-positive	**2.514** (1.182–5.347)	0.017*	**3.660** (1.602–8.360)	0.002*	**6.072** (2.675–13.779)	0.000*	**6.748** (2.833–16.072)	0.000*
No of organs affected (≥3)	**1.085** (0.604–1.951)	0.784			**1.331** (0.714–2.482)	0.368		
Metastatic sites								
Liver	**2.169** (1.165–4.041)	0.015*	**3.503** (1.734–7.078)	0.000*	**2.236** (1.156–4.324)	0.017*	**2.892** (1.448–5.775)	0.003*
Lung	**1.281** (0.729–2.251)	0.390			**1.025** (0.553–1.899)	0.937		
Bones	**1.359** (0.764–2.419)	0.297			**1.476** (0.782–2.787)	0.230		
Lymph nodes	**1.204** (0.674–2.150)	0.531			**1.110** (0.594–2.073)	0.744		
CNS	**0.590** (0.139–2.500)	0.473			**0.777** (0.183–3.305)	0.733		
Skin	**0.980** (0.235–4.081)	0.978			**1.594** (0.379–6.708)	0.525		
Type of first-line chemotherapy								
Taxane- or anthracyclin- single agents (reference)								
Taxane-based	**1.317** (0.576–3.010)	0.514			**1.247** (0.533–2.920)	0.611		
Combination without taxanes or anthracyclines	*2.533* (0.997–6.435)	*0.051*	**5.262** (1.814–15.264)	0.002*	**1.222** (0.467–3.202)	0.683		
CSC+/partial-EMT+ CTCs	*1.765* (0.983–3.169)	*0.057*	**2.283** (1.211–4.305)	0.011*	**2.223** (1.115–4.429)	0.023*	**2.228** (1.066–4.655)	0.033*

NOTE: Univariate and multivariate Cox regression analysis was performed to estimate the risk for relapse and death among HER2-negative patients with CTCs at baseline. Parameters showing statistical significance (*) or a trend for significance (Italics) in the univariate Cox regression analysis were subsequently included in a multivariate Cox proportional hazards regression model to evaluate whether they were independently associated with the risk of progression and death. The detection of CSC+/partial-EMT+ CTCs was an independent factor predicting for increased risk of relapse and death. Triple-negative tumors and liver metastases were associated with high risk for relapse and death, whereas combination therapy without taxanes or anthracyclines was predictive for increased risk for relapse.
Abbreviation: HR; hazard ratio (Bold).

expressed on normal blood cells; therefore, they should preferably be evaluated on single CTCs, instead of CTC-enriched cell populations. Using the current methodology, we further evaluated the differential expression patterns of these markers on single CTCs, and thus, we defined the clinically relevant CTC subset. This suggests that CTC analysis at the protein level is important for understanding their biological role.

In our study, low CTC numbers were in general detected which, considering the high CTC heterogeneity (6), may represent a limitation for their further phenotypic analysis. The hematopoietic marker CD45 was not included in the immunofluorescence panel due to the limitation of our method in using up to three markers; however, the specificity of the A45/B-B3 pan-cytokeratin antibody used in this study has been extensively demonstrated in previous work from our group (27–29, 31). Moreover, the association of the CSC$^+$/partial-EMT$^+$ subset with worse patient outcome is suggestive of an underlying malignant behavior. Another limitation of the current study is that the detection of CTCs was exclusively based on CK expression and thus, fully mesenchymal CTCs with complete loss of CK could not be identified. In addition, no functional assays were performed to confirm whether CTCs bearing the CSC$^+$/partial-EMT$^+$ phenotype were actually endowed with stem cell properties *in vitro* and/or *in vivo*. Nevertheless, we consider that it is clearly demonstrated that the simultaneous expression of distinct CSC and partial-EMT phenotypes on CTCs may identify an aggressive CTC subpopulation with clinical relevance in metastatic breast cancer.

In conclusion, the current study shows that the detection of CSC$^+$/partial-EMT$^+$ CTCs in the peripheral blood of patients with metastatic breast cancer before the start of first-line chemotherapy is an independent factor predicting for reduced PFS, whereas in the HER2-negative cohort, it is also predictive of decreased OS. The incidence of this CTC subpopulation significantly increased post-chemotherapy, which was further associated with nonresponse at the end of treatment and HER2-negative disease status. These results highlight the importance of CTC analysis for the detection of tumor cell subpopulations that could drive tumor progression. Moreover, they suggest that the detection of CSC$^+$/partial-EMT$^+$ CTCs could serve for the refinement of prognosis in patients with breast cancer undergoing first-line chemotherapy. Patients bearing this CTC population may not benefit from conventional chemotherapy regimens and are in need of novel targeted therapies against molecules and pathways involved in the regulation of stemness and EMT states. Indeed, agents targeting CSCs directly or via reversing EMT are currently in phase I/II clinical trials (40), and thus the real-time evaluation of CSC and EMT markers at the CTC level could be used for the selection of patients who could benefit the most.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.A. Papadaki, G. Stoupis, V. Georgoulias, S. Agelaki
Development of methodology: M.A. Papadaki, P.A. Theodoropoulos
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.A. Papadaki, S. Agelaki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.A. Papadaki, P.A. Theodoropoulos, D. Mavroudis, S. Agelaki
Writing, review, and/or revision of the manuscript: M.A. Papadaki, P.A. Theodoropoulos, D. Mavroudis, V. Georgoulias, S. Agelaki
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Acknowledgments

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