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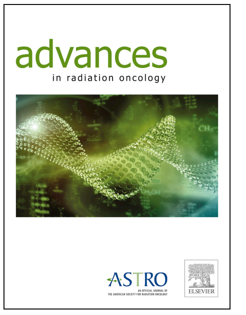

Prospective assessment of the association between circulating tumor cells and control of brain disease after focal radiotherapy of breast cancer brain metastases

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Prospective assessment of the association between circulating tumor cells and control of brain disease after focal radiotherapy of breast cancer brain metastases

Short Running Title: Circulating tumor cells and brain metastases

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Summary

- Predicting the risk of early distant brain failure (DBF) is a useful resource for management decisions in patients who are candidates to local treatment of brain metastasis.
- Prospective assessment of the association between circulating tumor cells (CTC) and brain disease control after stereotactic radiotherapy/radiosurgery (SRT) for breast cancer brain metastasis (BCBM).
- CTC may have a role as a biomarker of DBF and subsequent guider between focal or whole-brain radiotherapy in patients with BCBM.

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Prospective assessment of the association between circulating tumor cells and control of brain disease after focal radiotherapy of breast cancer brain metastases.

ABSTRACT

Introduction**:** Predicting the risk of early distant brain failure (DBF) is in demand for management decisions in patients who are candidates to local treatment of brain metastases. This study aims to analyze the association between circulating tumor cells (CTC) and brain disease control after stereotactic radiotherapy/radiosurgery (SRT) for breast cancer brain metastasis (BCBM).

Methods: Prospective assessment of CTC before (CTC1) and 4–5 weeks after (CTC2) SRT and its relations with the number of new lesions suggestive of BCBM before SRT (NL). CTC were quantified and analyzed by immunocytochemistry to evaluate the expression of the proteins COX2, EGFR, ST6GALNAC5, NOTCH1 and HER2. Distant brain failure-free survival (DBFFS), the primay endpoint, diffuse distant brain failure-free survival (D-DBFFS) and overall survival (OS) were estimated. Analysis for DBF within 6 months, with death as competing risk, was performed.

Results: Patients were included between 2016 and 2018. CTC were detected in all 39 patients before and in 34 of 35 patients after SRT. After median follow-up of 16.6 months, median DBFFS, D-DBFFS and OS were 15.3, 14.1 and 19.5 months, respectively. DBF at 6 months was 40% with CTC1 ≤ 0.5 and 8.82% with CTC1 > 0.5 CTC/mL (P = .007) and of D-DBF at 6 months was 40% with CTC1 ≤ 0.5 and 0 with CTC1 > 0.5 CTC/mL (P = .005) and 25% with NL/CTC1 > 6.8 and 2.65% with NL/CTC1 ≤ 6.8 (P = .063). On multivariate analysis, DBFFS was inferior with CTC1 ≤ 0.5 (HR 8.27, 95% CI, 2.12–32.3; P = .002) and D-DBFFS was inferior with CTC1 ≤ 0.5 (HR 10.22, 95% CI, 1.99–52.41; P = .005). Protein expression was not associated with outcomes.

Conclusions: These data suggest that CTC1 and NL/CTC1 may have a role as a biomarker of early diffuse DBF and subsequent guide between focal or whole-brain radiotherapy in patients with BCBM.

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KEYWORDS: Biomarkers, Tumor. Brain Neoplasms. Circulating Tumor Cells. Radiosurgery.

INTRODUCTION

Breast cancer brain metastases (BCBM) have been reported in 18-30% of patients with metastatic disease,[1] with an increasing incidence related to the evolution of brain imaging and more effective control of extracranial disease as a consequence of improvements in systemic therapy and the resulting decrease in overall mortality.[2] The advances in the management of BCBM have led to a median overall survival (OS) of 16 months for all patients and 36 months in the best prognostic group from a large contemporary cohort.[3]

In this context, predicting the risk of early distant brain failure (DBF) is a useful and demanding resource for management decisions in patients who are candidates to local treatment of BCBM. Selecting focal stereotactic radiotherapy (SRT) or whole-brain radiotherapy (WBRT) is a clinical conundrum between optimizing intracranial tumor control and avoiding potential deterioration of cognitive function and quality of life.[4,5]

Risk score, nomogram and prognostic metric for DBF after initial treatment with upfront SRT have been developed.[6-8] However, despite the large patient population and multi-institutional validation, none is disease-specific and all of them are retrospective and based on non-contemporary cohorts. Within this context, we hypothesized whether the evaluation of a biological marker of micrometastatic disease could predict the DBF and help clinicians to decide between SRT or WBRT for BCBM. This study aims to analyze the association between circulating tumor cells (CTC) and control of brain disease after SRT for BM.

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METHODS

Study Design and Participants

Prospective assessment of CTC before (CTC1) and 4-5 weeks after (CTC2) SRT for BCBM and its relations with the number of new lesions suggestive of BCBM before SRT (NL). Eligibility criteria included adult patients (≥ 18 years of age) with BCBM candidates to SRT, who were, priority, those with oligometastaic disease (< four lesions) and no more than ten lesions, expected survival > 6 months defined by the diagnosis-specific graded prognostic assessment (DS-GPA) or prior WBRT. SRT or resection of BCBM before the CTC1 was allowed. Exclusion criteria included pregnant patients, those who had underwent WBRT less than 30 days before blood sample was collected or received any systemic therapy less than 7 days before blood sample was collected.

This study was approved by the Institutional Review Board and all participants provided written informed consent and followed the Reporting of Tumor Marker Studies (REMARK) guidelines.

Procedures

Participants underwent magnetic resonance imaging (MRI) and computed tomography (CT)-based simulation and were immobilized using a stereotactic mask. SRT was performed with single stereotactic radiosurgery (SRS) or stereotactic fractionated radiotherapy (SFRT) depending on the size and location of the target volume following evidence-based experience published.[9,10] All patients were treated within 7 days after MRI simulation for SRT planning with a Varian TrueBeam^{TM} linear accelerator with micromultileaf colimator, cone-beam CT and robotic couch.

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Assessments

Venous blood samples for CTC1 and CTC2 analysis were timely collected on the same day of the simulation MRI and the first follow-up MRI, respectively. The ISET® (Isolation by SizE of Tumors; Rarecells, France) was used to quantify and evaluate CTC as described.[11] Briefly, 10 mL of blood was collected on EDTA tubes and kept under homogenenization for up 4 hours at room temperature to avoid blood coagulation. Then, the blood was diluted 1:10 with the ISET filtration buffer, transferred to the ISET block and filtered through a polycarbonate membrane with calibrated, 8-µm-diameter, cylindrical pores. The ISET system is based on the principle that most of white blood cells are the smallest cells of the body and that CTC are larger than 8 µm. After the filtration, membranes were washed once with phosphate-buffered saline, decoupled of the block and stored at -20 ºC until time of analysis. CTC were counted per 1 mL of blood and characterized according to five criteria: negativity for CD45 staining, nucleus size > 12 µm, hyperchromatic and irregular nuclei, visible cytoplasm and a nuclear to cytoplasm ratio > 80%. Immunocytochemistry was performed to evaluate the expression of the proteins COX2, EGFR, ST6GALNAC5, that are mediators of CTC passage through the blood-brain barrier,[12] and NOTCH1 and HER2, which are associated with a metastatic competency to the brain (Fig 1).[13]

Follow-up acquired-volumetric post-contrast MRI was obtained 4-5 weeks after SRT, then every 3 months during the first year and every 4 months in the second year, unless clinically indicated an earlier time point. Imaging evaluators were blinded to CTC analysis and vice-versa.

Endpoints

DBF was defined as any new lesion suggestive of BCBM that developed outside the previous planning target volume, not present on prior scans and visible in minimum two projections on MRI, following the Response Assessment in Neuro-Oncology Brain Metastases working group.[14] Diffuse DBF (D-DBF) was defined as progression with more than four new BCBM or meningeal carcinomatosis, a more representative endpoint of the potential indication for salvage WBRT. OS was defined as time from date of SRT to date of death. DBF-free survival (DBFFS) and diffuse DBFFS (D-DBFFS) were defined from date of SRT to date of either DBF, D-DBF or death.

Statistical Analysis

The required sample size was defined following the prediction of 15 events (DBF) per variable (CTC) for time-to-event endpoint (DBFFS).[15] Estimating that the events would occur in half of the participants and taking in account the eventual dropout and loss of possible loss of follow-up, the target enrollment was 40 patients.

The baseline characteristics were expressed as absolute and relative frequencies for qualitative variables and as the median, minimum and maximum for quantitative variables. Distant brain failure-free survival (DBFFS), the primary endpoint, diffuse distant brain failure-free survival (D-DBFFS) and overall survival (OS) were estimated by Kaplan-Meier estimator.[16] Log-rank tests were applied to compare the survival curves and the optimal cut-off values were determined following Lausen and Schumacher.[17] The Cox semiparametric proportional hazards model was fitted to assess which variables would be associated to the endpoints.[18] Variables who achieved significance level of 0.2 in single regression were used at the multiple regression models. The final model was obtained using the stepwise backward method (likelihood ratio) with criteria for entry $P < 0.05$ and removal $P > 0.10$.

The assumption of proportional hazards was assessed based on the so-called Schoenfeld residuals. There was evidence that covariates had a constant effect over time in all cases. In addition, competing risk analysis for DBF in the presence of death was applied. The cumulative incidence function was estimated and the Gray’s test was considered to compare the curves. We fitted univariate sub-distribution hazards of an event for different variables according to the Fine-Gray model, which is a Cox type proportional sub-distribution hazards model.[19]

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The significance level was fixed at 5% for all tests. Statistical analyses were performed using R software version 3.5 (R Foundation for Statistical Computing, Austria). The study closed in February 2018 and data set was locked on October 30, 2018.

RESULTS

Participants

Between November 2016 and February 2018, 40 women were enrolled and 39 accrued (1 withdrew from study). Baseline characteristics are listed in Table 1.

Radiotherapy

A total of 119 BCBM were irradiated and the median number of BCBM per patient was 2 (1-15), with a median volume of 0.9 cc (0.027-39.18). SRT was performed as SRS or SFRT in 27 (69%) and 12 (31%) of patients, respectively. The median prescribed dose was 20 (15-22) Gy and 27.5 (25-30) Gy with SRS and SFRT, respectively. Adjuvant SRT was performed in 4 surgical cavities in 4 patients, all of them underwent SFRT with a dose of 25 Gy in 5 fractions. Only one patient, with 3 previous SRS in contiguous areas, evolved with a lesion suggestive of radionecrosis 3 months after SFRT. The actuarial brain local control at 6 and 12 months after SRT was 100% and 97.93%, respectively.

Circulating tumor cells

The detection rate of CTC1 and CTC2 was, respectively, 100% in the 39 patients before SRT and 97% (34/35) in the 35 patients after SRT (4 deaths between CTC1 and CTC2). The median CTC1 and CTC2 was 2 CTC/mL and 2.33 CTC/mL, respectively ($P = .357$). The expressions of the proteins in CTC1 and CTC2 are listed in Table 2.

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Regarding the expression of HER2, there was a discrepancy between the immunophenotype of the primary tumor and CTC1 in 15 of the 32 tested patients: 14 of the 15 patients with HER2-positive immunophenotype had negative expression in the CTC1 and 1 of the 17 without HER2-positive immunophenotype had positive expression of HER2 in the CTC1. Among the 27 patients tested in the CTC2, there was disagreement in 14: 12 of the 14 patients with HER2-positive immunophenotype had negative expression in CTC2 and 2 of the 13 without HER2-positive immunophenotype had positive expression of HER2 in CTC2.

Among the 15 and 14 patients with HER2-positive immunophenotype on primary tumor that were tested for the expression of the proteins in, respectively, CTC1 and CTC2, 10 patients had negative expression of HER2 both in CTC1 and CTC2.

Distant Brain Failure

After a median follow-up of 14.6 months (95% CI, 11.1-18.1) in the 36 evaluable patients, there were 15 patients with DBF, being 6 with D-DBF (3 with progression with more than 4 new BCBM and 3 with leptomeningeal carcinomatosis). The median DBFFS and D-DBFFS was 15.3 months (95% CI, 12.2-not reached) and not reached, respectively.

The mean time to D-DBF in the 6 patients was 6.2 (1-12) months and the salvage treatment was performed in 4 patients: SRS in 1 patient with more than 4 new BCBM and WBRT in 2 patients with more than 4 new BCBM and 1 patient with leptomeningeal carcinomatosis.

The median DBFFS was 6 months in patients with CTC1 ≤ 0.5 CTC/mL and not reached in patients with CTC1 > 0.5 CTC/mL (HR, 4.97; 95% CI, 1.48-16.69; P = .0041) and the median D- DBFFS was 6 months in patients with CTC1 ≤ 0.5 CTC/mL and not reached in patients with CTC1 > 0.5 CTC/mL (HR, 10.22; 95% CI, 1.99-52.4; P = .005).

The median DBFFS was 7 months in patients with immunophenotype triple negative and not reached in patients with immunophenotypes luminal B and HER2-positive (HR, 0.25; 95% CI, 0.07-0.89; P = .03) and it was 7.47

months in patients with DS-GPA ≤ 3 and not reached in patients with DS-GPA > 3 (HR, 0.34; 95% CI, 0.12-0.95; P = .04).

The median DBFFS was not reached in patients with NL ≤ 5 and 10.6 months in patients with NL > 5 (HR, 3.60; 95% CI, 1.08-12.05; P = .037) and the median D-DBFFS was not reached in patients with NL ≤ 6 and 10.6 months in patients with NL > 6 (HR, 10.72; 95% CI, 2.13-53.82; P = .004). The median D-DBFFS was 12.1 months in patients with NL/CTC1 > 6.8 and not reached in patients with NL/CTC1 ≤ 6.8 (HR, 7.37; 95% CI, 1.34-40.5; P = .022).

The cumulative incidence of DBF at 6 months, with death as a competing risk factor, was 40% in patients with ≤ 0.5 CTC/mL and 8.82% in patients with CTC1 > 0.5 CTC/mL (P = .007; Fig 1A) and of D-DBF at 6 months was 40% in patients with ≤ 0.5 CTC/mL and 0 in patients with CTC1 > 0.5 CTC/mL (P = .005; Fig 1B) and 25% in patients with NL/CTC1 > 6.8 and 2.65% with NL/CTC1 ≤ 6.8 (P = .063; Fig 2).

On multivariate analysis, after the Cox proportional selection and stepwise regression, DBFFS was inferior in patients with CTC1 ≤ 0.5 CTC/mL (HR 8.27, 95% CI, 2.12–32.3; P = .002) and superior in patients with immunophenotype HER2-positive (HR 0.128, 95% CI, 0.025–0.534; P = .013), and D-DBFFS was inferior in patients with CTC1 ≤ 0.5 CTC/mL (HR 10.22, 95% CI, 1.99–52.41; P = .005)

There was no significative association between DBFFS/ D-DBFFS and CTC2, number of extracranial metastases (ECM) sites (1 versus ≥ 2) or kinetics of CTC (CTC2/CTC1). The expression of the proteins COX2, EGFR, ST6GALNAC5 and NOTCH1 in CTC1 and CTC2 were not associated with DBFFS and D-DBFFS. However, there was a trend to longer DBFFS in patients that expressed HER2 in CTC1 and CTC2 (Fig 3A and 3B).

Overall Survival

After a median follow-up of 16.6 months (95% CI, 14.8-18.4) in the 39 evaluable patients, there were 16 deaths, being 11 (68%) due to extracranial

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progression, mainly in the lung (9 out of 11). The median OS was 19.5 months (95% CI, 16.1-22.9).

The median OS was 8.6 months in patients with CTC1 ≤ 0.5 CTC/mL and 19.5 months in patients with CTC1 > 0.5 CTC/mL (HR, 3.07; 95% CI, 0.95-9.82; P = .047); 4.8 months in patients with immunophenotype triple negative and not reached in patients with immunophenotypes luminal B and HER2-positive (HR, 0.15; 95% CI, 0.04-0.5; P = .002); 19.5 months in patients with DS-GPA > 2 7.6 months in patients with DS-GPA ≤ 2 (HR, 0.23; 95% CI, 0.08-0.65; P = .006) and 8.6 months in patients with NL/CTC1 > 2.2 and 19.5 months in patients with CTC1 ≤ 2.2 (HR, 3.32; 95% CI, 1.19-9.26; P = .02; Fig 4)

On multivariate analysis, after the Cox proportional selection and stepwise regression, OS was superior in patients with NL/CTC1 ≤ 2.2 CTC/mL (HR 0.159, 95% CI, 0.050–0.505; P = .002) and superior in patients with immunophenotype HER2-positive (HR 0.073, 95% CI, 0.018–0.288; P < .0001), and luminal B (HR 0.224, 95% CI, 0.062–0.816; P = .023).

There was no significative association between OS and CTC2, number of extracranial metastases (ECM) sites (1 versus ≥ 2) or kinetics of CTC (CTC2/CTC1). The expression of the proteins COX2, EGFR, ST6GALNAC5 and NOTCH1 in CTC1 and CTC2 were not associated with OS. However, there was also a trend to longer OS in patients that expressed HER2 in CTC1 and CTC2 (Fig 3C and 3D).

DISCUSSION

This translational study showed that CTC were detectable in almost all patients and that among women with BCBM, those with a lower number of CTC (≤ 0.5 CTC/mL) before the SRT were significantly more likely to develop early DBF and D-DBF. Additionally, the ratio NL/CTC before SRT was a potential prognostic factor of D-DBF and an independent prognostic factor of OS. These results are promising and may be applicable in a recurrent clinical dilemma that is the decision between SRT or WBRT in order to optimize the control of BCBM and mitigate toxicity.[20,21]

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The high observed rates of CTC detection may be related to the ISET method of isolation by filtration when compared to CellSearch system (Veridex, USA), which is the most used and based on the separation of cells expressing epithelial markers. During cancer cell dissemination, especially in the epithelial to mesenchymal transition, the epithelial surface markers can be downregulated. Therefore, a lower detection rate may be observed in a method in which the CTC detection and isolation relies only on epithelial markers positivity.[22,23] The ISET method has been validated in several published studies with different types of cancer, providing high sensitivity (1 CTC/mL) and specificity (100%).[24]

A significant association between the number of CTC before treatment and survival results has already been established; it is an independent predictor of progression-free survival and OS, with an inverse relation, in patients with metastatic breast cancer. [25,26] Still, there is a unique clinical study that evaluated the impact of CTC on BCBM outcome. In a preplanned analysis of the LANDSCAPE phase II trial, patients with HER2-positive metastatic breast cancer with BCBM without previous WBRT who received first-line combination of lapatinib and capecitabine had CTC detected (CellSearch) at baseline and day 21. The central nervous system objective response and 1-year OS rate were significantly higher in patients with no CTC at day 21, but there was no difference in time to progression, an outcome that involved the evaluation of new brain metastases.[27]

Despite being counterintuitive, our finding of significant association, with a direct relation, between the number of CTC and DBFFS was reported in exploratory analysis of few retrospective studies. Undetectable CTC status was positively correlated with presence of BCBM and OS in a series of patients with metastatic breast cancer.[28] Likewise, in a cohort of patients with brain metastases of non-small cell lung cancer, patients with isolated metastases to the brain were less frequently identified as CTC-positive compared to patients with multiple metastatic sites, including the brain, although CTC were still predictive for OS.[29] More recently, an update of the breast DS-GPA revealed that time from primary diagnosis to BCBM was shorter in patients without ECM compared to those with ECM, suggesting that some patients may have occult

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BCBM at presentation of early-stage breast cancer and/or a more brain-metastatic tumor phenotype.[3] Besides that, there was no relation between the burden of extracranial disease, represented by the number of ECM sites, with DBF and OS in our study, which is in contrast with recent findings of higher incidence of BCBM in patients with greater number of metastatic sites.[30]

Therefore, beyond quantity, a qualitative analysis of CTC may refine the prediction of brain disease control. For this purpose, we evaluated the ratio NL/CTC, that embodies a qualitative indicator, as the greater the ratio, we can infer that a smaller number of CTC is associated with a greater of number of BCBM and, then, these CTC probably generate more brain metastasis. In fact, NL/CTC1 was a potential prognostic factor of D-DBF, besides an independent prognostic factor of OS. Regarding the expressions of proteins that eventually could characterize BCBM-associated CTC in this study, HER2 was the only associated with a trend to longer DBFFS and OS, both in CTC1 and CTC2. Interestingly, HER2 was one of four markers that composed BCBM signature of CTC that were highly invasive and capable of generating brain and lung metastases in a patient-derived xenograft mouse model,[13] and 9 out of 16 deaths in our cohort were due to ECM progression in the lung. This is coherent with a shorter OS in patients with a NL/CTC1 > 2.2, suggesting that this sort of CTC is prone to brain and lung progression.

In this context, the possibility of spontaneous interconversion of HER2 phenotypes in the CTC, irrespective of the HER2 status of the primary breast cancer,[31] highlights the potential predictive and prognostic impact of phenotypic characterization of CTC. Additionally, in patients with HER2-positive immunophenotype on primary tumor that had negative expression of HER2 in CTC, the conversion of the phenotype may be associated with a response to the anti-HER2 targeted therapies. Of note, among the 20 patients with HER2-positive immunophenotype on primary tumor in our study, 15 were on anti-HER2 targeted therapies before SRT, being 8 of them on dual blockade.

After evaluating the multiple factors associated with DBF and reviewing our results and clinical and experimental evidence from the recent literature, we hypothesize that, among multiple other possibilities, in patients with BMBC

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there is a development of a pre-metastatic brain environment that involves the infiltration of immunosuppressive neutrophils and the reduction of cytotoxic T cells,[32] in addition to the infiltration of myeloid cells that produce chemokines and attract other myeloid cells and CTC, with consequent proliferation of brain metastasis.[33] Myeloid cells are stimulated by COX2 from primary tumors and a high expression of COX2 was observed in CTC1 and CTC2 in our data. Considering that COX2 is associated with intercompartmental migration between the brain, cerebrospinal fluid and blood,[34] it is plausible that the brain environment already amenable to the formation of metastases tends to attract CTC to the local and reduce their amount in the bloodstream. Thus, patients with BCBM and a lower CTC count in the blood would have a higher risk of new brain metastasis, since it is likely that the volume of CTC is in the brain compartment. On the other hand, patients with a higher number of CTC in the blood would have a mechanism of evasion from the brain attraction. The way forward to continue this investigation and test our hypothesis is to carry out a study to evaluate and compare CTC in the blood and cerebrospinal fluid of patients with BCBM.

These results should be considered in the circumstances of the limitations of the research. The small sample size may have led to a biased overestimated analysis and the inclusion of multiple immunophenotypes of breast cancer with different propensity to develop brain metastases was a causal factor of the heterogeneity of the results. The immunocytochemistry performed to evaluate the expression of multiple proteins in CTC is a challenging process, with a sensitivity variability and risk of cross-reactivity with the distinct antibodies. Additionally, the different systemic therapies used may have influenced both the number of CTC and the brain disease control, although unlikely for the latter. From another perspective, this was a prospective and pragmatic study that accrued only patients with breast cancer, without the inherent biases from retrospective analysis with different primary tumors that developed risk score, nomogram or prognostic metric to preditc DBF after SRT.[6-8] While adding some evidence in a few explored topic, the data presented herein are hypothesis generating and further prospective validation is required.

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In conclusion, our findings indicate that CTC were detectable in almost all patients with BCBM. CTC before SRT was an independent prognostic factor of DBFFS and D-DBFFS and NL/CTC before SRT was an independent prognostic factor of OS and a potential prognostic factor of D-DBF at 6 months. These data suggest that CTC and NL/CTC1 may have a role as a biomarker of early D-DBF, and subsequent guider between focal or whole-brain radiotherapy in patients with BCBM.

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FIGURES CAPTIONS

Fig 1. Pictures of CTC isolated from Immunostaining of CTC from patients with metastatic breast cancer and its relations with the 8 µm pores (asterisks) of the ISET membranes. A and B) CTC stained with HER2 (arrows), visualized by DAB (diaminobenzidine). C and D) CTC visualized with haematoxylin (arrows). E and F) Leucoytes from patients visualized with haematoxylin (arrows). Images were taken at x400 magnification using a light microscope (Research System Microscope BX61 – Olympus, Tokyo, Japan) coupled to a digital camera (SC100 – Olympus, Tokyo, Japan).

Fig 2. Cumulative incidence of (A) DBF, (B) D-DBF stratified by CTC1 (≤ 0.5 or > 0.5 CTC/mL) and D-DBF stratified by NL/CTC1 (≤ 6.8 or > 6.8) based on the Fine-Gray model.

Fig 3. Kaplan-Meier plot for DBFFS stratified by HER2 expression in (A) CTC1 and (B) CTC2 and for OS stratified by HER2 expression in (C) CTC1 and (D) CTC2.

Fig 4. Kaplan-Meier plot for OS stratified by NL/CTC1.

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Table 1. Baseline Characteristics

Median age, years (range)	54 (34-70)
Immunophenotype (%)	
HER2-positive	20 (51)
Luminal B	12 (31)
Triple negative	7 (18)
DS-GPA (%)	
0-1	1 (2.5)
1.5-2	6 (15.5)
2.5-3	6 (15.5)
3.5-4	26 (66.5)
KPS (%)	
70-80	7 (18)
90-100	32 (82)
ECM (%)	
Absent	6 (15.5)
Present	33 (84.5)
ECM status (%)	
Absent	6 (15.5)
Progressive	17 (43.5)
Stable	16 (41)

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Number of ECM sites (%)	
None	6 (15.5)
1	29 (74.5)
2	2 (5)
3	2 (5)
Previous treatment to the brain (%)	
None	18 (46)
SRT	9 (23)
Surgery	5 (13)
WBRT	4 (10)
Surgery and SRT or WBRT	3 (8)
Systemic therapy before CTC1 (%)	
None	3 (8)
Hormonal therapy	9 (23)
Chemotherapy	12 (31)
HER2-Targeted therapy	15 (38)

Abbreviations: DS-GPA, diagnosis-specific graded prognostic assessment; ECM, extracranial metastases; KPS, Karnofsky performance score; SRT, focal stereotactic radiotherapy; WBRT, whole-brain radiotherapy.

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Table 2. Frequencies of the expression of the proteins in CTC1 and CTC2.

Proteins	CTC1			CTC2		
	Category	n	%	Category	n	%
COX2	Negative	1	3.6	Negative	1	4
	Positive	27	96.4	Positive	24	96
	Total	28	100	Total	25	100
EGFR	Negative	22	78.6	Negative	9	50
	Positive	6	21.4	Positive	9	50
	Total	28	100	Total	18	100
ST6GALNAC5	Negative	12	46.2	Negative	6	33.3
	Positive	14	53.8	Positive	12	66.7
	Total	26	100	Total	18	100
NOTCH1	Negative	13	40.6	Negative	11	40.7
	Positive	19	59.4	Positive	16	59.3
	Total	32	100	Total	27	100
HER2	Negative	29	90.6	Negative	19	70.4
	Positive	3	9.4	Positive	8	29.6
	Total	32	100	Total	27	100

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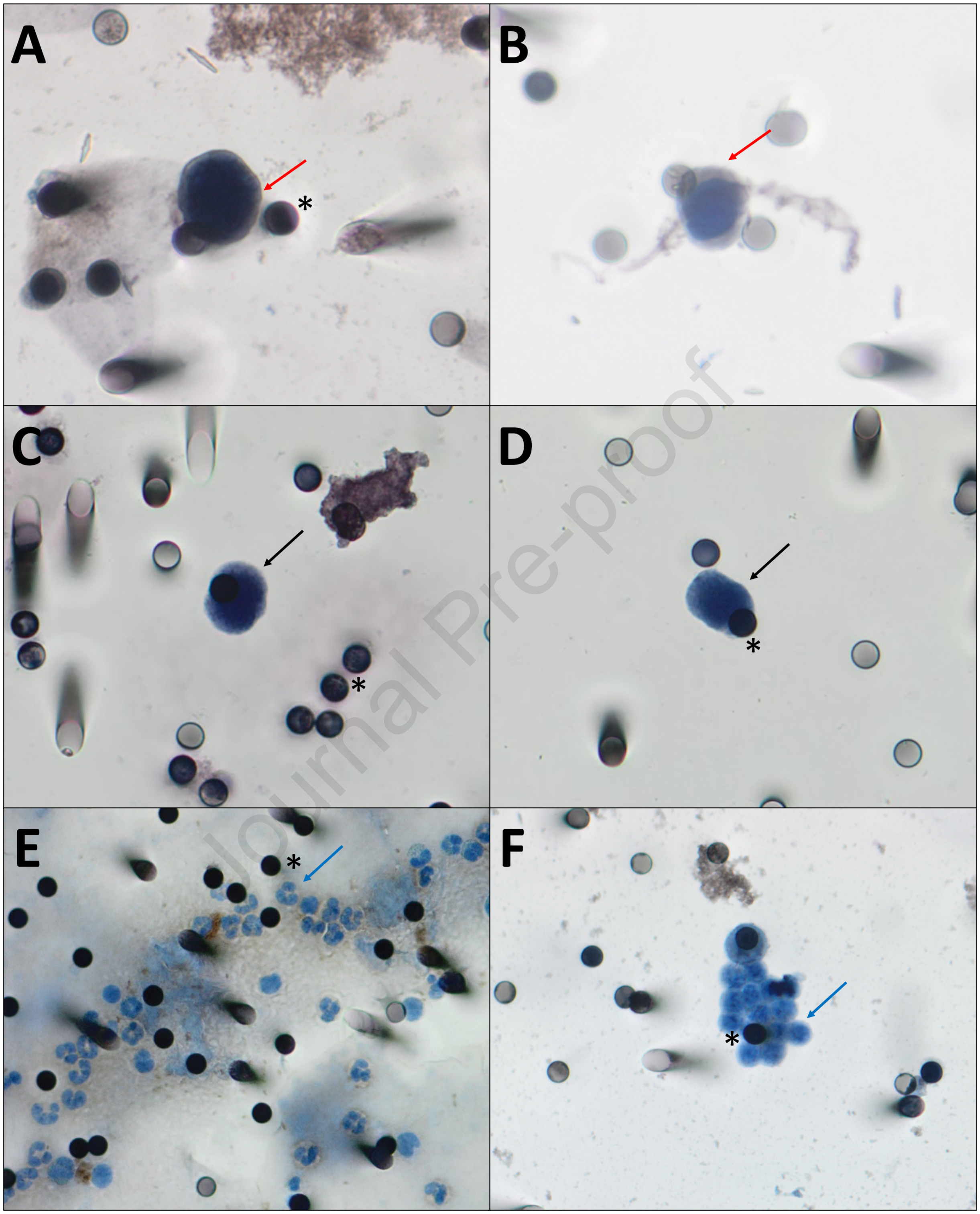

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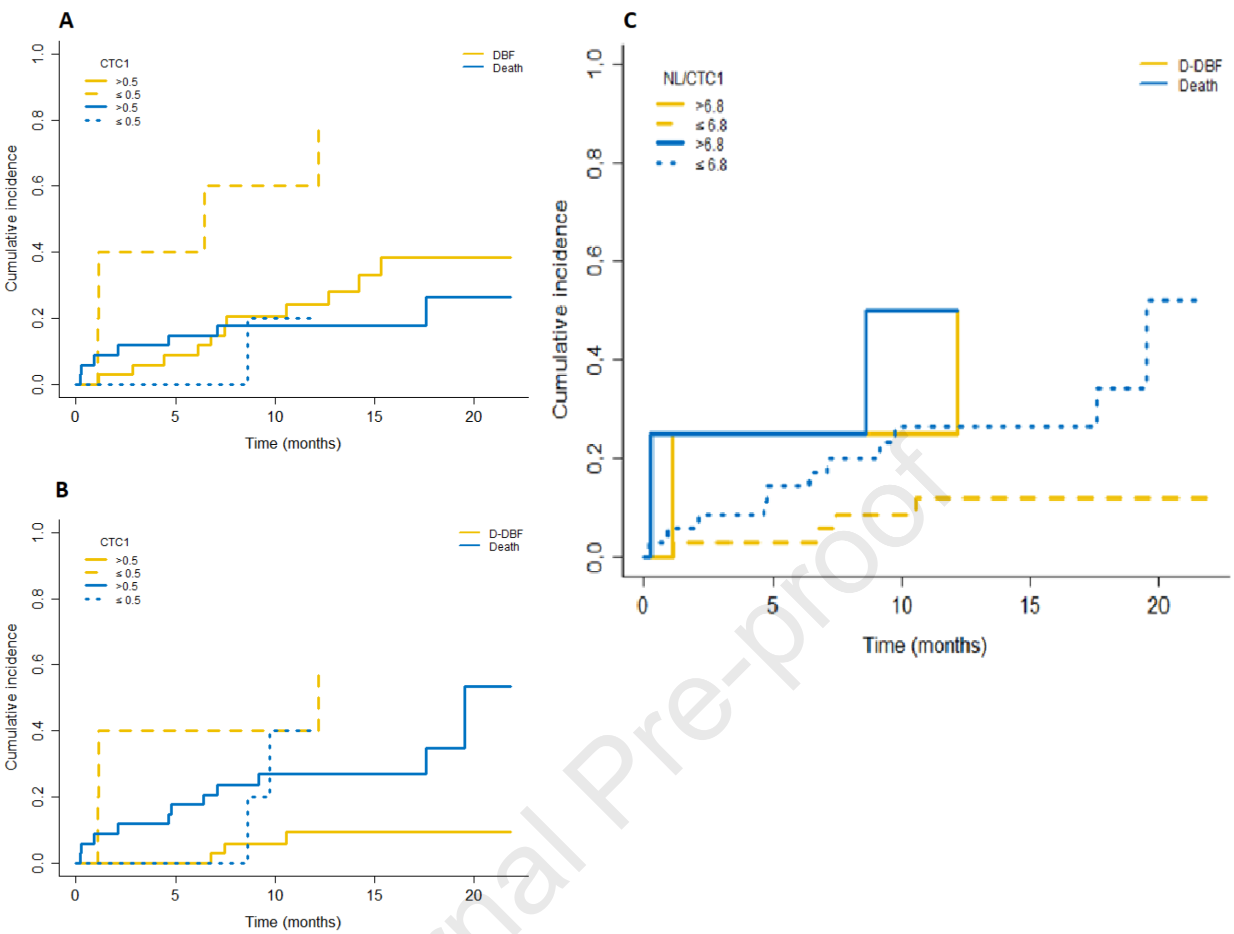

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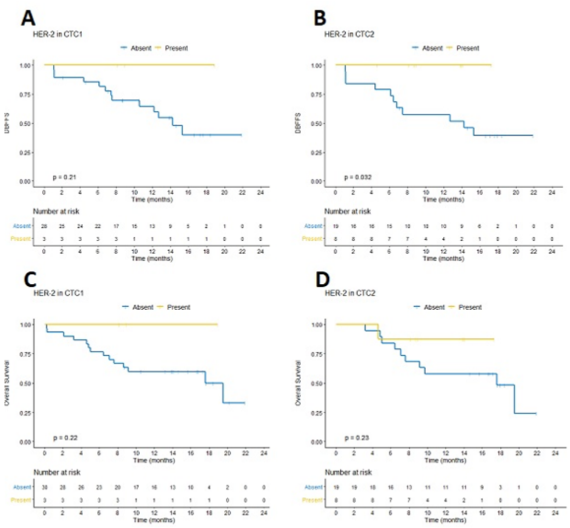

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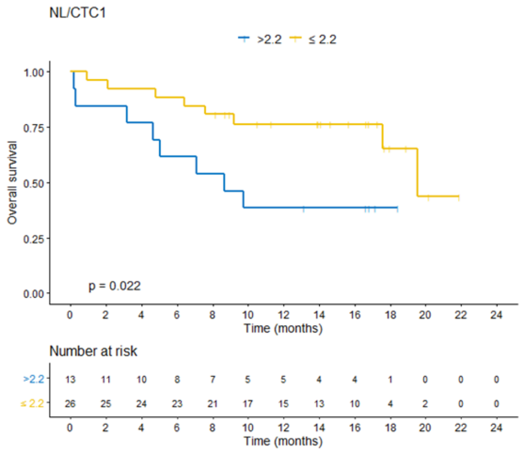

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