



Circulating tumor cells predict early skeletal-related events in patients with bone metastases from breast cancer

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ABSTRACT

Background: Bone metastases and following skeletal-related events (SREs) are frequent in metastatic breast cancer (MBC) and are a major cause of disability. Circulating tumor cells (CTCs) are prognostic in MBC and are more frequent in patients with bone metastases (BMs). We investigated whether CTCs could be quantitative predictive factors of SREs in this setting.

Methods: CTC analysis was performed using CellSearch technology within the first month of BM diagnosis and blood samples containing ≥ 5 CTCs/7.5 mL were considered positive. Patients were then prospectively followed-up until first onset of a SRE or death.

Results: In 145 BM-MBC patients, mean CTC number was 295/7.5 mL and 60% of patients had ≥ 5 CTCs. SREs occurred in 82 (57%) patients. After a median follow-up of 105 months, median time-to-SRE (TT-SRE), progression-free survival (PFS) and overall survival (OS) from CTC analysis were 18, 5, and 14 months, respectively. In multivariable analysis, CTCs ≥ 5 predicted a shorter TT-SRE (median ttSRE 11 vs 31 months, HR 2.08, 95%CI 1.26-3.40, $p = .004$). CTC ≥ 5 were also an independent predictor of shorter PFS (HR 2.72, 95%CI 1.86-3.95, $p < 0.0005$) and worse OS (HR 3.16, 95%CI 2.183-4.576, $p < 0.0005$).

Conclusion: In this series of MBC patients with BMs and a long follow-up, the detection of CTCs in peripheral blood was not only associated with a worse prognosis in terms of PFS and OS, but was also predictive of an increased risk of skeletal-related events.

1. Background

Bone is the first site of metastasis in 25%-40% of breast cancer (BC) patients but up to 80% of metastatic breast cancer (MBC) patients will develop bone metastases during the disease course [1,2]. This incurable condition leads to impaired quality of life (QOL) because of skeletal-related events (SREs) [3] and to reduced survival [4].

The latency between the diagnosis of BC and the onset of a bone metastasis greatly varies between months and years or even decades [5,6] suggesting that the bone microenvironment represents an ideal soil

for both dormancy and proliferation of cancer cells.

Skeletal-related events, including pathological fractures, spinal cord compression, hypercalcemia of malignancy, and the need for palliative radiotherapy or orthopedic surgery to bone, represent major clinical complications in patients with bone metastatic breast cancer [1,7]. The occurrence of SREs is associated with severe bone pain, reduced mobility, loss of functional independence, and a substantial deterioration in quality of life [1,8]. Moreover, SREs frequently require hospitalization and multidisciplinary interventions, resulting in increased healthcare burden and costs. Previous studies demonstrated that the risk

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of developing SREs is influenced by both disease-related and patient-related factors, including the extent and pattern of bone involvement, osteolytic activity, performance status, age, and biochemical markers of bone turnover such as lactate dehydrogenase (LDH) and alkaline phosphatase [1,9]. Importantly, the development of SREs has also been associated with shorter survival outcomes in MBC patients, emphasizing the need for early identification of individuals at higher risk [9]. Although bone-targeted agents, such as bisphosphonates and denosumab, have significantly reduced the incidence and delayed the onset of SREs [1,10], these complications still occur in a substantial proportion of patients, highlighting the need for additional predictive biomarkers able to improve risk stratification and therapeutic management.

Circulating tumor cells (CTCs) $\geq 5/7.5$ mL of peripheral blood (PB) as detected by the CellSearch™ method have been described in approximately 50% of MBC patients [11] and have shown validity both as a predictive marker of response to therapy and as a prognostic factor [12]. In patients with bone metastases higher numbers of CTCs are displayed compared to other visceral metastatic sites and an extensive bone involvement directly correlates with even higher levels of CTCs [13].

The number and type of bone metastases along with patient-based features (including age, pain composite score, performance status, LDH and baseline alkaline phosphatase) have been described as independent prognostic markers of SREs in MBC patients [14]. However, no studies focused on the relationship between CTCs and SREs in MBC patients with bone metastases. Given that CTCs levels are higher in these patients, we hypothesized that CTCs could be quantitative potential predictive markers of SREs in this setting, thus providing a rationale for the development of strategies aimed at the early diagnosis and timely management of SREs in patients with metastatic breast cancer. Therefore, given the absence of previous studies specifically investigating the association between CTC burden and skeletal-related events in metastatic breast cancer, we sought to address this knowledge gap through a retrospective analysis of a well-characterized cohort of patients with bone metastases and long-term follow-up, evaluating whether baseline CTC count could serve as a predictive biomarker for the occurrence of skeletal-related events.

2. Methods

2.1. Study design and patients

In this paper we analyzed a subgroup of MBC patients included in a wider prospective trial of CTCs evaluation in patients with different solid neoplasms conducted at the Spedali Civili di Brescia from 2009 to 2012 [15–18]. The study has been conducted according to the Declaration of Helsinki and has been approved by the Institutional Review Board (NP605-2009). Each patient signed a specific informed consent form before the analysis of CTCs. Patients were managed outside an interventional clinical trial and CTCs information had no impact on the therapeutic decisions taken by the multidisciplinary team of the Institutional Breast Cancer Unit.

The primary objective was to investigate the correlation between CTC levels and SREs, and secondary objective was to confirm the prognostic significance of CTCs in terms of survival outcomes in MBC patients with bone metastases. After CTC sampling, patients were prospectively followed-up. Minimal requirements for analytical eligibility in this study were: diagnosis of MBC with histologically- and/or radiologically-confirmed bone metastases; at least one valid CTC count; availability of pathological and clinical records and follow-up data. Patients with a second primary neoplasm different from MBC were excluded from the analysis. No prospective or retrospective molecular profiling was performed to investigate the presence of genomic alterations or other molecular drivers in the study population.

2.2. Clinical evaluation of bone metastases and SREs

Bone metastases were identified based on of surgical records, bone biopsies or imaging techniques data including X-rays, computed tomography (CT), magnetic resonance imaging (MRI), technetium-99 m hydroxymethylene bone scintigraphy (^{99}Tc -bone scintigraphy) and fluorine-18-fluorode-oxyglucose positron-emission tomography/computed tomography (^{18}F FDG-PET/CT) as clinically indicated.

A trephine BoMa biopsy was performed only when clinically indicated and all patients signed a specific informed consent before the procedure. BoMa biopsy was performed from unilateral posterior iliac crest with an 8-gauge needle after local anesthesia. Alternatively, a bone biopsy was obtained during orthopedic surgical interventions or by a percutaneous computed tomography (CT)-guided fine needle biopsy.

We defined SREs as both true clinical complications — including pathologic fractures, spinal cord or nerve root compression, and hypercalcemia — and the therapeutic interventions used to treat them, namely surgery and radiotherapy. We considered synchronous SREs that appeared within the first three months after bone metastases diagnosis.

2.3. CTCs analysis

For all the patients in this analysis, CTC sampling was performed within the first month of diagnosis of bone metastases. CTC analysis was conducted using the FDA-cleared CellSearch technology (Menarini Silicon Biosystems) according to the manufacturer's instructions. Briefly, PB from MBC patients was drawn into 10 mL evacuated tubes which contained a cell preservative (CellSave Preservative Tubes, Menarini Silicon Biosystems), maintained at room temperature and processed for CTC enumeration within 96 h from collection. Epithelial cells were enriched immunomagnetically using a ferrofluid with antibodies against the epithelial cell adhesion molecule (EpCAM). EpCAM-positive cells were labeled with the nuclear dye 4,6-diamidino-2-phenylindole (DAPI) and monoclonal antibodies specific for the leukocyte common antigen CD45 and cytokeratins 8, 18, 19. CTCs were defined as CD45-negative, cytokeratin-positive cells with an intact nucleus and were enumerated by trained operators. Blood samples containing ≥ 5 CTCs/7.5 ml were considered CTC-positive, consistent with the standardized cut-off derived from prior landmark studies and subsequently adopted in the literature [11,19].

2.4. Statistical analysis

Descriptive statistics were used for patient's characteristics. Continuous variables were categorized using predefined cutpoints. Categorical variables were analyzed with the Chi-square test or the Fisher's test. Associations of continuous and categorical variables were assessed with the Mann-Whitney *U* Test. Outcome measures were calculated from the date of CTC analysis and included: time-to-SRE (TT-SRE), progression-free survival (PFS) and overall survival (OS). SREs included hypercalcemia, pathological bone fractures and spinal cord compressions. Survival curves were generated with the Kaplan-Meier method and stratified by clinical covariates and CTC values. Differences among curves were evaluated with the log-rank test. Median follow-up was calculated with the inverse Kaplan-Meier method. All reported *p* values are two-sided and *p* values less than .05 were considered statistically significant. Potential impact of CTC levels and of single clinical variables on outcome measures (TT-SRE, PFS, OS) was estimated with the Cox proportional-hazard model. Results are given as hazard ratios (HRs) with 95% confidence intervals (95% CIs). Variables significantly associated with outcome measures at a *p* level of significance $\leq .1$ were included in a multivariable Cox model. The SPSS software (v23.00; SPSS, Chicago, IL, USA) was used for statistical analysis.

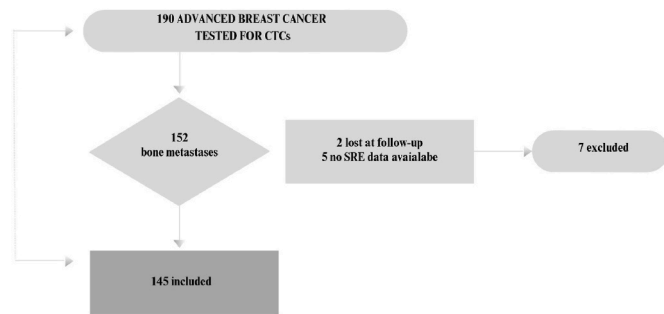


Fig. 1. CONSORT flow diagram of the study.

The diagram illustrates the selection process of the study participants. Of the 190 advanced breast cancer patients tested for circulating tumor cells (CTCs), 152 had bone metastases. Two patients were excluded due to loss to follow-up, while an additional 5 patients were excluded due to the unavailability of skeletal-related event (SRE) data. Consequently, a total of 145 patients were included in the final analysis.

3. Results

3.1. Patients' characteristics

From January 2009 to April 2012, 190 MBC patients sequentially treated at the Department of Medical Oncology, Spedali Civili of Brescia were prospectively studied with CTC analysis. Of these, 145 patients met the inclusion criteria for the present analysis (Fig. 1). Patient characteristics are summarized in Table 1. Patients were included consecutively and performed CTC sampling and analysis within the first month of diagnosis of bone metastases. The majority (83%) of patients had a luminal histology and 53% of patients had a Ki67 $\geq 20\%$. Approximately 17% of patients were metastatic at diagnosis while 32% and 51% of patients developed metastases within or after 4 years from diagnosis, respectively. For the 120 (83%) non-metastatic patients at diagnosis, the median time-to first metastasis was 66 months (range 1-370). Median age at diagnosis of metastatic disease was 58 years (range 33-86). At study entry, 96 (66%) and 49 (34%) patients had an ECOG PS 0-1 and ≥ 2 , respectively. Ten (7%) patients were treatment-naïve and the whole study population received a median of 1 (range 0-13) and 2 (range 0-7) lines of hormonal treatment and chemotherapy, respectively.

3.2. Skeletal metastases, CTCs and SREs

Bone was the first site of metastatic spread in 86% of cases and all patients developed bone metastases within a median time of 50 months from initial diagnosis. The metastatic pattern included other visceral sites such as liver (46%), lymphnodes and locoregional soft tissues (41%), lung and pleura (40%) and central nervous system [CNS] (10%) with a median number of 2 metastatic sites. Bone involvement was limited (1-2 sites) and extensive (>2 sites) in 37 (26%) and in 108 (74%) patients, respectively. Bone metastases were predominantly osteolytic in 78% of cases and localized in the axial skeleton (Table 1). At study entry, CTCs ≥ 1 , ≥ 5 and ≥ 50 were detected in 77%, 60% and 35% of patients, respectively. Mean and median number of CTCs were 295 CTCs (SD ± 1204.3) and 17 CTCs (range 0-11064) (Table 1). CTCs ≥ 5 were more frequent in patients with ≥ 3 metastatic sites ($p .03$), CNS metastases ($p .04$), bone metastases as first site of MBC ($p .05$) and extensive bone involvement ($p .001$) (Table 2). A diagnostic BoMa biopsy was conducted in 37 (26%) bone metastatic patients with extensive skeletal involvement. Twenty of 21 (95%) positive cases had a CTC count of ≥ 5 . When BoMa involvement occurred, it was associated with very high CTC numbers (CTC $\geq 1000/7.5$ mL, $p .051$).

SREs were documented in 82 (57%) of patients and included 3 (4%) spinal cord compressions, 16 (20%) cases of hypercalcemia (any serum level above normal), 29 (35%) pathological bone fractures and 34 (41%)

Table 1

Clinico-pathological patients characteristics at CTC evaluation/bone metastases diagnosis.

Characteristic	MBC patients (N = 145) N (%)
Median age [range], years	58 [33-86]
<60 years	80 (55)
≥ 60 years	65 (45)
Tumour subtype	
Luminal	120 (83)
Non-luminal	25 (17)
- Her2-positive	15 (10)
- Triple-negative	10 (7)
Histological grade (available in 144 patients)	
I	14 (10)
II	69 (48)
III	61 (42)
ECOG Performance status at CTC analysis	
0	4 (3)
1	92 (63)
2	29 (20)
3	9 (6)
4	11 (8)
ECOG Performance status at CTC analysis	
0-1	96 (66)
≥ 2	49 (34)
Ki-67 value (available in 134 patients)	
median % [range]	20 [1-80]
<20%	63 (47)
$\geq 20\%$	71 (53)
Metastasis-free interval	
median [range], months	51 [0-370]
0 months (stage IV at diagnosis)	25 (17)
1-48 months	46 (32)
>48 months	74 (51)
First metastatic site	
Bone	124 (86)
Other	21 (14)
Metastatic sites	
Bone	145 (100)
Liver	67 (46)
Lung/pleura	58 (40)
CNS	14 (10)
Locoregional	59 (41)
No. of metastatic sites	
median [range]	2 [1-7]
1	42 (29)
2-3	87 (60)
>3	16 (11)
Skeletal involvement	
limited (1-2 sites)	37 (26)
extensive (≥ 3 sites)	108 (74)
Number of previous chemotherapy lines	
0	27 (19)
1-3	95 (65)
>3	23 (16)
Number of previous HT lines	
0	24 (17)
1-3	100 (69)
>3	21 (14)
CTC evaluation	145/145 (100)
Mean CTC number/7.5 mL ($\pm$ SD)	295 ($\pm$ 1204)
Median CTC number/7.5 mL (range)	17 (0-11064)
CTC count	
0 CTC	33/145 (23)
1-5 CTC	29/145 (20)
6-50 CTC	34/145 (23)
≥ 51 CTC	49/145 (34)
^{18} F-DG-PET/CT	72/145 (50)
Negative	7/72 (10)
Positive	65/72 (90)
Bone marrow biopsy	37/145 (26)
Negative	16/37 (43)
Positive	21/37 (57)

Table 2Associations between clinico-pathological characteristics and SREs or CTCs ≥ 5 .

Category	SREs (%)	p	CTC ≥ 5 (%)	p
Age				
<60 years	45/80 (56)	1.0	44/80 (55)	.397
≥ 60 years	37/65 (57)		41/65 (63)	
ECOG PS				
0-1	42/96 (44)	.597	42/96 (44)	<.0005
≥ 2	19/49 (39)		43/49 (88)	
Tumour subtype				
Luminal	71/120 (59)	.188	72/120 (60)	.507
Non-Luminal	11/25 (44)		13/25 (52)	
Ki-67 value (available in 134 patients)				
<20%	35/63 (56)	.485	36/63 (57)	.862
$\geq 20\%$	44/71 (51)		42/71 (59)	
Metastasis-free interval				
Synchronous	15/25 (60)	.825	18/25 (72)	.181
metachronous	67/120 (56)		67/120 (56)	
First metastatic site				
Bone	72/124 (58)	.476	77/124 (62)	.054
Other	10/21 (48)		8/21 (38)	
Metastatic sites (except bone)				
Liver	37/67 (55)	.867	42/67 (63)	.400
Lung/pleura	32/58 (55)	.865	30/58 (52)	.174
CNS	6/14 (43)	.396	12/14 (86)	.043
Locoregional	37/59 (63)	.236	40/59 (68)	.086
No. of metastatic sites				
1	24/42 (57)	.854	18/42 (43)	.033
2-3	50/87 (58)		55/87 (64)	
>3	8/16 (50)		12/16 (75)	
Skeletal involvement				
limited (1-2 sites)	18/37 (49)	.337	13/37 (35)	.001
extensive (≥ 3 sites)	64/108 (45)		72/108 (67)	
Number of previous chemotherapy lines				
0	18/27 (67)	.395	20/27 (74)	.193
1-3	53/95 (56)		52/95 (55)	
>3	11/23 (48)		13/23 (57)	
Number of previous HT lines				
0	13/24 (54)	.343	14/24 (58)	.812
1-3	60/100 (60)		60/100 (60)	
>3	9/21 (43)		11/21 (52)	
Skeletal related events				
Yes	-	-	48/82 (59)	1.0
No	-	-	37/63 (60)	
CTC				
<5	34/60 (57)	1.0	-	-
≥ 5	48/85 (57)			

Table 3

Skeletal related events incidence, type and treatment.

Characteristic	MBC patients (N = 145) N (%)
Skeletal related event	
Yes	82/145 (57)
No	63/145 (43)
SRE type	
Hypercalcemia	16/82 (20)
Spinal cord compression	3/82 (4)
Pathologic bone fracture	29/82 (35)
Orthopedic surgical intervention	0/82 (0)
Palliative radiation on bone	34/82 (41)
SRE medical treatment	
Denosumab	3/36 (8)
Biphosphonates	33/36 (92)
Zoledronic Acid	27/36 (75)
Pamidronate	6/36 (17)

of palliative radiotherapy on bone metastases. In 41/82 (50%) patients, SREs were synchronous at the date of CTC analysis and in 36/61 (59%) they occurred after a median of 17.2 months from the date of CTC analysis. In the whole series median time-to-SRE from CTC count was 18 months (range 0-156 months). Among patients requiring medical management for SREs, biphosphonates were the most frequently

administered agents (92%), whereas denosumab was used in only a small proportion of cases (8%). (Table 3).

3.3. Time-to-SRE and survival endpoints

After a median follow-up of 105 months from initial breast cancer diagnosis (last update January 2026), all patients had at least one disease progression and 140 (96%) were dead. Median TT-SRE, PFS and OS from CTC analysis for the whole series were 18 months [0-156], 5 months [0-156] and 14 months [1-157], respectively (Fig. 2). Predictors of shorter time-to-SRE at univariate analysis included luminal tumor subtype (HR .52, 95%CI .275-.995, p .048), extensive bone involvement (HR 1.90, 95%CI 1.116-3.244, p .18) and CTC ≥ 5 (HR 2.39, 95%CI 1.472-3.890, p < 0.0005). At multivariable analysis, only CTC ≥ 5 remained as an independent predictor of shorter TT-SRE (HR 2.08, 95%CI 1.266-3.403, p .004) (Table 4). Independent predictors of worse PFS at multivariate Cox analysis included the presence of visceral metastases (HR 1.54, 95%CI 1.061-2.243, p .023) and CTC ≥ 5 (HR 2.72, 95%CI 1.865-3.955, p < 0.0005) (Table 5). Independent predictors of worse OS at multivariate Cox analysis included the presence of visceral metastases (HR 1.72, 95%CI 1.174-2.512, p .005) and CTC ≥ 5 (HR 3.16, 95%CI 2.183-4.576, p < 0.0005) (Table 6).

4. Discussion

Skeletal-related events are common in BC patients with skeletal metastases and are major contributors of reduced QOL, morbidity and mortality. However, while identification of patients at high risk of SRE is of paramount importance, in clinical practice there are no validated factors to identify such high-risk patients during the course of disease. This study analyzed CTCs as potential predictive factors of skeletal-related events in bone metastatic BC patients.

In our series, SREs occurred in 57% of patients, with a median time-to-SRE from CTC analysis of 18 months. Nearly 60% of patients had CTCs ≥ 5 as detected by the CellSearch system. CTCs ≥ 5 were more frequent in patients with bone as first metastatic site, extensive skeletal involvement (>2 sites), CNS metastases, and a worse performance status (ECOG PS ≥ 2). Importantly, although CTCs ≥ 5 were not directly associated with a higher frequency of SREs at baseline, they were predictive of a significantly shorter time to first SRE and independently increased the risk of developing SREs at multivariable analysis (HR 2.08, p = 0.004). These findings suggest that elevated CTC levels may identify a subgroup of patients with biologically more aggressive bone disease and a higher propensity to develop skeletal complications earlier during the disease course. Importantly, the prognostic role of CTC enumeration was not limited to skeletal complications. Patients with CTC counts ≥ 5 /7.5 mL also experienced significantly shorter progression-free survival and overall survival, and CTC positivity remained an independent predictor of TT-SRE, PFS, and OS in multivariable analyses. Taken together, these findings indicate that baseline CTC evaluation correlates with clinically meaningful outcomes and may contribute to the identification of patients with a more aggressive disease phenotype who are at increased risk of early skeletal complications and poorer prognosis.

Several limitations should be considered when interpreting our findings and before implementing CTC assessment in routine clinical practice. First, although CTC analysis was prospectively performed, the present study represents a retrospective analysis of a single-center cohort and CTC enumeration was based on a single baseline assessment, preventing evaluation of dynamic changes during treatment and disease evolution. Second, patients underwent CTC evaluation at different time points during the course of metastatic disease. However, since previous studies have consistently demonstrated that CTC enumeration retains its prognostic value regardless of the timing of assessment [19], we believe that this heterogeneity is unlikely to have substantially affected our findings. Third, no molecular characterization of CTCs or tumor genomic profiling was available; therefore, we could

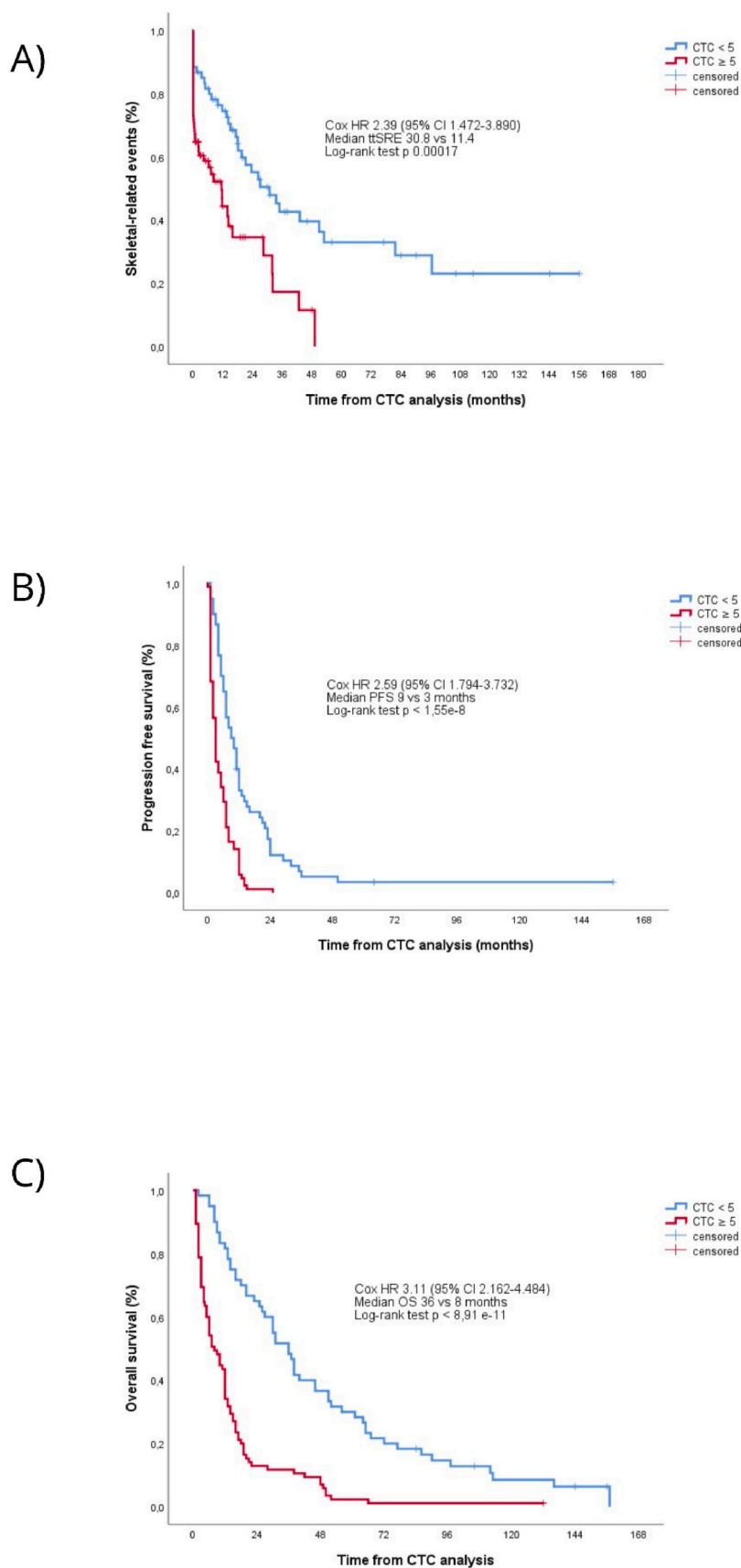


Fig. 2. Impact of circulating tumor cells (CTCs) on clinical outcomes (A) Kaplan-Meier curve for time to skeletal-related events (SRE) according to CTC count (<5 vs ≥ 5). (B) Kaplan-Meier curve for progression-free survival (PFS) by CTC count (<5 vs ≥ 5). (C) Kaplan-Meier curve for overall survival (OS) based on CTC count (<5 vs ≥ 5).

Table 4

Uni-/Multivariate analyses of prognostic factors for Time to SRE in MBC with bone metastases.

Variable		Univariate analysis				Multivariate analysis		
		ttSRE months	HR	95% CI	p	HR	95% CI	p
Age	<60	14.2	.900	.581-1.393	.636	-	-	-
	≥60	26.3						
Tumor Subtype	Luminal	15.1	.523	.275-.995	.048	.572	.298-1.099	.094
	Non-Luminal	42.7						
Ki67	<20%	30.8	1.56	.996-2.451	.052	-	-	-
	≥20%	11.7						
Grade	I	50.9	2.00	.888-4.518	.094	-	-	-
	II	14.2						
	III	15.9						
Bone involvement	Limited (1-2 sites)	49.1	1.90	1.116-3.244	.018	1.67	.966-2.893	.066
	Extensive (≥3 sites)	13.8						
Bone mets	metachronous	19.6	1.72	.974-3.046	.061	-	-	-
	synchronous	7.4						
Visceral metastases	no	28.4	1.50	.922-2.454	.102	-	-	-
	yes	14.2						
CTC	<5	30.8	2.39	1.472-3.890	<.0005	2.08	1.266-3.403	.004
	≥5	11.4						

Table 5

Uni-/Multivariate analyses of prognostic factors for PFS in MBC with bone metastases.

Variable		Univariate analysis				Multivariate analysis		
		PFS months	HR	95% CI	p	HR	95% CI	p
Age	<60	5.0	.834	.598-1.164	.286	-	-	-
	≥60	6.0						
Tumor Subtype	Luminal	5.0	.762	.486-1.196	.238	-	-	-
	Non-Luminal	6.0						
Ki67	<20%	6.0	1.26	.893-1.786	.187	-	-	-
	≥20%	6.0						
Grade	I	4.0	1.40	.770-2.544	.271	-	-	-
	II	5.0						
	III	7.0						
Bone involvement	Limited (1-2 sites)	7.0	1.22	.833-1.779	.309	-	-	-
	Extensive (≥3 sites)	5.0						
Bone mets	metachronous	5.0	1.14	.733-1.774	.562	-	-	-
	synchronous	3.0						
Visceral metastases	no	7.0	1.48	1.017-2.143	.040	1.54	1.061-2.243	.023
	yes	4.0						
CTC	<5	9.0	2.59	1.794-3.732	<.0005	2.72	1.865-3.955	<.0005
	≥5	3.0						
SRE	yes	4.0	.980	.699-1.373	.905	-	-	-
	no	6.0						

Table 6

Uni-/Multivariate analyses of prognostic factors for OS in MBC with bone metastases.

Variable		Univariate analysis				Multivariate analysis		
		OS months	HR	95% CI	p	HR	95% CI	p
Age	<60	12.0	.926	.663-1.294	.652	-	-	-
	≥60	16.0						
Tumor Subtype	Luminal	14.0	.792	.501-1.251	.317	-	-	-
	Non-Luminal	13.0						
Ki67	<20%	16.0	1.27	.893-1.794	.185	-	-	-
	≥20%	13.0						
Grade	I	18.0	1.32	.736-2.348	.355	-	-	-
	II	14.0						
	III	13.0						
Bone involvement	Limited (1-2 sites)	30.0	1.35	.921-1.973	.125	-	-	-
	Extensive (≥3 sites)	12.0						
Bone mets	metachronous	15.0	1.56	.998-2.421	.051	-	-	-
	synchronous	7.0						
Visceral metastases	no	21.0	1.70	1.165-2.483	.006	1.72	1.174-2.512	.005
	yes	12.0						
CTC	<5	36.0	3.11	2.162-4.484	<.0005	3.16	2.183-4.576	<.0005
	≥5	8.0						
SRE	yes	10	.794	.566-1.114	.183	-	-	-
	no	17						

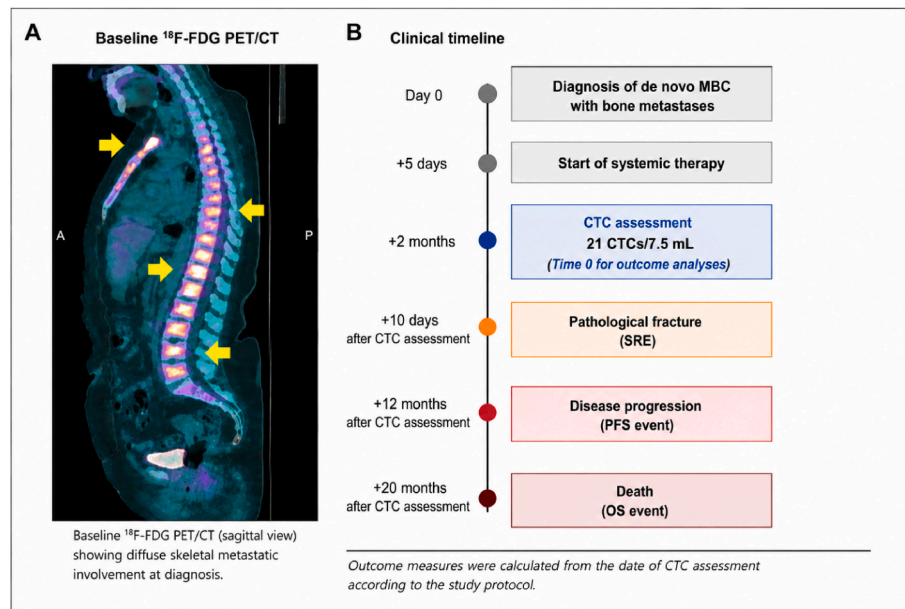


Fig. 3. Representative clinical course of a patient with elevated baseline CTC count and subsequent adverse clinical outcomes. This case illustrates how an elevated baseline CTC count (21 CTCs/7.5 mL), measured 2 months after de novo metastatic breast cancer with bone involvement, was followed by an early skeletal-related event (10 days), disease progression at 12 months, and death at 20 months. It exemplifies the potential ability of CTC enumeration to anticipate adverse clinical outcomes.

not investigate whether specific molecular alterations contributed to the increased risk of skeletal complications observed in patients with elevated CTC counts. Finally, patients were treated according to the standards of care adopted between 2009 and 2012. Bone-targeted agents were administered after the occurrence of skeletal-related events rather than prophylactically, and denosumab was used in only a minority of patients. Nevertheless, these aspects should also be interpreted in light of the distinctive strength of our study, which, to the best of our knowledge, represents the largest cohort of breast cancer patients with bone metastases prospectively followed for nearly 10 years after a single CTC assessment. Although the CellSearch® platform remains the only FDA-cleared and clinically validated method for CTC enumeration, prospective multicenter studies are warranted to establish whether CTC-guided clinical decision-making can improve patient outcomes before its implementation in routine clinical practice.

Our results of CTCs in 60% of bone metastatic BC patients are in line with a seminal study from the MD Anderson research group [20] that reported CTCs ≥ 5 in 61% of patients with bone metastases compared to 16% of patients without bone metastases. Furthermore, they reported that extensive bone involvement correlated with higher CTC numbers and that this had a worse prognostic outcome, challenging the common belief of the bone as an indolent metastatic site. As a hypothetical explanation for higher CTC count in bone disease they argued that in extensive BMs, BoMa infiltration could occur. In our series, a limited number of patients performed a BoMa biopsy. In the 21 patients with BoMa neoplastic infiltration, we found a not significant association with extensive bone involvement and a borderline association with very high (≥ 1000) CTC numbers suggesting that extensive BMs also involve the BoMa niche which, in turn, would act as a facilitator of CTC spread. However, these results are based on limited numbers of patients and should be taken with caution as hypotheses-generating.

Interestingly, elevated CTC count at the onset of bone metastases appeared to identify patients with a more aggressive disease phenotype. Experimental evidence suggests that mTOR signaling pathways regulate the balance between dormancy and proliferation of bone marrow-resident disseminated tumor cells and CTCs in breast cancer [20]. Therefore, our findings further support the concept that tumor biological activity may contribute not only to metastatic dissemination but

also to the earlier development of skeletal complications, with potential therapeutic implications in the era of CDK4/6 inhibitors [21].

Higher CTC levels were statistically associated with a worse ECOG performance status ≥ 2 and with an extensive skeletal involvement. Results from the largest pooled analysis of CTC individual data in MBC patients published to date, have identified CTC ≥ 5 as the best cut-off to identify two different prognostic categories within stage IV MBC (namely Stage IV_{indolent} [CTC<5] and Stage IV_{aggressive} [CTC ≥ 5]) in terms of PFS and OS [22]. By adopting the same stratification process we confirmed different survival strata across our series of bone metastatic BC patients. Therefore, patients with higher CTCs numbers intrinsically have a biological and clinical more aggressive disease that translates into a significant burden of disease signs and symptoms as measured by the ECOG performance status.

As SREs are preventable and most international guidelines advocate for early adoption of bone-directed agents, a CTC-guided prognostic stratification in MBC with bone metastases could address appropriate treatments in a tailored way to patients that can benefit most from these therapies. In this study, 61% of cases with luminal A/B cancers were associated with CTC ≥ 5 and those patients received 1-3 lines of hormonal therapy. Prolonged estrogen deprivation can increase androgens levels in postmenopausal breast cancer patients and expression of androgen receptor (AR) has been demonstrated on CTCs in breast cancer patients with bone metastases [23]. Several lines of evidence exist that bone metastases are more frequent in patients with endocrine-sensitive breast cancer and that endocrine interventions can alter the bone pre-metastatic niche [24]. Therefore, a liquid biopsy with AR-positive-CTCs should be explored as a specific dynamic marker of bone metastases and SREs in this histological subtype [25]. Finally, as the anti-RANK ligand (RANKL) monoclonal antibody, denosumab, is currently used for treatment of bone metastases and prevention of SREs, a CTC-based liquid biopsy with RANKL determination would represent an useful tool [26].

To further illustrate the potential clinical applicability of our findings, an exemplary case from our cohort is shown in Fig. 3. The patient presented with de novo metastatic breast cancer with bone involvement and underwent CTC assessment two months after diagnosis, revealing 21 CTCs/7.5 mL. Ten days after CTC evaluation, she experienced a

pathological fracture as her first skeletal-related event, followed by disease progression 12 months later and death after 20 months. Although this single case cannot establish causality, it exemplifies the temporal association between an elevated CTC count and adverse clinical outcomes observed throughout our study population, thereby facilitating the clinical interpretation of our findings.

In conclusion, in this series of MBC patients with bone metastases and long-term follow-up, we demonstrated that elevated CTC levels are associated with more extensive skeletal disease and independently predict a shorter time-to-SRE, as well as worse survival outcomes. Although CTCs are unlikely to represent direct mechanistic determinants of SREs, they may serve as clinically relevant biomarkers identifying patients at increased risk of early skeletal complications. Although the CellSearch methodology we used for CTC assessment has been progressively complemented by newer technologies, it still represents a robust and clinically validated approach for CTC enumeration; therefore our findings further contribute to the understanding of the complex interactions among primary BC, bone metastases, bone marrow dissemination and skeletal complications, and may support the design of future studies focused on personalized prevention and treatment strategies for SREs in metastatic breast cancer.

Statement

The present study has been conducted according to the Declaration of Helsinki and has been approved by the Institutional Review Board (NP605-2009). Each patient signed a specific informed consent form before the analysis of CTCs. Patients were managed outside an interventional clinical trial and CTCs information had no impact on the therapeutic decisions taken by the multidisciplinary team of the Institutional Breast Cancer Unit.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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