

# Circulating Tumor Cells With Epithelial-to-mesenchymal Transition Phenotypes Associated With Inferior Outcomes in Primary Breast Cancer

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**Abstract.** *Background/Aim:* Circulating tumor cells (CTCs) comprise a heterogeneous population of cancer cells with different clinical and biological value. The aim of this study was to evaluate the prognostic value of CTCs with an epithelial-mesenchymal transition (EMT) phenotype in primary breast cancer (PBC) patients. *Patients and Methods:* This study included 427 primary breast cancer patients. RNA extracted from CD45-depleted peripheral blood mononuclear cell (PBMCs) was evaluated for the expression of EMT transcription factors (TWIST1, SNAIL1, SLUG, ZEB1) by quantitative real time polymerase chain reaction (qRT-PCR). *Results:* In total, CTC EMT was detected in 77 (18.0%) patients. Patients without detectable CTC EMT in peripheral blood had significantly longer disease-free survival than patients with detectable CTC EMT. The prognostic value of CTC EMT was demonstrated in all subgroups of patients. *Conclusion:* CTCs with an EMT phenotype have a prognostic value in primary breast cancer.

Despite important breakthroughs in breast cancer prevention, diagnosis, and treatment, 5-10% of patients with

breast cancer present with metastasis at initial diagnosis, while approximately 20-30% of breast cancer patients develop metastasis during the course of the disease (1, 2). The metastatic cascade represents a multistep process involving the dissemination of tumor cells from the primary tumor site and their subsequent seeding to a distant location, where they can potentially generate a new cancerous growth (3). To execute the metastatic cascade, epithelial cancer cells must detach from the primary tumor, invade locally into surrounding tissues, intravasate into the peripheral circulation, survive during hematogenous transit, extravasate at distant tissues and subsequently create micrometastatic colonies that grow into new metastatic lesions (3).

An increasing number of studies have shown that several types of cancer cells often undergo epithelial-to-mesenchymal transition (EMT) to acquire tumor-initiating capabilities (4, 5). During EMT, epithelial cells upregulate mesenchymal gene expression patterns and down-regulate epithelial-related genes. Moreover, epithelial cells undergo reorganization of their cytoskeleton, loss of cell-cell contacts and apical-basal cell polarity, which results in increased motility of individual cells and the development of an invasive phenotype (6).

This switch in cell behavior is controlled by several signaling pathways, including TGF- $\beta$ 1, Wnt, Notch and Hedgehog signaling. The reprogramming of gene expression during EMT is mediated by overexpression of transcription factors (TFs), including TWIST1, SNAIL1, SLUG, ZEB1 and/or FOXC1/2 in mammary epithelial cells and/or breast cancer cells (4, 7).

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Numerous data suggest a close relationship between activation of EMT and the generation of circulating tumor cells (CTCs) (8-11). As described before, initiation and progression of EMT is closely associated with extracellular matrix remodeling and changes in cell–cell interactions, including increased expression of proteases that degrade the extracellular matrix. Finally, all of these mechanisms facilitate invasion and intravasation of epithelial cancer cells into the bloodstream (12, 13).

Furthermore, EMT has been closely connected to the stem cell phenotype and resistance to apoptotic signals, helping EMT-derived CTCs survive in foreign environments (5). EMT is associated with immunity, and activation of the EMT program induces T-regulatory cells and impairs dendritic cells, suggesting an immunosuppressive effect of EMT that might support cancer dissemination mediated by CTCs (14-17). Yang *et al.* showed that inhibition of the transcription factor TWIST, a master regulator of morphogenesis, in the highly metastatic 4T1 murine mammary cancer cell line reduced the metastatic burden and was also associated with a decreased number of CTCs in mice bearing xenograft mammary tumors. These results suggest a link among EMT, metastasis and the presence of CTCs (4). There is a continuum of CTC phenotypes that range from an epithelial phenotype to a mesenchymal phenotype, including those with a partial EMT phenotype (8, 9, 11), indicating that CTCs exhibit dynamic changes in their epithelial and mesenchymal composition (8).

Circulating tumor cells play a crucial role in the metastatic cascade, tumor dissemination and progression (3, 18). The prognostic value of CTCs was consistently demonstrated by a number of trials in metastatic as well as in primary breast cancer (19-23). Interestingly, all of these studies evaluated the prognostic value of CTCs that were defined based on the expression of epithelial markers. However, CTCs represent a heterogeneous population of cells, including CTCs with partial or complete EMT phenotypes. These subpopulations have different clinical and biological characteristics (18). While CTCs with an epithelial phenotype in breast cancer have been shown to be prognostically unfavorable, the prognostic value of CTCs with an EMT phenotype in primary breast cancer remains unknown.

In prior studies, our group and others showed that several established and robust methods for CTC detection, including the U.S. Food and Drug Administration-approved CellSearch® system (Veridex LLC, Warren, NJ, USA) and the real-time polymerase chain reaction-based AdnaTest Breast Cancer Select/Detect kit or AdnaTest™ (AdnaGen AG, Langenhagen, Germany), consistently showed the prognostic value of detecting CTCs but were unable to detect CTCs that underwent complete EMT (11). Therefore, we established a new CTC detection method that was based on the detection of EMT-TFs (11) and was able to detect CTCs with partial or complete EMT phenotypes. The aim of this study was to

evaluate the prognostic value of CTCs with an EMT phenotype in primary breast cancer.

## Patients and Methods

**Study patients.** This prospective translational study (Protocol TRU-SK 002; Chair: M. Mego) included 427 patients with stage I-III primary breast cancer (PBC) who were undergoing surgery. CTCs were detected in 5 ml of peripheral blood drawn on the morning of the surgical procedure. In all patients, complete diagnostic evaluation was performed to exclude the presence of distant metastasis. Patients with concurrent malignancy other than non-melanoma skin cancer in the previous 5 years were excluded. Data regarding age, tumor stage, histology, regional lymph node involvement, hormone receptor status, and HER2 status were also recorded for all patients.

This study was approved by the Institutional Review Board (IRB) of the National Cancer Institute of Slovakia and was conducted between March 2012 and February 2015. Each participant provided signed informed consent before study enrollment. Healthy donors (N=60) were age-matched women without breast cancer who were recruited and consented according to the IRB-approved protocol. Each healthy donor participant signed an informed consent.

**Detection of CTCs in peripheral blood.** CTCs were detected in peripheral blood by a quantitative real-time polymerase chain reaction (qRT-PCR)-based assay of peripheral blood that previously underwent CD45 positive (CD45+) cell depletion for CTC enrichment, as described previously. Briefly, peripheral blood samples were depleted of CD45+ leukocytes using the Rosette Sep™ kit (Stem Cell Technologies, Vancouver, BC, Canada). RNA isolated from CD45-depleted peripheral blood samples was reverse transcribed to cDNA and subsequently subjected to qRT-PCR to analyse expression of epithelial-mesenchymal transition (EMT-TF) genes (*TWIST1*, *SNAIL1*, *SLUG* and *ZEB1*) (24, 25). Patient samples with higher EMT-TF (*TWIST1*, *SNAIL1*, *SLUG* and *ZEB1*) gene transcript levels than those of healthy donors were scored as CTC EMT positive based on our preclinical study and human sample testing (11). These highest expression values in healthy donors were used as a “cut-off” to determine CTC positivity.

**Statistical analysis.** The patients’ characteristics were summarized using the median (range) for continuous variables and frequency (percentage) for categorical variables. The median follow-up period was calculated as the median observation time among all patients and among those who were still alive at the time of their last follow-up. Disease-free survival (DFS) was calculated from the date of CTC measurement to the date of disease recurrence (locoregional or distant), secondary cancer, death or last follow-up. DFS was estimated using the Kaplan–Meier product limit method and compared between groups by the log-rank test. Univariate analyses with Chi-squared or Fisher’s exact test were performed to find associations between prognostic factors and CTC status. A multivariate Cox proportional hazards model for DFS was used to assess differences in outcome on the basis of CTC EMT status (present vs. absent), hormone receptor status (positive for either vs. negative for both), HER-2 status (positive or negative), tumor size (T1 vs. T2 and higher), axillary lymph node involvement (N0 vs. N+) and Ki67 status (<20% vs. >20%). Stepwise regression techniques were used to build multivariate models using a significance level of 0.10 to remain in the model. All *p*-values

presented are two-sided, and associations were considered significant if the *p*-value was less than or equal to 0.05. Statistical analyses were performed using NCSS 11 Statistical Software (2016, NCSS, LLC., Kaysville, UT, USA, [ncss.com/software/ncss](http://ncss.com/software/ncss)).

## Results

The study population consisted of 427 primary breast cancer patients with a median age of 60 years (range=2-83 years). The patient characteristics are shown in Table I. There were 364 (85.2%) patients with estrogen receptor-positive (ER) and/or progesterone receptor-positive (PR) tumors; 66 (15.5%) patients with HER2/neu-amplified tumors. The majority of patients had good prognoses, with tumor sizes of less than 2 cm (69.1%), without axillary lymph node involvement (63.9%), and with low/intermediate grade (64.9%).

Data regarding adjuvant treatment were available for 384 (89.9%) patients. Adjuvant chemotherapy was administered to 220 (57.3%) patients. Fifty patients (13.0%) received adjuvant chemotherapy only, 149 (38.8%) patients received hormonal therapy only, while 170 (44.3%) patients received adjuvant chemotherapy and subsequent adjuvant hormonal therapy. Of the 327 patients with hormone receptor-positive tumors, 174 (53.2%) received adjuvant chemotherapy, and 315 (96.3%) received adjuvant hormonal therapy. Of the 58 HER2-positive patients, 15.1% of the total number of patients, 47 (81.0%) received adjuvant trastuzumab with chemotherapy, 8 (13.8%) received trastuzumab with hormonal treatment, and 3 (5.2%) did not receive any adjuvant treatment.

**CTC detection.** To detect overexpression of the EMT-inducing TF gene transcripts in PBC patients, we compared the expression levels in patient samples with those of HDs as described previously (see CTC detection in the Methods section) (11, 24, 25).

In each patient, CTCs were assessed in one sample of 5 ml of PB. In total, CTC EMT was detected in 77 (18.0%) patients. Compared to the highest levels of Snail and Zeb1 transcripts detected in HD samples, none of the patient samples overexpressed these gene transcripts. Among the patient samples, Twist1 and Slug transcripts were overexpressed in 74 (17.3%) and 4 (0.9%) samples, respectively. In one patient sample, there was overexpression of two EMT-inducing TF gene transcripts (Slug and Twist1), *e.g.*, expression of both genes was higher than the cut-off value in that sample.

**Association between CTC EMT and patient/tumor characteristics.** The patients' characteristics and their associations with CTC EMT are shown in Table II. Presence of CTC EMT was not associated with any patient/tumor characteristics except for p53 status (CTC EMT was present in 21.1% of p53 negative vs. 13.1% p53-positive tumors, *p*=0.05).

Table I. *Patients characteristics.*

	N	%
All patients	427	100.0
Histology		
Invasive ductal carcinoma	364	85.2
Other	63	14.8
Grade		
Low and intermediate	277	64.9
High grade	139	32.6
Unknown	11	2.6
T stage		
T1	295	69.1
>T1	132	30.9
N stage		
N0	273	63.9
N+	147	34.4
Unknown	7	1.6
Hormone receptor status (cut-off 1%)		
Negative for both	63	14.8
Positive for either	364	85.2
HER2 status		
Negative	361	84.5
Positive	66	15.5
Ki67 status		
<20%	257	60.2
>20%	168	39.3
Unknown	2	0.5
Molecular subtype		
Luminal A	215	50.4
Luminal B	100	23.4
HER2+	66	15.5
Triple negative	44	10.3
Unknown	2	0.5
p53 status		
Negative	266	62.3
Positive	160	37.5
Unknown	1	0.2
BCL-2 status		
Negative	118	27.6
Positive	308	72.1
Unknown	1	0.2
CTC_EMT		
Negative	350	82.0
Positive	77	18.0

**Prognostic value of CTC EMT in primary breast cancer.** At a median follow-up time of 55.0 months (range=4.9-76.7 months), 74 patients (17.3%) had experienced a DFS event, and 36 patients (8.4%) had died. Herein, we present DFS analysis due to the immaturity of overall survival data. Patients without detectable CTC EMT in the peripheral blood had significantly longer DFS than patients with detectable CTC EMT (HR=0.42, 95%CI=0.22-0.78, *p*=0.0003).

The estimated 2- and 5-year DFS for CTC EMT-negative vs. CTC EMT-positive patients was 94.6% and 85.5% vs. 88.3% and 59.6%, respectively (Figure 1). The prognostic

Table II. Association between patients/tumor characteristics and CTC\_EMT.

CTC_EMT	N	CTC positive		CTC negative		p-Value
		N	%	N	%	
Histology						
Invasive ductal carcinoma	364	69	19.0	295	81.0	0.29
Other	63	8	12.7	55	87.3	
Grade						
Low and intermediate	277	46	16.6	231	83.4	0.50
High grade	139	27	19.4	112	80.6	
Unknown	11					
T-stage						
T1	295	50	16.9	245	83.1	0.41
>T1	132	27	20.5	105	79.5	
N stage						
N0	273	45	16.5	228	83.5	0.42
N+	147	29	19.7	118	80.3	
Unknown	7					
Hormone receptor status (cut-off 1%)						
Negative for both	63	13	20.6	50	79.4	0.59
Positive for either	364	64	17.6	300	82.4	
HER2 status						
Negative	361	65	18.0	296	82.0	1.00
Positive	66	12	18.2	54	81.8	
Ki67 status (cut-off 20%)						
<20%	257	45	17.5	212	82.5	0.70
>20%	168	32	19.0	136	81.0	
Unknown	2					
Molecular subtype						
Luminal A	215	40	18.6	175	81.4	0.99
Luminal B	100	17	17.0	83	83.0	
HER2+	66	12	18.2	54	81.8	
Triple negative	44	8	18.2	36	81.8	
Unknown	2					
P53 status						
Negative	266	56	21.1	210	78.9	0.05
Positive	160	21	13.1	139	86.9	
Unknown	1					
BCL-2						
Negative	118	26	22.0	92	78.0	0.21
Positive	308	51	16.6	257	83.4	
Unknown	1					

value of CTC EMT was demonstrated in all subgroups of patients, and was most pronounced in the hormone receptor-positive, HER2-negative subgroup; however, in some subgroups, the differences did not reach statistical significance (Table III, Figure 2). The prognostic value of CTC EMT was demonstrated for patients treated with adjuvant chemotherapy, hormonal therapy or both modalities; however, this prognostic value was limited in patients to whom no adjuvant therapy was administered or who received adjuvant chemotherapy or hormonal therapy as the sole adjuvant modalities (Table IV). In multivariate analysis, the presence of CTC EMT, axillary nodal

involvement and hormone receptor status were independently associated with DFS (Table V).

## Discussion

In this prospective translational study, we demonstrated for the first time the prognostic value of CTCs with an EMT phenotype in primary breast cancer. The prognostic value of CTC EMT was demonstrated in all subgroups of patients, although some of these differences did not reach statistical significance. This observation could be influenced by the small sample size; however, the hazard

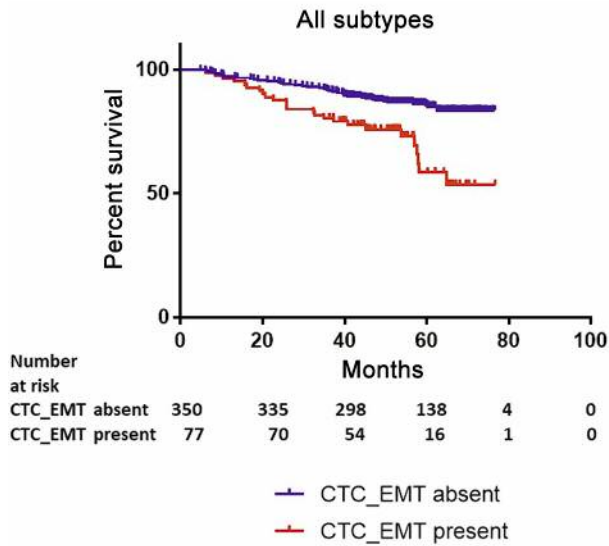


Figure 1. Kaplan–Meier estimates of probabilities of disease-free survival according to CTC\_EMT status ( $n=427$ ), Hazard ratio=0.42, 95%CI=0.22-0.78,  $p=0.0003$ .

ratios were consistent for all subgroups, ranging from 0.22 to 0.58. Surprisingly, the prognostic value of CTC EMT was most pronounced in the hormone receptor-positive and HER2-negative subgroups, which generally represent the patient population with the best outcome. The vast majority of these patients received adjuvant hormonal therapy, and approximately half of them received adjuvant chemotherapy as well, suggesting that current adjuvant treatment is not able to overcome treatment resistance associated with EMT and a stem cell-like phenotype. CTC EMT is related to cancer stem cell-like properties (9, 10) and to the resistance to conventional chemotherapy and radiation therapy. In a previous study, we observed an increased CTC EMT detection rate after neoadjuvant chemotherapy (11), while this study included treatment-naïve patients only. Therefore, the presence of CTC EMT was not affected by previous anticancer therapy, suggesting an intrinsic increase in the dissemination capabilities of tumors producing CTC EMT as well as an increase in their intrinsic treatment resistance.

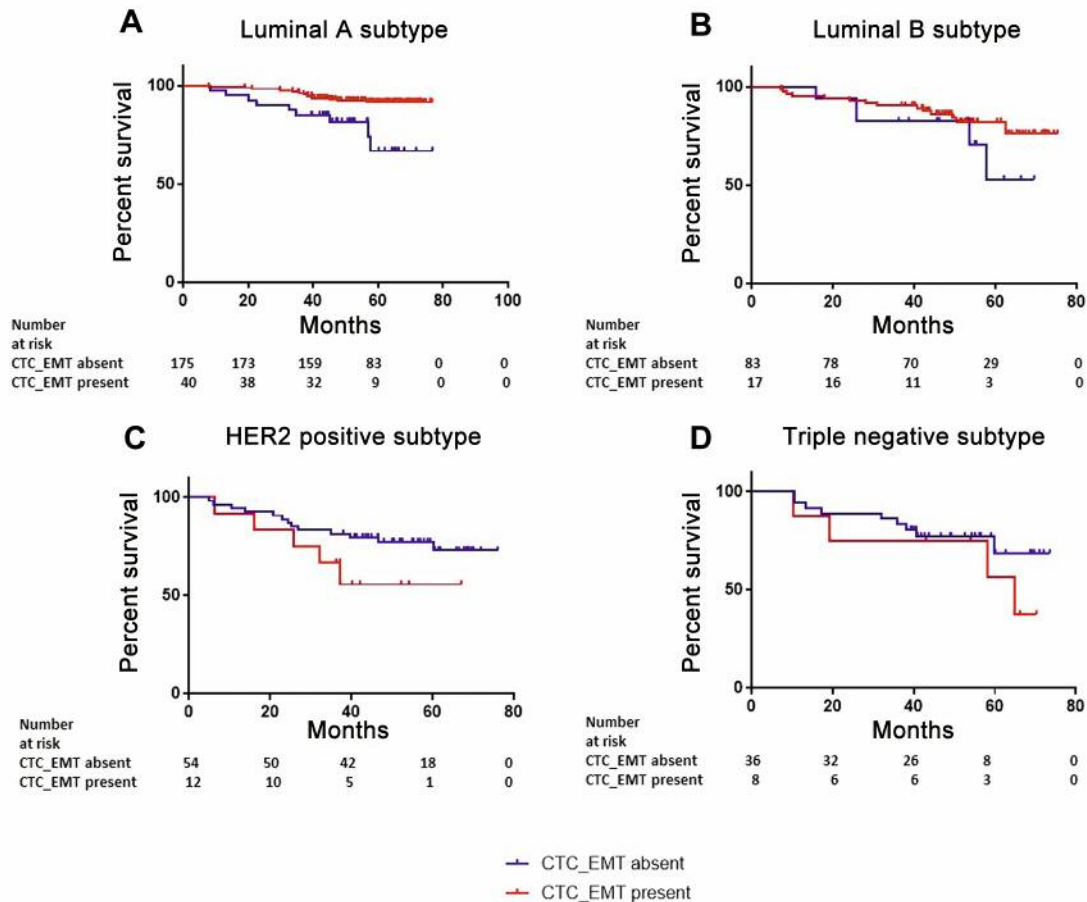


Figure 2. Kaplan–Meier estimates of probabilities of disease-free survival according to CTC\_EMT status in different molecular subtypes. A: Luminal A; B: Luminal B; C: HER2 positive; D: Triple negative.

Table III. Kaplan–Meier estimates of the disease-free survival by baseline CTC\_EMT groups of absent versus present.

	N	HR Low CI	95% High CI	95% p-Value
Overall				
CTC EMT absent	350	0.42	0.22	0.78
CTC EMT present	77			0.0003
Invasive ductal carcinoma				
CTC EMT absent	295	0.43	0.22	0.83
CTC EMT present	69			0.001
Other histology				
CTC EMT absent	55	0.34	0.05	2.20
CTC EMT present	8			0.10
Intermediate/low grade				
CTC EMT absent	231	0.37	0.13	1.05
CTC EMT present	46			0.01
High grade				
CTC EMT absent	112	0.50	0.23	1.08
CTC EMT present	27			0.03
T1 stage				
CTC EMT absent	245	0.42	0.18	0.99
CTC EMT present	50			0.009
T2 stage and higher				
CTC EMT absent	105	0.46	0.19	1.10
CTC EMT present	27			0.03
N0 stage				
CTC EMT absent	228	0.41	0.15	1.12
CTC EMT present	45			0.02
N+ stage				
CTC EMT absent	118	0.45	0.20	1.01
CTC EMT present	29			0.02
ER/PR positive for either				
CTC EMT absent	300	0.39	0.19	0.82
CTC EMT present	64			0.0009
ER/PR negative for both				
CTC EMT absent	50	0.57	0.19	1.71
CTC EMT present	13			0.24
HER positive				
CTC EMT absent	54	0.48	0.13	1.71
CTC EMT present	12			0.15
HER negative				
CTC EMT absent	296	0.40	0.20	0.82
CTC EMT present	65			0.0009
Ki67 low 20%				
CTC EMT absent	212	0.31	0.11	0.84
CTC EMT present	45			0.001
Ki67 high 20%				
CTC EMT absent	136	0.56	0.26	1.22
CTC EMT present	32			0.08
Triple negative				
CTC EMT absent	36	0.56	0.14	2.18
CTC EMT present	8			0.32
P53 negative				
CTC EMT absent	210	0.56	0.28	1.13
CTC EMT present	56			0.06
P53 positive				
CTC EMT absent	139	0.22	0.06	0.84
CTC EMT present	21			0.0002
BCL2 negative				
CTC EMT absent	92	0.40	0.16	0.99
CTC EMT present	26			0.01
BCL2 positive				
CTC EMT absent	257	0.47	0.21	1.07
CTC EMT present	51			0.02

HR: Hazard ratio; CI: confidence interval.

Table IV. Kaplan–Meier estimates of the disease-free survival by baseline CTC\_EMT and adjuvant treatment (N=384).

Variable	N	HR Low CI	95% High CI	95% p-Value
Overall				
CTC EMT absent	315	0.42	0.22	0.81
CTC EMT present	69			0.0007
Adjuvant chemotherapy				
CTC EMT absent	176	0.36	0.17	0.79
CTC EMT present	44			0.0008
No adjuvant chemotherapy				
CTC EMT absent	139	0.65	0.21	2.01
CTC EMT present	25			0.38
Chemotherapy + hormonal therapy				
CTC EMT absent	137	0.26	0.1	0.68
CTC EMT present	33			0.0001
Chemotherapy only				
CTC EMT absent	39	0.97	0.27	3.57
CTC EMT present	11			0.97
Hormonal therapy				
CTC EMT absent	264	0.34	0.15	0.76
CTC EMT present	55			0.0002
No hormonal therapy				
CTC EMT absent	51	0.91	0.32	2.55
CTC EMT present	14			0.86
Hormonal therapy only				
CTC EMT absent	127	0.73	0.18	2.94
CTC EMT present	22			0.63
No adjuvant treatment				
CTC EMT absent	12	0.85	0.16	4.52
CTC EMT present	3			0.84

HR: Hazard ratio; CI: confidence interval.

The presence of CTC EMT was not associated with any patient/tumor characteristics except for the p53 status, where tumors with no p53 expression (negative) had a higher probability of CTC EMT in peripheral blood. In previous studies, we showed that increased expression of MMP1 but not EMT TFs in cancer cells and/or tumor-associated stroma correlated with the presence of CTC EMT in peripheral blood (24, 26). Activation of MMP1 is closely connected to EMT and degradation of the basement membrane, processes that facilitate CTC release into peripheral blood circulation. In metastatic breast cancer, our previous data indicated an association between CTC EMT, HER2 status and cancer stem cell-like cells defined as ALDH<sup>+</sup>CD133<sup>+</sup> (10). The lack of association of CTC EMT with established prognostic factors, including nodal involvement, hormone receptor and/or HER2 status and, in contrast, their association with the abovementioned specialized biological processes suggest the unique, independent biological and prognostic value of the detected CTC EMT.

Data regarding CTCs should always be interpreted within the context of the detection method used (18). In the current study, we detected CTCs by qRT-PCR, based on the expression of four EMT-TFs with aCTC pre-enrichment step

Table V. Multivariate analysis of factors associated with disease-free survival.

Variable	Univariate analysis (HR (95%CI), <i>p</i> -Value)	Multivariate analysis (HR (95%CI), <i>p</i> -Value)
CTC with EMT phenotype	0.42 (0.22-0.78)	0.43 (0.26-0.71)
absent vs. present	<i>p</i> =0.0003	<i>p</i> =0.0009
N stage	0.38 (0.23-0.62)	0.38 (0.24-0.61)
N0 vs. N+	<i>p</i> <0.00001	<i>p</i> =0.0001
ER/PR status	0.37 (0.19-0.75)	0.37 (0.22-0.63)
positive for either vs. negative for both	<i>p</i> =0.0001	<i>p</i> =0.0002

utilizing CD45-negative selection. Unfortunately, peripheral blood depleted of CD45<sup>+</sup> cells does not necessarily contain only CTCs. Therefore, we defined CTC positivity based on a cut-off value that was established as the highest expression of the corresponding gene in a population of healthy donors. CTC EMT detection was mainly based on the detection of the Slug EMT-TF, while Twist contributed to the detection of CTC EMT in only 4 patients, and Snail1 and Zeb1 did not contribute to the detection of CTC EMT at all. We hypothesize that this observation is probably due to the high specificity of Slug for CTC EMT detection, as none of the healthy donors expressed Slug in leucocyte (CD45<sup>+</sup>)-depleted peripheral blood. Alternatively, high background expression of Snail1 and Zeb1 precluded the usefulness of these EMT-TFs for CTC EMT detection using the CD45-depletion method. At the same time, the detection of CTC EMT triggered by other EMT-TFs beyond Slug and Twist1 could be detected in some patients' subgroups, and their detection could lead to further improvement prognostication of these patients.

Several translational studies demonstrated activation of EMT in a subpopulation of CTCs, including expression of EMT-inducing transcription factors in CTCs (8-11, 27). In a study by Aktas *et al.*, several EMT-associated markers (TWIST1, AKT2 and PI3K $\alpha$ ) were detected in CTCs using a reverse-transcription (RT-PCR)-based assay, and this study demonstrated that at least one of the EMT markers was expressed in 62% of the CTCs (28). In other studies, it was shown that phosphorylated EGFR, HIF1 $\alpha$ , HER2 and PI3K/Akt signaling kinases, which can regulate EMT, were present in CTCs (29-31). However, these studies used epithelial markers for capturing CTCs, suggesting that CTCs with partial or complete EMT phenotypes might have been excluded from these analyses. Moreover, data regarding the prognostic value of CTCs undergoing EMT are very limited and in primary breast cancer they are absent. For example, Yu *et al.* showed that serial CTC monitoring in 11 patients suggested an association of mesenchymal CTCs with disease progression (8). In addition, in response to therapy and disease progression, patients displayed reversible CTC phenotype shifts between mesenchymal and epithelial states. Moreover, mesenchymal CTCs were observed as both single

cells and multicellular clusters, expressing known EMT regulators (8). In metastatic breast cancer, we showed the prognostic value of CTC EMT in patients undergoing high-dose chemotherapy with autologous stem cell support (32). In another study utilizing a DEPArray-based strategy for CTC detection, worse prognosis was associated with patients who had CTCs co-expressing epithelial and mesenchymal markers (33). Moreover, the presence of the EMT-related biomarker platin3 (PLS3) on CTCs was a prognostic biomarker in patients with stage I-III cancer, particularly in patients with luminal-type and triple-negative-type tumors (34).

## Conclusion

In conclusion, in this large prospective translational study, we showed, for the first time, that CTCs with an EMT phenotype that were detected before surgery in primary breast cancer patients had a prognostic value. We suggest that the presence of CTC EMT could lead to better identification of patients with an increased risk of recurrence, especially in primary breast cancer patients with hormone receptor-positive or HER2-negative tumors. Moreover, these data suggest that CTC EMT is able to identify patients with extremely poor prognosis and who will receive limited benefit from current adjuvant treatments. Further identification of signaling pathways activated in primary tumors associated with this CTC subpopulation could lead to identification of new therapeutic targets.

## Conflicts of Interest

There are no competing financial interests in relation to the work described in this manuscript.

## Authors' Contributions

M-M, J-R, M-C and J-M participated in the conception and design of this study. M-M performed the statistical analysis, and G-M, T-S and D-M were involved in CTC detection. M-K, J-B, and D-P were involved in the patient recruitment and performed the breast surgery. S-J was involved in the collection of clinical data. M-M drafted the article and all Authors reviewed it critically for its important intellectual content.

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