Dissemination of Circulating Tumor Cell Clusters Occurs Early in Non‐metastatic Breast Cancer Patients

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Research article

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Dissemination of circulating tumor cell clusters occurs early in non-metastatic breast cancer patients

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Abstract

Background: Metastatic spreading is promoted by cancer cell seeding from the primary tumor into the bloodstream. In patients with metastatic breast cancer (MBC), the clinical relevance of circulating tumor cell clusters (CTC-clusters) has been extensively reported, while their study in earlier stages is limited. Several methods, besides the FDA-cleared CellSearch®, limited to the detection of epithelial-enriched clusters, can be used for the detection of CTC-clusters. We hypothesize that resorting to marker-independent approaches can improve CTC-cluster detection.

Methods: Blood samples collected from healthy donors and spiked-in with tumor mammospheres, or from BC patients, were processed for CTC-cluster detection with 3 technologies: CellSearch®, CellSieve™ filters, ScreenCell® filters. The number of CTC-clusters was compared among the technologies and analyzed in relation to patient characteristics and outcome.

Results: In spiked-in samples, the 3 technologies showed similar capability of recover epithelial mammospheres, whereas, in a series of 19 clinical samples processed in parallel with the CellSearch® and CellSieve™ filters (that allow the detection of both epithelial and non-epithelial clusters), CTC-clusters were detected in 53% of samples with the CellSearch®, *versus* 79% and 84% with the CellSieve™, when considering only epithelial or both epithelial and non-epithelial clusters, respectively.

Next, blood samples from 37 non-metastatic breast cancer (NMBC) and 23 MBC patients were processed using ScreenCell® filters for attaining both unbiased enrichment and marker-independent identification of clusters based on cytomorphological criteria. At baseline, CTC-clusters were detected in 70% of NMBC cases and in 20% of MBC patients (median number= 2, range 0–20, *versus* 0, range 0-15, P=0.0015). Among NMBC patients, clusters were slightly higher in women with node-positive than node-negative status (0 *versus* 3, P=0.1110) and were more frequently

observed in women with luminal-like and triple-negative tumors than in patients with HER2-positive disease (median CTC-cluster number =4, 5, and 0 for luminal-like, triple-negative, and HER2-positive BC, respectively, *P*=0.0467).

Conclusions: We demonstrated that CTC-cluster detection can be improved by a marker-independent enrichment and identification, and we reported that CTC-clusters are more frequently detected in NMBC than in MBC patients, suggesting that dissemination of CTC-clusters is an early event in BC natural history.

Keywords: circulating tumor cell clusters, liquid biopsy, metastatic breast cancer, non-metastatic breast cancer, circulating tumor microemboli, size-based enrichment

Background

Metastatic spreading is the main cause for death in patients diagnosed with cancer. This process is promoted in its initial steps by cancer cell seeding from the primary tumor into the blood stream. Accordingly, a large amount of data has been collected across different tumor types linking the dissemination of circulating tumor cells (CTCs) with both poor prognosis and treatment failure/resistance [1].

Nonetheless, single CTCs are inefficient in sustaining metastatic dissemination as, to be able to colonize new sites, they must overcome numerous obstacles such as avoid anoikis, escape immunological control by circulating immune cells, resist to sharing stress due to fluid circulation, resulting in the fact that most CTCs do not survive long in the circulation [2, 3]. Therefore, being able to interact with other CTCs or with other cells by generating homo- or heterotypic CTC-clusters appears a biologically reasonable solution for increasing the metastatic potential of CTCs once they are facing the hostile blood environment.

Functional studies employing animal models and patient-derived data [4-7] definitely support a role of CTC-clusters in tumor dissemination and metastasis formation in breast cancer (BC). Such studies also offer hints on the biology of clusters revealing the mechanistic basis for their association with poor outcome and suggesting possible targets for specific treatments aiming at interfere with CTC-clusters formation and metastatic dissemination.

It is well known that metastatic dissemination occurs at early stages and is followed by a prolonged dormant status of these early disseminated cells [8-10]. This observation is supported by data demonstrating that enumeration of single CTCs predicts progression-free survival (PFS) and overall survival (OS) also in non-metastatic breast cancer (NMBC) patients (women with no evidence for distant metastases), both prior [11, 12] or after [13] breast surgery. Therefore, addressing the

presence of CTC-clusters in BC patients without clinically overt metastases holds promise to gain important hints about the dissemination process.

However, this issue has not yet been addressed and, in BC, most studies evaluating the clinical relevance of CTC-clusters have been limited to patients with metastatic or advanced disease [14-20]. Overall, these studies suggest a direct association between detection of CTC-clusters and poor clinical outcome, although the heterogeneous patient case series, technical issues in CTC-cluster enumeration and variable definitions of CTC-clusters must be taken into account as possible limitations and confounding factors.

Noteworthy, all the mentioned studies used the CellSearch® for CTC-cluster detection, which is possibly not the ideal method for CTC-cluster identification. The CellSearch® is a platform specifically developed for assuring high detection of single CTCs with epithelial features and for attaining standardization of their enumeration [21]. No data are instead available on its performance for CTC-cluster detection both in terms of recovery and of the integrity of isolated clusters. The CellSearch® approach includes a CTC-enrichment step employing ferrofluid nanoparticles with antibodies targeting EpCAM, which operates a selection in favor of clusters with exquisite epithelial features and possibly excludes larger CTC-clusters [22], that could result into an underestimation in CTC-cluster enumeration. Moreover, epithelial-to-mesenchymal transition (EMT) is recognized as an important driver of tumor invasion and metastatic dissemination [23], and literature data supported an increasing detection of mesenchymal markers in CTC-clusters compared to single CTCs in breast cancer patients [24]. Thus, investigating the use of epitope-independent methods, compared to the CellSearch®, for CTC-cluster detection is urgently needed to be able to fully appraise the actual clinical value of CTC-cluster in BC.

To tackle technical issues in CTC-cluster enumeration we compared, in a series of spiked-in and clinical samples, the number of CTC-clusters recovered using the CellSearch® platform and two size-exclusion methods based on a short-time filtration that allows for the detection of both epithelial

and non-epithelial CTC-clusters. Thereafter, we implemented the recovery of CTC-clusters by filtration in a prospective study involving patients with both NMBC and MBC to analyze CTC-cluster detection with respect to patient and primary tumor features.

Methods

Cell cultures and spiking experiments

The MCF7 breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM/F-12 (Lonza, Slough, UK) medium supplemented with 10% fetal bovine serum (Lonza). Mammospheres were derived from MCF7 cells cultured as floating cells in MammoCult™ (StemCellTechnologies, Vancouver, Canada), a serum-free culture medium, supplemented with Heparin Solution (StemCell Technologies) at final concentration of 4 µg/mL, and Hydrocortisone (StemCell Technologies) at final concentration of 0.48 µg/mL. The cells were maintained in non-adherent condition (Corning® Ultra-Low Attachment flask, Corning Inc., Corning, NY, USA) at 37 °C, in humidified 5% CO2 and 5% O2. Authentication of cell lines by STR DNA profiling analysis was performed by the Genomic Core Facility at Fondazione IRCCS Istituto Nazionale Tumori (INT). We adopt a *Mycoplasma* contamination testing policy employing an ELISA approach (MycoAlert mycoplasma detection kit, Lonza) for regular testing.

For the spiking experiments, either single MCF7 cells or single mammospheres were manually captured under an inverted microscope using a p10 micropipette and directly spiked into phosphate-buffered saline (PBS) supplemented with human serum albumin (HSA 3% w/v, to mimic protein concentration of plasma), or into healthy donor blood collected in either CellSave Preservative Tubes (Menarini Silicon Biosystems, Bologna, Italy) for CellSieve™ and CellSearch® processing, or in

K_2EDTA BD Vacutainer tubes (BD, Franklin Lakes, NJ, USA) for ScreenCell® processing. Spiked-in samples were processed following the same protocols used for clinical samples, described in paragraphs 2.2 and 2.5.

Comparison of CellSearch® and CellSieve™ filters for CTC-cluster detection in clinical samples

Blood samples (15 mL) were collected in CellSave Preservative Tubes (Menarini Silicon Biosystems) from patients with MBC treated at the Robert H Lurie Comprehensive Cancer Center at the Northwestern University (Chicago, IL, USA). All patients provided written informed consent to participate in the study, which was approved by the institutional review board at the Robert H. Lurie Comprehensive Cancer Center of Northwestern University (NUDB16Z01). Each sample was divided into 2 aliquots (7.5 mL each) and processed in parallel with the CellSearch® (Menarini Silicon Biosystems) and with CellSieve™ filters (Creatv MicroTech, Potomac, MD, USA) within 1 day from collection. For CellSearch® processing, the CELLSEARCH® Circulating Tumor Cell Kit (Menarini Silicon Biosystems) was used following the manufacturer's instructions. Briefly, after immunomagnetic enrichment based on EpCAM expression, enriched CTCs were stained with fluorescently-labeled antibodies against cytokeratins (CK) (8, 18 and 19) and CD45 and with DAPI. The number of CTC-clusters (groups of ≥ 2 $CK^{pos}/CD45^{neg}$ cells) was evaluated using the CELLTRACKS ANALYZER II® System (Menarini Silicon Biosystems) by a trained technician. For CellSieve™ filters processing, the CellSieve™ Enumeration Kit (Creatv MicroTech) was used following the manufacturer's instructions. The blood samples were filtered through a microporous membrane with pores of 7 µm diameter and subsequently stained with fluorescently-labeled antibodies against CK (8, 18 and 19), CD45 and CD31 (an endothelial marker used to exclude endothelial cell clusters) and with DAPI. The number of CTC-clusters (i.e. groups of ≥ 2

175 $CK^{pos}/CD45^{neg}$ or ≥2 $CK^{neg}/CD45^{neg}/CD31^{neg}$ cells for CK^{pos} and CK^{neg} CTC-clusters, respectively)
176 was evaluated using a fluorescence microscope.

177 **Case series & blood sample collection timing**

178 Women with a histologically confirmed diagnosis of stage II and III BC (NMBC) were recruited at
179 Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy) prior to start of neoadjuvant
180 treatment as for clinical practice, whereas women with stage IV BC (MBC) were recruited prior to
181 start of the first line of treatment. All patients provided written informed consent before undergoing
182 any procedures and the CTC study was approved by the INT Institutional Review Board and Ethics
183 Committee on February 19 2013.
184 Blood samples were longitudinally collected from patients with NMBC (i) before starting
185 neoadjuvant treatment, (ii) during treatment, (iii) at the end of treatment, and (iv) after surgery (from
186 3 to 27 weeks). Blood samples were collected from patients with MBC before starting the first line of
187 treatment.
188 Pathological complete response (pCR) was defined as the absence of cancer cells in the surgical
189 specimens of breast and lymph nodes. Partial response (PR) and stable disease (SD) referred to
190 clinical assessment of response to treatment according to the WHO criteria, hence a >50% tumor
191 shrinkage occurred for a PR, and >25% tumor increase for progressive disease (PD), whereas stable
192 disease was neither PR nor PD.

193 **Patho-biological characterization of tumors**

194 Hormone receptor status was evaluated according to the American Society of Clinical Oncology
195 guidelines [25]. HER2 status was considered negative when the immune-histochemical score was 0–
196 1, or 2+ with a negative chromogenic in situ hybridization result [26]. Ki-67 labeling index was
197 assessed by the MIB-1 monoclonal antibody by counting invasive cancer cells at the tumor

198 periphery, without focusing on hot-spots, as recommended by the International Ki-67 in Breast
199 Cancer Working Group [27].
200 The evaluation of tumor-infiltrating lymphocytes (TILs) was performed in full-face hematoxylin and
201 eosin sections from surgical or bioptic sample, strictly adhering to the criteria proposed by the TILs
202 Working Group [28]. Briefly, all mononuclear cells (i.e. lymphocytes and plasma cells) in the
203 stromal compartment within the borders of the invasive tumor were evaluated and reported as a
204 percentage. TILs outside the tumour border, around in situ component (DCIS) and normal breast
205 tissue, as well as in areas of necrosis, were excluded from the scoring.

206 **CTC-cluster enumeration by ScreenCell® filters**

207 Peripheral blood samples (9 mL), collected into K_2EDTA BD Vacutainer tubes (BD) using a 21G
208 needle, were stored at 4 °C in the dark and processed within 2.5 hours for CTC-cluster enrichment
209 using the ScreenCell® Cyto kit (ScreenCell, Sarcelles, France) [29] according to the manufacturer's
210 instructions, with slight modifications with respect to what previously described [30, 31]. Briefly,
211 three aliquots of 3.0 mL of whole blood per sample were separately mixed with 4 mL of a proprietary
212 red blood cell lysis and fixation buffer (ScreenCell® FC2 filtration buffer) and incubated for 8
213 minutes at room temperature. Each aliquot was filtered to isolate CTC-clusters using ScreenCell®
214 Cyto isolation supports (ISs), consisting in a microporous membrane with pores of 6.5 µm diameter.
215 After rinsing with PBS, ISs were air-dried and stained with Hematoxyilin Solution S (Merck,
216 Darmstadt, Germany) for 1 min and Shandon Eosin Y Aqueous Solution (Thermo Fisher Scientific
217 Inc., Waltham, MA, USA) for 30 seconds, at room temperature; or with May Grünwald (Merck
218 Millipore, Burlington, MA, USA; incubation for 2.5 min followed by a second incubation for 2.5 min
219 in May Grünwald diluted 1:2 with water) and Giemsa (Merck Millipore; diluted 1:10 with water, 10
220 min incubation) at room temperature. The stained ISs were sent to ScreenCell for evaluation by a
221 certified pathologist according to published criteria [32]. CTC-clusters were defined as clusters of ≥2

CTCs showing the criteria of malignancy: nuclear size ≥20 μm, nuclear-to-cytoplasmic ratio ≥0.75, irregular nuclear contours and nuclear hyperchromatism. In case the cytoplasm edges were not clearly visible inside the cluster (preventing nuclear-to-cytoplasmic ratio evaluation), malignancy identification was mainly based on nuclei appearance: nuclei scattered irregularly through the cluster and anisokaryosis (i.e. nuclei of variable sizes and shapes), in addition to nuclear size ≥20 μm and irregular nuclear membrane. Detailed guidelines for ScreenCell filter interpretation are described elsewhere [32]. Samples showing poor quality of cytology were excluded from the analysis. The total number of CTC-clusters for each sample was obtained by summing the CTC-clusters identified in the 3 ISs (corresponding to 9 mL of blood).

Statistical Analysis

Clinical and pathological variables were reported through descriptive analyses. Categorical variables were reported as frequency distribution, whereas continuous variables were described according to median and interquartile range (IQR). Differences in clusters distribution across subgroups of interest were tested through Mann–Whitney U test. Pairwise comparison between CellSearch® and CellSieve™ technologies, and across different timepoints during neoadjuvant therapy were performed though Wilcoxon sign-rank test. All reported *P*-values are two-sided.

Statistical analysis was conducted using the StataCorp 2016 Stata Statistical Software: Release 15.1 (College Station, Texas, USA), R (The R foundation for Statistical Computing. version 3.3.1) (2016-06-21) and JMP (SAS Institute, version 15).

Results

Comparison of different strategies for CTC-clusters identification

Technical validation of approaches used for CTC-cluster detection

To explore technical limitations of standard (CellSearch®) and filtration-based methods for CTC-cluster detection, spiking experiments were performed comparing size-exclusion approaches with the CellSearch® method (the currently most frequently used method in CTC-cluster studies). In particular, we compared 3 technologies: the CellSearch®, the CellSieve™ filters and the ScreenCell® filters. The latter two are very similar for the enrichment strategy (based on short-time filtration through a membrane with pores of 7 and 6.5 µm, respectively), but differ for the criteria employed for the identification of tumor cells. CellSieve™ filters include an identification based on marker expression similar to the one of the CellSearch® (CK^{pos} and $CD45^{neg}$ cells), whereas ScreenCell® filters' identification is based on cytomorphological evaluation.

Mammospheres derived from the MCF7 breast cancer cell line were used as a surrogate of CTC-clusters. For each technology, 10 mammospheres were spiked into healthy donor blood samples (n = 8), and subsequently processed for CTC-cluster enrichment. For ScreenCell® only, 2 spiking experiments were performed using PBS supplemented with HSA, instead of blood; this was done to test the stress associated with the filtration process itself, since, for this technology only, fresh blood is used for the spiking step and the presence of active immune cells from the donor might have an impact on mammospheres (for CellSearch® and CellSieve™ the blood is instead collected in CellSave tubes containing a preservative which fix blood cells). The number of recovered mammospheres for each of the 10 spiking experiments are reported in Table 1.

Table 1. Mammospheres identified in spiking experiments, using different detection methods

Sample ID	Enrichment technology	N. of recovered mammospheres
1	**CellSearch®**	7
2		8
3		6
4	**CellSieve™**	7
5		8
6		6
7	**ScreenCell®**	6
8		10
9*		10
10*		11

*For samples 9 and 10, mammospheres were spiked into PBS supplemented with HSA, instead of blood.

All three technologies showed similar recovery ranging from 60% to 100%. The impossibility of recovering 100% of the mammospheres in each sample suggests that a partial dissociation of the mammospheres occurred, as also supported by the presence of single tumor cells and fragments of mammospheres in the samples. However, disruption was not specifically induced by filtration, in fact all the samples with a 100% recovery were processed with filters.

Another possible concern about using filtration devices for CTC-cluster identification, is the possibility of the formation of aggregates on the filtration membrane during the processing, resulting in the identification of fictitious CTC-clusters. To exclude this possibility, we spiked single MCF7 cells in 3 healthy donor blood samples (n = 30 MCF7 cells per sample). The samples were processed with CellSieve™ filters and with ScreenCell® filters (2 and 1 sample, respectively). We observed the presence of only 1 aggregate of 2 tumor cells, on one CellSieve™ filter, indicating that filtration does not induce the formation of artifactual clusters.

CTC-cluster detection in clinical samples using an epithelial-based and a size-based approach

Once assessed that the ability of enriching clusters for the 3 technologies was similar, we next aimed at evaluating whether phenotypic heterogeneity of CTC-clusters in clinical samples (i.e. the presence of both epithelial and non-epithelial clusters) could have an impact on CTC-cluster detection by the epithelial marker-based CellSearch® platform, compared to the marker-independent and size-based approaches. In that respect, 19 blood samples collected from 16 patients with MBC were processed in parallel with CellSearch® and CellSieve™ filters (Fig. 1A). For this analysis, CellSieve™ was used as the representative among the 2 filtration methods, since its enrichment strategy is the same of ScreenCell filters (based on size), but its identification criteria are based on the detection of epithelial markers, and therefore allow for the distinction between epithelial and non-epithelial clusters (not possible with ScreenCell® filters).

Blood samples were collected from clinically selected patients with highly aggressive disease and during disease progression to increase the probability of CTC-cluster presence (in Additional file 1, patients' clinico-pathological characteristics are reported). For samples processed with the CellSearch®, only CTC-clusters expressing CK (CK^{pos} CTC-clusters, defined as groups of 2 or more cells showing CK^{pos} and $CD45^{neg}$ staining, Fig. 1B) could be detected, whereas for samples processed with CellSieve™ filters it was possible to identify both CK^{pos} and CK^{neg} clusters (Fig. 1C and 1D, respectively). CK^{neg} clusters were defined as groups of 2 or more cells showing a CK^{neg}, $CD45^{neg}$ and $CD31^{neg}$ staining (the latter marker allowing for the exclusion of endothelial cell clusters). CD31 expression was unexpectedly observed also in a few CK^{pos} CTC-clusters (Additional file 2). These clusters, being CK^{pos} and $CD45^{neg}$ were included in the analysis.

We detected ≥1 CK^{pos} CTC-clusters in 10 samples by using the CellSearch® and in 15 samples by using CellSieve™ filters (Additional file 3). Moreover, in the samples processed by filtration, CK^{neg} clusters were observed in 12 out of 18 evaluable samples, in 1 case alone and in 11 cases together

with CK^{pos} CTC-clusters. Overall, by considering 1 cluster per sample as positivity threshold, the positivity rate increased by using the size-based approach, going from 53 % (using the CellSearch®) to 79 % considering only CK^{pos} and 84 % considering both CK^{pos} and CK^{neg} clusters identified with CellSieve™ filters (Fig. 1E). Moreover, the absolute numbers of detected clusters were higher in samples processed with CellSieve™ filters than with the CellSearch® (Fig. 1F; Additional file 3). In samples processed with the CellSearch®, a median of 1 CK^{pos} CTC-cluster (interquantile range, IQR = 0-2; range 0-108) was identified, compared to a median of 3 CK^{pos} CTC-clusters (IQR 1-6; range 0-112) for samples processed with CellSieve™ filters ($P = 0.0293$). The increase in cluster counts for samples processed with CellSieve™ filters was even higher when considering CK^{pos} and CK^{neg} clusters together (median = 7, IQR 1-11; range 0-112, $P = 0.0038$).

These results suggest that by using a size-based and marker-independent approach it is possible to detect a higher number of clusters, allowing to identify them also in patients considered CTC-cluster negative by the CellSearch®. However, the observed phenotypic heterogeneity of clusters in BC patient samples, and in particular the presence of CK^{neg} clusters, highlighted an important limitation of CellSieve™ technology, which was able to enrich this type of clusters, but did not allow to reliably assess their malignancy (since they were only $DAPI^{pos}$). On the other hand, ScreenCell® technology had the same ability of enriching CK^{neg} clusters (since it is size-based as well), but its identification was based on cytomorphological evaluation and was therefore not dependent on the expression of any specific tumor markers. We therefore decided to use ScreenCell® filters to investigate the presence of CTC-clusters in both MBC and NMBC patients.

Detection of CTC-clusters by a size-based approach in patients with breast cancer

Patient characteristics

Between June 2014 and December 2015 a total of 37 and 23 patients with NMBC and MBC undergoing systemic treatment were enrolled in the study. Main clinical and pathological features are reported in Tables 2 and 3 (NMBC and MBC patients, respectively).

Table 2. Clinico-pathological characteristics of NMBC patients and CTC-clusters

	N	%	median CTC-clusters (range)	p	CTC-cluster + (%)	p
Age						
• <50	20	54.1	2.5 (0-20)	0.889	15 (75%)	0.719
• ≥50	17	45.9	2 (0-20)		11 (65%)	
Tumor size						
• T1-T2	21	56.8	4 (0-20)	0.180	16 (76%)	0.475
• ≥T3	16	43.2	1 (0-15)		10 (63%)	
Nodal status						
• N0	8	21.6	0 (0-12)	0.273	3 (37.5)	0.123
• N1	21	56.8	3 (0-20)		17 (81.1)	
• ≥N2	8	21.6	3 (0-20)		6 (75%)	
Histology						
• NST	35	94.6	2 (0-20)	0.322	15(68%)	
• Lobular	2	5.4	3 (0-15)		11(73%)	>0.99
Grade						
• 2	10	27.0	2 (0-15)	0.918	7 (70%)	>0.99
• 3	22	59.5	1.5 (0-20)		15 (68.2%)	
• Missing	5	13.5				
Ki67						
• <20	4	10.8	1.5 (0-12)	>0.10	2 (50%)	0.570
• ≥ 20	32	86.5	2 (0-20)		23 (72%)	
• Missing	1	2.7	-		-	
Subtype						
• HER2-positive	11	29.7	0 (0-8)	0.047	5 (45%)	0.111
• Triple negative	11	29.7	5 (0-20)		9 (82%)	
• Luminal-like	15	40.5	4 (0-20)		12 (80%)	
Type of neoadjuvant chemotherapy						
• Anthra/Taxane	32	86.5	2.5 (0-20)	0.984	22 (69%)	0.609
• CarboPt-based	5	13.5	1 (0-20)		4 (80%)	

337 **Table 3. Clinico-pathological characteristics of MBC patients**
338

	N	%
Age		
• <50	5	21.7
• ≥50	18	78.3
Histology		
• Ductal	15	65.2
• Lobular	2	8.7
• Other	6	26.1
Disease type at screening		
• Visceral	6	26.1
• Nonvisceral	12	52.2
• Missing	3	13.0
Hormone receptor status		
• ER -positive, PgR positive or both	18	78.3
• ER-negative and PgR-negative	5	21.7
HER2 status		
• Positive	1	4.3
• Negative	22	95.7
Metastatic disease at diagnosis		
• No	15	65.2
• Yes	8	34.8
Prior chemotherapy for metastatic disease		
• No	20	87.0
• Yes	3	13.0

339

340 The median age of NMBC patients treated with neoadjuvant chemotherapy (NAC) was 49 years
341 (range 26-84). At diagnosis, tumor size was 2-5 cm (cT2) in 20 patients (54 %), and > 5 cm (cT3-4)
342 in 16 patients (43 %). Clinical nodal status was positive (cN1-3) in 29 cases (78 %). No patients with
343 stage I BC were enrolled. Histological grade 3 was reported in 22 evaluable patients (60 %). Among
344 the 36 evaluable patients, the median Ki67 value was 40 %, with values ranging from 10 % to 90 %.

Thirty-two patients (86 %) had primary tumors with a Ki67 staining ≥ 20 %. Nine patients (24.3 %) reached a pathological complete response (pCR).

The median age of MBC patients was 68 years (range 29-84). The most common histological type was invasive ductal carcinoma (65 % of cases). Of the 23 patients included in the study, 6 (26 %) had visceral and 12 (52 %) had non-visceral involvement. Six patients (26 %) presented with de novo metastases. All patients, except three, had received no prior systemic treatment for metastatic disease.

CTC-clusters in patients with metastatic and non-metastatic breast cancer

To investigate the presence of CTC-clusters in our cohort of patients with MBC and NMBC, blood samples collected before starting systemic treatment underwent CTC-cluster enrichment by filtration, followed by a marker-independent CTC-cluster identification based on cytomorphological criteria using ScreenCell® filters (Fig. 2A). This simplified identification strategy requires only H&E staining rather than immunofluorescence, and it gives reliable results regarding cell malignancy, independently from the expression of specific markers. At baseline, in NMBC patients, 1 or more CTC-clusters were detected in 26/37 cases (70 %), with a median of 2 clusters per sample (range 0-20) (Fig. 2B). Among the 23 baseline samples collected from MBC patients, 3 samples were from pre-treated patients and one was not evaluable for CTC-cluster identification; CTC-clusters were detected in 4 of the 19 remaining samples (21 %), with a median of 0 CTC-clusters per sample (range 0-15). CTC-clusters were therefore more frequent and more abundant in patients with NMBC than MBC ($P = 0.0015$). In particular, patients with stage II BC showed a higher CTC-cluster count than patients with stage III and IV BC (Additional file 4A). Among patients with NMBC, a slightly higher number of CTC-clusters was detected in patients with node-positive status (Fig. 2C), although this difference was not statistically significant (median CTC-cluster number = 0 vs. 3 for node-negative vs. node-positive patients, $P = 0.1110$). CTC-clusters were more frequently observed in patients with luminal-like and triple negative BC than in patients with HER2-positive disease (median CTC-cluster

number = 4, 5, and 0 for luminal-like, triple-negative, and HER2-positive BC respectively, P = 0.0467) (Fig. 2D). For 25 patients for whom a primary tumor tissue sample was available, the presence of CTC-clusters was analyzed with respect to the presence of tumor-infiltrating lymphocytes (TILs) at the primary tumor site but no difference in CTC-cluster counts was observed between patients presenting a high or low level of TILs (median CTC-cluster number = 3 vs. 2 for patients with < 12 % vs. ≥ 12 % TILs, P = 0.5392) (Additional file 4B).

These results indicate that CTC-clusters are present in early stages in BC patients and are more frequent than in MBC patients. Among NMBC patients, CTC-clusters are more abundant in the blood of patients with HER2-negative disease.

Longitudinal evaluation of CTC-clusters during neoadjuvant therapy

To further investigate the clinical relevance of CTC-clusters in NMBC patients, longitudinal blood samples collected at baseline (N = 37), during (N = 30), at the end (N = 14) of NAC and after surgery (N = 18) were analyzed (Fig.3A). The median number of detected CTC-clusters at baseline was 2 (range 0-20), during treatment (DT) was 1 (range 0-97), and at the end of treatment (EOT) was 3 (range 0-116). Thus, CTC-clusters did not decrease during NAC, but instead increased in some patients. Overall, no significant differences were observed in DT and EOT with respect to baseline. On the other hand, a significant decrease was observed from DT to surgery (P = 0.0448) and EOT to surgery (P = 0.0208). Only a slight decrease was instead observed between baseline and surgery (P = 0.0678). The median number of CTC-clusters after surgery was 0 (range 0-20).

At baseline, numerically less clusters were observed in NAC-responders, i.e. patients with complete disappearance or a reduction of primary tumor volume of at least 50% after NAC, as compared to non-responders, i.e. patients with stable disease after NAC: 1 cluster (range 0-20) *versus* 4 clusters (range 0-12), respectively (P = 0.58). The presence of CTC-clusters at baseline was not significantly

associated with pCR (Additional file 5A). However, patients without clusters at baseline reported a numerically higher pCR rate as compared with those presenting with clusters, 27% versus 23%, respectively. Moreover, after surgery, a significantly lower number of clusters was observed in patients with pathological complete or partial response *versus* stable disease ($P = 0.0208$) (Additional file 5B). As of May 15, 2020, a total of 10 out of 37 NMBC patients relapsed. No difference in baseline or post treatment distribution of clusters was reported among patients with or without a relapse. At the same date, 4 out of 19 evaluable MBC patients had died, notably the negative predictive value of clusters at baseline in this case was as high as 86%, but the data is merely explorative due to the small sample size.

We present two examples of patients who responded to NAC but did not achieve pCR, illustrating the cluster's dynamics during treatment.

Patient A (Fig. 3B) was diagnosed with a 40 mm ductal carcinoma of the right breast, G3 ER, PgR and HER2 negative, 90% Ki67. Bone scan and liver ultrasound were negative for distant involvement. She was further staged with positron emission tomography (PET) scan that confirmed a breast primary lesion with a standardized uptake value (SUV) of 22.5 and no loco-regional involvement. The patient was therefore enrolled in a NAC clinical trial and received 4 cycles of Doxorubicin 60 mg/m2 together with Paclitaxel 200 mg/m2 q21. No clusters were detectable at baseline. The first PET evaluation showed a dramatic drop in metabolic activity (SUV 3.7), with 5 clusters detectable in the peripheral blood. Eribulin 1.23 mg/m2 was then started and 4 cycles were administered with a 1, 8, q21 schedule. The subsequent PET scan showed further metabolic response with a 3.7 SUV, while an increase in clusters was observed (35 clusters). She then underwent quadrantectomy with 17 mm residual disease and absence of nodal involvement (ypT1c, N0). ER was 2%, PgR and HER2 were negative, Ki67 was confirmed at 90%. Filter based enumeration after surgery showed a complete clearance of detectable clusters. The patient was then started on adjuvant

CMF (Cyclophosphamide 600 mg/m2, methotrexate 40 mg/m2 and 5-Fluorouracil 600 mg/m2) but died 7 months after surgery for noncancer-related causes without any detectable local or distant relapse.

Patient B was diagnosed with a screening-detected lobular carcinoma of the left breast, G2, ER 20%, PgR 10%, HER2 negative and Ki67 10%. The baseline breast magnetic resonance (MRI) showed a multifocal primary tumor with a 38 mm main lesion and a 3 mm satellite lesion, while distant metastases were excluded through PET scan. Baseline clusters enumeration was 3. A Doxorubicin 60 mg/m2 and Paclitaxel 200 mg/m2 q21 based NAC was started. Breast MRI after 4 cycles showed a partial regression. Clusters were not detectable. CMF was administered for 4 cycles. While the breast MRI showed further radiological response, 116 clusters were detected in the peripheral blood. The patient underwent quadrantectomy with 3 mm residual disease and 2 metastatic lymph nodes out of 7 analyzed (ypT1a N1a). ER was 90 %, PgR and HER2 were negative, Ki-67 was 5 %. After bone scan restaging, she was started on adjuvant Letrozole 2.5mg, which is still ongoing without evidence of distant or local recurrence.

Discussion

In the current study, we have challenged the most frequently used technical approach for CTC-cluster detection in BC, the CellSearch®, by comparing it with methods based on size exclusion. We report that filtration allowed to detect a higher number of clusters in the blood of BC patients. Moreover, by using a filtration-based approach to analyze blood samples prospectively collected from NMBC and MBC patients, we observed that CTC-clusters were more frequently detected in NMBC than in MBC patients, and that molecular subtypes affected their presence in NMBC. Finally, the presence of

clusters before starting neoadjuvant treatment did not associate with pCR and their numbers increased during treatment, but dropped after surgery.

To the best of our knowledge, this is the first study specifically comparing CTC-cluster detection by CellSearch® and by a validated filtration-based technique [33], in patients with BC. Such a comparison has instead been performed in small-cell lung cancer patients, by using in parallel the CellSearch® and the ISET filtration approach, showing similar results [22]. Indeed in lung cancer patients no clusters were detected with the CellSearch®, whereas they were found in 50% of samples from stage IIIB/IV patients processed with the ISET. The findings were explained by the authors as a possible failure of the immune-magnetic enrichment step in the CellSearch® protocol to capture large size clusters. However, additional considerations can be made regarding the increased CTC-cluster detection attained by using filtration, both in our study and in that by Krebs and colleagues. Strong positivity for mesenchymal, with concomitant weak positivity for epithelial markers, has been reported for CTC-clusters isolated from patients with advanced breast cancer [24]. Thus, an increase in cluster detection is not surprising when using methods that do not relay on the expression of epithelial markers, and which are not limited to the detection of epithelial clusters only. Conversely, the observed increased detection of epithelial clusters (CK^{pos}) is an unexpected finding. A possible explanation is that CK^{pos} CTC-clusters can also include cells undergoing EMT and therefore expressing a mixed phenotype rather than a frankly epithelial one. Since the CellSearch® detects only CTC-clusters expressing both EpCAM and CK, but EpCAM expression is lost early during EMT [23, 34] the CellSearch® could miss CK^{pos} CTC-clusters that are going through the EMT. This hypothesis could not be verified in the present study since the expression of mesenchymal markers was not assessed. However, it is supported by the results of the spiking experiments showing that when using frankly epithelial mammospheres (expressing both EpCAM and CK), CellSearch yielded comparable recovery rates as filtration devices.

462 Besides filters, other marker-independent technologies such as the ^{HB}CTC-Chip [4, 24], the Cluster-
463 Chip [35] and the Parsortix™ [6], have been employed for CTC-cluster studies, but mainly focusing
464 on functional aspects rather than on pure translational purposes. In fact, despite a number of studies
465 have described new technical tools specifically dedicated to CTC-cluster detection [for a review see
466 36], none of these innovative methods is widely available to clinical research centers. In this context,
467 simpler technologies, as those based on size-exclusion would represent a more affordable approach,
468 easily transferable to clinical studies that might help in elucidating the role of CTC-clusters in
469 different clinical contexts.
470 Indeed, here we applied an easy-to-use filtration-based approach to investigate the relevance of
471 CTC-clusters in 37 NMBC and in 23 MBC patients. The ScreenCell® technology was chosen since
472 its validity has already been reported both for single CTCs and for clusters both when identified
473 based on cytomorphological criteria only, [37, 38] or based on marker expression [39].
474 Overall we report that, in baseline samples collected at the beginning of NAC, the detection of at
475 least 1 CTC-cluster occurred at least 3-times more frequently in women with early breast cancer than
476 in women beginning first line treatment for MBC (a result that we also observed in our previous pilot
477 study, which was comparing ScreenCell® with AdnaTest technology [30]). Although, due to the
478 small case series, we have not done a formal analysis to exclude a bias due to different distribution of
479 molecular subtypes between the two groups, molecular subtype linked effects would have impacted
480 the data in opposite direction than observed. Thus our findings support the concept that dissemination
481 of CTC-clusters is an early event in NMBC patients, rather than an event occurring during metastatic
482 progression, as might have been expected by the high metastatic potential of clusters [4]. Since
483 dissemination is proven to occur early in breast cancer [8, 9], and indeed single CTCs hold
484 prognostic value also in NMBC women [11-13], the more frequent presence of CTC-clusters and the
485 higher number of clusters seen in early rather than later steps of the disease is intriguing.

Nevertheless, many questions on clinical and biological aspects still remain to be answered. We observed that molecular subtypes affect the prevalence of CTC-clusters. In particular CTC-clusters were found to be significantly more frequent in women bearing HER2-negative tumors, a result that may appear as contra-intuitive since HER2-positive tumors are more aggressive and are frequently associated with stemness markers [40]. Moreover, we have noticed that luminal-like tumors release high number of clusters, a finding possibly linked to their late relapse-pattern and to a more efficient promotion of dormancy within the clusters from patients with ER+ tumors [41]. Overall, this suggests that clusters should be studied in molecularly homogeneous populations, although this could not be done in this study, due to the limited number of patients.

In our cohort of NMBC patients, the detection of clusters did not correlate with the likelihood of achieving pCR, a finding already reported in the literature for CTCs [12, 38]. Moreover, during the course of treatment a trend towards an increase in CTC-clusters rather than a decrease was observed, as also described in another study using ScreenCell® filters [38]. Indeed, only after surgery we actually observed a decrease in the number of clusters. Although a significantly higher number of clusters persisting after surgery was detected in patients with a pathologically non-responding disease (median 3, IQR 1 – 11.5 *vs* 0, IQR 0-1 for non-responders and responders, respectively).

Thus, it may be speculated that in NMBC, clusters formation is related to the presence and characteristics of primary tumor, and the neoadjuvant treatment has a different effect on the primary tumor and on clusters. Moreover, despite this study is not properly powered to detect differences in disease-free survival and no association was observed between relapse and CTC-clusters at baseline, it is intriguing to think of potential applications of cluster enumeration after surgery as a completion of pathological staging to assess the overall combined response to systemic and locoregional treatments.

Notably, a discrepancy between cluster dynamics and imaging was observed. As consistently shown by the index cases, clusters generally increased during NAC notwithstanding the concomitant

radiological and metabolic response. On the other hand, patients that did not show response to NAC had a significantly higher number of clusters after surgery. This suggests a more nuanced role of clusters in NMBC with respect to that of epithelial clusters in the metastatic setting.

A crucial question raised by our results deals with the phenotype and the actual composition of clusters. Having used a size-based approach for cluster enrichments and morphological criteria for the detection, we were confident about the malignancy of the clusters, but we lost the information regarding their epithelial/non-epithelial phenotype. However, since in the numerous studies run with the CellSearch® in women with early disease, massive presence of clusters has not been reported, we speculate that clusters detected in the current study are not frankly epithelial, but rather with a mesenchymal or with a mixed phenotype.

Regarding the cluster composition, the role of inflammatory cells remains to be addressed. Indeed, cooperation and crosstalk with other blood cells play a relevant role in increasing the metastasis-promoting efficiency of cluster [7, 42-45]. However, in the current study, we did not find an association between TILs evaluated on the primary tumor and CTC-clusters, thus the possible interaction between inflammatory cells and CTC-clusters warrants further studies.

Finally, the observation that clusters do not disappear with the neoadjuvant treatment (and thus possibly also persist after adjuvant treatment), support the need to develop treatment strategies specifically designed at interfering with clusters [6, 46]. Such strategies would be promising especially if CTC-clusters isolated in NMBC patients would prove to hold metastatic potential, a still unanswered question worth to be addressed in the future.

We are aware of the study limits due to the small size and heterogeneity of the case series, although its strength may be linked to the fact that these represent real-world patients, prospectively collected within the daily clinical practice.

Conclusions

This study represents a small snapshot on CTC-cluster detection methods and on the prevalence of clusters in BC patients at different disease stages. Nonetheless, it highlights the possible bias linked to inadequate methods for cluster detection, a technical bias that is worth to be considered in future translational studies. In addition, we report a new observation on the fact that CTC-clusters are frequent in women with NMBC. This represents a provocative finding that needs to be addressed in future studies on larger series of cancer patients, homogeneous with respect to molecular subtype. Finally, the observation that CTC-clusters do not disappear during neoadjuvant treatment, foster the importance of developing treatments specifically aimed at interfering with them.

List of abbreviations

BC: breast cancer; CK: cytokeratin; CTC: circulating tumor cell; DT: during treatment; EMT: epithelial-to-mesenchymal transition; EOT: end of treatment; HSA: human serum albumin; IQR: interquartile range; IS: isolation support; MBC: metastatic breast cancer; MRI: magnetic resonance imaging; NAC: neoadjuvant chemotherapy; NMBC: non-metastatic breast cancer; OS: overall survival; pCR: pathological complete response; PBS: phosphate-buffered saline; PD: progressive disease; PET: positron emission tomography; PFS: progression free survival; PR: partial response; SD: stable disease; SUV: standardized uptake value; TIL: tumor-infiltrating lymphocytes.

Declarations

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board and Ethics Committee of Fondazione IRCCS Istituto Nazionale dei Tumori on February 19, 2013, and by the Institutional Review Board at the Robert H. Lurie Comprehensive Cancer Center of Northwestern University (NUDB16Z01). The patients/participants provided their written informed consent to participate in this study.

Consent for publication

Not Applicable.

Data Availability Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare no competing interests.

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Authors' Contributions

Conception and design of the study: V.C.; C.R.; S.D.C.; M.G.D.

Acquisition of data: C.R.; S.D.C.; R.M.; A.M.; M.V.; A.V.; P.D.; Y.Z.; C.D.; G.S.; S.F.; G.P.

Analysis and/or interpretation of data: C.R.; S.D.C.; L.G.; M.C.; V.C.; M.G.D.

Drafting the manuscript: C.R.; V.C.; L.G.; S.D.C.

Revising the manuscript critically: R.M.; A.V.; P.D.; Y.Z.; M.V.; A.M.; C.D.; G.S.; S.F.; G.P.; M.C.; M.G.D.

All authors approved the final version of the manuscript.

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Janine Wechsler (ScreenCell, Sarcelles, France) counted the CTC-clusters present on ScreenCell filters, without knowledge of the clinical data.

Daniel Adams provided technical support for cluster detection on CellSieve filters.

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Figure Legends

Figure 1. Comparison of CellSearch and CellSieve for CTC-cluster detection in clinical samples.

(**A**) Nineteen blood samples collected from patients with MBC were processed in parallel with CellSearch and CellSieve for the detection of CTC-clusters. (**B**) Representative image of a CK^{pos} CTC-cluster detected by CellSearch (green = CK; pink = DAPI; 10X magnification). (**C-D**) Representative images of a CK^{pos} (**C**) and a CK^{neg} (**D**) cluster detected by CellSieve (green = CK; blue = DAPI; yellow = CD45; the white arrows indicate 2 $CD45^{neg}/CK^{neg}$ cells inside the cluster). (**E**) Doughnut plot showing the percentages of samples containing CK^{pos} CTC-clusters (blue) analyzed by CellSearch (outer circle, 53 %) and CellSieve (inner circle, 79 %). Positivity threshold was set at 1 CTC-cluster/7.5 mL of blood. The percentage of CellSieve samples containing only CK^{neg} clusters are shown in orange (5 %). (**F**) Spaghetti plot showing the numbers of CTC-clusters detected in each sample analyzed by CellSearch and CellSieve. For CellSieve samples, both the counts of CK^{pos} CTC-clusters only and of CK^{pos} plus CK^{neg} clusters (CellSieve total) are reported (colors are arbitrary assigned for increasing readability of the graph only).

Figure 2. Detection of CTC-clusters in patients with early and metastatic breast cancer.

(**A**) Representative images of CTC-clusters enriched by filtration using ScreenCell filters. The list of cytomorphological criteria used for the identification of CTC-clusters is reported in the inset. (**B – D**) Boxplots reporting the number of CTC-clusters detected in baseline samples collected from NMBC vs. MBC patients (**B**); and in baseline samples collected from NMBC patients, according to the patients' nodal status (**C**) and to the disease subtype (**D**).

Figure 3. CTC-cluster evaluation during neoadjuvant therapy in early breast cancer patients. (**A**) Violin plot showing the number of CTC-clusters detected in samples longitudinally collected from 37 NMBC patients. CTC-clusters were evaluated before starting neoadjuvant treatment (Baseline), during (DT), at the end of therapy (EOT), and after surgery (Surgery). The colors indicate the BC subtype (blue = HER2-positive; purple = luminal-like; red = triple-negative) while the gray shadow indicates the density of samples for the corresponding CTC-cluster number. The detailed description of 2 index cases is reported in panels **B** and **C**. TNBC = triple-negative breast cancer; AT = Antracyclines, Taxanes; CMF = Cyclophosphamide, Methotrexate, Fluorouracil; pCR = pathological complete response.

Additional files

Additional file 1 (.DOCX): Table reporting the clinico-pathological characteristics of the patients' cohort for technological comparison.

Additional file 2 (.TIFF): Figure showing CTC-clusters expressing CD31.

Additional file 3 (.DOCX): Table reporting CTC-cluster count in samples processed in parallel with CellSearch and CellSieve filters.

Additional file 4 (.TIFF): Figure showing the association of CTC-clusters with clinico-pathological characteristics in patients with NMBC.

Additional file 5 (.TIFF): Figure showing the association of CTC-clusters with outcome in NMBC patients.

Figures

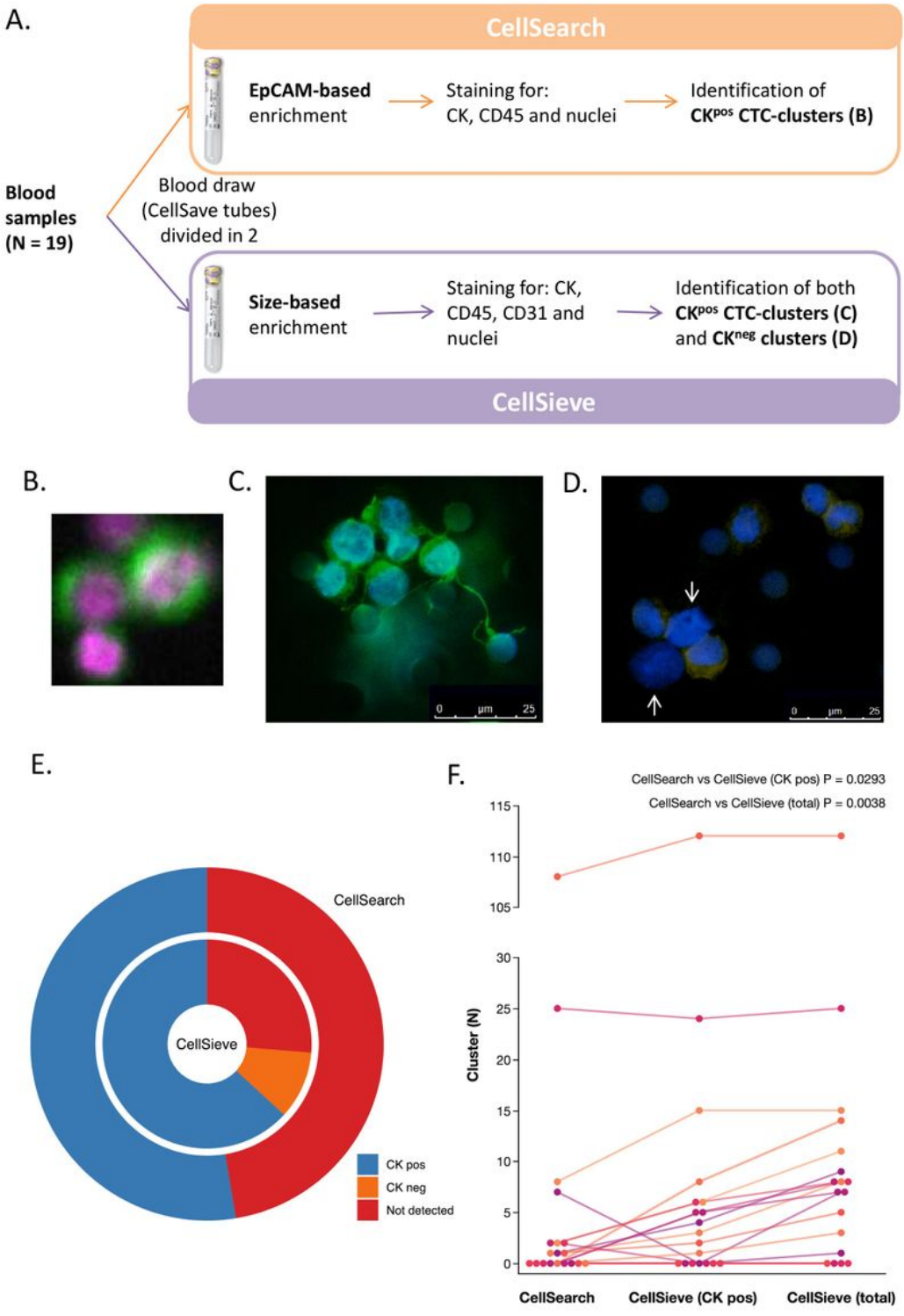

Figure 1

Comparison of CellSearch and CellSieve for CTC-cluster detection in clinical samples. (A) Nineteen blood samples collected from patients with MBC were processed in parallel with CellSearch and CellSieve for the detection of CTC-clusters. (B) Representative image of a CKpos CTC-cluster detected by CellSearch

(green = CK; pink = DAPI; 10X magnification). (C-D) Representative images of a CKpos (C) and a CKneg (D) cluster detected by CellSieve (green = CK; blue = DAPI; yellow = CD45; the white arrows indicate 2 CD45neg/CKneg cells inside the cluster). (E) Doughnut plot showing the percentages of samples containing CKpos CTC-clusters (blue) analyzed by CellSearch (outer circle, 53 %) and CellSieve (inner circle, 79 %). Positivity threshold was set at 1 CTC-cluster/7.5 mL of blood. The percentage of CellSieve samples containing only CKneg clusters are shown in orange (5 %). (F) Spaghetti plot showing the numbers of CTC-clusters detected in each sample analyzed by CellSearch and CellSieve. For CellSieve samples, both the counts of CKpos CTC-clusters only and of CKpos plus CKneg clusters (CellSieve total) are reported (colors are arbitrary assigned for increasing readability of the graph only).

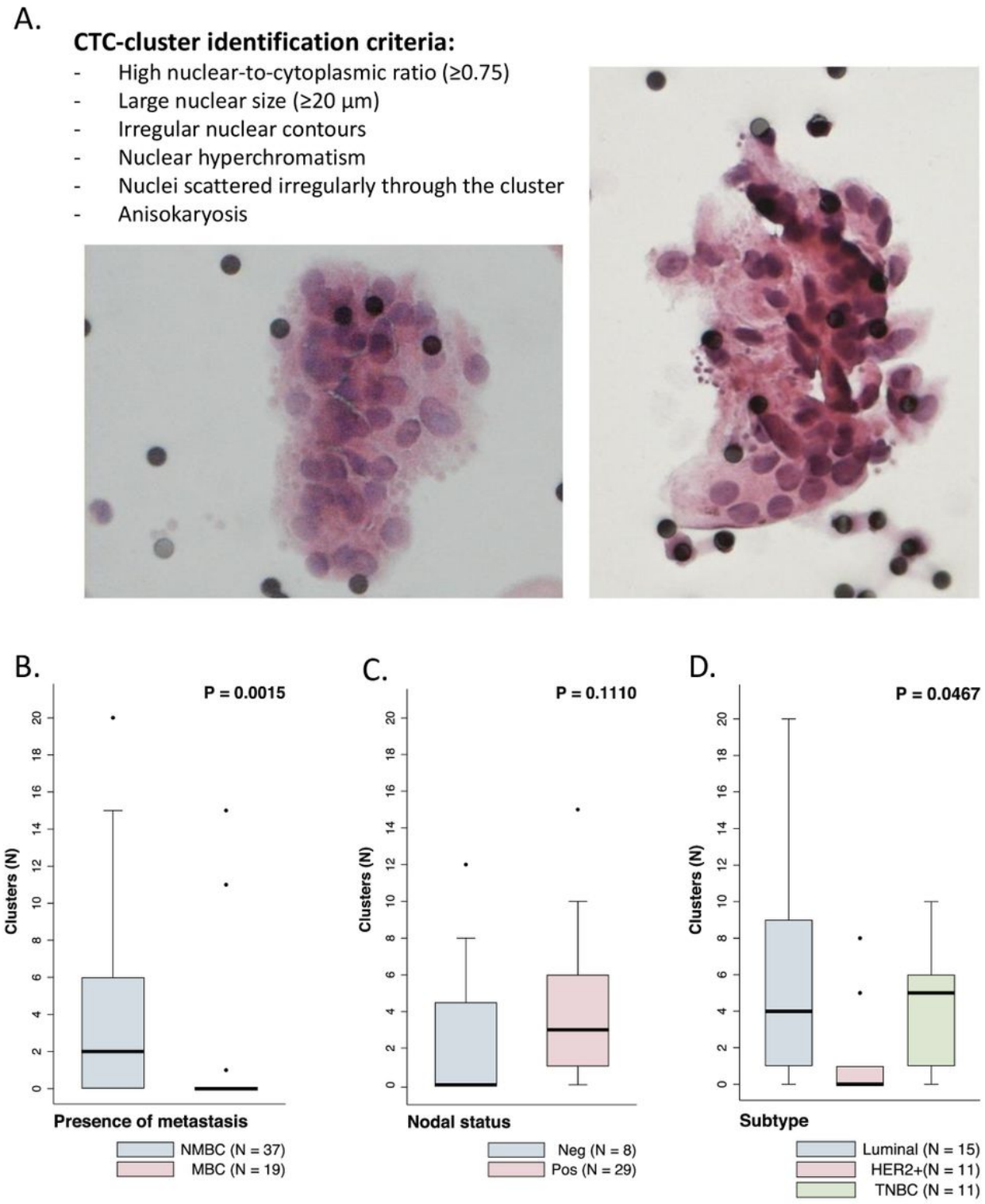

Figure 2

Detection of CTC-clusters in patients with early and metastatic breast cancer. (A) Representative images of CTC-clusters enriched by filtration using ScreenCell filters. The list of cytomorphological criteria used for the identification of CTC-clusters is reported in the inset. (B – D) Boxplots reporting the number of CTC-clusters detected in baseline samples collected from NMBC vs. MBC patients (B); and in baseline

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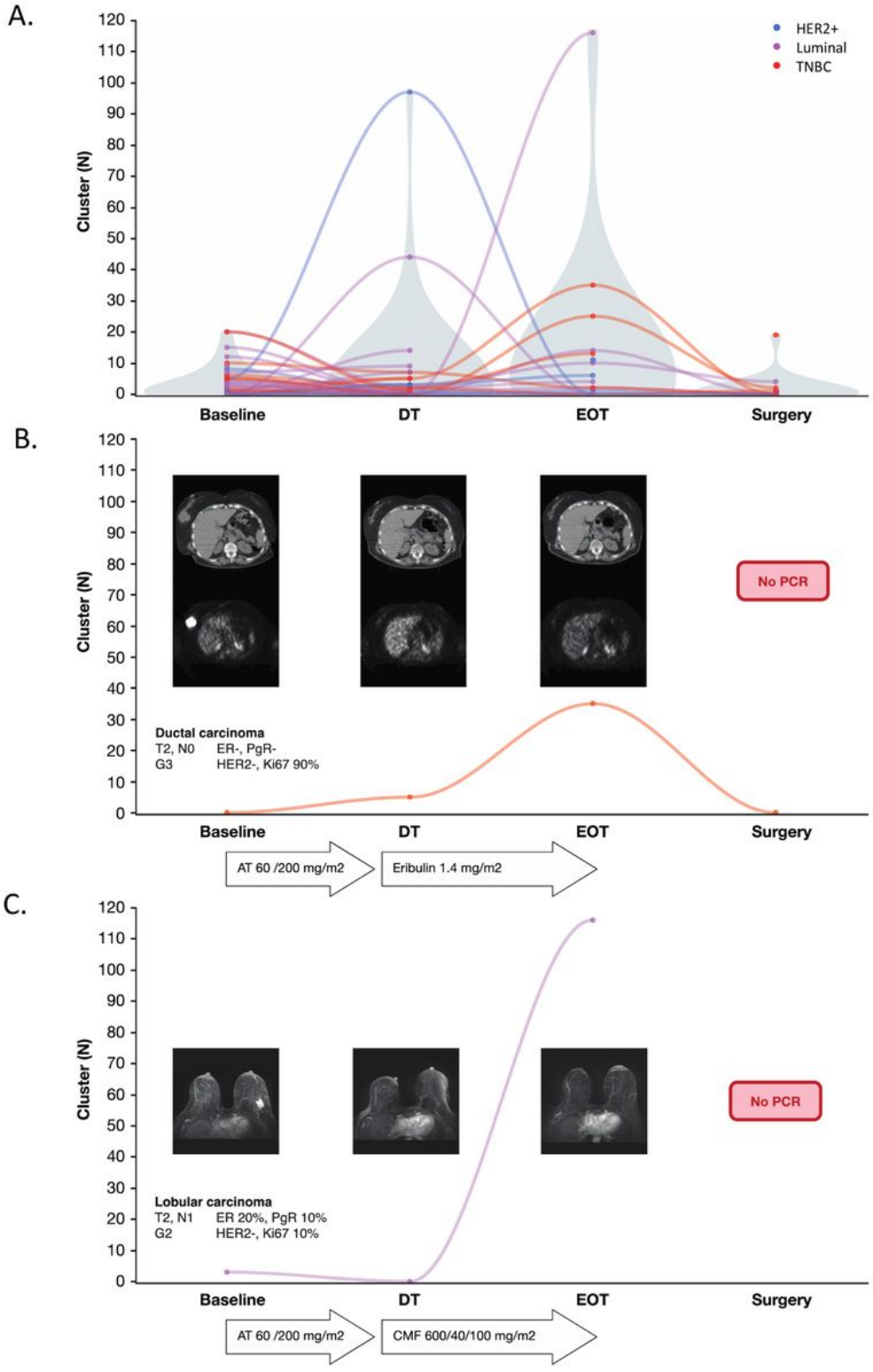

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
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- Additionalfile3.docx
- Additionalfile4.tiff
- Additionalfile5.tiff