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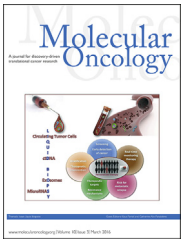

Review

Circulating tumor cells in breast cancer☆

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ABSTRACT

Over the past decade, technically reliable circulating tumor cell (CTC) detection methods allowed the collection of large datasets of CTC counts in cancer patients. These data can be used either as a dynamic prognostic biomarker or as tumor material for "liquid biopsy". Breast cancer appears to be the cancer type in which CTC have been the most extensively studied so far, with level-of-evidence-1 studies supporting the clinical validity of CTC count in both early and metastatic stage. This review summarizes and discusses the clinical results obtained in breast cancer patients, the issues faced by the molecular characterization of CTC and the biological findings about cancer biology and metastasis that were obtained from CTC.

1. Introduction

At the end of the XXth century, numerous studies investigated the role of bone marrow disseminated tumor cells in the micrometastatic process of breast cancer (BC). The establishment of robust detection techniques in the mid-2000's, shifted the interest towards the circulating tumor cells (CTCs) that are detected in blood of BC patients. Ten years after the first evidence supporting the clinical validity of CTC detection in BC, our review aims to recapitulate the key concepts and observations made on CTC as a biomarker for breast cancers.

2. CTCs as a marker of micrometastatic spread in early BC

2.1. *Micrometastasis biomarkers in early BC*

After the first reports in the early 90's (Pantel et al., 1993, 1991), several groups developed different in-house techniques to detect disseminated tumor cells (DTC) in the bone-marrow of early BC patients, mostly based on epithelial cell staining and cytological visual screening (Pantel et al., 2009; Vincent-Salomon et al., 2008). A large pooled analysis performed on 4703 individual patient data demonstrated that disseminated

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tumor cells were a significant prognostic factor for disease-free survival and overall survival in early BC (Braun et al., 2005). Although standardized cytological criteria have been set up, bone marrow DTC detection remained labor-intensive and only limited attempts were initiated to demonstrate its clinical utility (Diel et al., 2008; Hoffmann et al., 2011; Naume et al., 2014). Bone-marrow DTC detection has therefore not been implemented in the routine clinical workup of early BC patients. Nevertheless, the level-of-evidence 1 prognostic impact of bone marrow DTC was acknowledged in the 2010 TNM classification of BC, which introduced a cM0(i+) class for patients with no clinical or radiographic evidence of distant metastases but with tumors cells detected in the bone marrow (i.e. DTC), in blood (i.e. CTCs) or in distant non-regional lymph nodes. At that time, only a few reports on CTC detection in non-metastatic BC were published, with heterogeneous techniques. Molecular detection methods (*i.e.* detection of epithelial mRNA in blood mononucleated cells, which development has been since discontinued because of limited specificity) suggested a prognostic impact of CTCs in early BC patients (Ignatiadis et al., 2007; Pierga et al., 2007). The first cytological approaches to detect CTCs in the peripheral blood were similar to the one used for detection of bone marrow DTC. Most of these first attempts did not report significant prognostic impact (Pierga et al., 2004).

Studies reporting the synchronous detection of CTC in blood and DTC in bone marrow of BC patients are recapitulated in Table 1. In most studies, the detection of isolated cancer cells in the blood and in the bone-marrow were significantly correlated, with concordance rates ranging from 66 to 94%. Discrepant cases (patients with tumor cells detected only in blood or bone marrow) can be mostly attributed to the limited sensitivity of the detection techniques. Moreover, according to our current understanding of metastasis as a multistep process, the various biological processes involved may also be in part responsible for the discrepancy observed. Dissemination of cancer cell in the blood corresponds to one of the first step of the metastatic process (when cancer cells detach from the primary tumor and intravasate), while micrometastasis in a distant organ, like the bone marrow, reflects a more advanced stage (Bidard et al., 2013c, 2008).

2.2. *Clinical validity in early BC*

The first evidences supporting the clinical validity of detecting ≥1 CTC (using the US Food and Drug Administration (FDA) -cleared cytological CellSearch® technique) as a prognostic biomarker in non-metastatic BC have been reported in patients treated by neoadjuvant chemotherapy (i.e. before surgical removal of the tumor). A remarkable homogeneity of detection rates across studies was observed, with the exception of the inflammatory BC subtype. Table 2 summarizes the studies published on early BC, as of December 2015. Strikingly, in opposition to most of the currently used clinicopathological prognostic factors, CTC detection studies using the CellSearch® technique showed no association with the response to therapy, measured by the completion of a pathological complete response (which corresponds to the complete eradication of invasive tumor cells by neoadjuvant chemotherapy; Table 2). An international meta-analysis of CTC detection in neoadjuvant-treated BC patients has been initiated recently ("IMENEO" study). This study aims to demonstrate the clinical validity of CTC count as an independent prognostic factor with a level-of-evidence 1. In stark contrast with CellSearch®-based studies, a study conducted on 91 non-metastatic patients with the Maintrack© cytology-based CTC detection system reported extraordinarily high CTC concentrations (up to 100 CTC per mm^3, which is similar to the number of monocytes). This study also showed that different patterns of CTC decrease during neoadjuvant therapy were associated with pathological complete response (Pachmann et al., 2008). These results however need to be replicated.

Studies conducted in the adjuvant setting (i.e. after surgical removal) also reported a significant prognostic impact of ≥1 CTC detection, with the same technique. In the German multicentric study SUCCESS more than 2000 patients who were eligible for adjuvant chemotherapy were screened for CTC before the start of chemotherapy (Rack et al., 2014). The overall CTC detection rate before chemotherapy (21.5%) was marginally correlated with the nodal status (19.6% and 22.4% in node-negative and node-positive tumors, respectively) but not with any other tumor characteristics. After the completion of adjuvant chemotherapy, a rather similar CTC positivity rate was found (22.1%). CTC positivity before chemotherapy was confirmed as an independent prognostic marker for disease-

Table 1 – Concordance between bone-marrow disseminated tumor cells (DTC) and CTC detection in breast cancer.

References	Year	Stage	Tech.	N pts	Conc.	p Value correlation	Detection rate		Prognostic impact		Multiv.
							CTC	DTC	CTC	DTC	
Pierga et al. (2004)	2004	I–IV	ICC	114	66%	<0.001	24%	59%	NS	DFS	Yes
Wiedswang et al. (2006)	2005	I–III	ICC + IMS	341	81%	0.1	10%	14%	DFS OS	DFS OS	–
Benoy et al. (2006)	2006	I–IV	RT-PCR	148	68%	0.3	15%	28%	NS	OS	Yes (for DTC)
Fehm et al. (2009)	2009	I–III	ICC/RT-PCR	414	72%	0.05	13%	24%	–	–	–
Daskalaki et al. (2009)	2009	I–II	RT-PCR	165	94%	<0.001	55%	58%	OS	OS	Yes
Banys et al. (2012)	2011	I–III	ICC/RT-PCR	209	74%	0.03	21%	15%	–	–	–
Molloy et al. (2011)	2011	I–II	ICC/RT-PCR	733	80%	<0.01	8%	12%	DFS OS	DFS OS	Yes
Schindlbeck et al. (2013)	2013	I–IV	ICC/CellS®	202	71%	0.002	20%	28%	OS	NS	–

Stage: BC stage; Tech.: techniques used: Conc.: concordance rate; ICC: immunocytostaining; CellS®: CellSearch®; IMS: immunomagnetic selection; "–": not done; DFS: disease-free survival; OS: overall survival; NS: Not significant; Multiv.: multivariate analysis of prognostic impact. Studies with less than 100 patients are not mentioned in the table.

Table 2 – CTC detection in BC treated by neoadjuvant therapy.

Trial name (if any) References	Patients number tumor type	CTC detection rate (≥1CTC/7.5 ml)				Correlation between CTC detection and		Prognostic impact	
		Before neo. tt.	During neo. tt.	After neo. tt., before surgery	After neo. tt., after surgery	Tumor characteristics	Pathological complete response	Disease-free survival	Overall survival
REMAGUS02 Pierga et al. (2008); Bidard et al. (2010, 2013)	N = 115 all subtypes	23%	–	17%	–	No	No	Yes	Yes
GEPARQUATTRO Riethdorf et al. (2010b)	N = 213 all subtypes	22%	–	11%	–	No	No	–	–
GEPARQUINTO Riethdorf et al. (2010a)	N = 364 all subtypes	23%	14%	11%	–	No	No	–	–
Gunma Hospital Horiguchi et al. (2012)	N = 95 all subtypes	19%	–	–	–	No	No	No	–
NEOAVA Mathiesen et al. (2013)	N = 90 HER2-negative	18%	19%	11%	–	No	No	–	–
NEOZOTAC Onstenk et al. (2015a)	N = 68 HER2-negative	18%	16%	–	–	No	No	–	–
NEOALLTO Azim et al. (2013)	N = 51 HER2-positive	11%	10%	16%	–	No	No	–	–
MD Anderson Hall et al. (2015a)	N = 57 triple negative	–	–	30%	–	No	No	Yes	Yes
BEVERLY02 Pierga et al. (2012b, 2015b)	N = 52 HER2-positive inflammatory	37%	13%	7%	15%	No	No	Yes	Yes
BEVERLY01 Pierga et al. (2015a)	N = 92 HER2-negative inflammatory	40%	6%	11%	3%	No	No	Yes	Yes
MD Anderson Mego et al. (2015)	N = 77 all subtypes inflammatory	54%	–	–	–	No	No	No	No
MD Anderson Hall et al. (2015b)	N = 63 all subtypes inflammatory	–	–	27%	–	No	No	Yes	No

Studies that specifically enrolled inflammatory breast cancer patients are displayed at the bottom of the table. Neo. tt.: neoadjuvant therapy.

free survival (Hazard Ratio: 2.11, 95%CI = [1.49–2.99]) and overall survival HR: 2.18; 95%CI = [1.32–3.59]. Higher number of CTC detected at baseline (≥5 CTC) was associated with a worse prognosis, while CTC positivity after chemotherapy had a marginally significant prognostic impact on survival. A single center study conducted at the MD Anderson Cancer Center also reported a significant impact of CTC positivity in 304 patients (detection rate: 24%) (Lucci et al., 2012). A pooled analysis included most of the afore-mentioned studies in adjuvant and neoadjuvant settings and reported ≥1 CTC per 7.5 ml of blood (using the CellSearch® system) as an independent prognostic factor (Janni et al., 2016).

Building on these results, the interventional trial TREAT CTC (NCT01548677), coordinated by the European Organization for Research and Treatment of Cancer (EORTC), is a large phase II study testing the effect of trastuzumab on CTC detection and survival in HER2-negative, M0(i+) early BC (study design has been reviewed elsewhere (Bidard et al., 2013b)). This clinical utility trial is currently ongoing in several European countries.

In the meantime, it is important to note that, in most studies, the amplitude of the CTC-positivity individual risk of BC relapse and death, as measured by hazard ratio, is not inferior to that of the usual prognostic factors (tumor size, grade, proliferation, node involvement…) that are currently taken into account for the adjuvant treatment decision. Moreover, CTC count is neither correlated to these usual prognostic factors nor to the primary tumor response to neoadjuvant chemotherapy. For example, in inflammatory BC, CTC count complements pathological complete response to identify patients with excellent prognosis despite the BC initial aggressive features (Pierga et al., 2015b). CTC detection from blood tests being a reliable tool to estimate the metastatic risk, it is very likely that CTC count has a significant role to play in the future as a metastasis-associated prognostic biomarker in early BC patients.

3. CTC as a dynamic prognostic factor in metastatic BC

3.1. *Clinical validity in metastatic BC*

In 2004, a first study reported a significant clinical validity of CTC count in metastatic BC with the CellSearch® system (Cristofanilli et al., 2004). One hundred seventy-seven patients starting a new line of treatment had their CTC count assessed at baseline and after few weeks of treatment. A threshold to distinguish patients with short versus long-progression free survival was investigated, set up at ≥5 CTC/7.5 ml of blood on the basis of a training cohort and confirmed in a validation cohort. This prognostic value was also observed during treatment (Hayes et al., 2006). By combining dichotomized CTC count (high or low) at two time-points (baseline and after 1 cycle of treatment), 4 different progression-free survival profiles were obtained. The worse prognosis was seen in patients with high CTC count at both time points, as expected. Interestingly, patients with a high CTC count at baseline but a low CTC count after one cycle of therapy had a much better prognosis, almost similar to that of patients with low CTC count at baseline. Together with analytical validity data, these clinical data of CTC count as a dynamic prognostic biomarker prompted the FDA to clear the CellSearch® technique as an "aid in the monitoring of patients with metastatic breast cancer". The initial claim was that CTC count monitoring during therapy would allow early detection of resistance to therapy and ultimately improve the management of metastatic BC patients.

Several reports were then published, most of them of limited size (level of evidence II–III) and with some discordant results. The IC 2006-04 study was the first observational study specifically designed and powered (267 patients) with CTC count as the primary endpoint (level of evidence 1 study) and confirmed most of the initial findings (Pierga et al., 2012a). Ten years after the seminal study, a pooled analysis of 1944 individual patient data finally established undisputable results on CTC in metastatic BC and demonstrated for the first time the superiority of CTC count over serum tumor markers (CEA, CA15.3). First of all, ≥1 CTC can be detected in about 70% of stage IV BC patients and CTC count is associated with performance status, number of metastatic sites, elevated LDH, elevated serum tumor markers but not with tumor subtype. In addition, CTC count is a dynamic prognostic marker of progression-free survival and overall survival. Hazard ratio of survival between high and low CTC counts increases together with the threshold used to define high CTC count. Finally, in contrast to serum tumor markers, adding CTC count and its change during therapy to an optimized clinico-pathological model significantly increases the prognostic value of the model (Bidard et al., 2014).

3.2. *Clinical utility in metastatic BC*

The first trial initiated to demonstrate the clinical utility of early CTC changes after one cycle of chemotherapy was conducted in the US by the SouthWest Oncology Group. In the SWOG S0500 trial metastatic BC patients whose CTC count stays above 5 CTC/7.5 ml of blood after the first cycle of the first line of chemotherapy were eventually randomized to an early switch to the second line of chemotherapy. Such switch was hypothesized to greatly improve the patients overall survival compared to the standard imaging-based management (targeted hazard ratio: 0.6). The study results were published in 2014 and no survival difference was seen between the two arms (Smerage et al., 2014). To explain these negative results, it has been discussed by the study investigators that second line chemotherapy is unlikely to have a significant effect (even when introduced earlier on the basis of elevated CTC count) on BCs that have a primary resistance to first line chemotherapy. Other comments have been made on the trial's design and concepts (Alunni-Fabbroni et al., 2014; Bidard and Pierga, 2015). On the basis of these negative results, the 2015 American Society of Clinical Oncology clinical practice guidelines for CTC count considered reasonable for clinicians to not use CTC count in women with metastatic BC (Van Poznak et al., 2015). While this negative trial had a major impact on the use of CTC count by US clinicians, two other clinical utility trials based on CTC count are currently ongoing in France:

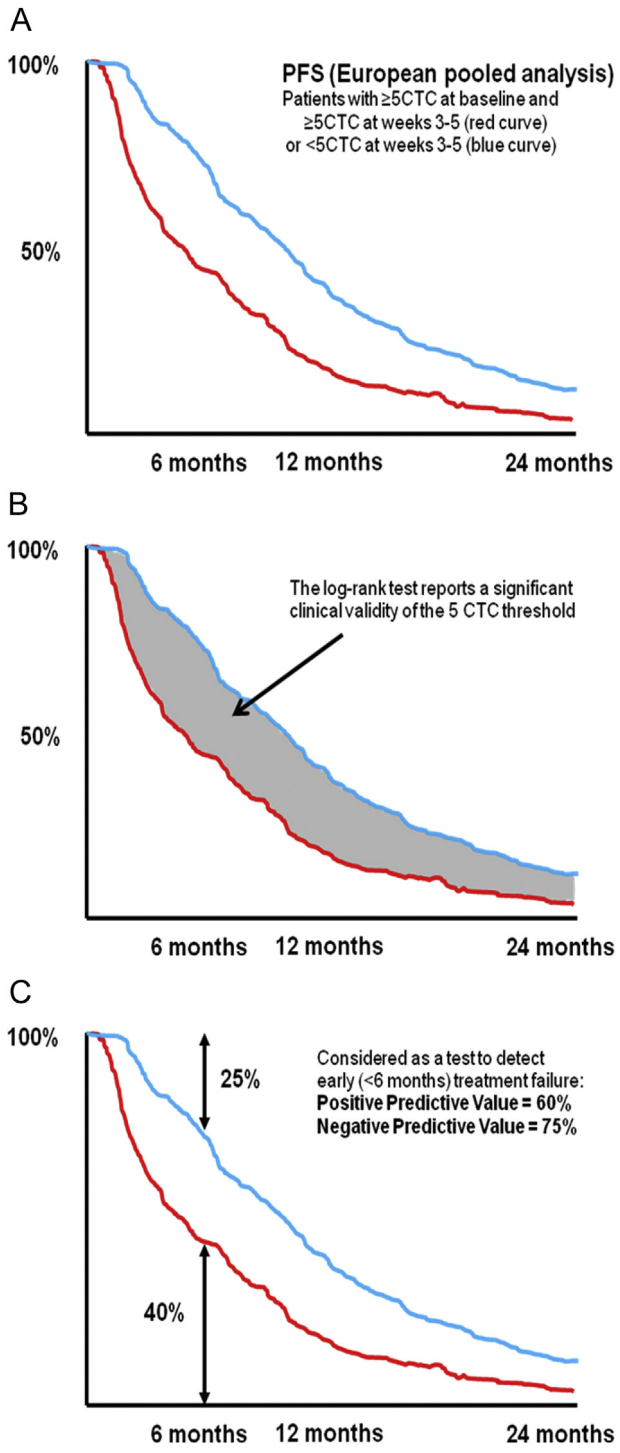

Figure 1 – Thresholds with clinical validity may have limited clinical utility. A: Progression-Free Survival (PFS) curves of metastatic BC patients with ≥5 CTC/7.5 ml at baseline, according the CTC count at weeks 3–5: the red curve displays the PFS of patients who retains a CTC count ≥5 CTC/7.5 ml after 3–5 weeks on treatment and the blue curve displays the PFS of patients in whom the CTC count decreases <5 CTC/7.5 ml after 3–5 weeks on treatment (curves inspired from the European pooled analysis (Bidard et al., 2014)). B: Statistically significant log-rank test between the two PFS curves, confirming the clinical validity of the ≥5 CTC/7.5 ml threshold. C: Using ≥5 CTC/7.5 ml after 3–5 weeks on treatment as a predictive test of early (<6 months) tumor progression, only 60% of patients with a positive test (i.e. ≥5 CTC/7.5 ml) have a PFS shorter than 6 months, while 75% of patients with <5 CTC/7.5 ml after 3–5 weeks on treatment have a PFS >6 months. The positive and negative predictive values of the 5 CTC/7.5 ml threshold are therefore limited when used as a test to predict early tumor progression.

- The "CirCe01" trial (NCT01349842) is also based on the early changes of CTC count, but patients are enrolled before the start of third line of chemotherapy and followed with the CTC test throughout the successive lines of chemotherapy.
- The "STIC CTC" trial (NCT01710605) investigates the clinical utility of the prognostic value of baseline CTC count. In this trial, the choice of the first line of treatment (hormone therapy or chemotherapy) for relapsing hormone-positive BC is determined either by the clinician or by the baseline CTC count.

The study designs have been reported elsewhere (Bidard et al., 2013b), and the results are expected within the next 2 years.

4. Quantitative aspects of CTC detection

4.1. *Detection rate*

Numerous CTC detection techniques have been developed so far (Ignatiadis et al., 2015; Parkinson et al., 2012), but only a few of them have been used in clinical cohorts of relevant size. Among these technologies, some very promising approaches, such as the microfluidic-based "lab-on-a-chip", have not yet confirmed their better sensitivity in large cohorts (Nagrath et al., 2007). As cancer cell lines do not recapitulate the phenotypic heterogeneity (in size, protein expression, stiffness…) of real CTC, preclinical reports relying on cancer cell lines spiking assays to estimate the sensitivity of CTC detection techniques are of limited clinical interest. In that regard, direct comparisons between techniques in real patients are more valuable. In BC patients, such comparison showed that filter-based and different EpCAM enrichment-based detection techniques have a globally similar CTC detection rate, although important differences were seen at the individual level (Farace et al., 2011; Magbanua et al., 2015). Another approach to increase the CTC detection rate is to screen larger volumes of blood (Fischer et al., 2013; Stoecklein et al., 2015), although higher numbers of CTC detected does not always translate into a better clinical validity or utility. As an example, a head-to-head comparison of the Adnatest® (multiple antibodies-based immunoenrichment followed by epithelial mRNAs detection) and the CellSearch® system showed that, in contrast to the CellSearch® system, the Adnastest® system had low/no prognostic value in metastatic BC patients (Müller et al., 2012).

4.2. *Reproducibility*

Reproducibility issues are also crucial for CTC detection, and very few techniques clearly demonstrated their intra-and inter-laboratory reproducibility. The FDA clearance of the

CellSearch® system for CTC detection in metastatic BC was primarily based on the review of *in vitro* performances of the system. Later, clinical data tested its reproducibility on metastatic (Kraan et al., 2011) and early BC (Ignatiadis et al., 2014) and reported an acceptable even not perfect inter-laboratory reproducibility. These limited, yet existing, reproducibility issues in addition to statistical fluctuations related to rare events (Poisson's law) led to the threshold of ≥5 CTC/7.5 ml of blood. The use of the CellSearch® system was also restricted to BC metastatic patients only, despite clinical validity of the data obtained with the ≥1 CTC/7.5 ml threshold in early BC patients. To lower these reproducibility issues, a weekly inter-laboratory review of detected CTC has been therefore initiated in the above-mentioned TREAT CTC trial, which relies on detection of CTC in early BC patients.

4.3. *Threshold-related issues*

Finally, quantitative approaches of CTC detection in BC have been strongly restricted by the early establishment of the ≥5 CTC/7.5 ml threshold separating patients into two groups with "shorter" or "longer" survival. That threshold was set on the basis of the reproducibility issues described above but also because 5 CTC/7.5 ml was the median CTC count, thus maximizing the log-rank test results (Cristofanilli et al., 2004). Further data, obtained in the large European pooled analysis of CTC in metastatic BC patients, showed that the survival hazard-ratio increases together with the CTC count, with no clear threshold (Bidard et al., 2014). Furthermore, it is critical to point out that the ≥5 CTC/7.5 ml threshold is an overall prognostic factor in metastatic BC (endpoint: global survival curves) but was never optimized to detect early tumor progression (endpoint: resistance to chemotherapy with tumor progression within the first 3–4 months, Figure 1). In the run-in phase of the phase III CirCe01 trial (NCT01349842), which allows for a CTC-based management of chemotherapy in advanced metastatic setting, we observed that among patients with ≥5 CTC/7.5 ml at baseline, a composite criteria (<5 CTC/7.5 ml or relative decrease ≥−70% of the baseline CTC count) showed better prognostication for early tumor progression (within 4 months) than the proposed <5 CTC/7.5 ml threshold (Helissey et al., 2015). The role played by this threshold-related issue in the SWOG0500 trial failure is potentially critical, as a non-negligible percentage of patients in the CTC arm (around 25%, according to the published progression-free survival curves (Smerage et al., 2014)) have been switched off a first line chemotherapy that would have delayed tumor progression for >6 months. We therefore recommend that thresholds for clinical validity should be distinguished, whenever needed, from those intended for clinical utility.

5. CTCs as a tool for liquid biopsy

5.1. *Successes and pitfalls of CTC characterization*

As any other tumor material (surgical specimen, biopsy, fine needle aspirate…), CTC can be characterized with "omics" analyses: genomics, either focused on copy-number profiles (Kanwar et al., 2015; Neves et al., 2014; Polzer et al., 2014), *ERBB2* amplification (Krishnamurthy et al., 2013; Mayer et al., 2011), point mutations (Deng et al., 2014; Fernandez et al., 2014; Markou et al., 2014; Pestrin et al., 2015; Schneck et al., 2013) or on DNA methylation (Chimonidou et al., 2013a, 2013b, 2011), transcriptomics (Mostert et al., 2015; Onstenk et al., 2015b; Powell et al., 2012; Sieuwerts et al., 2011) and proteomics. Among proteins of potential therapeutic interest, the estrogen and progesterone receptors (Nadal et al., 2012; Somlo et al., 2011), HER2 (Ligthart et al., 2013), Ki67 (Paoletti et al., 2015) and apoptosis and DNA-repair related proteins (Garcia-Villa et al., 2012; Smerage et al., 2013) have been investigated, together with PDL1 expression on CTC (Mazel et al., 2015) and many other potential biomarkers.

While these approaches are useful for the understanding of the biological bases of the metastatic process, it is critical to understand that these characterizations face many potential flaws. First, molecular characterization can be performed on single CTC or on the CTC bulk. Single CTC approach requires sophisticated tools for CTC sorting, generally based on microfluidics (Autebert et al., 2012), with a long turnaround per sample. Then single-cell molecular approaches have to be used on isolated CTC, while the sensitivity and specificity of these approaches are not fully controlled. Strikingly, many studies based on single CTC approach are reporting high discrepancy rates between CTCs and matched tumor deposits (primary tumor or metastases), but also among CTCs. *PIK3CA* mutation status, when studied in CTC, was shown to display "molecular heterogeneity" (Pestrin et al., 2015), "heterogeneity and discordance" (Deng et al., 2014), or to "change with time" (Markou et al., 2014). As *PIK3CA* mutations are thought to be clonal in BC, it is unclear whether these high discrepancy rates are due to either the limited sensitivity of single cell characterization techniques, leading to false negative results, or to a real major intercellular heterogeneity, previously ignored. Similar examples of questionable heterogeneity can be found in proteomic analyses of CTC, many of these using non-standardized immunocytofluorescence. We demonstrated with HER2 immunofluorescence of BC CTC that the reliability of these approaches clearly is a direct function of the number of CTC studied (Ligthart et al., 2013), and that low CTC count is a major flaw for the translation of CTC characterization into a trustworthy clinical tool. In contrast to single CTC characterization, quality of population-based approaches depends on the CTC/background leukocytes ratio and focus on patients with elevated CTC counts. Beyond any biological findings, these patients with extremely elevated CTC count (e.g. >100,000 CTC/7.5 ml in Heidary et al. (2014)) are rare and have a gloomy prognosis, limiting the interest of such approaches in clinics.

5.2. *HER2, a key target in BC CTC*

Notwithstanding the above-mentioned limitations of CTC molecular characterization, CTC-based HER2 status assessment became of primary interest 10 years ago when a first report showed that *ERBB2*-amplified CTC were detected at the time of tumor progression in HER2-negative metastatic BC patients (Meng et al., 2004). It is currently unclear whether changes of HER2 status are mostly due to inaccurate HER2

status assessment of the primary tumor or to the metastatic growth of a HER2-positive subclone initially "hidden" within HER2-negative primary tumor. In both cases, however, the addition of anti-HER2 drugs might demonstrate some clinical benefit. Two prospective trials are currently ongoing to demonstrate the clinical benefit of anti-HER2 therapy in patients with metastatic BC considered as HER2-negative and HER2-positive CTC. The German DETECTIII study is a phase 3 study, comparing the lapatinib and chemotherapy combination vs chemotherapy alone in patients having at least 1 CTC with strong HER2 immunocytofluorescence (NCT01619111). A recent update on this study stated that about 19% of HER2-negative metastatic BC patients were screened with at least 1 CTC with strong HER2 fluorescence (Janni, 2015a). In contrast, the French CirCe T-DM1 study is a single arm phase 2 study in which metastatic BC patients with HER2-amplified CTC (HER2/CEP17 FISH) receive T-DM1 as single therapy (NCT01975142). In this study, the discrepancy rate (patients with HER2-negative BC harboring HER2-positive CTC) is lower than in the DETECTIII trial, around 5–8% (Bidard, 2015, p. 1). Details on these trials have been published elsewhere (Bidard et al., 2013b; Schramm et al., 2015).

6. Biology of CTCs

6.1. CTC detection and BC features

CTC represent a unique opportunity to look at the metastatic process in patients and to study the molecular (genomic, transcriptomic and proteomic) processes associated with the spread of tumor cells throughout the body (Bednarz-Knoll et al., 2011). Several biological parameters that could influence CTC release and/or CTC detection have been investigated. As mentioned above, CTC detection is globally correlated with the tumor burden, and therefore more frequent in patients with metastatic BC. Among the non-metastatic BC, inflammatory BC display very high CTC levels (Hall et al., 2015b; Pierga et al., 2012b), contributing to the poor prognosis of these aggressive BC. We found no overt association between CTC count at metastatic relapse and BC clinical subtypes (hormone receptor-positive, HER2-positive, triple-negative) in the European pooled analysis (Bidard et al., 2014). However, the use of anti-HER2 therapies was associated with a major and prolonged drop of the CTC count in HER2-positive metastatic breast cancer, as also reported in neoadjuvant study on inflammatory BC (Pierga et al., 2012b). The association of CTC and another blood-derived cancer biomarker, the circulating tumor DNA (ctDNA), has been investigated in two cohorts of limited size (Dawson et al., 2013; Madic et al., 2015). As in other tumor types, CTC and ctDNA are globally correlated in BC, but ctDNA can be detected in more patients. In metastatic BC patients, it is unclear whether ctDNA levels present a stronger prognostic impact than CTC. In contrast to Dawson et al., we reported that the quantification by two next-generation sequencing technologies of cell-free *TP53* mutations in metastatic triple negative BC, before the start of a new line of therapy, had no significant prognostic impact. In this study, baseline CTC detection demonstrated its prognostic impact despite the limited number of patients, suggesting that the prognostic information conveyed by ctDNA might be inferior, or at least not superior, to that of CTC (Madic et al., 2015). Other biologically and clinically relevant differences between CTC and ctDNA have been reviewed elsewhere (Bidard et al., 2013a).

6.2. CTC and epithelial–mesenchymal transition

The presence of epithelial–mesenchymal transition (EMT) features on BC CTC has been investigated in several cohorts of limited size. EMT is a process by which carcinoma cells can reprogram and acquire some cellular properties mostly seen in mesenchymal cells, such as motility, invasion and absence of cell polarity. EMT is thought to be induced by the tumor microenvironment (e.g. following the release of growth factors) or in response to hypoxia, and leads to a local spread of cancer cells. Epithelial cells that have undergone EMT may express mesenchymal markers while reducing the level of expression of epithelial markers such as EpCAM (Thiery, 2002). For these EMT-related investigations on CTC, it is critical to clearly isolate CTC from leukocytes, as normal blood cells are of mesenchymal origin. In a study with high resolution imaging of isolated CTC in a microfluidic chip, mesenchymal CTC from BC patients were isolated, either as single cells or as cell clusters, and showed to express EMT-related genes (Yu et al., 2013). Interestingly, epithelial and mesenchymal patterns were different across tumor subtypes and were also dynamic during therapy, mesenchymal CTC being frequently observed at the time of tumor progression. Several other studies reported some mesenchymal traits on detected BC CTC (Armstrong et al., 2011; Mego et al., 2015a,b, 2012; Papadaki et al., 2014), but the clinical impact of these cells has not been demonstrated yet. Stemness-related features have been also detected on BC CTC (Raimondi et al., 2011). The impact of EMT-associated EpCAM downregulation on CTC detection by EpCAM-dependent techniques, such as the CellSearch® system, has been repeatedly reported. Although most of BC subtypes can be efficiently captured by EpCAM-based assays (Ring et al., 2015), several EMT-related antigens have been successfully investigated in addition to EpCAM-based immunoselection as an effective way to increase CTC detection (Mostert et al., 2012, 2011; Onstenk et al., 2015a; Satelli et al., 2015).

6.3. Alive CTC

Live CTC can be also detected by different techniques (Ignatiadis et al., 2015). The epithelial immunospot EPISPOT® technique allows for the quantification of live CTC based on their ability to secrete proteins related to epithelial lineage (Alix-Panabières and Pantel, 2015), and demonstrated a significant prognostic impact in a cohort of 254 metastatic BC patients (Ramirez et al., 2014). The success of short-term culture of CTC was also shown to be a function of antitumor treatment efficacy in the corresponding patient (Khoo et al., 2015). A few articles also reported the establishment of CTC-derived xenografts (Maheswaran and Haber, 2015; Rossi et al., 2014). Conversely, CTC can be also detected and studied in "regular" xenografts (*i.e.* patient-derived or cell line-derived

xenografts); such approaches might decipher the biological determinants of CTC release by "regular" BC xenograft, CTC-derived BC xenograft or even by BC primary tumor (Baccelli et al., 2013; Giuliano et al., 2015; Javaid et al., 2015). In a preliminary study, pre-existing activation of DNA damage response pathways in CTC was associated with resistance to chemotherapies that were otherwise efficient on the matched primary tumor bulk (Gong et al., 2015), a preclinical finding that might explain why the primary tumor response to chemotherapy does not influence the prognostic impact of CTC in early BC.

7. Conclusion

CTC detection proved to be a significant prognostic factor in both early and metastatic BC but evidences supporting its clinical utility are still expected, through numerous ongoing trials. While ctDNA is apparently a better material for the characterization of the cancer mutational landscape (Alix-Panabières and Pantel, 2013; Bidard et al., 2013a), the intrinsic biological role of CTC as metastatic "seeds" and its corresponding clinical prognostic impact guarantee the use of CTC as a major biological and clinical tool in BC patients.

Conflict of interest

The authors have no conflict of interest.

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