Chapter 2
Circulating Tumour Cells in Primary Disease: The Seed for Metastasis

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Abstract Metastatic dissemination is the most common cause of death in cancer patients. Traditionally, dissemination has been considered a late event; however it has been suggested that at least in some tumours, cells might leave the primary lesion early in its development. To escape from the primary, tumour cells undergo total or partial epithelial to mesenchymal transition (EMT), become able to unmoor from surrounding epithelial cells and reach the bloodstream. Alone or in groups (clusters), these circulating tumour cells will use their phenotypical flexibility, including properties associated with EMT, stemness, resistance to anoikis and dormancy to survive in the bloodstream, reach, invade and colonise distant organs. In recent years, these cells, which can be detected in the blood or in the bone marrow in the early disease setting, have been studied as prognostic markers as well as a potential source of dynamic information regarding tumour characteristics to guide treatment decisions. Ongoing clinical trials are evaluating the clinical utility of circulating tumour cells.

Keywords Circulating tumour cells • Cell clusters • Epithelial-mesenchymal transition • Dormancy • Reseeding • Phenotypical flexibility • Stemness • Liquid biopsy • Heterogeneity • HER2 status discordance

Introduction

Despite the advances in the treatment of breast cancer (BC) achieved in the last 30 years, development of metastatic disease is still a major cause of death for women diagnosed with BC [1]. Metastasis formation is a highly inefficient process in which tumour cells leave the primary tumour, enter the bloodstream, invade other organs and colonise them, forming secondary tumours (metastasis) [2–4]. Technological advances have allowed the detection of tumour cells circulating in the bloodstream—called circulating tumour cells (CTCs)—and of tumour cells that have invaded other organs called disseminated tumour cells (DTCs) [5–7].

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Both CTCs and DTCs have been the focus of intense translational research in the last 20 years. Interest in them is driven by the insights they can provide into the underpinnings of the process of cancer dissemination, leading possibly to new therapeutic targets, as well as how they may inform us on the dynamic changes in tumour genotype and phenotype that occur during treatment [6, 8, 9].

In this chapter, we provide a review of the findings from CTC research that have led to a better understanding of the dissemination process with special emphasis on BC. We focus on the contributions of CTC research to the key concepts of epithelial to mesenchymal transition (EMT), stemness, dormancy and self-seeding, as well as the potential role of CTC clusters in dissemination. Finally, we discuss studies on the prognostic significance of CTCs/DTCs and on characterisation of these cells and ongoing interventional trials testing CTCs as tools to improve outcome of patients with early BC.

Circulating Tumour Cells and the Biology of Metastasis

Definition of Circulating Tumour Cells

CTCs were first detected in BC patients 150 years ago [10] and are shed by the primary tumour during the dissemination process. They are rare events detected in the bloodstream (1 for every 10^6/10^7 mononuclear cells). CTCs can leave the primary tumour either alone (single CTCs) or in clusters with other tumour cells and/or surrounding stromal cells and matrix (respectively, homotypic or heterotypic CTC clusters, Fig. 2.1) [6, 7, 11]. Though several aspects of CTC/DTC biology are not entirely understood, one characteristic of these cells is phenotypical plasticity, i.e. the ability to undergo phenotypic changes in order to survive [7, 12–14].

Models of Metastatic Progression

Models of metastatic progression have evolved from the nineteenth century onwards and are generally based on the assumption that cells harbouring genetic or epigenetic alterations evolve within a Darwinian framework, acquiring progressively the malignant phenotype [15]. In BC specifically the original Halstedian model assumed progression in a local, centrifugal fashion before invasion of distant organs, stressing as a consequence local versus systemic therapeutic approaches [16, 17]. Over time, with increasing knowledge on tumour biology including studies on CTC/DTCs, our understanding has evolved significantly and today emphasizes the systemic nature of BC [3, 18].

The Linear and Parallel Progression Models

The model of linear progression from preinvasive to invasive and to metastatic tumour assumes that metastasis results from pre-existing highly metastatic rare cells within the primary tumour [19]. This model is supported by the clinical observation

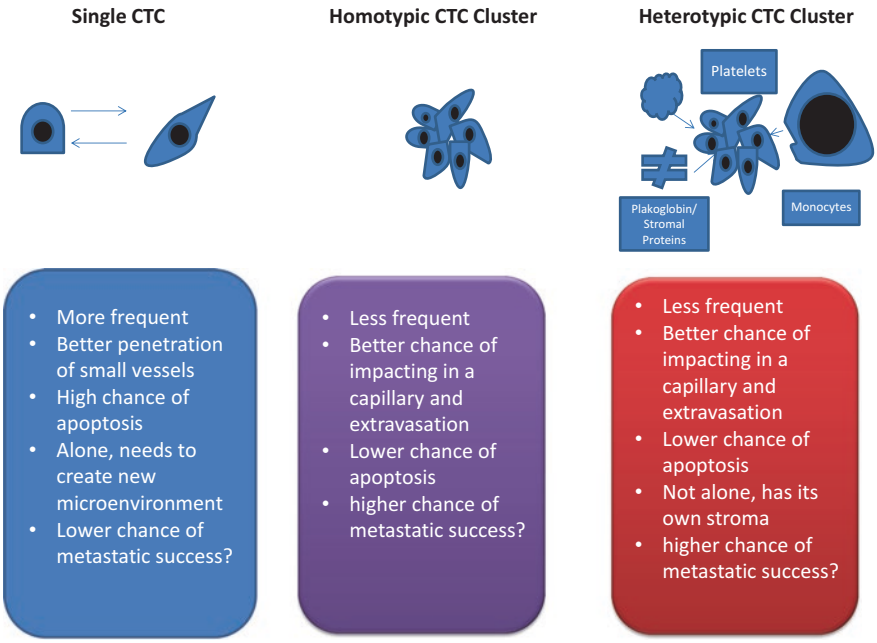

Fig. 2.1 Comparison between single CTC, homotypic clusters and heterotypic clusters

that larger tumours tend to be more often metastatic at presentation or to recur more frequently post-curative treatment than smaller tumours and is the basis for current screening and treatment guidelines [20, 21]. Though the linear model emphasises the systemic nature of cancer [3], CTCs and DTCs are deemed to be late events, the product of successive generations of cells undergoing natural selection within the primary tumour, acquiring most if not all of capabilities necessary to successfully disseminate and eventually leaving the primary to form distant metastasis [19, 22].

However, the linear model alone does not adequately explain a number of clinical scenarios. Though patients with T2–4 or N-positive BC have a higher chance of developing local or distant recurrences, still, 4% of patients with tumours smaller than 1 cm will die from cancer in 10 years [23]. Moreover, evidence from clinical studies on CTCs/DTCs and from studies using murine models suggests that dissemination may start earlier than previously thought [18, 24, 25].

In one study by Banis and collegues, 16% out of 404 patients with ductal carcinoma in situ (DCIS) scheduled to undergo surgery had detectable DTCs in the BM. No correlation with lymph node positivity or survival was found [26]. The same group as well as other investigators have found similar results in smaller studies of patients with DCIS or microinvasive disease [27, 28]. In the landmark meta-analysis published in 2005 with a total of 4703 patients, 25.2% of patients with T1 tumours had detectable DTCs in the BM [29]. Indirect clinical evidence for early dissemination has also been provided by studies on the adverse prognostic value of

CTC detection in patients with early breast cancer [30, 31]. We have detected CTCs in the bloodstream of 9% (6/73) of women with DCIS/LCIS [32].

Beyond the above clinical studies, there is evidence from preclinical studies that dissemination might occur early on during the process of metastasis.

Podsypanina and colleagues showed that non-cancerous murine mammary cells injected into the bloodstream can survive inside lung tissue. These cells can undergo malignant transformation forming lung metastasis completely independently from the existence of a primary tumour [33].

Another group studied the development of metastasis before the appearance of primary tumour in a model of BC in mice that are carriers of the polyoma virus middle T oncogene. The development of primary BC in these mice happened in stages—first hyperplasia around 26 days after birth, followed by solid masses developed at around 60 days and finally invasive tumours by 116 days. In contrast, cultures of lung tissue showed cells capable of forming tumour colonies at day 26. These cells were morphologically similar to mammary cancer cells and expressed cancer stem cell (CSC) markers CD44 and Sca1 [34].

In a landmark study using mice that have a constitutionally activated human epidermal growth factor receptor 2 (HER2) gene, Hüsemann and colleagues showed that DTCs can be detected in the bone marrow of mice during the preinvasive phase of tumour progression [24]. The mice develop mammary hyperplasia between weeks 7 and 9, in situ lesions by 14–18 and overtly invasive disease by 23–30 weeks. In the BM, DTCs were already detectable in some cases as early as week 4. Interestingly, as primaries grew in size, the number of DTCs as well as the number of genetic alterations in these cells remained fairly stable.

Taken together, these data suggest that cells might leave the primary tumour early during tumour development and evolve in parallel with cells in the primary tumour (parallel progression model). This model implies genomic heterogeneity between primary tumour and distant metastasis, since there is a parallel evolution in the primary and distant site. This model, if true, challenges our capacity to infer genomic profiles for micrometastatic disease based on the analyses of the primary tumour and might at least partly explain why some patients with early BC relapse and die despite adjuvant systemic treatment. On the other hand, the fact that adjuvant treatment based on primary tumour characteristics is effective in reducing relapses and the existence of effective primary tumour-based gene signatures that predict the risk of distant relapse [35] suggests that the parallel progression model alone cannot explain a significant proportion of BC cases.

Tumour Self-Seeding

Self-seeding is another complementary hypothesis that has been supported by recent experimental evidence, which postulates that CTCs and DTCs, now "veterans" of the hard evolutionary struggle of invasion, survival in the bloodstream and colonisation of distant organs, return to the primary tumour, an environment likely to prove more welcoming to them [36–38].

The pivotal studies conducted by Kim and colleagues were designed to evaluate this intriguing hypothesis [39]. In a murine model, the investigators injected two mammary glands with a metastatic BC cell line, one marked with GFP-luciferase (donor) and another without (recipient). After 60 days recipient tumours had extensive seeding by donor cells (up to a quarter of total mass). In a further study the same group of researchers showed that this 'homing" effect is possibly achieved through chemo-attractants such as interleukin-6 and interleukin-8. Interestingly, the rate of growth in the seeded primary was accelerated mainly by increased number of cells through a paracrine effect, suggesting that self-seeding can induce increased growth in the primary [39].

Zhang and colleagues in a series of experiments using an osteosarcoma murine model encountered very similar results [40]. The hypothesis that interleukin signalling mediates self-seeding has been further strengthened by a recent publication by the same group showing that suppression of IL-6 halts self-seeding [41].

Apart from the above preclinical studies, indirect evidence supporting the hypothesis of self-seeding has been provided by a clinical study in which 3072 patients undergoing surgery for BC were submitted to a BM biopsy. 732 (24%) had DTCs, 139 patients experienced local recurrence and 48 of these (35%) were DTC positive. Being DTC positive was significantly associated with a higher risk of local recurrence [42], suggesting that DTCs can influence local recurrence.

Steps in the Invasive: Metastatic Cascade

The success of a CTC/DTC is measured by its capacity in actually generating metastasis. In order to achieve this, a complex series of events must take place called the invasive-metastatic cascade. This process involves cancer cells as well as multiple other actors and can be divided into several steps [3, 43].

Local Invasion and Intravasation

In epithelial tumours, cells will face severe challenges before they are able to disseminate. To reach the bloodstream, cancer cells must first break through the barrier formed by surrounding cells, underlying stroma and endothelial cells. There is no single path to tissue invasion, with multiple mechanisms both active and passive coming into play to result either in individual invasion or collective invasion and intravasation, mirroring the two forms in which CTCs may be detected—single or clustered [43, 44]. In both, however, the process of EMT is fundamental.

Epithelial-Mesenchymal Transition

Epithelial cells share characteristics that make them well adapted to their function of forming cell barriers. Apical-basal polarity and tight cell-to-cell binding through desmosomes and between cell and basal layer through hemidesmosomes allow for

their organisation into highly structured sheets of cells, such as in the skin or the intra-ductal epithelium of the breast [45]. However, during physiological situations such as organogenesis and wound healing, epithelial cells must be able to move alone and in groups as well as to change shape, abilities more often associated with mesenchymal cells [46]. This process of phenotypic plasticity through which epithelial cells lose their epithelial characteristics and acquire those of mesenchymal cells is named epithelial-mesenchymal transition (EMT). EMT is driven by cell-to-cell communication through various cytokines—such as between epithelial cells and cancer-associated fibroblasts or macrophages. This interaction leads to activation of pathways such as transforming growth factor beta (TGFbeta), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) signalling pathways [47, 48], by inducing one of the EMT-associated transcription factors, including TWIST1, SNAI1 or SLUG [49]. To test the importance of cytokine milieu produced by activated monocytes (MCs) in inducing EMT, Cohen and colleagues exposed in vitro cell lines from both inflammatory BC and non-inflammatory BC to activated MCs, with results showing increased cellular motility as well as a morphological and protein expression shift towards a mesenchymal phenotype.

For a cancer cell seeking to leave the primary tumour (or preinvasive lesion) and to cross the endothelium to reach the intravascular space [50], EMT is instrumental [13, 51]. Complete EMT (where all epithelial characteristics are lost) can lead to individual invasion. The cell loses traditional epithelial polarity and proteins (E-cadherin, plakoglobin and integrins) that form the attachment organelles, freeing it to enter conjunctive tissue underneath. During this process it acquires one of the two possible phenotypes:

1. A mesenchymal-like shape with lamellipodia and filopodia granting ability to adhere to the extracellular matrix (ECM) through N-cadherin: These cells produce enzymes such as the metalloproteinases (MMP) that play several key roles in BC progression, including the degradation of the ECM, of cell-to-cell junctions, stimulation of cell motility and interestingly the induction of EMT directly and indirectly (by influencing surrounding cancer-associated cells) [52, 53]. Thus, it can be said of these cells that through intercellular communication they become true "microenvironment engineers" with the goal of constructing the environment that maximises their chance of invasion [54].
2. Amoeboid, with round-shaped cells that can pass through gaps formed by other cells in the ECM (macrophages, for instance): It is MMP independent, and tightly regulated through interplay between RHO kinase and the GTPase RAC [55]. The relevance of this mode of movement in vivo is still controversial since artificial models of ECM microenvironment may not be accurate, possibly allowing for cellular motility that would not be possible in vivo [56, 57], leading some experts to challenge the invasive potential of this phenotype.

Recent studies demonstrate the existence of populations of CTCs that express markers for EMT, and are not detected through traditional EpCAM-based methods [58, 59]. Their study has been expanded by the increasing perception of their special clinical and biological relevance. Other mesenchymal markers are being used to detect them, such as vimentin, Twist and N-cadherin [60].

In a recent study in 149 patients with early BC, Cierna and colleagues sought to investigate the presence of CTCs with EMT markers, as well as their correlation with MMP1 in tumour tissue and stroma [61]. CTCs with epithelial markers were present in 8.7% of patients and CTCs with epithelial-mesenchymal transition (EMT) markers in 13.4% of patients. Patients with CTC/EMT in peripheral blood had significantly increased expression of MMP1 in tumour cells and tumour-associated stroma ($p = 0.05$) than those of patients without CTC/EMT. MMP1 expression was associated with tumour features such as high grade or high proliferation (Ki 67 > 20%), both markers of worse prognosis.

In an animal model of CTC EMT composed of mice bearing metastatic BC grafts from human cancer, Gorges and colleagues were able to detect CTCs with EMT markers that an EpCAM-based method could not detect [58]. To test the theory that cells injected into the bloodstream of a mouse would down-regulate EpCAM (a phenomenon used here as a surrogate marker for EMT), they injected tumour cells that expressed epithelial markers, and looked for them for 30 min to 4 h after injection. CTCs showed morphological changes and down-regulated EpCAM within 30 min from injection. Some cells, however, presented expression patterns that were mixed. The above results suggest that the regulation of phenotypic changes in tumour cells is a dynamic process [58].

Partial EMT, a process that leads to cells with mixed epithelial-mesenchymal features, might be the most efficient pathway to dissemination [57]. While complete EMT leads more likely to the shedding of single cells, in partial EMT clumps or clusters of between 2 and 50 cells will be shed together with parts of their microenvironment, including cancer-associated cells and ECM, possibly leading to survival and colonisation advantages [11, 57, 62, 63].

To reach the bloodstream cells must breach blood vessels, a step facilitated by tumour stimulation of the formation of new vessels that are tortuous and “leaky”. The MMPs that participate in previous steps are also important in this process of formation of new vessels and constant remodelling [64].

Survival in the Circulation and Extravasation

Dissemination is an inefficient process, and most CTCs will not successfully form metastasis [65]. In practice, most CTCs detected are apoptotic [66]. Different phenomena account for this high failure rate and are connected to challenges the CTC will face in the bloodstream.

Single Circulating Tumour Cells

Once in the bloodstream, different mechanisms can lead to the apoptosis of singe CTCs such as anoikis and the shear stress exerted by the blood current. The high failure rate at this phase is well exemplified by a study by Tarin and colleagues performed in 1984 on 15 cancer patients with neoplastic ascites. Peritoneovenous

shunts were placed to alleviate ascites and as a consequence a high number of tumour cells were injected into the circulation. Surprisingly, very few patients went on to develop solid metastasis in the following months or even years, in one case [67].

Anoikis can be defined as apoptosis induced by disconnection from the ECM and from surrounding cells; it seems to be a built-in safeguard against unmoored epithelial cells that can disrupt tissue cohesion. It is therefore a process that all successful tumour cells must evade to survive. Many of the signalling pathways harnessed for increased proliferation in cancer can confer resistance to anoikis, such as EGFR. More significantly, EMT (total or partial) also can confer resistance to it [7, 68].

The shear stress produced by circulation of blood is recognised as being capable of inducing phenotypic changes in endothelial cells that are fundamental to normal vessel function but also to impact the dissemination process [69, 70]. Recent experiments in colon and prostate cancer CTCs suggest that shear force can induce apoptosis via TRAIL in time (duration of shear stress) and dose (strength of shear stress) dependent fashion [71]. This observation may have clinical implication as TRAIL receptor inducers are now in phase I and II testing [72].

Finally, the immune system plays a role likely by clearing cells through natural killer lymphocytes as well as other immune cells. As shown by an experiment by Steinert and colleagues in colon cancer CTCs adaptive pressure caused by immune cells gives rise to CTCs with a CD47 hyper-expressing phenotype that inhibits the activity of immune cells [73].

Clustered Circulating Tumour Cells

The existence of CTC clusters has in the past been used, among other arguments, to question the in vivo relevance of EMT, but, as we have previously discussed, they are not incompatible. Clusters allow for higher ability to survive, resistance to anoikis and higher chance of adhering at secondary sites [7, 74]. Recent evidence suggests that clusters are more capable to lead to successful colonisation compared to single CTCs [11].

CTC clusters are widely present in different epithelial tumours, and can potentially be formed through cells leaving the tumour together or aggregating in the bloodstream [75]. One experiment in an immunodeficient mice model, using a cell line of lung metastatic cells engineered to express either green fluorescent protein or m-cherry in a 1:1 ratio, was conducted by Aceto and colleagues. Cells were injected into the fat pads of mice. Search for CTCs in their blood once overt tumours were present at the injection site uncovered both clustered (2.6%) and single cells (97.4%). Ninety-one percent of the clusters were positive for both protein markers. Nine percent of clusters were of single origin, and none of the single-origin clusters were larger than three cells (suggesting that clusters are not the product of intravessel proliferation of one cell). An examination of the mice's lungs showed that 53% of metastases were of dual origin (and therefore were the product of clusters)

and 47% of single origin. This suggests that while single CTCs are more common, clusters successfully form metastasis 50 times more often. In a second experiment, the same group investigated the issue of the origin of clusters—formation by random aggregation in the bloodstream versus group exit from the primary. The same differently marked cells were injected, this time in different fat pads. A total of 96% of resulting clusters were of single colour, suggesting that intravascular formation of a cluster is a minor phenomenon. Another provocative result of this experiment came from the analysis of the primary. Up to 5% of the cells in each primary carried the opposite colour tag, suggesting that cells can invade the primary lesion (self-seeding). Further experiments by the same authors showed that clusters displayed higher resistance to apoptosis and faster clearance from blood—probably secondary to capillary entrapment that may favour metastasisation. In the blood of ten patients with metastatic BC the same authors were able to define that the gene encoding the protein plakoglobin is highly overexpressed in clusters [11].

The enhanced ability of CTC clusters to form metastasis has been corroborated by three recently published studies. In a cohort of 115 patients, single CTCs and clusters were detected in 36 (31.3%) and 20 (17.4%) of patients, respectively. Though both findings predicted worse prognosis, it was in patients where both were present that the risk of progression was the highest. In another study, in an advanced triple-negative breast cancer population, CTCs (both single and clustered) were correlated with worse prognosis. Interestingly cells detected in clusters had a much lower chance of being apoptotic [76]. In lung cancer one study reported similar findings on cluster cell apoptosis, as well as, significantly, that clusters expressed mesenchymal markers in a heterogeneous manner (vimentin and E-cadherin), compatible with different cells undergoing EMT to different degrees [77].

Plakoglobin is one of the molecular components of the cell-to-cell junctures called desmosomes and plays a vital role in maintaining the cohesiveness of CTC clusters. Suppression of plakoglobin expression has been suggested to inhibit the formation of clusters and metastasisation [11]. This result nicely complements work from Holen and colleagues on the role of plakoglobin in invasion. Blocking plakoglobin expression led, in vitro, to reduced adhesion between cells and enhanced invasion through an artificial model of the basement membrane. In vivo suppression led to greater growth of the primary, but no formation of metastasis, despite evidence of more than a 2.5-fold increase in CTCs [78].

What hypothesis can be drawn from the above studies? It seems that clusters of cells that undergo partial EMT and thus keep enough adhesive capabilities to maintain cohesion and even stroma inside the bloodstream can better survive in the bloodstream [11, 62, 79]. These cells may be more adept at initiating metastasis. Associating in the bloodstream with platelets and other cells remains as important as it was in invasion or as it will be during colonisation [80]. The paradoxical role of plakoglobin, however, on the one hand during invasion and exit from primary acting as a barrier, and then as an essential component for effective dissemination, shows that, for CTCs, increased metastatic potential may be associated not only with numbers, but also with phenotypical flexibility, upregulating and downregulating pathways as their expression becomes necessary or deleterious. For

extravasation, as well as for other steps in the metastatic cascade, this flexibility is associated with the ability to disrupt normal micro-vessel permeability and is mediated via the secretion of paracrine factors such as angiopoietins and MMPs [3].

Formation of Metastasis

Once extravasation has occurred tumour cells will face the challenge of colonisation. To do so successfully a few key abilities are vital, including the ability to influence the new microenvironment as well as related different cellular states, such as dormancy and stemness.

Disseminated Tumour Cells in the Bone Marrow

The invasion of the BM in BC is a long established fact. As a model of metastatic invasion of tissues and organs, BM is highly valuable, though with the caveat that the application to the clinic is hampered by the fact that BM aspiration is not a routine part of clinical practice in BC [81].

As previously discussed, studies suggest that BM DTCs are present early on during BC development. However, it seems that not all DTCs are capable of colonisation, as is shown by Braun and colleagues in a combined analysis of 4703 BC patients with detectable DTCs with early or locally advanced disease. Of these, despite DTCs being a negative prognostic factor for this cohort, after 10 years <50% had recurred [29].

The crucial bottleneck in this step is the ability to influence a foreign microenvironment. A number of studies have shown that specific genetic signatures facilitate the colonisation by DTCs of different organs and are likely associated with the capacity of DTCs to effectively communicate with surrounding cells in order to achieve colonisation [3]. When unable to do so DTCs may be eliminated or enter a quiescent state called dormancy.

Dormancy

Dormancy is a reversible state of mitotic arrest (or alternatively very slow rate of mitosis) first defined in the 1940–1950s [82]. It allows DTCs to endure unfavourable environments so that they can be able to colonise at a later stage. From a clinical standpoint, it is at the heart of resistance to adjuvant therapy and of late recurrences in BC [12, 14, 83].

There remains a large area of uncertainty regarding the mechanisms that induce dormancy and especially that cause reactivation from dormancy. Dormancy has been suggested to be the results of microenvironmental factors associated with limited angiogenesis and active immune response. Altered signalling through pathways such as the RAS-MEK-ERK/MAPK and PI(3)K/AKT can also induce dormancy.

Therefore, it is possible that dormancy may be a programmed reaction by a DTC installed into a microenvironment in which it cannot thrive, likely due to ineffective communication with surrounding cells [84].

Some interesting experiments on CTC/DTCs have shed some light into this phenomenon. In a model of dormancy, Naumov and colleagues used two different murine BC DTC lines, one slowly metastatic, with large numbers of dormant cells, and another rapidly disseminating, with a small number of dormant cells. Adriamycin treatment was not effective in the dormant subgroups of both cell lines, suggesting that dormancy is a mechanism of adjuvant chemotherapy resistance in BC. This finding correlates with clinical scenarios in triple-negative breast cancer (TNBC) and ER-positive BC during post-treatment follow-up. TNBC is more aggressive and highly responsive to chemotherapy and seldom recurs after 5 years, suggesting (following the above model) a low ratio of dormant cells. In ER-positive tumours, specially luminal A, there is low sensitivity to chemotherapy and a tendency towards late relapses, bespeaking of a high number of dormant cells [85, 86].

In one study investigating the angiogenic phenotype, Rogers and colleagues showed that in vitro, a cell line of liposarcoma that was originally not capable of angiogenesis formed small tumours, but at around 125 days acquired the capacity of producing larger vessels (angiogenic switch), going on thereafter to rapidly proliferate. However, in each consecutive cell generation, 6–8% of new cells reverted to the non-angiogenic dormant state. This underscores that, like EMT/MET, dormancy is a state of cancer cells and that cancer cells can go in and out, as needed [87].

In a study with 36 patients 7 years post-mastectomy, Meng and colleagues showed that 13 (36%) had detectable CTCs, up to 22 years after treatment. CTC clearance is approximately 1–2.4 h, so it is reasonable to assume that the origin of these cells is in slowly replicating/dormant groups of DTCs where the number of divisions is matched by the number of cells that undergo apoptosis or are shed [88].

Stemness

Cancer stem cell (CSC) theory, also called tumour-initiating cell theory, postulates that a subgroup among cancer cells is responsible for the formation of metastasis. Since earlier discussed studies show that CTCs when injected into fat pads lead to formation of local tumours, it is reasonable to assume that a subpopulation of CTCs and DTCs have properties of tumour-initiating cells. The main characteristics of these cells are their ability for self-renewal and for formation of non-CSC progeny [89], as well as their resistance to chemotherapy. At least in some instances, CSC may not be a separate category of cells, but indeed a transient state that can be activated and deactivated, and that is closely connected to EMT [90]. There are several studies in BC, showing that significant portions of CTCs and DTCs express CSC markers.

Aktas and colleagues analysed blood samples from 39 patients with BC and showed that 80% of patients with CTCs overexpressed EMT markers or the CSC marker ALDH1 [91]. In another study, Giordano and colleagues studied samples from 29 women with MBC, and found a correlation between CTCs with EMT

markers and CSC markers [92]. This association between EMT and stemness was investigated in depth by Mani et al., in a pivotal series of experiments [93]. Murine immortalised cells that were previously not able to form tumours were stimulated to undergo EMT. The resulting cells had the expected morphological and protein expression patterns, and were also largely $CD44^{high}/CD24^{low}$, a phenotype strongly associated with CSC. These cells were able to form mammospheres (a standard in vitro surrogate for tumour-forming potential). Furthermore, analyses of these mammospheres showed that only 4–6% of cells were capable of forming new ones. In another experiment, a population of CSC was analysed, looking for EMT markers. Results showed that these cells expressed EMT-related genes at levels similar to CTCs that had undergone EMT [93]. One study of 66 patients with early BC found that 48.6% of patients had DTCs and that 43% of detected DTCs had stem cell-like phenotype [94]. Patients with TNBC had a higher chance of having DTCs with stem cell-like phenotype in the BM [94]. Multiple other studies have confirmed that CTCs with EMT and stem cell-like markers can be found in the bloodstream of BC patients [95, 96]. Moreover, bone marrow DTCs with stem cell-like phenotype have shown to be an unfavourable prognostic marker in BC [97].

Taken together these results show that a subpopulation of CTCs and DTCs express stem cell-like markers, and that stemness may be a state associated with EMT that can be acquired by tumour cells.

Clinical Applications of Circulating Tumour Cells

The clinical potential of CTCs/DTCs has been intensively studied in the last 15 years [98] and multiple detection methods are available, that have been recently summarised [6, 99]. CTCs in the early setting can be used (Fig. 2.2):

1. As screening tools
2. As prognostic factors
3. For identifying therapeutic targets
4. For monitoring response to treatment

These applications may be achieved via enumeration or characterisation of CTCs at the deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein levels.

Circulating Tumour Cells as Prognostic Factors

In early BC a large number of studies have demonstrated the adverse prognostic value of CTC/DTC detection (Table 2.1) [30, 31, 100–109, 112–124, 126].

Zhang and colleagues have performed a meta-analysis of both early disease and metastatic trials, totalling 49 studies and 6825 patients [129]. In early disease CTC detection was associated with worse DFS and OS (HR 2.86 and 2.78, respectively).

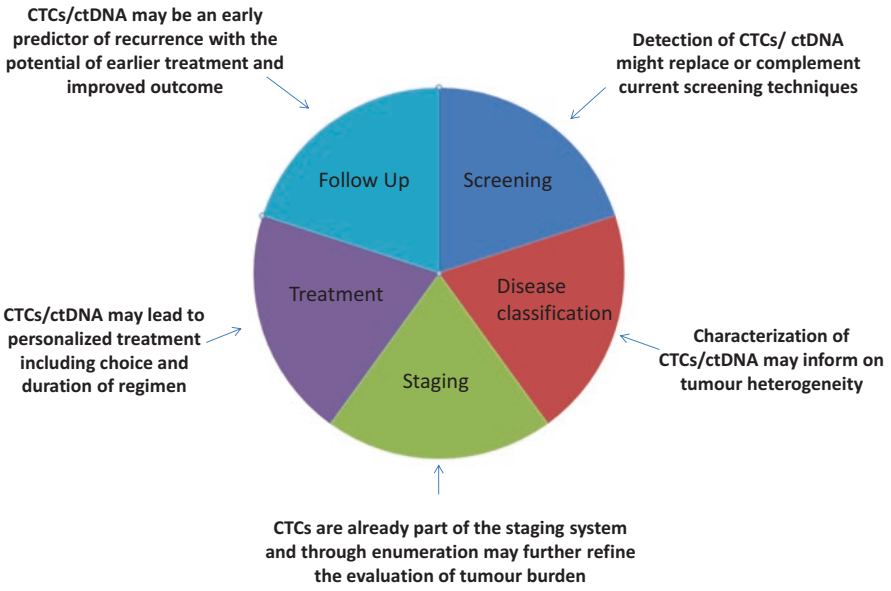

Fig. 2.2 Potential clinical applications of CTCs/ctDNA

In a recently published pooled analysis of individual data from 3173 patients with early BC, Janni and colleagues detected CTCs in 20.2% of the patients. Detection of CTCs was an independent prognostic factor for DFS, distant disease-free survival (DDFS) and OS. Patients with detectable CTCs had more often larger tumours, greater lymph node involvement and a higher tumour grade [126].

In the largest study to date in early breast cancer, Rack and colleagues evaluated retrospectively 1492 pre- and post-adjuvant chemotherapy. Before chemotherapy 21.5% were CTC positive and after chemotherapy 22.4%. The presence of CTCs was significantly correlated with worse DFS and OS. Interestingly a higher number of detectable CTCs (more than 5 per 30 mL of blood) determined worse outcomes [123]. Another, smaller prospective study on 302 patients with 24% positive samples had similar results [121].

This data as well as smaller studies (Table 2.1) and data on DTCs in the same setting [29] have led to a change in BC staging, with the creation of the cM0(i+) category, though this is seldom used in clinical practice at this point in time [98].

Circulating Tumour Cell Characterisation

Characterisation studies with CTCs hold the potential of more precise guidance to treatment than simple enumeration. Via the investigation of different markers CTC subpopulations may be identified that determine a worse outcome or that may

Table 2.1 Clinical studies with CTCs in early breast cancer

Author	Detection method	Number of patients	Timing	Detection rate	Prognostic	Marker
Stathopoulos et al. [100]	RT-PCR	111	Pre- and post-chemotherapy	36%	No	CK-19 mRNA
Gaforio et al. [101][a]	ICC	92	Pre-chemotherapy	62%	PFS and OS	CK
Xenidis et al. [102]	RT-PCR	161	Post chemotherapy	27.3	RFS	CK-19 mRNA
Giatromanolaki et al. [103]	RT-PCR	100	Pre-chemotherapy	33%	RFS	CK-19 mRNA
Pierga et al. [104][a]	ICC	114	Post-chemotherapy	24.5%	No	CKs 8, 18, 19
Masuda et al. [105]	RT-PCR	206	Pre-chemotherapy	18%	RFS	CK-7
Benoy et al. [106][a]	RT-PCR	148	Pre-treatment	28,30%	No	CK-19 mRNA, mammaglobin
Ntoulia et al. [107]	RT-PCR	101	Pre-chemotherapy	13.9%	RFS	Mammaglobin A
Wiedswang et al. [108]	ICC	341	Pre-chemotherapy	10%	RFS and OS	AE1 and AE3
Wong et al. [109][a]	ICC	131	During chemotherapy	42,50%	No	CK-8
Xenidis et al. [110]	RT-PCR	167	Pre-chemotherapy	21,60%	RFS and OS	CK-19 mRNA
Ignatiadis et al. [30]	RT-PCR	444	Pre-chemotherapy	40.8%	RFS and OS	CK-19 mRNA
Xenidis et al. [111]	RT-PCR	119	Post-chemotherapy	18,15%	RFS and OS	CK-19 mRNA
Ignatiadis et al. [112]	RT-PCR	175	Pre-chemotherapy	41,1%/8%/28.6%	see text	CK-19, mammaglobin A and HER2
Tkaczuk et al. [113][a]	ICC	105	Pre- and post-chemotherapy	30% and 56%—post-24 months	OS	Multi-CK
Daskalaki et al. [114]	RT-PCR	165	Pre- and post-chemotherapy	55.2%	OS	CK-19mRNA
Marques et al. [115]	RT-PCR	321	Pre- and post-chemotherapy	55.1%	No	Mammaglobin mRNA
Serrano et al. [116][a]	ICC	71	Pre- and post-chemotherapy	66%	PFS and OS	CK

Xenidis et al. [31]	RT-PCR	437	Pre- and post-chemotherapy	41%/32%, respectively	RFS and OS	CK-19 mRNA
Bidard et al. [117]	CellSearch	115	Pre- and post-chemotherapy	23%	RFS and OS	CK-8, 18, 19 and CD45
Chen et al. [118]	RT-PCR	50	Pre-chemotherapy	54%	RFS	CK-19, mammaglobin and CEA
Molloy et al. [119]	RT-PCR	82	Pre-chemotherapy	20%	RFS	CK19, p1B, EGP-2, PS2, mammaglobin and SBEM
Molloy et al. [120]	RT-PCR	733	Pre-treatment	7.9%	RFS and OS	CK19, p1B, EGP-2, PS2, mammaglobin and SBEM
Lucci et al. [121]	CellSearch	302	Pre-chemotherapy	24%	RFS and OS	CK-8, 18, 19 and CD45
Aktas et al. [122]	AdnaTest	68	Pre-chemotherapy	31%	No	AdnaTest BC, EMT and SC
Rack et al. [123]	CellSearch	2026	Pre- and post-chemotherapy	21,5%/22.1%	RFS and OS	CK-8, 18, 19 and CD45
Tryfonidis et al. [124]	RT-PCR	223	Pre- and post-chemotherapy	44.4%/40.3	RFS and OS	CK-19 mRNA
Hartkopf et al. [125]	AdnaTest/ CellSearch	202/383	Pre-chemotherapy	9%/5%	RFS for CellSearch	CK-8, 18, 19, CD45 and AdnaTest BC
Janni et al. [126]	CellSearch	3173	Pre-chemotherapy	20.2%	RFS and OS	CK-8,18,19 and CD45
Kasimir-Bauer et al. [127]	AdnaTest	376	Pre-chemotherapy	22%	RFS	AdnaTest BC
Kasimir-Bauer et al. [128]	AdnaTest	135	Pre- and post-chemotherapy	24%/8%	No	AdnaTest BC, EMT and SC

[a]Both early and metastatic, *OS* overall survival, *CK* cytokeratin, *RT-PCR* reverse transcription-polymerase chain reaction, *ICC* immunocytochemistry, *RFS* relapse-free survival, *PFS* progression-free survival, *OS* overall survival, *BC* breast cancer, *EMT* epithelial-mesenchymal transition, *SC* stem cell, *CEA* carcinoembryogenic antigen

Table 2.2 Studies on HER2 status discordance between primary tumour and CTCs

Author	Number of patients	Disease setting	Method of detection	Number of discordant cases
Meng et al. [88]	57	Early and advanced	ICC	8
Riethdorf et al. [142]	213 and 207	Early	CellSearch	8 and 3
Lang et al. [140]	92	Early	CellSearch	0
Fehm et al. [136]	67	Advanced	AdnaTest	8
Wülfing et al. [144]	42	Early	ICC	12
Ignatiadis et al. [32]	174	Preinvasive and early	CellSearch	5
Pestrin et al. [141]	66	Advanced	CellSearch	8
Flores et al. [137]	75	Advanced	CellSearch	10
Kallergi et al. [138]	24	Early	ICC	10
Wallwiener et al. [143]	107	Advanced	ICC	27
Krishnamurthy et al. [139]	95	Early and advanced	CEE	5
Apostolaki et al. [134]	214	Early	RT-PCR	40
Apostolaki et al. [135]	2016	Early	RT-PCR	47
Ignatiadis et al. [30]	185	Early	RT-PCR	28

suggest additional specific treatment. We have demonstrated that different CTC subpopulations have different prognosis by studying 175 early BC patients using a three-marker (mammoglobin, HER2 and CK19) reverse transcriptase polymerase chain reaction (RT-PCR) (Table 2.1) [112].

Multiple markers have been investigated in both BC and other tumours that have led to ongoing interventional trials. Protein markers that have been investigated include HER2 and PDL1 [31, 32, 112, 130]. RNA expression and DNA mutation detection in CTCs have been performed in advanced prostate cancer and BC [131, 132].

For CTC characterisation, HER2 attracted attention because of the availability of specific agents of proven clinical value [133]. In parallel, it was uncovered that in some cases of HER2-negative primary tumours, HER2+ CTCs were detectable [30, 32, 88, 134–143] (Table 2.2).

Interventional Studies

Hypothetically, since CTC detection is a surrogate marker for replicating tumour cells, the detection of remaining CTCs after a course of chemotherapy can be taken as signifying suboptimal efficacy in the adjuvant or metastatic settings, and thus be used to select patients that need more extensive or different treatment. In parallel the existence of HER2-discordant CTC subpopulations has raised interest

in trastuzumab treatment for CTC-positive women with an HER2-negative primary. This hypothesis has been explored and is under exploration in various trials [145] (Table 2.2).

Bozionelou and colleagues led a pilot study on 30 patients with advanced BC and detectable CTCs/DTCs 1 month post-latest treatment. Patients received trastuzumab alone. Of 20 patients receiving weekly trastuzumab, after 4 weeks 75% of patients had no detectable CTCs/DTCs and stopped treatment. Of 10 patients receiving 3-weekly trastuzumab, 60% had no detectable CTCs/DTCs and stopped treatment [146].

A similar single-centre study was conducted on the adjuvant setting, where 75 patients with HER2-negative primary tumour and detectable CTCs were randomised between post-adjuvant trastuzumab for 6 cycles or regular follow-up. Interestingly 89% of patients had HER2 overexpression in detected cells. 75% of patients in the treatment arm were CTC negative after the end of treatment, compared to 17.9% after the same time period in the control arm. Treatment arm was also significantly superior to control in terms of relapse-free survival [147].

An ongoing multicentre trial, the "Treat CTC" (NCT01548677), aims to confirm and extend the above results using the CellSearch technology. This is a phase II that is currently randomising HER2-negative BC patients with detectable CTCs irrespective of HER2 status after surgery and (neo)adjuvant chemotherapy to either 6 cycles of trastuzumab or observation. The primary objective of this trial is the rate of CTC detection post-treatment. The rationale and the design of the Treat CTC trial including the results of the pilot phase have been recently published [148].

Naume et al. aimed to evaluate the clinical utility of bone marrow DTCs in a large adjuvant trial in BC patients. 1066 patients were submitted to bone marrow biopsy after completing standard adjuvant chemotherapy with 5-FU, epirubicin and cyclophosphamide (FEC). A total of 7.2% were positive and went on to receive 6 cycles of docetaxel. Among these, 20.8% had persistent DTCs in bone marrow biopsy following docetaxel. Post-docetaxel, DTC-negative patients had similar prognosis to the group who were negative after FEC only, suggesting the effectiveness of this strategy [149].

Conclusions

Though recent advances in CTC/DTC research have shed new light in this process of dissemination, many hypotheses remain to be further tested. At this point in time it has been suggested that at least in some patients the detection and characterisation of CTCs/DTCs may provide additional information to that provided by the primary tumour alone. Ongoing or future studies using CTCs or newer more sensitive tools such as circulating tumour DNA will explore the question of tailoring adjuvant treatment for women with early breast cancer based on the detection of minimal residual disease. These studies, if positive, may change clinical practice in the early setting from a "static" primary biopsy-centric model to a "dynamic" liquid biopsy-centric model.

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