

Circulating tumor cells in breast cancer patients

Minireview

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Circulating tumor cells (CTCs) represent a potential non-invasive substitute for real-time tissue biopsy. Moreover, detection of CTCs in breast cancer patients has been reported as a strong prognostic factor. Biomarkers on CTCs have been analysed and correlated to tissue biopsies from breast cancer patients. Discordance in expression of potential biomarkers between primary tumor, metastatic sites and CTCs has been observed. Potential analytical confounding factors include lack of analytical consistency, varying sensitivities and specificities of used assays and differences in analytical ranges among various reported studies. Besides, clonal evolution within primary tumor (and metastatic sites) that leads to intratumor heterogeneity must be accounted for. Nevertheless, several on-going trials are exploring CTCs detection and biomarker profiling in view of personalising cancer treatment based on these real-time results. In this work, we will review CTCs in breast cancer patients and focus on identification of novel prognostic biomarkers.

Key words: circulating tumor cells, breast cancer, biomarkers

Breast cancer the most common malignancy diagnosed in women – an estimated 1.67 million new cases were diagnosed world-wide in 2012, with Western Europe having the highest incidence of 96 per 100,000 (Figure 1).

In Slovakia, 2600 new cases were diagnosed in 2012. Breast cancer currently ranks as a second cause of death from cancer (lung cancer being first) in developed countries. [1] There is no known prevention for breast cancer and an estimated life-time (80 years) absolute risk of development of breast cancer is 1 in 8 to 1 in 10 women. Fortunately, during the early decades of life, the odds are lower (1 in 228 for 30-year old woman). [2] Despite advances in screening, diagnosis and treatment, significant portion of patients is diagnosed in advanced stages or develops metastatic disease. Median survival of stage IV disease is about 28 months, depending on type and site of disease. [3] This failure has been associated with different tumor factors and biology, including various subtypes of breast cancer (e.g. luminal, basal), distinct cell populations in a tumor (intratumor heterogeneity) and existence of cancer stem cells that are in part responsible for tumor resistance, dormancy and spread. [4]

Metastatic cascade

Cancer is characterised by Darwinian evolution. [5] Next-generation sequencing (NGS) studies show that clonal evolution occurs within primary tumor and continues in metastatic disease as well. These cell clones can disseminate into distant organs where they can readily develop further metastatic foci or can remain dormant. Tumor dormancy is thought to be responsible for late recurrences, for example in luminal breast cancer type. Different hypotheses have been proposed regarding origin and metastases-forming capability of escaping cells into circulation. However invasion-metastatic cascade is a common cascade that tumor cells must complete to exit primary tumor and establish a new metastatic deposit at a distant site. It involves local invasion through extracellular matrix (ECM), intravasation into circulation, migration through blood stream, arrest and extravasation into distant organs, survival and re-initiation of their proliferative potential. [6]

Millions of tumor cells are shed daily into circulation, however experimental data suggests that only tiny fraction of these

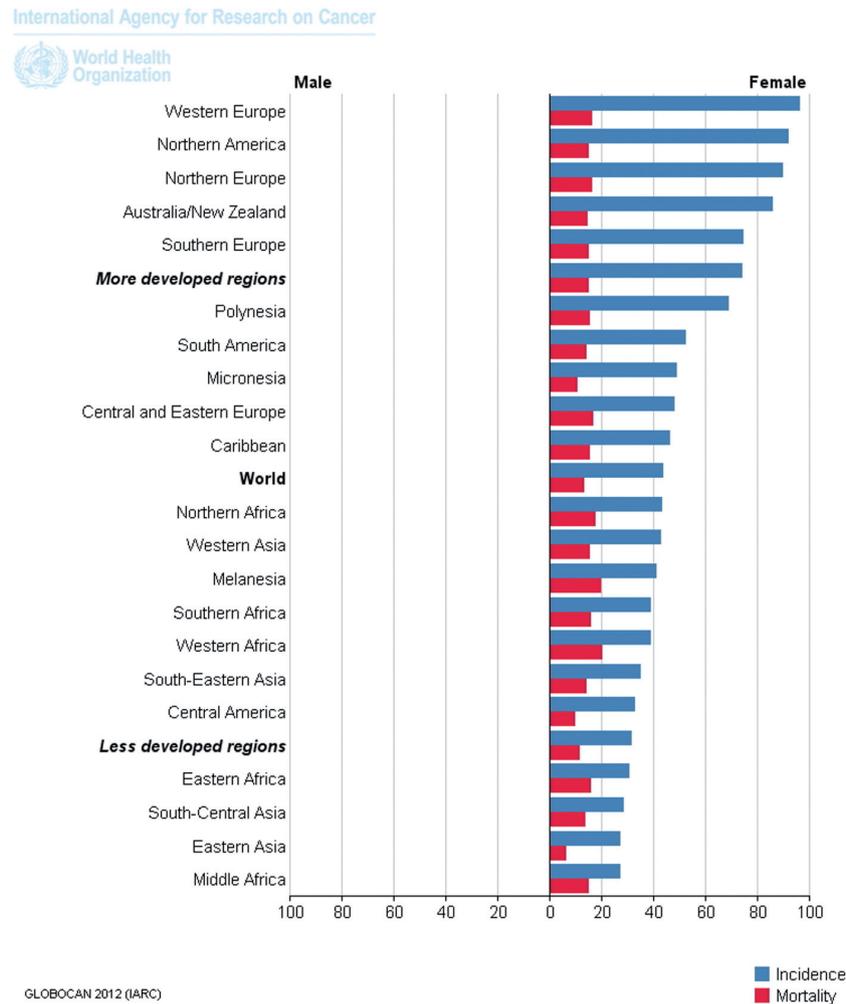


Figure 1. Estimated age-standardised rates (World) per 100,000. From [1]

circulating tumor cells (CTCs) are able to form macroscopic metastases. [7]

Local invasion. Local invasion is first step in the metastatic process. Cancer cells must breach ECM, which requires changes in cell-cell and cell-ECM adhesion molecules. Two different types of individual cell invasion have been recognised: mesenchymal invasion (integrin-dependent, typical of dedifferentiated epithelial cancers) and amoeboid invasion (integrin-independent, typical of lymphoma) with possibility of interconversion between them. [8]

In mesenchymal invasion, down-regulation of E-cadherin with up regulation of N-cadherin is observed. Invasion is further helped by proteolytic degradation of ECM, mediated by matrix metalloproteinases (MMPs) and the urokinase plasminogen activator system (uPA). A significant number of CTCs display amplification of uPA receptor which is responsible for liberation of active plasmin and subsequent degradation of ECM. [9] In mesenchymal-cell migration, cancer cells undergo cell-biological program

known as epithelial-mesenchymal transition (EMT), in which epithelial cells loose cell-cell contacts, cell polarity, acquire mesenchymal-gene expression and down-regulate epithelial-associated genes. EMT program dissociates cells from epithelial sheets into individual cells exhibiting multiple mesenchymal attributes and heightened invasiveness. [10] EMT has also been associated with stem-cell phenotype and resistance to apoptosis. Consequently, CTCs with EMT phenotype are more capable of survival in different environments (e.g. blood stream) and are more proficient colonising distant sites. [11] It was shown that induction of EMT in mammary epithelial cells results in acquisition of stem-cell properties (e.g. chemoresistance) and conversely, reversing EMT (that is mesenchymal-to-epithelial transition (MET)), leads to sensitisation of cancer stem cells to cancer treatment. [12] EMT switch is associated with enhanced migration, cell adhesion facilitating extravasation and neovasculogenesis. Several extracellular molecules induce EMT (e.g. Notch, TGF- β , Wnt), which relay signals intracellularly through

MAPK, PI3K, NF- κ B and others to act on transcription factors such as Snail, Slug, Twist, SIP1 and others. Consequently, several EMT genes are downregulated (E-cadherin, cytokeratin, Claudins) and mesenchymal markers upregulated (N-cadherin, vimentin, MMPs, collagens). [13] In humans, EMT phenotype was detected in several cancers and studies have also reported reduced expression of epithelial markers and increase in mesenchymal gene expression on breast cancer CTCs. [14-17] Blocking expression of Twist in 4T1 mammary cell line reduced metastatic burden and CTCs in mice with these xenograft tumors, linking EMT, CTCs and presence of metastasis. [18] Another elegant study used dynamic *in vivo* model of EMT using vimentin as a marker of mesenchymal phenotype. Clear heterogeneity of vimentin in the primary tumor was observed and CTCs in blood of mice were detected as early as day 8 after inoculation. Vimentin was expressed in the CTCs from all mice, however Snail and Slug expression was found in minority of samples. Intravascular tumor emboli also expressed vimentin, suggesting EMT phenotype is associated with intravasation and formation of CTCs. [19] In large lung metastases, vimentin expression was again heterogeneous with homogenous expression of E-cadherin, as in primary tumors. It is thought that following extravasation and in the colonisation process, CTCs undergo re-differentiation to an epithelial phenotype and form colonies with similar characteristics as primary tumor. It is possible that in this transformation process, some CTCs might keep their mesenchymal and stem-cell properties. This is supported by the fact that emboli in the lungs in previous experiment still expressed vimentin, suggesting that some EMT characteristics remain during extravasation, but MET occurs for further metastatic growth. Further data to support the role of EMT in the dissemination of human breast cancer come from the work of Yu *et al.* [20] Rare primary tumor cells exhibiting both epithelial and mesenchymal markers were identified, and serial monitoring of CTCs with mesenchymal phenotype was associated with disease progression. These cells were expressing known EMT regulators, such as TGF- β and FOXC1 transcription factors.

Intravasation and survival in circulation. Intravasation of invasive cells is influenced by various humoral factors, such as transforming growth factor- β (TGF β), epidermal growth factors from tumor-associated macrophages and vascular endothelial growth factors (VEGFs). The neovasculature generated by carcinoma is abnormal, lacks extensive pericyte coverage and no longer provides a physical barrier that would restricts transendothelial cancer cell intravasation. [21] CTCs in circulation are challenged by hostile environment – hemodynamic forces, immune cells (e.g. natural killer cells) and absence of adhesion to ECM, which is essential for epithelial cells. Normally, epithelial cells undergo anoikis, a form of apoptosis activated by loss of adhesion to ECM. On the other hand, it is likely that CTCs spend only a brief time in circulation owing to their large size compared to capillaries. [6] Additionally, carcinoma cells form large tumor emboli by

interaction with blood coagulation system and platelets. The binding of tumor cells through tissue factors, fibrin, L- and P-selectins to platelets enables them to shield themselves from physical forces in blood stream, evade immune system and lodge themselves as tumor microemboli.

Kim *et al.* observed in breast and colon xenograft models re-infiltration of primary tumor by cells from its metastatic sites, a process called “self-seeding”. [22] Self-seeding requires ability of primary tumor to attract its CTCs and CTCs to be able to re-infiltrate primary tumor site. It is also possible that re-infiltrating CTCs may provide growth advantage to primary tumor by changing its metastasis virulence genes (these genes CTCs acquired/accumulated at distant sites).

Arrest and colonisation. Theoretically, CTCs can disseminate to any site given the anatomical layout of vasculature. Still, it has been observed in the clinic that certain cancers metastasise to only a limited subset of target organs. Certainly, some CTCs are capable of specific adhesive interactions, such as metadherin expression on breast cancer cells homing to lungs. [23] In order to extravasate, CTCs secrete various factors inducing vascular permeability and disrupting endothelial cell-cell junctions in healthy microvasculature. For example, MMP-1 and MMP-2, as well as angiopoietin-like-4 and cyclooxygenase-2 (COX-2) disrupt pulmonary vasculature for breast CTCs to extravasate in lung parenchyma. [24] Tumor cells can also express chemokine receptors that guide tumor cells to specific sites. [25] *Psaila et al.* have argued for establishment of “premetastatic niche” whereby hematopoietic progenitor cells from bone marrow are mobilised by primary tumor signals and interact with fibronectin at future metastatic sites. They further modify local microenvironment through secretion MMP-9 that liberates chemoattractant molecules from ECM (e.g. stromal cell-derived factor-1). [26]

Metastasis formation. Large number of CTCs can be detected in the bloodstream of cancer patients. [27] However, vast majority of CTCs do not form metastatic foci, a point demonstrated by 15 ovarian cancer patients with peritoneovenous shunts for malignant ascites. These patients received millions of cancer cells into their circulation, but failed to develop overtly metastatic disease. On autopsy, some patients did not have any metastases, others have small tumor deposits in many organs and some have more metastatic foci only in organs already involved prior to placement of shunt. [28] These data suggest that for CTCs, only very few complete all steps of metastatic cascade and form macroscopic metastases. In some patients with breast carcinoma, late relapses, often decades after primary surgery, have been observed. This could be explained by cell dormancy, gradual accumulation of genetic and/or epigenetic changes required for colonisation or by changes in microenvironment.

CTCs in breast cancer

Presence of CTCs in peripheral blood represent surrogate marker for tumor self-seeding potential. They play pivotal

role in tumor progression and dissemination. The detection, enumeration and characterisation of CTCs should guide clinicians about cancer prognosis, help in prediction of treatment effectiveness and provide superior method for detection of disease progression. Furthermore, ability to identify sub-population of CTCs with stem-cell properties should direct targeted treatments and affect metastatic cascade. [29] There are several challenges in detection and characterisation of CTCs. They represent rare cells in the background of millions of nucleated blood cells; therefore enrichment step is needed before detection of CTCs. However, due to its heterogeneity, preselected marker for enrichment will not detect CTCs without this marker, i.e. CTCs with mesenchymal phenotype will be missed if they do not express selected epithelial marker. Lastly, there is a limitation on blood volume that can be taken from a patient in view of increasing yield of CTCs. CTCs were initially described almost 150 years ago as cancer cells in blood of deceased patient. [30] More than 50 platforms have been used for detection of CTCs; the only FDA-cleared system is CellSearch from Veridex, USA. Table 1 gives overview of some commercially available or frequently used systems.

A novel method of detecting CTCs *in vivo* is being developed. A GILUPI CellCollector is antibody (e.g. anti-EpCAM-antibody) coated wire inserted into cubital vein of patient for 30 minutes, thereby coming into contact with large volume of patient blood. Following enrichment, further CTCs detection is carried out by various methods (immunocytologically, *in vivo* cell culture (EPISPOT) or using molecular technologies). Common method of enrichment is based on immunomagnetic isolation. Usually, magnetic beads are labelled with antibodies specific for EpCAM (epithelial adhesion molecule) for positive selection and anti-CD45 antibody (anti-leukocyte) for negative selection. Labelled cells are then separated by strong magnetic fields. This method is used in FDA-approved CellSearch system for CTC detection. However, this technology may miss cells that do not express selected antigens. Although negative selection might abrogate

this, additional processing (e.g. red cell lysis) may lead to further loss of CTCs. Several other platforms use antibodies (EpCAM) immobilised on solid-surfaces (e.g. CTC-chip) and combine these with filtration methods (e.g. On-Q-ity Chip). Further enhancement of CTC capture efficiency was studied using P-/E-selectin in addition to EpCAM selection. More than 3-fold enhancement of CTC selection was by described using these additional adhesion molecules. [31] Another methods use filtration based on size of cells and their deformability – more deformable erythrocytes and leucocytes pass via small (5 μm) gaps, but stiffer and larger tumor cells are tapped. Combination of anti-EpCAM selection, sedimentation and microfiltration was described as having recovery rate up to 99% for MCF-7 cancer cells. [32] Comparison of various commercial platforms for CTC detection was performed. [33-35] CytoTrack (laser scanning of labelled cells) and CellSearch (EpCAM based) systems showed comparable ability to identify CTCs *in vitro* with a recovery rate of almost 70% for MCF-7 cells. CellSieve method (a microfiltration system) isolated larger population of CK+/CD45- cells than CellSearch. Using nuclear morphology and specific CK staining pattern, a distinct subpopulation of CTCs correlated with that found by CellSearch. AdnaTest BreastCancer Select/Detect (detection of gene transcripts GA 733-2, MUC-1 and HER2) showed equivalent sensitivity to that of CellSearch system in detecting > 2 CTCs. AdnaTest complements the CellSearch system in allowing assessment of genomic markers. In CellSearch system, cytokeratin and DAPI-positive, CD45-negative cells are enumerated semiautomatically using fluorescent microscope. In AdnaTest BreastCancer Select/Detect, mRNA is isolated from selected cells (based on magnetic particles), transcribed into cDNA and subjected to multiplex PCR for tumor-associated gene expression. If cells are isolated by size, they are further stained by CK-antibodies and specific surface markers for that particular tumor. Enumeration is done by light or fluorescent microscopy. All these different technologies might detect different CTCs populations. Analytical validity (accuracy of

Table 1. Platforms for capture of CTCs

EpCAM-affinity based	
CellSearch system	Iron particles with CTCs removed by magnets. DAPI+ (nuclear staining), CK8,18,19+, CD45-, 4 μm in size
AdnaTest	Antigen selection and further RT-PCR analysis for tumor associated genes (EpCAM, MUC-1, HER-2)
On-Q-ity Chip	Microfabricated columns coated with EpCAM antibodies and gradient sizing
MACS (magnetic activated cell sorting system)	Magnetic beads with anti-EpCAM or tumor epitopes
Physical properties-based	
Density gradient centrifugation	Heavier neutrophils and erythrocytes migrate to bottom, tumor cells and mononuclear cells are at top
ScreenCell	Filtration based on cell size
ApoStream	Uses electrical property differentiation between normal blood and tumor cells
DEPArray	Electrical property differentiation. Image-based selection
Others	
EPISPOT (Epithelial immunospot)	Depletion of CD45+, enrichment of CXCR4+ cells, ELISA based detection of secreted proteins, viable cells only
FAST (Fibre-optic array scanning technology)	Ultra-fast digital microscopy detecting immunofluorescently labelled cells 500x faster than digital microscopy

measurement) of these tests is challenging. Indeed, characterization of CTCs in respect to EMT is also a challenge – there are no specific molecular markers, mesenchymal markers (e.g. vimentin) might lead to high false positive results and selection based on epithelial markers would be unsatisfactory. [19] CTC enrichment limited to EpCAM positive cells may miss CTCs that do not present this specific marker, such as those with EMT phenotype. Several signaling pathways are responsible for EMT, such as TGF β , Wnt, Notch and others. They induce transcriptional factors such as Snail-1, Slug, Twist, ZEB1, 2 and others. Twist1, in cooperation with BMI1, promotes cancer dedifferentiation and metastasis. [36] For example, commercially available AdnaTest EMT/StemCellDetect detects TWIST1, Akt-2 and PI3K α transcripts and contains primer for stem-cell detection, ALDH1. Several authors used detection of CTC subpopulations with AdnaTest or other mesenchymal markers (vimentin, N-cadherin, SNAIL). [15, 37-40] Collectively, they found that CTCs are not uniform population; they can co-express one or both markers and exist in transitional state from epithelial to mesenchymal phenotype. Furthermore, clinical validity of CTCs is only recently being explored by several trials. CTC enumeration is supported as prognostic marker in breast cancer and its role as predictive biomarker is promising. [41-44]

Prognostic role of CTCs. A cut-off ≥ 5 CTCs in 7.5ml of patient blood was used in pivotal study on 177 women with metastatic breast cancer before starting new line of chemotherapy and at the first follow-up visit. Patients with higher than 5 CTCs had shorter median PFS and OS (2.7 vs 7 months and 10.1 vs >18 months, $p < 0.001$). [41] Increased in survival of patients with <5 CTCs in blood was independent of histology, HR status, molecular subtype, line of treatment or whether patient had recurrent or newly diagnosed metastatic disease. CTCs analysed at mid-treatment in retrospective analysis were compared with metabolic response using 18-FDG PET/CT scanning. Detection of five or more CTCs during treatment accurately predicted prognosis of metastatic breast cancer patients beyond metabolic response. [45] Prognostic usefulness was further confirmed in two large retrospective analyses. Six studies totalling 841 patients predicted for worse PFS and OS at baseline and at various time points up to 21 weeks from initiation of anticancer treatment independently of type or line of chemotherapy and site of metastases. [46] Another pooled analysis compiled 1944 patients starting new line of treatment and having CTC quantification done by validated CellSearch method. This study also confirmed independent prognostic effect of CTC count on PFS and OS. Moreover, increases in CTCs during treatment were associated with shortened survival and conversely, decreases in CTCs with treatment prolonged PFS and OS. [47] Given these data, serial CTCs enumeration could be used as a surrogate marker for patient prognosis. Recent publication also confirmed value of CTCs in newly diagnosed inflammatory breast cancer. [48] Data are less clear in patients undergoing targeted treatment. It is thought that antiangiogenic compounds interfere with

CTCs extravasation or induce EMT phenotype, rendering CTCs enumeration difficult. [49, 50] Prognostic role of CTCs during treatment with HER-2 targeted agents is inconsistent. It is plausible that effective anti-HER-2 treatment eliminates CTCs bearing HER-2 amplification. [51, 52]

A cut-off ≥ 1 CTCs in 7.5ml of patient blood was set for early stage breast cancer. [44] CTCs were detected in approximately 20% of patients in adjuvant setting in a large multicenter prospective German study (SUCCESS). Patients with lymph node metastases were more often CTC positive but other clinicopathologic characteristics or treatment were not associated with CTC positivity. There was no difference in CTC count before and after chemotherapy. The presence of CTCs was significantly predictive of reduced three year disease-free survival (DFS) at 87.9% vs 94.2% for CTC negative patients, $p < 0.001$. It is interesting to note that in subgroup analysis, presence of CTCs reduced DFS in all node positive patients, but not in node negatives. [53] Presence of CTCs was associated with reduced breast-cancer specific and overall survival. Also, persistence of CTCs after adjuvant chemotherapy was significantly associated with reduced DFS and with trend toward reduced OS. This fact might be relevant in the clinic, CTCs persistence might select patients whose tumors are resistant to standard cytotoxic adjuvant chemotherapy. Another prospective study on 302 early breast cancer patients reached similar conclusions. One or more CTCs were identified in 24% patients, and their presence predicted worse PFS and OS. [44] In a pooled analysis of 3172 early stage patients, CTCs were detected in 20% and associated with larger tumors, lymph node positivity and higher grade. CTCs remained independent prognostic factor in multivariate analysis for poor PFS (HR 1.8) and OS (HR 2.1, $p < 0.001$ both). [54]

Predictive role of CTCs. There are several trials on-going investigating treatment tailoring based on CTCs (Table2). [55] Characterisation of CTCs properties could lead to useful predictive biomarkers and would represent real-time biopsies in cancer patients undergoing treatment.

SWOG S0500 is first prospective trial with reported results evaluating whether early treatment switch based on persistence of CTCs would improve outcome. One hundred twenty three patients with ≥ 5 CTCs/7.5ml blood that persisted after one cycle of first-line chemotherapy were randomised to continue initial treatment until radiographic progression or to change treatment (physician choice). Changing treatment based on persistence of CTCs did not improve their OS (HR 1.0). Lack of effective treatment options for these patients might be plausible explanation. These patients might be better served by molecular analysis of CTCs and determination whether patients are eligible for trial with targeted treatment. Prognostic value of CTCs was confirmed: median OS of 35 months was seen in patients with low CTCs prior starting chemotherapy, compared to only 13 months for those with persistent high counts. Patients whose elevated CTCs dropped after one cycle of chemotherapy had intermediate prognosis with OS of 23 months ($p < 0.001$). [56]

Table 2. Selected randomized trials evaluating clinical utility of CTCs

Trial	Setting	Objective	No. of patients planned	Results	Country Reference
SWOG 0500	MBC, ≥ 5 CTCs/7.5ml blood before and after 1 cycle of 1 st line chemo	Compare OS between early switch vs waiting for radiological progression	595 eligible, 123 randomised	No change in OS	USA [56]
STIC CTC METABREAST	MBC, ER+, HER-2-, 1 st line HT if CTC <5 or chemo if ≥ 5	Compare PFS and economic impact between physician choice and CTC-driven choice of treatment	994	on-going	France [57]
COMETI P2	MBC, ER+, HER-2-, 2 nd line of HT	Observational study of endocrine therapy index and its impact on radiographic progression and PFS	200	on-going	USA, Canada [58]
CirCe01	MBC, ER+, HER-2-, 3 rd line of chemo	Compare OS between early switch vs waiting for radiological progression	568	on-going	France [59]
Detect III	MBC, HER-2-, up to 3 rd line of chemo, ≥ 1 HER-2+ CTC/7.5ml blood	CTC clearance rate with addition of lapatinib to chemotherapy in HER-2+CTC	120 to randomise	on-going	Germany [60]
Detect IV	MBC, ≥ 1 CTCs/7.5ml blood, ER+, HER-2-, for everolimus or for eribulin ER-, HER-2-	Impact on PFS with addition of everolimus to HT or eribulin	520	on-going	Germany [61]
Treat-CTC	EBC, ≥ 1 CTCs/15ml blood, HER-2-post chemo	Compare CTC detection after 18 wks of treatment with trastuzumab vs observation	174 to randomise	on-going	EORTC [62]

In the French STIC CTC trial, almost 1000 MBC patients that express HR and are negative for HER-2 are planned to accrue. Randomisation will be between standard arm of physician choice of hormone treatment or chemotherapy based on current guidelines versus CTC arm where patients with low CTC (<5 cells/7.5ml) will be treated with less aggressive approach (hormone treatment) and those with ≥ 5 cells/7.5ml will receive chemotherapy. This trial has been designed to show non-inferiority of CTC arm for PFS and superiority of CTC arm for medico-economics endpoints. [57] Preliminary results (presented as oral congress communication, Crete, 2014) showed that in 33% patients randomised in investigational arm, CTC level determination led to a change of treatment.

COMETI P2 is on-going study in North America that is taking into consideration not only CTC count but also the biology of CTC. Four biomarkers are assessed on isolated CTCs in the CellSearch system: ER, Bcl2, HER-2 and Ki67. An endocrine therapy index (ETI) score is calculated based on number of CTCs and staining intensity of these four biomarkers. Two hundred patients proceeding to second line HT will be observed for early radiographic progression and PFS and these time points will be correlated to their endocrine therapy index score. It is hypothesised that CTC-ETI will be predictive of endocrine responsiveness or resistance. [58]

The French CirCe01 is another attempt to compare early switch of chemotherapy in non-responding patients based on elevated CTCs after first cycle of chemotherapy. Moreover, CTCs will be evaluated after first cycle of each new line of chemotherapy treatment. Patients with persistently elevated CTCs will change chemotherapy after only one cycle of therapy. Standard arm will involve radiological and clinical assessments for progression. [59] It is hoped that early discontinuation of useless

chemotherapies in this very palliative setting will bring palliative care to patients sooner and also reduce treatment costs.

DETECT III trial is evaluating addition of lapatinib, a HER-2 targeting agent, to conventional treatment in initially HER-2 negative MBC (on biopsy) who have at least one CTC that is HER-2 positive. As HER-2-negative primary tumors can develop HER-2-positive CTCs during progression from 9-24%, a question is if these patients will benefit from anti-HER-2 targeted treatments. [63, 64] The study primary endpoint was recently modified from PFS (and randomisation of over 200 patients) to clearance of CTCs with only 120 patients need to be randomised. [60] A spin-off study DETECT IV is recruiting patients those are HER-2 negative and have HER-2 negative at least 1 CTC/7.5 blood. These patients are then assigned to addition of everolimus to endocrine treatment (if HT would be their treatment otherwise) or newly approved drug eribulin to those who would be considered for cytotoxic treatment. PFS of both groups is primary endpoint. [61]

EORTC trial Treat-CTC is investigating addition of adjuvant trastuzumab to women with HER-2 negative tumors who have completed assigned adjuvant chemotherapy and still have persistent CTCs (so called cM0(i+) patients). They are randomised to observation (standard arm) or addition of anti-HER-2 targeted treatment. After six courses CTC count is repeated and compared. Recurrence-free survival is secondary endpoint. [62] The rationale comes from several observation; benefit of adjuvant trastuzumab might not be confined to HER-2 overexpressing tumors (as seen in subgroup analysis from NSABP B31 and N9831 trials), and mouse models suggesting trastuzumab might target cancer stem-cell population. [65, 66] Further evidence for efficacy comes from randomised phase II study where HER-2 negative EBC patients with persistent CTC that were

double stained for CK+/HER-2+ with CK 19+ mRNA. DFS was significantly higher for trastuzumab treated patients ($p=0.008$). [67]

These trials represent attempts to definite a place for CTC testing in clinical practice in early and metastatic breast cancer patients. Each of them proposes different, complementary design that will guide further research into this new biological tool with hope to improve lives of breast cancer patients.

Potential biomarkers in primary tumor associated with CTCs

Although clinical utility of CTCs enumeration is being recognised in various trials, in future CTCs might provide “liquid biopsy” for molecular screening programs and treatment tailoring. Whole-exome sequencing and NGS have already been explored, as well as drug-sensitivity testing of cultured CTCs *ex vivo*. [68-70] Currently, clinically useful tissue biomarkers in primary breast tumor are hormonal receptor status (ER, PR), HER-2, proliferation index Ki-67 and grade of the tumor, other factors are clinical – extent of disease, its localisation, patient age and performance status. CTCs would present a unique biomarker, as they represent part of patient’s tumor burden that could be assessed for target antigens. Moreover, their phenotypic properties might be associated with specific biomarkers in primary tumors that lead to more aggressive behaviour of cancer.

In two prospective studies evaluating (repeated) biopsies of metastatic disease, discordance rates between primary tumor (removed previously) and new metastases were about 10% for HER-2 and 10-16% for ER. These biopsies brought change of treatment in 14-18% of patients. [71, 72]

HER-2. Evaluation of HER-2 on CTCs is possible through the use of spare channel of the CellSearch system. HER-2 positive CTCs were reported from 24-33% in studies evaluating CTCs in neoadjuvant setting and in metastatic disease. [73, 74] Concordance of HER-2 status between primary tumor and baseline was 86-87% for HER-2 negative or positive primary tissue, respectively. Overall, 18% of patients acquired HER-2 positivity during treatment. Generally, presence of HER-2 positive CTCs was associated with inferior outcomes compared to HER-2 negative or no CTCs. Lighthart *et al.* in prospective study evaluating HER-2 CTCs in adjuvant and metastatic patients defined CTCs HER-2 positivity as overexpression in 75% of CTCs with ≥ 5 CTCs. Using this cut-off, 9% of M1 patients that were HER-2 negative had HER-2 positive CTC status and conversely 29% with HER-2 positive primary had negative HER-2 CTCs. [64] As detailed above, HER-2 evaluation on CTCs and its impact on patient management is being further studied in clinical trials.

ER, PR. ER and PR receptors are established biomarkers in breast cancer, however only 30% respond to HT in registration studies of aromatase inhibitors. [75, 76] Heterogeneous populations of CTCs with different hormonal phenotypes were described in breast cancer patients. [77] Primary breast tumors are classified as ER positive if at least 1% of cells stain

on immunohistochemical investigation. Thus, ER-negative CTCs might originate from ER-negative (primary) cells, or these clones might be selected under pressure from hormonal therapy. This CTC heterogeneity might be partly responsible for failure of HT. Fifty percent of CTCs showed mixture of both ER positivity and ER-negativity in ER-positive primary tumor. In the same study ER-negative CTCs were found in 19% of patients harbouring ER-positive primary tumor. [78] By culturing CTCs *ex vivo* and establishing CTC-derived cell lines, it was possible to identify activating ESR-1 mutations in three of six CTCs lines from pre-treated patients with aromatase inhibitors. These ESR-1 mutant cell lines were resistant to tamoxifen and fulvestrant, but resistance was overcome by HSP90 inhibitor. [70] Discordance rates between primary tumor and ER positive CTCs vary from 40-77% in some studies. These wide variations might be also due to methodological issues, differences in cut-off limits for ER/PR receptors in CTCs as well as primary samples. [79, 80]

Other biomarkers. Other potential biomarker that was already detected in cultured CTCs is PIK3CA, alpha catalytic subunit of phosphatidylinositol 3-kinase. PIK3CA mutations are frequent mutations in breast cancer; up to 21% were detected in 504 samples from German neoadjuvant studies. It was demonstrated that these patients are less likely to achieve pCR rates with neoadjuvant chemotherapy and trastuzumab. [81] Analysis of PI3K pathways markers could influence patients for selection of PI3K/AKT/mTOR pathway inhibitors. Detection of PIK3CA was also achieved from circulating free DNA, when these samples were compared to up-to-date tumor biopsies, concordance rate of 100% was detected. If comparison was done on archived tumor tissue, discordance rate rose to 30%. [82] These results suggest that trials investigating PIK3CA inhibitors should optimally select patients on current mutational status rather than on archival tissue. PIK3CA mutations were detected in 35% of CTCs by combination of allele-specific, asymmetric rapid PCR and melting analysis. [83] It was also confirmed in this study that PIK3CA mutation status can change during disease progression and is associated with inferior survival. Evaluation of PIK3CA in CTCs appears to be clinically plausible strategy, however with a caveat that there may be a mutational heterogeneity within one sample or at different time points. [84]

Evaluation of phospho-PI3K, along with EGFR, HER-2 and phospho-Akt in early and metastatic breast cancer patients revealed similar levels of phospho-PI3K and phospho-Akt in both disease subgroups. Phospho-EGFR was observed in higher percentage in metastatic patients (33 vs 86%). [85] Furthermore, it was found out that EGFR is co-expressed with HER-2 and both phospho- kinases. This pathway could be used as a target for novel agents.

Another potential biomarker that is currently under study is TOP2A, a DNA topoisomerase II alpha. Amplifications in TOP2A gene were associated with responsiveness to anthracycline (DNA topoisomerase 2 inhibitor) chemotherapy. TOP2A amplifications was analysed in one study on

CTCs. Concordance rates were around 70% and no HER-2 co-amplification was detected in CTCs. After three cycles of anthracycline treatment, co-amplification of TOP2A and HER-2 was detected in one patient with initially negative tumor for both of these markers. [77] Such changes might be clinically relevant, as patients with co-amplifications of HER-2 and TOP2A may benefit with anti-HER agents and anthracycline-based regimens.

Expression of Notch ligands (Delta-like and Jagged) has been reported in triple-negative breast cancer and blocking agents this signalling pathway have entered development. Similarly, inhibitors of other signalling pathways involved in breast cancer tumorigenesis, such as Hedgehog and Wnt have entered development. Other potential biomarkers might include multidrug resistance-associated protein (MRP), which is related to more aggressive behaviour and resistance to treatment and IGF-1 and IGF-1R (insulin growth factor receptor), related to radioresistance and local recurrence. [86, 87] Recent study has investigated MMP1 expression in primary tumors and phenotypic changes in CTCs. In multivariate analysis, CTCs with EMT phenotype and tumor grade were associated with MMP1 expression in cancer cells, while Ki-67 and CTCs with EMT phenotype were independently associated with MMP1 expression in tumor associated stroma. [88] Other potential marker associated with tumor expansion is VEGF, vascular endothelial growth factor. Overexpression of VEGFs produces cell proliferation and changes in tumor vasculature and would facilitate CTC release. Interleukin-8 and its cognate receptors, CXCR1 and CXCR2 are important in regulating breast cancer stem-like cells. IL-8 activates EGFR and HER-2 and is associated with poor prognosis in breast cancer. [89] Fibroblast growth factors and their receptors (FGFR) promote several steps in metastatic pathway, including EMT, invasion and angiogenesis. [90] Recently truncated FGFR1- β splice variant was identified in cell cultures after exposure to TGF- β and same variant was confirmed on patient-derived tumor samples. Its expression was important in metastatic outgrowth. [91] Other potential markers associated with CTCs might be vimentin (as described in previous section), fibronectin, claudin-4, SPARC, CAIX, Plastin3 and Glut-1. [92] Aurora-A kinase has also been implicated in EMT pathway responsible for the development of distant metastases in ER α (+) breast cancer cells. [93]

However, data that show clinical utility of these biomarkers are yet unavailable. Clinical utility will also depend on prevalence of patients in whose such an approach would be feasible.

Conclusions

Despite heterogeneity of breast cancer, current therapeutic strategies are based on the samples from primary tissues. Few validated biomarkers exist that guide our treatment decisions. Many patients with seeming good prognosis (small tumors, no lymph node involvement) that are treated less

aggressively in adjuvant setting recur, on the other hand it is possible that we are over treating substantial majority of women in adjuvant setting. Circulating tumor cells represent promising biomarker in personalising treatment for cancer patients. CTCs have already demonstrated its prognostic value in several trials in early as well as advanced breast cancer. Validation of assays for CTCs bearing certain biomarkers would be clinically useful in term of diagnosis, prognosis and therapy failure. Randomised clinical trials with standardised methodologies are urgently needed. Besides established therapeutic targets (ER, HER2) and other potential markers present on CTCs (PIK3CA), other markers involved in metastatic cascade and self-seeding should be further identified with their relation to CTCs. Interruption of metastatic cascade on several levels, targeting CTCs as well as biomarker-driven phenotype of primary tumor might significantly change outcome of breast cancer patients.

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