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# Micrometastatic disease in breast cancer: Clinical implications

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ABSTRACT

The presence of bone marrow disseminated tumour cells (DTCs) was shown to predict poor clinical outcome in early breast cancer. However, peripheral blood is easier to obtain and allows for serial monitoring of minimal residual disease. Towards this aim, circulating tumour cells (CTCs) in the blood are detected using either direct methods, mainly antibody-based assays (immunocytochemistry, immunofluorescence and flow cytometry), or indirect methods, mainly nucleic acid-based assays (detection of mRNA transcripts by reverse transcriptase polymerase chain reaction, RT-PCR). The detection of CTCs using RT-PCR for CK19 was shown to be an independent prognostic factor in women with early breast cancer. Furthermore, considerable progress has been accomplished in genotyping, phenotyping and profiling micrometastatic cells. The challenge now is to integrate minimal residual disease as a prognostic and predictive tool in the management of breast cancer. This requires the standardisation of micrometastatic cell detection and characterisation, which will allow the incorporation of CTCs/DTCs into prospective clinical trials testing their clinical utility.

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## 1. Introduction

The TNM system used for staging breast cancer does not accurately identify prognosis for some women and especially for those with small, axillary node-negative tumours who still may relapse and die of breast cancer.[1] Therefore, many investigators have hypothesised that the detection of micrometastases in the bone marrow (disseminated tumour cells, DTCs)[2–4] or peripheral blood (circulating tumour cells, CTCs)[5–7] might provide additional prognostic information beyond TNM system. Furthermore, the micrometastatic cells that are undetectable by the classical imaging and laboratory studies (minimal residual disease, MRD), when present after potentially curative surgery, are thought to contribute to disease relapse, and therefore are the obvious targets of adjuvant treatment strategies. Consequently, the study of these cells, apart from the impact on refining prognosis, has the exciting potential of individualising adjuvant treatment for women with breast cancer.

Bone marrow DTCs were shown to have less advanced genomic changes than primary tumour cells, suggesting that tumour cell dissemination occurs early in the course of the disease, and therefore breast cancer, even when diagnosed at an early stage, should be considered in many patients as a systemic illness.[8,9] However, the American Society of Clinical Oncology (ASCO) 2007 recommendations for breast cancer tumour markers suggested that the present data are insufficient to recommend assessment of bone marrow DTCs for the management of patients with breast cancer.[10] Similarly, the panel concluded that the measurement of CTCs should

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not be used to make the diagnosis or to influence any treatment decisions in patients with breast cancer.[10] However, this is the first time that DTCs/CTCs have been included in the ASCO guidelines, and the panel concludes that the data are intriguing and should be further evaluated in future studies.[10] Here, we will critically review the literature concerning the clinical implications of DTCs/CTCs and present the potential that the study of MRD might have in changing the way we manage breast cancer. Micrometastasis in the axillary lymph nodes (included in the last TNM edition[11]) is beyond the scope of the present review.

## 2. Definition of occult tumour cells

Occult tumour epithelial cells found in the bone marrow and peripheral blood of women with breast cancer are termed as disseminated tumour cells (DTCs) and circulating tumour cells (CTCs), respectively. DTCs and CTCs are only rarely found in the bone marrow and peripheral blood of otherwise healthy women. Several investigators have provided evidence that most detectable epithelial cells in the bone marrow[9,12,13] or peripheral blood[14] of women with breast cancer harbour genomic alterations characteristic of malignant cells.

## 3. Methods of detection of occult tumour cells

By definition, micrometastatic cells are undetectable with standard haematoxylin-eosin staining. These cells have been detected usually after an initial enrichment step (density gradients such as Ficoll/Hypaque, OncoQuick, filtration, immunomagnetic selection techniques such as magnetic affinity cell sorting or magnetic beads) using either direct methods, mainly antibody-based assays (immunocytochemistry, immunofluorescence and flow cytometry), or indirect methods, mainly nucleic acid-based assays (mRNA transcripts by RT-PCR)[15–20] for an epithelial marker (see below). Several investigators have compared different detection methods.[21,22] The nucleic acid-based assays have generally been considered more sensitive, whilst immunocytochemistry has the additional advantage of allowing the assessment of the morphology of the stained cells.

A semi-automated system based on immunofluorescence and flow cytometry, the CellSearch™ system (Veridex, Warren, New Jersey, USA), has been cleared by the FDA as an aid in the monitoring of patients with metastatic breast, colorectal and prostate cancers. However, the clinical utility of monitoring CTCs with the CellSearch system remains to be proven. The peripheral blood sample is enriched for cells expressing the epithelial cell adhesion molecule (EpCAM) with antibody-coated magnetic beads, and the cells are labelled with the fluorescent nucleic acid dye 4,2-diamidino-2-phenylindole dihydrochloride. Fluorescently labelled monoclonal antibodies specific for leucocytes (CD45-allophycocyan) and epithelial cells (cytokeratin 8,18,19-phycoerythrin) are used to distinguish epithelial cells from leucocytes.[23]

Since the preferred method of CTCs detection, automated digital microscopy (ADM), is too slow to scan large substrate areas, Krivacic et al. developed an approach that uses fibre-optic array scanning technology (FAST).[24] FAST cytometry enabled a 500-fold speed-up over ADM with comparable sensitivity and superior specificity.[24] The combination of FAST and ADM allowed the investigators to detect rare epithelial cells from whole unseparated blood after immunofluorescence staining with a pan-cytokeratin antibody.

Another method for the detection of circulating epithelial tumour cells, CETCs, from whole unseparated blood uses laser scanning cytometer after staining with anti-EpCAM and anti-CD45 fluorescent antibodies (MAINTRAC™).[25]

Recently, Nagrath et al. described the development of a microfluidic platform (the 'CTC-chip') capable of efficiently and selectively separating viable CTCs from peripheral blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions and without requiring prelabelling or processing of samples (CellPoint Diagnostics, California, USA).[26] The 'CTC-chip' can detect and isolate rare CTCs with high purity from small blood volumes (2–3 ml).[26]

For the detection of CTCs/DTCs, different markers have been chosen based on their expression in epithelial cells but not in mesenchymal cells (epithelial-specific markers) or based on their specific expression in breast tissue (breast tissue-specific markers). Amongst these markers, cytokeratins (CKs), which are intermediate filament keratins found in the cytoskeleton of epithelial cells, have been most extensively used.[27] The CKs more commonly studied in breast cancer are CK19 and CK8,18. However, false-positive results have been observed using either nucleic acid-based or antibody-based assays.[16] Contaminating genomic DNA during RNA extraction, illegitimate expression or stimulation of CTCs/DTCs' markers in mononuclear cells or lymphocytes by cytokines and the presence of CK19 pseudogenes have been responsible for false-positive results when using nucleic acid-based assays.[16,28–31] The use of quantitative RT-PCR which can sometimes discriminate low illegitimate background expression from the higher levels found in breast cancer as well as the design of primers that do not amplify genomic DNA or pseudogenes might partly address and resolve the above-mentioned concerns.[32] Similar limitations have been described using antibody-based techniques. Many of the antibodies directed at epithelial and breast cancer cells are known to also stain occasionally haematopoietic cells displaying illegitimate expression of cytokeratins (CK19) or MUC1. Non-specific staining of plasma cells can also occur due to alkaline phosphatase reaction against the kappa and lambda light chains on the cell surface.[16] Optimising the antibodies and using the appropriate negative controls in staining experiments have been employed to overcome the above-mentioned problems.

## 4. Diagnosis

Several studies have used CTCs detection as a tool to assist breast cancer diagnosis.[33,34] Reinholz et al. reported that molecular detection of CTCs can be used in combination with mammography and physical examination for the early detection of breast cancer. They used mammaglobin-A (MGB1) and B305D-C genes to construct a diagnostic test that correctly identified breast cancer in women undergoing biopsy for

mammographically detected breast abnormalities with a sensitivity of 70.5% and a specificity of 81%.[33] Chen et al. developed a membrane array-based method that simultaneously detects multiple peripheral blood mRNA markers.[34] The assay achieved a sensitivity of 80.6% and a specificity of 83.8% for breast cancer diagnosis.[34]

## 5. Prognosis

DTCs and CTCs have been extensively studied for their impact on prognosis estimation in breast cancer (see Table 1).

### 5.1. *Metastatic breast cancer*

Most studies reporting on the prognostic value of CTCs in patients with metastatic breast cancer have used the CellSearch™ system. The presence of $\geqslant 5$ CTCs per 7.5 ml of whole blood in 177 patients with measurable metastatic breast cancer before a new treatment was started was an independent predictor of progression-free survival (PFS) and overall survival (OS).[35] Furthermore, CTCs' detection by CellSearch was suggested to be a superior surrogate end-point than the current radiology imaging studies for assessing the response to treatment and predicting OS in metastatic breast cancer patients.[36] To provide further evidence that CTC detection can improve clinical outcome in metastatic breast cancer, the Southwest Oncology Group (SWOG) has launched a phase III trial (ClinicalTrials.gov NCT00382018) to test the strategy of changing chemotherapy compared with continuing the same chemotherapy for metastatic breast cancer patients who have elevated CTC levels at the first follow-up assessment. Several other investigators have reported other less standardised or validated antibody- or nucleic acid-based assays to detect CTCs or DTCs in metastatic breast cancer.[37–41]

### 5.2. *Early breast cancer*

#### 5.2.1. *DTCs*

Several studies have evaluated the prognostic value of bone marrow DTCs in early breast cancer, and some of them have failed to demonstrate an independent prognostic value of DTCs when controlled for the 'well-known' primary tumour and clinical characteristics.[2,3,42–48] However, a meta-analysis involving 4703 early breast cancer patients provided adequate statistical power to address this question.[4] DTCs were detected in 30.6% of the patients at the time of primary surgery and their detection was an independent prognostic factor for poor outcome. For the subset of low-risk patients with pT1N0 tumours ($n = 1036$), the presence of DTCs was associated with an increased risk of distant metastasis and death during the first 5 years.[4] However, the ASCO 2007 recommendations concluded that these retrospective data do not justify differential recommendations for adjuvant therapy[10] for a patient with bone marrow micrometastases.

Apart from the above-mentioned meta-analysis, where DTCs were examined at the time of primary surgery, Janni et al. reported that the persistence of DTCs during recurrence-free follow-up in patients with breast cancer was an independent prognostic factor for short recurrence-free survival (RFS) and OS.[49]

#### 5.2.2. *CTCs*

Stathopoulou et al. first reported that the detection by nested RT-PCR of CK19mRNA-positive cells in the peripheral blood of early breast cancer women was an independent prognostic factor for worse disease-free survival (DFS) and OS.[5] Later on, the same investigators developed a real-time RT-PCR for the quantification of CK19mRNA transcripts.[50] Xenidis et al. used the above-mentioned assay and detected CK19mRNA-positive cells in the peripheral blood of 21.6% of 167 patients with axillary lymph node-negative breast cancer before the administration of adjuvant chemotherapy. Their presence was an independent prognostic factor for worse DFS and OS.[6] In an expanded cohort of 444 women with stages I–III breast cancer, Ignatiadis et al. detected CK19mRNA-positive cells by the same real-time RT-PCR assay in the blood of 40.8% of patients before adjuvant chemotherapy.[7] The presence of these cells was an independent prognostic factor for short DFS and OS. Furthermore, Xenidis et al. demonstrated that CK19mRNA-positive cells were detected post adjuvant chemotherapy in 32.7% of 450 early breast cancer patients, and their presence was an independent prognostic factor for reduced DFS and OS.[51] Several other investigators have reported on the molecular or immunocytochemical detection of CTCs using cytokeratins.[52,53]

In the SUCCESS trial, >1 CTC/23 ml of blood was detected by the CellSearch System in 9.5% and 8.7% of 1500 node-positive and high-risk node-negative early breast cancer patients before and after adjuvant chemotherapy, respectively.[54] After a 12-month median follow-up, detection of >1 CTC/23 ml after but not before adjuvant chemotherapy was associated with shorter disease-free and overall survival.[54] Pachmann et al. used the MAINTRAC technology and identified 1–100,000 circulating epithelial cells (CETCs)/ml of peripheral blood in women with early breast cancer.[55] All 91 women had detectable CETCs, and a 10-fold increase in CETCs numbers between blood samples drawn before and after adjuvant chemotherapy was an independent predictor of relapse.

There are obvious and significant differences in CTCs' detection rates between the molecular techniques, the CellSearch and the MAINTRAC platform, which could only be addressed through a direct comparison of these technologies in the same patient population with early breast cancer.

#### 5.2.3. *Detection of DTCs/CTCs: is it always prognostically relevant?*

Approximately 30–50% of patients with breast cancer, whose bone marrow contains DTCs, will develop clinically apparent breast cancer metastases during a 5- to 10-year period of follow-up although even patients without DTCs may relapse and die of breast cancer.[4] Similarly, only 30% of patients with CK19mRNA-positive CTCs relapse, whereas 15% of patients without these cells still relapse and die of breast cancer after a 5-year median follow-up.[7] For patients who relapse with no detectable DTCs/CTCs, this could be explained by sampling error or could be attributed to the suboptimal sensitivity of the assays or cytokeratins as a marker of occult tumour cells. Indeed, tumour cell dissemination has been linked to the epithelial–mesenchymal transition and the loss of epithelial cell markers.[56] On the other hand, for patients with DTCs/CTCs who did not relapse, this could be due to the detection of

**Table 1 – Representative studies on the prognostic value of DTCs (A), CTCs (B) and DTCs versus CTCs (C) in breast cancer.**

| Patients | | Methods | | | | | Results | | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Stage | Patients (no.) | Tissue | Volume (ml) | Enrichment/ detection method | Marker | Cut-off | Detection rate (%) | Independent prognostic value | |
| DTCs | | | | | | | | | |
| *Panel A* | | | | | | | | | |
| I–III | 4703[1] | BM | NR | Ficoll/ICC | CK, MUC | $\geqslant$1DTCs/1–4 $\times 10^6$ MNCs | 30.6 | DFS, DDFS, BCSS, OS | [4] |
| I–III | 552 | BM | NR | Ficoll/ICC | CK | $\geqslant$1DTCs/2 $\times 10^6$ MNCs | 36 | DMFS, OS | [96] |
| I–III | 228 | BM | 3–8 | Ficoll/ICC | CK | $\geqslant$1DTCs/$10^6$ MNCs | 12.7 | RFS, OS | [49] |
| I–II[2] | 484 | BM | 10–20 | Ficoll/ICC | CK | $\geqslant$1DTCs/2–4 smears | 30.8 | NR | [46] |
| I–II | 128 | BM | NR | Ficoll/ICC | CK | $\geqslant$5DTCs/2 $\times 10^6$ MNCs | 13.3 | NR | [47] |
| I–III | 727 | BM | 40–50 | Ficoll/ICC | MUC | $\geqslant$1DTCs/4 $\times 10^6$ MNCs | 43.3 | DDFS, OS | [2] |
| I–III | 350 | BM | 16–32 | Ficoll/ICC | MUC | $\geqslant$1DTCs/10–25 smears | 25 | No | [43] |
| I–III | 52 | BM | NR | Ficoll/ICC | CK18, HER2 | $\geqslant$1DTCs/4 $\times 10^5$ MNCs | CK+: 100<br>CK+/HER2+: 60 | OS | [70] |
| I–III | 137 | BM | 10–20 | Ficoll/F | CK, HER2 | $\geqslant$1DTCs/$10^6$ MNCs | CK+: 34, CK+/HER2+: 12 | NR | [71] |
| CTCs | | | | | | | | | |
| *Panel B* | | | | | | | | | |
| IV | 177 | PB | 7.5 | IS | CK | $\geqslant$5CTCs/7.5 ml | 49 | PFS, OS | [35] |
| IV | 103 | PB | 24 | Ficoll/RT-PCR | Multi[3] | Based on healthy women | 29.1 | No | [38] |
| I–IV | 123 | PB | 5 | Filtration/ICC | CK8 | I–III: $\geqslant$4 CTCs/5 ml<br>IV: $\geqslant$13 CTCs/5 ml | I–III: 25<br>IV: 11.6 | I–III: No<br>IV: TTP | [37] |
| I–IV | 65 | PB | 10 | EL/RT-PCR | Multi[4] | [6] | 69 | NR | [74] |
| IV | 25 | PB | 10 | Ficoll/IS/PCR ELISA | Telomerase | Based on healthy women | 84 | NR | [41] |
| I–III | 148 | PB | 10 | Ficoll/RT-PCR | CK19 | Nested[7] | 29.7 | DFI, OS | [5] |
| I–III | 444 | PB | 20 | Ficoll/RT-PCR | CK19 | 0.6MCF7 Eq/5 μg RNA | 40.8 | DFS, OS | [7] |
| I–II[2] | 167 | PB | 20 | Ficoll/RT-PCR | CK19 | 0.6MCF7 Eq/5 μg RNA | 21.6 | DFS, OS | [6] |
| I–III | 175 | PB | 20 | Ficoll/RT-PCR | Multi[5] | 0.6MCF7 Eq/5 μgRNA, Nested[6] | 44 | DFS | [76] |
| I–III | 101 | PB | 20 | Ficoll/RT-PCR | MGB1 | Nested | 13.9 | DFI | [64] |
| I–II | 214 | PB | 20 | Ficoll/RT-PCR | HER2 | Nested | 21 | DFI | [67] |
| I–III | 35 | PB | 50 | IS/ICC | CK, HER2 | $\geqslant$1 CTCs/50 ml | 48.6 | No | [72] |
| I–III | 1500 | PB | 22.5 | Ficoll/IS/IF (CellSearch™) | CK 8, 18, 19 | >1 CTC/22.5 ml | 9.5 | NR | [54] |
| I–III | 91 | PB | 7.5 | EL/IF | EPCAM, CD45 | 10-fold change in CETS during treatment | 100 | RFS | [55] |
| I–IV | 109 | PB | 5 | Ficoll/RT-PCR | CK19 | Nested | 44 | NR | [52] |
| I–IV | 94 | PB | 10 | IS/RT-PCR | MUC | Ct $\leqslant$ 37 | 37 | NR | [60] |
| NED | 310 | PB | 10 | EL/RT-PCR | MGB1 | Nested | 2 | No | [62] |
| I–IV | 62 | PB | 10 | Ficoll/RT-PCR | EGFRvIII | Nested | 43.5 | NR | [68] |

(*continued on next page*)

**Table 1 – continued**

| Patients | | DTCs | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Methods | | | | Results | | | Reference |
| Stage | Patients (no.) | Tissue | Volume (ml) | Enrichment/ detection method | Marker | Cut-off | Detection rate (%) | Independent prognostic value | |
| | | | | DTCs versus CTCs | | | | | |
| *Panel C* | | | | | | | | | |
| NED | 341 | PB | 40 | Ficoll, IS, ICC | CK | $\geqslant$1 CTCs/10 × $10^6$MNCs | CTCs: 10 | NR | [85] |
| | | BM | | | | $\geqslant$1 DTCs/2 × $10^6$ MNCs | DTCs:14 | | |
| I–IV | 114 | PB | 7–14 | Ficoll, ICC | CK | $\geqslant$1 CTCs/3 × $10^6$MNCs | CTCs: 24.5 | DTCs for DFS | [86] |
| | | BM | 3–5 | | | $\geqslant$1 DTCs/3 × $10^6$ MNCs | DTCs: 59 | | |
| I–IV | 148 | PB | 9 | Ficoll, RT-PCR | CK19 | Based on healthy women | PB: 15 | CK19+DTCs for OS | [87] |
| | | BM | 9 | | MGB1 | | BM: 28 | | |

NED, non-evidence of disease; BM, bone marrow; PB, peripheral blood; NR, not reported; CTCs, peripheral blood circulating tumour cells; DTCs, bone marrow disseminated tumour cells; MNCs, mononuclear cells; ICC, immunocytochemistry; IF, immunofluorescence; IS, immunomagnetic separation; EL, erythrocyte lysis; CK, cytokeratin; Muc, mucin; MGB1, mammaglobin-A; EGFRvIII, epidermal growth factor receptor variant-III; 4703[1], this meta-analysis includes most of the patients reported as separate studies in Table 1A; I–II[2], node-negative tumours; multi-marker[3] (CK19, p1B, PS2 and EGP2); multi-marker[4] [human chorionic gonadotropin (hCG), c-Met, 134-*N*-acetylgalactosaminyl-transferase (GalNAc-T) and tumour-associated antigen (MAGE-A3)]; multi-marker[5] (CK19, MGB1 and HER2); [6], for a patient to be considered CTC-positive, at least one mRNA marker should be positive; DFS, disease-free survival; DDFS, distant disease-free survival; BCSS, breast cancer-specific survival; OS, overall survival; DMFS, distant metastasis-free survival; RF, recurrence-free survival; DFI, disease-free interval; PFS, progression-free survival; TTP, time-to-progression; RT-PCR, reverse transcriptase polymerase chain reaction; Ct, cycle threshold; Nested[6], in case of nested RT-PCR, results are expressed as detectable or non-detectable; Eq, equivalents.

apoptotic cells, or cells that are not able to self-renew and generate metastases. In the case of bone marrow DTCs, this could also be attributed to interactions between the surrounding bone marrow stroma and DTCs which might favour or not the development of clinically apparent metastases.[57] Moreover, microarray and comparative genomic hybridisation (CGH) studies have shown that breast cancer is a genetically heterogeneous disease,[58,59] and that even the DTCs of any given patient with early breast cancer may exhibit diverse genomic profiles.[13] Therefore, in order to improve the sensitivity/specificity of DTCs/CTCs' detection as a prognostic tool as well as to define subpopulations of DTCs/CTCs with aggressive biological behaviour, which could be used more precisely as surrogate markers for relapse, several approaches have been employed.

5.2.3.1. *Other markers (apart from cytokeratins) for detection of MRD.* Several investigators have used different markers such as mucins, mammaglobin-A (MGB1), maspin, carcinoembryonic antigen (CEA), HER2, EGFRvIII and cathepsin D for the detection of micrometastatic cells.[60–69] Since HER2 oncoprotein has been associated with an aggressive biological behaviour in breast cancer, several groups have studied the expression of HER2 on micrometastatic cells. Braun et al. first reported that HER2 was immunocytochemically co-expressed in 31 of 52 women with detectable CK18-positive DTCs.[70] Patients with HER2-positive DTCs had significantly shorter OS compared with patients with HER2-negative DTCs.[70] Solomayer et al. used double immunofluorescence and reported HER2 positivity in 20 of 46 patients with cytokeratin-positive DTCs.[71] Apostolaki et al. used nested RT-PCR to detect peripheral blood HER2mRNA-positive cells in 21% of 214 patients with early breast cancer after the administration of adjuvant chemotherapy; their detection was an independent prognostic factor for reduced DFI.[67] Wulfing et al. used double immunocytochemical staining to identify HER2-positive CTCs that correlated with shorter DFS and OS in early breast cancer patients treated with adjuvant chemotherapy or hormonotherapy.[72] Moreover, Ignatiadis et al. have shown that women with the CK19mRNA-positive/HER2mRNA-positive molecular profile in the blood had shorter DFS compared with CK19mRNA-positive/HER2mRNA-negative patients.[73] Although the above-mentioned studies have used different methodologies for the detection and characterisation of CTCs/DTCs' HER2 status, HER2-positive cells have been consistently detected in approximately half of early breast cancer patients presenting CTCs/DTCs, and these patients had worse prognosis than their counterparts with CTCs/DTCs not expressing HER2.

Based on the heterogeneity of CTCs/DTCs, several multi-marker RT-PCR assays have been reported.[33,74–77] Taback et al. used human chorionic gonadotropin (hCG), oncogene receptor (c-Met), 134-*N*-acetylgalactosaminyl-transferase and a tumour-associated antigen (MAGE-A3) to develop a multi-marker RT-PCR assay combined with an electrochemiluminescence-automated detection system for the detection of CTCs in breast cancer.[74] Zehentner et al. chose four different markers, namely mammaglobin-A, B305D, GABRP and B726P, to develop a multi-marker, real-time RT-PCR assay for the detection of CTCs in breast cancer patients.[75] Mikhitarian et al. used a panel of 8 genes for the molecular detection of

DTCs and CTCs [mammaglobin-A, CEA, CK19, PIP, muc1, PSE, Erb (DTCs only) and EpCAM (CTCs only)].[77] However, no correlation between CTCs' detection using the above-mentioned multi-marker assays and clinical outcome have been reported. Recently, Ignatiadis et al. reported for the first time that the use of a multi-marker (CK19, mammaglobin-A and HER2) RT-PCR to detect CTCs predicted poor clinical outcome in early breast cancer patients and that this assay had an increased accuracy as compared to CTCs' detection by real-time RT-PCR for CK19 alone.[76]

5.2.3.2. *Assessment of the proliferative potential of micrometastatic cells*. Two different groups have reported that the proliferative potential of bone marrow DTCs in *ex vivo* cultures correlated with clinical outcome of cancer patients.[78,79] Indeed, a strong expansion of tumour cells in culture was correlated to an increased rate of cancer-related death.

5.2.3.3. *Assays to distinguish between apoptotic and non-apoptotic or viable micrometastatic cells*. In general, when using the nucleic acid-based assays, the detection of an mRNA transcript in a blood sample suggests the presence of a viable cell since the viability of RNA once released from cells is poor and the presence of mRNA suggests an active transcription machinery. Moreover, Alix-Panabieres et al. used epithelial immunospot (ELISPOT) to detect viable, non-apoptotic CTCs.[80] The assay allowed the detection of protein secretion at the individual cell level. Fehm et al. detected apoptotic DTCs using immunocytochemistry in 36 of 157 (23%) breast cancer patients.[81] Interestingly, the response to therapy in breast cancer patients was reflected by the presence of apoptotic DTCs.

5.2.3.4. *Study of micrometastatic cells with 'stem cell-like' phenotype*. Given the recent identification of breast cancer tumour-initiating cells with the CD44+CD24–/low phenotype,[82] it could be hypothesised that micrometastatic cells with this phenotype might represent a prognostically relevant subpopulation. Balic et al. employed triple-staining immunocytochemistry to evaluate 50 cytokeratin-positive bone marrow specimens from early breast cancer patients for the presence of DTCs with the above-mentioned putative stem cell phenotype.[83] This phenotype was detected in all CK+ specimens, and its median prevalence was 65% amongst all DTCs detected per patient compared with primary tumours, where this phenotype was observed in <10% of tumour cells.[83] A similar study that detected CTCs with 'stem cell-like' phenotype has recently been reported.[84]

5.2.4. *DTCs versus CTCs*

Compared with the bone marrow, peripheral blood sampling is easier and more acceptable by the patients and their treating physicians. Therefore, an important question is whether peripheral blood sampling can replace bone marrow aspiration for the evaluation of MRD. Wiedswang et al. compared the prognostic value of CTCs versus that of DTCs detected by immunocytochemistry in 341 breast cancer patients with sampling performed at a median follow-up of 40 months after the initial operation.[85] Although both CTCs (10% of the patients) and DTCs (14% of the patients) were significantly associated with clinical outcome, DTCs were more informative than CTCs.[85] Pierga et al. compared the detection of cytokeratin-positive CTCs versus that of DTCs with an automatic-assisted immunocytochemical detection system in a cohort of 114 breast cancer patients.[86] In non-metastatic patients ($n = 75$), the presence of DTCs but not CTCs was prognostic for poor DFS.[86] In another study, investigators performed real-time RT-PCR for the detection of CK19 and mammaglobin-A in 148 patients with early and metastatic breast cancer.[87] Patients with either an elevated CK19 or mammaglobin-A expression level in the bone marrow but not in the peripheral blood had worse OS.[87] Although the above-mentioned studies suggest that DTCs are prognostically superior to CTCs in early breast cancer, the best validated techniques for the detection of bone marrow DTCs (i.e. immunocytochemistry[4]) and peripheral blood CTCs (i.e. real-time RT-PCR for CK19[6,7]) have never been directly compared.

5.2.5. *MRD and breast cancer molecular subtypes*

Ignatiadis et al. reported recently the first study that examined the prognostic value of micrometastatic cells in relation to early breast cancer molecular subtypes.[7] The presence of CK19mRNA-positive CTCs predicted worse outcome in patients with oestrogen receptor (ER)-negative but not ER-positive early breast cancer, despite the similar proportions of patients with CK19 mRNA-positive CTCs in both subgroups. Moreover, the presence of CK19mRNA-positive CTCs was associated with shorter DFS and OS in the triple-negative and HER2-positive subgroups, but not in the ER-positive/HER2-negative subgroups.[7] Interestingly, according to most primary tumour gene expression signatures, the majority of ER-negative tumours are assigned to the poor-prognosis group, whereas ER-positive tumours comprise a mixture of poor- and good-prognosis tumours.[59,88] Therefore, it would be interesting to prospectively assess the hypothesis that by combining information from primary tumour gene expression profiling and the detection of micrometastatic cells, we could further improve prognosis in early breast cancer.

## 6. Prediction

Apart from refining prognosis, the most exciting field is studying the role of MRD as a predictive tool. There is experimental evidence that the micrometastatic cells have less advanced genomic alterations than primary tumour cells.[9] Since the micrometastatic cells are the true targets of adjuvant systemic treatment, it might be important to consider when choosing therapy, not only the characteristics of the primary tumour, but also those of micrometastatic cells in order to improve outcome of early breast cancer patients.

### 6.1. *DTCs/CTCs phenotyping, profiling and genotyping*

Several investigators have tried to phenotype individual micrometastatic cells. Meng et al. used a sensitive blood test to capture CTCs and evaluate their *HER2* gene status by fluorescence *in situ* hybridisation.[89] They reported that 9 of 24

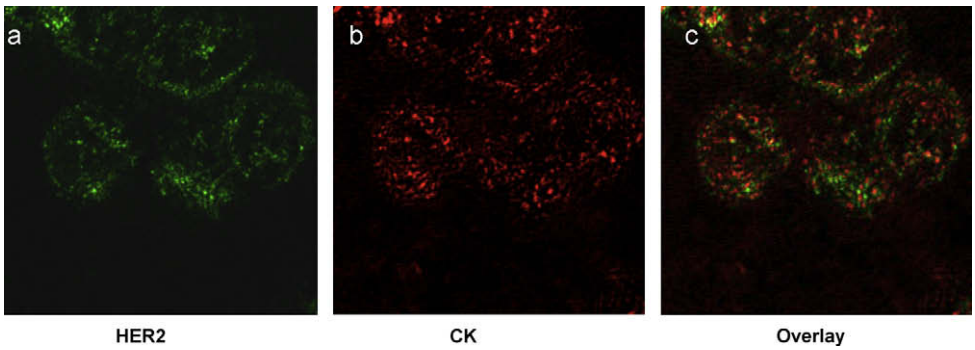


**Fig. 1 – Representative images of confocal laser scanning microscopic sections of a cluster of HER2-positive micrometastatic cells. PBMCs cytospins were double stained with polyclonal pan-cytokeratin (red) and monoclonal HER2 (green) antibodies. Magnification (×400). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

breast cancer patients with HER2-negative primary tumours had acquired HER2 gene amplification in their CTCs during cancer progression.[89] Meng et al. also demonstrated a marked tendency for co-amplification of HER2 and urokinase plasminogen activator receptor (uPAR) genes on individual CTCs.[90] On the other hand, Kallergi et al. provided evidence of enhanced expression of activated signalling kinases as well as of HER2 on individual CTCs.[91] As shown in Fig. 1, a cluster of HER2-positive micrometastatic cells from a woman with breast cancer is visualised after double immunofluorescence staining. Moreover, Smirnov et al. using microarray technology identified global gene expression profiles from CTCs of metastatic cancer patients and created a list of CTC-specific genes.[92] Intriguing results have demonstrated that gene expression profiling of single cells is feasible with oligonucleotide arrays.[93] Finally, using the CellPoint platform, Maheswaran et al. detected EGFR mutations in CTCs from patients with lung cancer treated with gefitinib. This study provides proof of principle for the feasibility of minimally invasive (blood sample instead of tumour biopsy) serial monitoring of tumour cell genotypes during treatment.[94] Based on these data, it seems that CTCs' phenotyping/profiling/genotyping not only may be crucial for identifying new targets which could be used to eliminate MRD, but also could serve as a less invasive and therefore more feasible real-time monitoring system to asses evolution of genetic changes on tumour cells with potential prognostic and therapeutic implications.

### 6.2. *Chemotherapy and hormonal therapy*

Several studies have demonstrated that CTCs/DTCs are relatively resistant to chemotherapy,[95–99] probably due to their low proliferative potential.[100] Concerning hormonal therapy and CTCs, Xenidis et al. showed that the persistent detection of CK19mRNA-positive cells in 119 patients with hormone receptor-positive tumours during tamoxifen administration was an independent prognostic factor for short DFS and OS.[101] Therefore, the persistent detection of CK19mRNA-positive cells during adjuvant tamoxifen administration should be further investigated as an indicator of tamoxifen resistance and the need to switch to an alternative adjuvant hormonal therapy such as an aromatase inhibitor.

### 6.3. *New targeted agents*

Since conventional chemotherapy and hormonal therapy cannot eliminate all micrometastatic cells in all patients, several investigators have targeted DTDs/CTCs using monoclonal antibodies. The monoclonal antibody 17-1A (against the epithelial adhesion molecule EpCAM) resulted in a marked reduction of EpCAM+/CK+ DTCs in patients with advanced breast cancer.[102,103] Moreover, Bozionellou et al. reported that a short course of trastuzumab could eliminate chemotherapy- and hormonotherapy-resistant CK19mRNA- and HER2mRNA-positive CTCs and DTCs in 20 (67%) of 30 patients with early and metastatic breast cancer.[104] Similar data have been reported in a xenograft SCID mice model.[105] Furthermore, HER2-positive CTCs/DTCs have been reported in patients with HER2-negative primary tumours.[71–73] Therefore, it would be interesting to prospectively test the hypothesis that this subpopulation of women could also benefit from adjuvant trastuzumab administration.

## 7. DTCs/CTCs as prognostic and predictive tool. Is it ready for prime time?

CTCs can serve as a real-time biopsy to evaluate and monitor treatment response since their detection can be easily repeated at different time intervals, whereas this is not the case for bone marrow DTCs. Therefore, most ongoing and planned clinical studies use peripheral blood CTCs instead of bone marrow DTCs in order to study MRD as a prognostic and predictive tool in breast cancer. Although there are emerging data that DTCs/CTCs might be, in the near future, exciting new prognostic and predictive tools for individualising breast cancer treatment, several problems have to be addressed before they could be used in clinical practice.

a. Detection of DTCs/CTCs is not yet standardised. Several efforts have been made towards this direction during the last year.[106–108] However, many investigators have

reported DTCs/CTCs detection in breast cancer patients, starting from different bone marrow/peripheral blood volumes per patient and using different enrichment procedures and different methods or epithelial tumour markers for their detection, thus making comparison across studies extremely difficult (Table 1). DTCs have primarily been detected using immunocytochemistry with anti-cytokeratin or anti-mucin antibodies. CTCs detection has also mainly relied on CKs. However, even studies using the same method and marker did not use common standardised procedures (e.g. different primers, amplification conditions and platforms for the RT-PCR, or different antibodies, protocols and detection systems for the immunocytochemistry or immunofluorescence). Therefore, the assays used have in many cases been suboptimally standardised with low reproducibility, whereas the cut-off values chosen for defining DTCs/CTCs positivity have not been adequately validated.

b. Published studies on DTCs/CTCs are flawed by many problems. Many studies reporting on CTCs had poor statistical designs, suffering from small sample sizes, were retrospective and have not been reported in a rigorous fashion.[109] Therefore, it is important that authors publishing in the field of CTCs/DTCs should adhere to reporting recommendations for tumour marker studies.[109]
c. No published studies have as yet shown that by using DTCs/CTCs as a prognostic and/or predictive biomarker we can improve clinical outcome of patients with breast cancer. Henry et al. published a tumour marker development flow chart, in which they described how a tumour marker can reach clinical practice. First, an accurate method to measure the tumour marker has to be developed. Then, a preliminary, preclinical hypothesis for this marker should be validated, ideally in archived specimens from prospective trials.[110] Finally, a prospective clinical trial should be designed so that treatment decisions in the experimental arm are based, at least in part, on CTCs/DTCs. In this way, definitive proof will be provided that DTCs/CTCs can be used to improve clinical outcome in breast cancer.

Although a number of hurdles remain to be overcome before the evaluation of minimal residual disease becomes standard practice for patients diagnosed with breast cancer, advances in the field promise to revolutionise the way oncologists approach clinical trial design, prognostication and treatment decision-making.

## Conflict of interest statement

None declared.

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## REFERENCES

1. Carter CL, Allen C, Henson DE. Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer* 1989;**63**:181–7.
2. Diel IJ, Kaufmann M, Costa SD, et al. Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status. *J Natl Cancer Inst* 1996;**88**:1652–8.
3. Braun S, Pantel K, Muller P, et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. *N Engl J Med* 2000;**342**:525–33.
4. Braun S, Vogl FD, Naume B, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. *N Engl J Med* 2005;**353**:793–802.
5. Stathopoulou A, Vlachonikolis I, Mavroudis D, et al. Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. *J Clin Oncol* 2002;**20**:3404–12.
6. Xenidis N, Perraki M, Kafousi M, et al. Predictive and prognostic value of peripheral blood cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in node-negative breast cancer patients. *J Clin Oncol* 2006;**24**:3756–62.
7. Ignatiadis M, Xenidis N, Perraki M, et al. Different prognostic value of cytokeratin-19 mRNA-positive circulating tumor cells according to estrogen receptor and HER2 status in early breast cancer. *J Clin Oncol* 2007;**25**:5194–202.
8. Husemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. *Cancer Cell* 2008;**13**:58–68.
9. Schardt JA, Meyer M, Hartmann CH, et al. Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. *Cancer Cell* 2005;**8**:227–39.
10. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol* 2007;**25**:5287–312.
11. Singletary SE, Allred C, Ashley P, et al. Revision of the American Joint Committee on Cancer staging system for breast cancer. *J Clin Oncol* 2002;**20**:3628–36.
12. Klein CA, Schmidt-Kittler O, Schardt JA, Pantel K, Speicher MR, Riethmuller G. Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. *Proc Natl Acad Sci USA* 1999;**96**:4494–9.
13. Klein CA, Blankenstein TJ, Schmidt-Kittler O, et al. Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. *Lancet* 2002;**360**:683–9.
14. Fehm T, Sagalowsky A, Clifford E, et al. Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. *Clin Cancer Res* 2002;**8**:2073–84.
15. Slade MJ, Coombes RC. The clinical significance of disseminated tumor cells in breast cancer. *Nat Clin Pract Oncol* 2007;**4**:30–41.
16. Lacroix M. Significance, detection and markers of disseminated breast cancer cells. *Endocr Relat Cancer* 2006;**13**:1033–67.
17. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer* 2004;**4**:448–56.
18. Ignatiadis M, Georgoulias V, Mavroudis D. Circulating tumor cells in breast cancer. *Curr Opin Obstet Gynecol* 2008;**20**:55–60.
19. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer* 2008;**8**:329–40.

20. Hayes DF, Smerage J. Is there a role for circulating tumor cells in the management of breast cancer? *Clin Cancer Res* 2008;**14**:3646–50.
21. Ring AE, Zabaglo L, Ormerod MG, Smith IE, Dowsett M. Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. *Br J Cancer* 2005;**92**:906–12.
22. Smith BM, Slade MJ, English J, et al. Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. *J Clin Oncol* 2000;**18**:1432–9.
23. Kagan M, Howard D, Bendele T, et al. A sample preparation and analysis system for identification of circulating tumor cells. *J Clin Ligand Assay* 2002;**25**:104–10.
24. Krivacic RT, Ladanyi A, Curry DN, et al. A rare-cell detector for cancer. *Proc Natl Acad Sci USA* 2004;**101**:10501–4.
25. Pachmann K, Clement JH, Schneider CP, et al. Standardized quantification of circulating peripheral tumor cells from lung and breast cancer. *Clin Chem Lab Med* 2005;**43**:617–27.
26. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 2007;**450**:1235–9.
27. Pantel K, Cote RJ, Fodstad O. Detection and clinical importance of micrometastatic disease. *J Natl Cancer Inst* 1999;**91**:1113–24.
28. Jung R, Kruger W, Hosch S, et al. Specificity of reverse transcriptase polymerase chain reaction assays designed for the detection of circulating cancer cells is influenced by cytokines in vivo and in vitro. *Br J Cancer* 1998;**78**:1194–8.
29. Ring A, Smith IE, Dowsett M. Circulating tumour cells in breast cancer. *Lancet Oncol* 2004;**5**:79–88.
30. Zippelius A, Kufer P, Honold G, et al. Limitations of reverse-transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cancer cells in bone marrow. *J Clin Oncol* 1997;**15**:2701–8.
31. Kowalewska M, Chechlinska M, Markowicz S, Kober P, Nowak R. The relevance of RT-PCR detection of disseminated tumour cells is hampered by the expression of markers regarded as tumour-specific in activated lymphocytes. *Eur J Cancer* 2006;**42**:2671–4.
32. Stathopoulou A, Ntoulia M, Perraki M, et al. A highly specific real-time RT-PCR method for the quantitative determination of CK-19 mRNA positive cells in peripheral blood of patients with operable breast cancer. *Int J Cancer* 2006;**119**:1654–9.
33. Reinholz MM, Nibbe A, Jonart LM, et al. Evaluation of a panel of tumor markers for molecular detection of circulating cancer cells in women with suspected breast cancer. *Clin Cancer Res* 2005;**11**:3722–32.
34. Chen CC, Hou MF, Wang JY, et al. Simultaneous detection of multiple mRNA markers CK19, CEA, c-Met, Her2/neu and hMAM with membrane array, an innovative technique with a great potential for breast cancer diagnosis. *Cancer Lett* 2006;**240**:279–88.
35. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004;**351**:781–91.
36. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging–predicting overall survival in metastatic breast cancer. *Clin Cancer Res* 2006;**12**:6403–9.
37. Wong NS, Kahn HJ, Zhang L, et al. Prognostic significance of circulating tumour cells enumerated after filtration enrichment in early and metastatic breast cancer patients. *Breast Cancer Res Treat* 2006;**99**:63–9.
38. Bosma AJ, Weigelt B, Lambrechts AC, et al. Detection of circulating breast tumor cells by differential expression of marker genes. *Clin Cancer Res* 2002;**8**:1871–7.
39. Baker MK, Mikhitarian K, Osta W, et al. Molecular detection of breast cancer cells in the peripheral blood of advanced-stage breast cancer patients using multimarker real-time reverse transcription-polymerase chain reaction and a novel porous barrier density gradient centrifugation technology. *Clin Cancer Res* 2003;**9**:4865–71.
40. Bidard FC, Vincent-Salomon A, Sigal-Zafrani B, et al. Prognosis of women with stage IV breast cancer depends on detection of circulating tumor cells rather than disseminated tumor cells. *Ann Oncol* 2008;**19**:496–500.
41. Soria JC, Gauthier LR, Raymond E, et al. Molecular detection of telomerase-positive circulating epithelial cells in metastatic breast cancer patients. *Clin Cancer Res* 1999;**5**:971–5.
42. Cote RJ, Rosen PP, Lesser ML, Old LJ, Osborne MP. Prediction of early relapse in patients with operable breast cancer by detection of occult bone marrow micrometastases. *J Clin Oncol* 1991;**9**:1749–56.
43. Mansi JL, Gogas H, Bliss JM, Gazet JC, Berger U, Coombes RC. Outcome of primary-breast-cancer patients with micrometastases: a long-term follow-up study. *Lancet* 1999;**354**:197–202.
44. Funke I, Schraut W. Meta-analyses of studies on bone marrow micrometastases: an independent prognostic impact remains to be substantiated. *J Clin Oncol* 1998;**16**:557–66.
45. Gebauer G, Fehm T, Merkle E, Jaeger W, Mitze M. Micrometastases in axillary lymph nodes and bone marrow of lymph node-negative breast cancer patients – prognostic relevance after 10 years. *Anticancer Res* 2003;**23**:4319–24.
46. Gerber B, Krause A, Muller H, et al. Simultaneous immunohistochemical detection of tumor cells in lymph nodes and bone marrow aspirates in breast cancer and its correlation with other prognostic factors. *J Clin Oncol* 2001;**19**:960–71.
47. Landys K, Persson S, Kovarik J, Hultborn R, Holmberg E. Prognostic value of bone marrow biopsy in operable breast cancer patients at the time of initial diagnosis: results of a 20-year median follow-up. *Breast Cancer Res Treat* 1998;**49**:27–33.
48. Bidard FC, Vincent-Salomon A, Gomme S, et al. Disseminated tumor cells of breast cancer patients: a strong prognostic factor for distant and local relapse. *Clin Cancer Res* 2008;**14**:3306–11.
49. Janni W, Rack B, Schindlbeck C, et al. The persistence of isolated tumor cells in bone marrow from patients with breast carcinoma predicts an increased risk for recurrence. *Cancer* 2005;**103**:884–91.
50. Stathopoulou A, Gizi A, Perraki M, et al. Real-time quantification of CK-19 mRNA-positive cells in peripheral blood of breast cancer patients using the lightcycler system. *Clin Cancer Res* 2003;**9**:5145–51.
51. Xenidis N, Apostolaki S, Perraki M, et al. Circulating CK-19 mRNA (+) cells in patients with stage I and II breast cancer after the completion of adjuvant chemotherapy: evaluation of their prognostic relevance. *Breast Cancer Res Treat* 2007;**106**(Suppl. 1) [abstract 109].
52. Kahn HJ, Yang LY, Blondal J, et al. RT-PCR amplification of CK19 mRNA in the blood of breast cancer patients: correlation with established prognostic parameters. *Breast Cancer Res Treat* 2000;**60**:143–51.
53. Witzig TE, Bossy B, Kimlinger T, et al. Detection of circulating cytokeratin-positive cells in the blood of breast cancer patients using immunomagnetic enrichment and digital microscopy. *Clin Cancer Res* 2002;**8**:1085–91.
54. Rack B, Schindlbeck C, Schneeweiss A, et al. Prognostic relevance of circulating tumor cells (CTCs) in peripheral blood of breast cancer patients before and after adjuvant

chemotherapy: the German SUCCESS-Trial. *Proc Am Soc Clin Oncol* 2008:26 [abstract 503].
55. Pachmann K, Camara O, Kavallaris A, et al. Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. *J Clin Oncol* 2008;**26**:1208–15.
56. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. *Nat Rev Cancer* 2005;**5**:744–9.
57. Guirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer* 2007;**7**:834–46.
58. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000;**406**:747–52.
59. Ignatiadis M, Desmedt C. Predicting risk of breast cancer recurrence using gene-expression profiling. *Pharmacogenomics* 2007;**8**:101–11.
60. De Cremoux P, Extra JM, Denis MG, et al. Detection of MUC1-expressing mammary carcinoma cells in the peripheral blood of breast cancer patients by real-time polymerase chain reaction. *Clin Cancer Res* 2000;**6**:3117–22.
61. Zach O, Kasparu H, Krieger O, Hehenwarter W, Girschikofsky M, Lutz D. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via a nested reverse transcriptase polymerase chain reaction assay for mammaglobin mRNA. *J Clin Oncol* 1999;**17**:2015–9.
62. Zach O, Kasparu H, Wagner H, Krieger O, Lutz D. Prognostic value of tumour cell detection in peripheral blood of breast cancer patients. *Acta Med Austriaca Suppl* 2002;**59**:32–4.
63. Silva AL, Tome MJ, Correia AE, Passos-Coelho JL. Human mammaglobin RT-PCR assay for detection of occult breast cancer cells in hematopoietic products. *Ann Oncol* 2002;**13**:422–9.
64. Ntoulia M, Stathopoulou A, Ignatiadis M, et al. Detection of mammaglobin A-mRNA-positive circulating tumor cells in peripheral blood of patients with operable breast cancer with nested RT-PCR. *Clin Biochem* 2006;**39**:879–87.
65. Stathopoulou A, Mavroudis D, Perraki M, et al. Molecular detection of cancer cells in the peripheral blood of patients with breast cancer: comparison of CK-19, CEA and maspin as detection markers. *Anticancer Res* 2003;**23**:1883–90.
66. Brandt B, Roetger A, Heidl S, et al. Isolation of blood-borne epithelium-derived c-erbB-2 oncoprotein-positive clustered cells from the peripheral blood of breast cancer patients. *Int J Cancer* 1998;**76**:824–8.
67. Apostolaki S, Perraki M, Pallis A, et al. Circulating HER2 mRNA-positive cells in the peripheral blood of patients with stage I and II breast cancer after the administration of adjuvant chemotherapy: evaluation of their clinical relevance. *Ann Oncol* 2007;**18**:851–8.
68. Silva HA, Abraul E, Raimundo D, et al. Molecular detection of EGFRvIII-positive cells in the peripheral blood of breast cancer patients. *Eur J Cancer* 2006;**42**:2617–22.
69. Solomayer EF, Diel IJ, Meyberg GC, et al. Prognostic relevance of cathepsin D detection in micrometastatic cells in the bone marrow of patients with primary breast cancer. *Breast Cancer Res Treat* 1998;**49**:145–54.
70. Braun S, Schlimok G, Heumos I, et al. ErbB2 overexpression on occult metastatic cells in bone marrow predicts poor clinical outcome of stage I–III breast cancer patients. *Cancer Res* 2001;**61**:1890–5.
71. Solomayer EF, Becker S, Pergola-Becker G, et al. Comparison of HER2 status between primary tumor and disseminated tumor cells in primary breast cancer patients. *Breast Cancer Res Treat* 2006;**98**:179–84.
72. Wulfing P, Borchard J, Buerger H, et al. HER2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients. *Clin Cancer Res* 2006;**12**:1715–20.
73. Ignatiadis M, Perraki M, Apostolaki S, et al. Molecular detection and prognostic value of circulating CK-19 mRNA- and HER2 mRNA-positive cells in the peripheral blood of women with early breast cancer. *Clin Breast Cancer* 2007;**7**:883–9.
74. Taback B, Chan AD, Kuo CT, et al. Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. *Cancer Res* 2001;**61**:8845–50.
75. Zehentner BK, Secrist H, Hayes DC, et al. Detection of circulating tumor cells in peripheral blood of breast cancer patients during or after therapy using a multigene real-time RT-PCR assay. *Mol Diagn Ther* 2006;**10**:41–7.
76. Ignatiadis M, Kallergi G, Ntoulia M, et al. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. *Clin Cancer Res* 2008;**14**:2593–600.
77. Mikhitarian K, Martin RH, Ruppel MB, et al. Detection of mammaglobin mRNA in peripheral blood is associated with high grade breast cancer: interim results of a prospective cohort study. *BMC Cancer* 2008;**8**:55.
78. Solakoglu O, Maierhofer C, Lahr G, et al. Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors. *Proc Natl Acad Sci USA* 2002;**99**:2246–51.
79. Pierga JY, Bonneton C, Magdelenat H, et al. Clinical significance of proliferative potential of occult metastatic cells in bone marrow of patients with breast cancer. *Br J Cancer* 2003;**89**:539–45.
80. Alix-Panabieres C, Vendrell JP, Pelle O, et al. Detection and characterization of putative metastatic precursor cells in cancer patients. *Clin Chem* 2007;**53**:537–9.
81. Fehm T, Becker S, Becker-Pergola G, et al. Presence of apoptotic and nonapoptotic disseminated tumor cells reflects the response to neoadjuvant systemic therapy in breast cancer. *Breast Cancer Res* 2006;**8**:R60.
82. Al-Hajj M, Wicha MS, ito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003;**100**:3983–8.
83. Balic M, Lin H, Young L, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* 2006;**12**:5615–21.
84. Theodoropoulos PA, Polioudaki E, Sanidas E, Agelaki S, Mavroudis D, Georgoulias V. Detection of circulating tumor cells with breast cancer stem cell-like phenotype in blood samples of patients with breast cancer. *AACR Annual Meeting* 2008 [abstract 2008].
85. Wiedswang G, Borgen E, Schirmer C, et al. Comparison of the clinical significance of occult tumor cells in blood and bone marrow in breast cancer. *Int J Cancer* 2006;**118**:2013–9.
86. Pierga JY, Bonneton C, Vincent-Salomon A, et al. Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. *Clin Cancer Res* 2004;**10**:1392–400.
87. Benoy IH, Elst H, Philips M, et al. Real-time RT-PCR detection of disseminated tumour cells in bone marrow has superior prognostic significance in comparison with circulating tumour cells in patients with breast cancer. *Br J Cancer* 2006;**94**:672–80.
88. Sotiriou C, Piccart MJ. Taking gene-expression profiling to the clinic: when will molecular signatures become relevant to patient care? *Nat Rev Cancer* 2007;**7**:545–53.
89. Meng S, Tripathy D, Shete S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. *Proc Natl Acad Sci USA* 2004;**101**:9393–8.

90. Meng S, Tripathy D, Shete S, et al. UPAR and HER-2 gene status in individual breast cancer cells from blood and tissues. *Proc Natl Acad Sci USA* 2006;**103**:17361–5.
91. Kallergi G, Mavroudis D, Georgoulias V, Stournaras C. Phosphorylation of FAK, PI-3K, and impaired actin organization in CK-positive micrometastatic breast cancer cells. *Mol Med* 2007;**13**:79–88.
92. Smirnov DA, Zweitzig DR, Foulk BW, et al. Global gene expression profiling of circulating tumor cells. *Cancer Res* 2005;**65**:4993–7.
93. Hartmann CH, Klein CA. Gene expression profiling of single cells on large-scale oligonucleotide arrays. *Nucleic Acids Res* 2006;**34**:e143.
94. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;**359**:366–77.
95. Xenidis N, Mavroudis D, Apostolaki S, et al. Effect of adjuvant treatment on the circulating CK19mRNA positive tumor cells in patients with early stage breast cancer. *Proc Am Soc Clin Oncol* 2006;**24**(18S) [abstract 20012].
96. Braun S, Kentenich C, Janni W, et al. Lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow of high-risk breast cancer patients. *J Clin Oncol* 2000;**18**:80–6.
97. Xenidis N, Vlachonikolis I, Mavroudis D, et al. Peripheral blood circulating cytokeratin-19 mRNA-positive cells after the completion of adjuvant chemotherapy in patients with operable breast cancer. *Ann Oncol* 2003;**14**:849–55.
98. Becker S, Solomayer E, Becker-Pergola G, Wallwiener D, Fehm T. Primary systemic therapy does not eradicate disseminated tumor cells in breast cancer patients. *Breast Cancer Res Treat* 2007;**106**:239–43.
99. Slade MJ, Singh A, Smith BM, et al. Persistence of bone marrow micrometastases in patients receiving adjuvant therapy for breast cancer: results at 4 years. *Int J Cancer* 2005;**114**:94–100.
100. Pantel K, Schlimok G, Braun S, et al. Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. *J Natl Cancer Inst* 1993;**85**:1419–24.
101. Xenidis N, Markos V, Apostolaki S, et al. Clinical relevance of circulating CK-19 mRNA-positive cells detected during the adjuvant tamoxifen treatment in patients with early breast cancer. *Ann Oncol* 2007;**18**:1623–31.
102. Braun S, Hepp F, Kentenich CR, et al. Monoclonal antibody therapy with edrecolomab in breast cancer patients: monitoring of elimination of disseminated cytokeratin-positive tumor cells in bone marrow. *Clin Cancer Res* 1999;**5**:3999–4004.
103. Kirchner EM, Gerhards R, Voigtmann R. Sequential immunochemotherapy and edrecolomab in the adjuvant therapy of breast cancer: reduction of 17-1A-positive disseminated tumour cells. *Ann Oncol* 2002;**13**:1044–8.
104. Bozionellou V, Mavroudis D, Perraki M, et al. Trastuzumab administration can effectively target chemotherapy-resistant cytokeratin-19 messenger RNA-positive tumor cells in the peripheral blood and bone marrow of patients with breast cancer. *Clin Cancer Res* 2004;**10**:8185–94.
105. Barok M, Balazs M, Nagy P, et al. Trastuzumab decreases the number of circulating and disseminated tumor cells despite trastuzumab resistance of the primary tumor. *Cancer Lett* 2008;**260**:198–208.
106. Borgen E, Naume B, Nesland JM, et al. Standardization of the immunocytochemical detection of cancer cells in BM and blood: I. Establishment of objective criteria for the evaluation of immunostained cells. *Cytotherapy* 1999;**1**:377–88.
107. Fehm T, Solomayer EF, Meng S, et al. Methods for isolating circulating epithelial cells and criteria for their classification as carcinoma cells. *Cytotherapy* 2005;**7**:171–85.
108. Fehm T, Braun S, Muller V, et al. A concept for the standardized detection of disseminated tumor cells in bone marrow from patients with primary breast cancer and its clinical implementation. *Cancer* 2006;**107**:885–92.
109. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies. *J Clin Oncol* 2005;**23**:9067–72.
110. Henry NL, Hayes DF. Uses and abuses of tumor markers in the diagnosis, monitoring, and treatment of primary and metastatic breast cancer. *Oncologist* 2006;**11**:541–52.