

Review

# Profiling of Invasive Breast Carcinoma Circulating Tumour Cells—Are We Ready for the ‘Liquid’ Revolution?

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**Abstract:** As dissemination through blood and lymph is the critical step of the metastatic cascade, circulating tumour cells (CTCs) have attracted wide attention as a potential surrogate marker to monitor progression into metastatic disease and response to therapy. In patients with invasive breast carcinoma (IBC), CTCs are being considered nowadays as a valid counterpart for the assessment of known prognostic and predictive factors. Molecular characterization of CTCs using protein detection, genomic and transcriptomic panels allows to depict IBC biology. Such molecular profiling of circulating cells with increased metastatic abilities appears to be essential, especially after tumour resection, as well as in advanced disseminated disease, when information crucial for identification of therapeutic targets becomes unobtainable from the primary site. If CTCs are truly representative of primary tumours and metastases, characterization of the molecular profile of this easily accessible ‘biopsy’ might be of prime importance for clinical practice in IBC patients. This review summarizes available data on feasibility and documented benefits of monitoring of essential IBC biological features in CTCs, with special reference to multifactorial proteomic, genomic, and transcriptomic panels of known prognostic or predictive value.

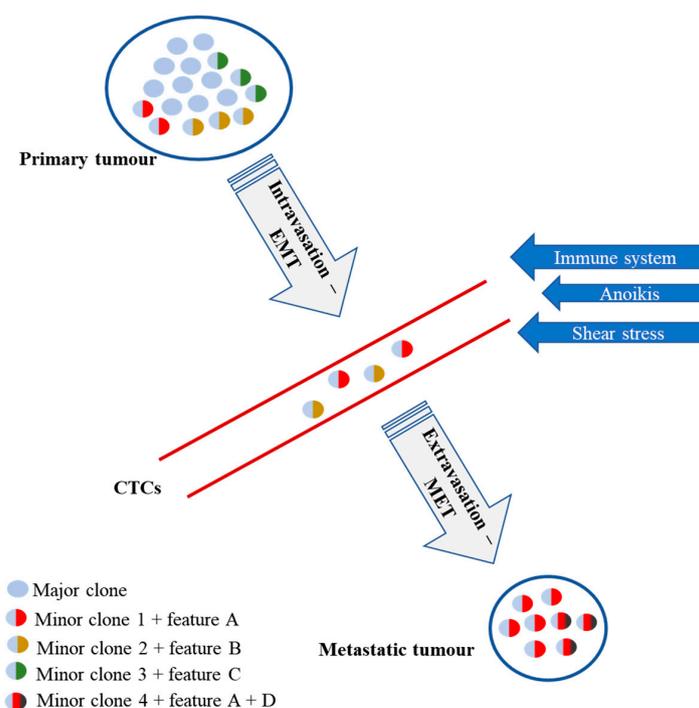
**Keywords:** invasive breast cancer; circulating tumour cells; predictive/prognostic multiparametric panels; gene signatures; single cells analysis

## 1. Introduction

Invasive breast carcinoma (IBC) is the most frequent and the deadliest cancer in women worldwide; however, identification of its predictive features and application of this knowledge in clinical practice have brought a huge progress in effective management of IBC patients [1–4]. The factors predicting disease course encompass both routinely assessed pathological (lymph node status, grading, tumour size and extent) and biological characteristics, predominantly the steroid hormone receptor status (Estrogen Receptor (ER), Progesterone Receptor (PR)), as well as human epidermal growth factor receptor 2 (HER2) expression/amplification status and Ki67 proliferation index [2].

The main cause of invasive breast carcinoma (IBC)-related deaths is systemic spread of cancer cells and formation of metastases. These arise from minor subpopulations characterized by specific phenotypic traits, not representative of the bulk of primary tumour, and thus likely no to be targeted by the administered therapy (Figure 1) [5–7]. Tumour heterogeneity might, therefore,

cause misinterpretation of therapeutic effects and should be appropriately addressed in evaluation of response to the treatment [8,9]. The need for monitoring of disease course and real-time assessment of therapy efficacy led to development of the so-called ‘liquid biopsies’, which would allow for early identification of disease progression (prognostic value) and selection of therapy (predictive value) [10–13]. Analysis of circulating tumour DNA (ctDNA), free circulating microRNAs (fc-miRNAs) and circulating tumour cells (CTCs), as major types of ‘liquid biopsy’, has generated interest in such personalized therapeutic approach [10–12,14–17]. Due to relatively small fragment size/amount of DNA, ctDNA can be used only for defining genomic changes related to cancer, such as mutation status or copy number variations, but not for detection of chromosomal aberrations [10,11,13,18,19]. Development of fc-microRNAs panels of prognostic or predictive value is the highlight of current translational research, however it provides only scarce information about tumour heterogeneity [20–23]. This can be assessed uniquely in the examination of CTCs. In IBC, the majority of therapeutic decisions is based on transcriptomic (gene expression), proteomic (status of HER2 and hormone receptors), or genomic profiling of cancer cells [24–26]. The aim of this review is to summarize current state of knowledge regarding multifactorial proteomic, genomic and transcriptomic panels applied to the analysis of CTCs and, when available, their potential clinical utility in IBC.



**Figure 1.** Heterogeneity of circulating tumour cells (CTCs). Major clone and minor clone 3 of the primary tumour do not shed CTCs, thus do not contribute to metastases. Two minor clones (1 and 2) with acquired genotypic and phenotypic changes A and B disseminate via hematogenous/lymphatic pathway, facing several stress insults. Only minor clone 1 is able to quit the blood/lymph stream and form metastasis in a distant site, where it acquires additional changes (D), generating minor clone 4. EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition.

## 2. Multifactorial Prognostic and Predictive Panels in Invasive Breast Cancer

Assessment of ER, PR, HER2, and Ki67 statuses allows to classify IBC into four biologically and clinically distinct subtypes: luminal A, luminal B, HER2-enriched, or triple negative [27–29]. This so-called intrinsic IBC classification has been reproduced by hierarchical cluster analyses of gene expression profiles, favoured by modern approach to IBC subtyping, enabling a more comprehensive evaluation of mRNA levels of genes included in molecular signatures established in microarray experiments [27,30–32]. The PAM50 is the most widely used IBC gene signature and is considered

a robust counterpart of classification based on immunohistochemical subtyping [30,33–37]. In the last years, many more predictive panels have been constructed and introduced to the management of IBC patients [32,38–41]. They were developed on the basis of either PAM50 genes (e.g. Prosigna) or genes selection guided by the outcome data (e.g., 70-gene signature—MammaPrint, 21-gene recurrence score—OncotypeDx, Breast Cancer Index, and multigenetic Endopredict) [38–45]. The actual advantage of multigenetic expression panels over routinely used predictive factors is still being disputed; however, according to both the World Health Organization’s (WHO) and St. Gallen’s expert consensus, multigenic panels are regarded as, at least, equally valuable (Table 1) [2,44].

### 3. Inter- and Intratumoural Heterogeneity of IBC

IBC is a highly heterogeneous disease and this heterogeneity is apparent between and within tumour-bearing patients (the “intertumoural” and “intratumoural” heterogeneity, respectively) [27–29,31,37,46]. The intertumoural heterogeneity of IBC concerns various histopathological classes, tumour grades or the aforementioned intrinsic molecular subtypes. As in all cancers, the intratumoural heterogeneity of IBC results from the dynamic changes that occur during tumour development, which involve gradual accumulation of diverse genetic, epigenetic, and phenotypic transformations combined with clonal expansion and selection (Figure 1) [46–49]. Progression into metastatic disease is associated with further phenotypic alterations, which are transient and reversible, so the similarity between primary tumours and synchronous metastases naïve to treatment can be expected and, indeed, is often observed [50,51]. The acquired transient molecular traits attributable to the process of dissemination remain poorly characterized, leaving a question mark against phenotypic concordance of circulating cells ‘in transit’ with primary or secondary tumours, or both [46–49,52,53]. The analysis of CTCs, so-called ‘liquid biopsy’ would, therefore, give an insight into the biology of the metastatic cascade providing ‘the missing link’ in molecular characterisation of tumour evolution. Moreover, it may serve as a valid counterpart for the assessment of prognostic and predictive factors in patients with IBC. This would be of prime importance for clinical practice, especially in advanced disease, where analysis of metastatic cells should provide information essential for identification of therapeutic targets, otherwise unobtainable from primary tumours.

### 4. CTCs—Clinical Potential and Limitations

The existence of CTCs was first proposed in the nineteenth century [54], but due to technological obstacles, the experimental examination of CTCs has become feasible only in recent years [52,55]. Although representative of only a small population of the primary tumour, CTCs are regarded as the prime means of disease dissemination and understanding of their biology is essential. Phenotypically CTCs are highly heterogeneous. This is due to the ways of entering the bloodstream (active vs. passive) but, above all, to molecular traits transiently acquired to meet demands of the rapidly changing microenvironment. Furthermore, CTCs subpopulation contains single cells as well as clusters [56–58], which exhibit intermediate EMT-phenotype, i.e., pro-survival and migratory abilities of mesenchymal cells and cell-cell interaction profile of epithelial cells [12]. As demonstrated by Aceto et al., these features might be responsible for up to 50-times higher metastatic abilities of CTC clusters than single cells [57]. Only 0.1% of single CTCs survive more than 24 h in the bloodstream (the half time of CTCs is estimated to range from 1 to 3 h), and only less than 0.01% of these cells have the ability to produce metastasis [48,49,59,60]. Identification of this most aggressive, metastasis initiating population of CTCs, represents both challenge and promise of modern diagnostics.

### 5. Methods of CTCs isolation

A number of methods have been developed for CTCs enrichment and isolations, with comprehensive summary published in a recent review [61]. CTCs analysis can be performed on enriched (with depletion of blood cells) or non-enriched samples (without depletion of blood cells), depending on the CTCs detection strategy, the throughput of the method and the type of marker to be analysed (DNA, RNA, or protein). Because gene expression profiles are usually tested in the bulk

samples, high CTCs enrichment is preferred, what reduces background signals from the contaminating blood cells [62–64]. When proteins are analysed, each cell positive for the selected CTCs markers (potential CTC) is visually inspected, so the enrichment allows for reducing the number of cells to be screened and application of lower-throughput methods.

Enrichment of IBC CTCs can be achieved by either positive or negative selection. In the positive selection (using e.g., CellSearch, AdnaTest, MACS, MagSweeper technologies), epithelial surface markers such as EpCAM are used (additionally MUC-1 in the AdnaTest) [65–68]. Elimination of mesenchymal-like CTCs, cells that lost epithelial features, i.e. undergone complete or partial EMT, from the sample is a severe limitation of the method [69]. In the negative selection, blood cells are removed by depletion of cells positive for CD45 [62,70] or a panel of markers: CD45, CD16, CD19, CD163, CD235a/GYP (as in MINDEC strategy) [71]. Although the purity of the sample may get compromised (the main limitation of the method), enrichment of cancer cells independently of their EMT status is the method's great advantage. Isolation of mesenchymal CTCs is also possible in non-enriched CTCs samples (whole blood or PBMC blood fraction), where the risk of CTCs loss in a process of positive or negative selection is limited. However, in practice, these methods rely mostly on epithelial markers (most often epithelial cytokeratins) for detection of CTCs (e.g., CytoTrack [72], FASTCell™ [73], HD-CTC [74]). Inclusion of antibodies against specific mesenchymal markers in the CTCs staining panel would largely improve performance of these methods.

For genomic and transcriptomic characterization of CTCs, the purity of the CTCs-enriched fraction is the most important factor, since background signal might interfere with the readout of the molecular assay. For genomic test, the sensitivity of the assay for mutation detection in the background of wild-type allele should be considered [75,76]; though, in practice, genomic testing of CTCs is usually performed at the single cell level. In the transcriptomic assays, expression of genes included in the molecular test should be analysed in a number of control samples to adjust for background expression level and apply a cut-off value, when appropriate [62–64,77].

## 6. The Prognostic and Predictive Value of CTCs Detection and Quantification in Patients with IBC

The most aggressive clone in the primary tumour does not necessarily arise from the most abundant population of tumour cells, thus it is vital to reveal the 'identity' of clones with a high metastatic potential (Figure 1) [78,79]. As CTCs are being identified with these highly invasive and usually infrequent yet responsible for spread of disease cells, clinical trials are being designed to detect and assess CTCs for clinical decision making. The majority of studies of circulating cells have been focused on the possible prognostic and predictive value of CTCs detection and quantification. CTCs presence has been found an independent poor prognostic factor IBC [52]. Bidard et al. conducted an individual data analysis of 1944 metastatic IBC patients and showed an independent prognostic association between CTCs quantity and both progression-free (PFS) and overall survival (OS) [14]. This was confirmed in a large systematic review with meta-analysis by Yan et al., who included 6712 IBC patients from 50 studies and showed a strong predictive value of CTCs status during the treatment for both PFS and OS [80]. The count of CTCs correlated negatively with treatment results in IBC patients and their presence either before or after chemotherapy was linked with a poor prognosis [52,80–89].

Despite very promising results on prognostic value of CTCs quantification and detection, modification of applied therapy based on CTCs' enumeration was found of no impact on OS and even PFS of patients in the, so far, most important SWOG0500 trial [90]. However, there were some doubts regarding the design of the study, as there were strong discrepancies in chemotherapeutic regimens applied in patients from different study arms, which puts in question the actual predictive value of CTCs quantification. In summary, the available data strongly suggest that not only a quantitative evaluation, but also a detailed characterization of CTCs' biological traits is required to justify their future clinical application as close counterparts of primary or metastatic tumours, or both.

## 7. Assessment of Multiparametric Panels in IBC CTCs

### 7.1. Protein Level

On the protein level, practical assessment of CTCs' biological features is currently limited to the key phenotypic IBC characteristics features (expression of ER, PR, and HER2) [73,91–95]. Majority of the studies reports a considerable disparity in the ER/PR/HER2 status between CTCs and primary or metastatic tumour cells but accumulated data are conflicting [53,91–93,96–101]. Aktas et al. and Babayan et al. demonstrated that expression of ER and PR in CTCs was lost or downregulated when compared to the ER/PR positive primary or metastatic tumours [92,93]. This finding was not confirmed by Kalinsky et al. and Fehm et al., who reported a similarity of the ER/PR profile between CTCs and primary/metastatic tumours [91,102]. Complicating matter even further, a recent analysis by Jordan et al. demonstrated that subsets of cells displaying opposite phenotypic features, e.g., HER2-positive and HER2-negative, may exist simultaneously among the CTCs population from the same patient [53].

Assessment of heterogeneity of CTCs is currently considered the biggest challenge in evaluation of a true predictive value of IBC CTCs for clinical practice [49,103,104]. The research aimed at valuation of the prognostic or predictive usefulness of CTCs' ER/PR/HER2 status is limited. The biggest and most up-to-date study by Beije et al. [105–109] did not show either predictive or prognostic role of ER or HER2 status in 154 patients with metastatic IBC and this was confirmed by other reports [93,101,103,105]. In contrast, studies by Onstenk et al., Wallwiener et al., Hayashi et al., Paoletti et al. (inventor of a CTC-Endocrine therapy index (CTC-ETI)) or a meta-analysis by Wang et al. showed HER2 or ER levels in IBC CTCs as weak, but significant prognostic and predictive indicators [106–110]. To summarize, although technologically feasible, evaluation of ER/PR/HER2 status in IBC CTCs has not proved yet to be of a reliable prognostic and predictive value (Table 1) [93,104].

**Table 1.** Prognostic and predictive value of biological markers in invasive breast carcinoma—primary tumour vs. CTCs. IBC, invasive breast carcinoma; CTCs, circulating tumour cells; PFS, progression-free survival; OS, overall survival.

Analyte	Marker	Prognostic/Predictive Value in IBC-Primary Tumour	Successfully Applied in CTCs	Prognostic or Predictive Value when Examined in CTCs	Reference
DNA	HER2 amplification	Strong predictive value.	Yes, can be robustly assessed.	No confirmed prognostic/predictive value in metastatic breast cancer patients treated with ado-trastuzumab emtansine [111].	[112–116]
	PIK3CA gain-of-function mutation	Prognostic factor linked to good prognosis; not applied in routine clinical practice.	Yes, can be robustly assessed.	Not assessed.	[112,117,118]
	TP53 loss-of-function mutation	Prognostic factor linked to poor prognosis; no predictive value in routine clinical practice.	Yes, can be robustly assessed.	Not assessed.	[117,119]
	RB1	Prognostic factor linked to poor prognosis. Predictive value—low RB1 expression in triple negative/ER-negative breast cancers related to good prognosis in patients treated with chemotherapy.	Yes, can be assessed.	Not assessed.	[118]
	ESR1 mutations	Prognostic factor linked to poor prognosis, potentially to be applied in clinics as a negative predictive factor (hormone resistance).	Yes, can be robustly assessed.	Not assessed.	[119]
	Ion AmpliSeq™ Cancer Hotspot Panel v2	Not assessed.	Yes, can be robustly assessed.	Not assessed.	[10,120]
RNA	ESR1/PGR	Both receptors routinely examined at protein level. Discrepancies between mRNA and protein expression frequently observed, but mRNA evaluation also shown of prognostic/ predictive value.	Yes, can be robustly assessed.	Prognostic value like in primary tumour, discrepant results of predictive value.	[93,105,107].
	HER2	Discrepancies between mRNA and protein levels seen in nearly 25% of patients. Protein examination routinely applied in clinics. mRNA also of both prognostic and predictive value.	Yes, can be robustly assessed.	HER2-positive CTCs are linked to poor prognosis in terms of both OS and PFS.	[108,109,121–123]
	EMT pathway molecules	Association between high levels of mesenchymal markers frequently reported. No predictive value or validated clinical application.	Yes, but efficiency of protocol/s still to be improved.	High frequency of mesenchymal CTCs linked to poor prognosis. No data on predictive value.	[62,97,99,124,125]
	PAM50	Prognostic and predictive value comparable to standard predictive factors, useful in clinical practice.	No report on coverage of all genes; single reports on partial assessment of the signature	Not assessed.	[91,126–128]
	Prosigna	Routinely applied predictive panel in clinics.	No, cannot be robustly applied.	Not assessed.	[126,127]
	Other panels, including EndoPredict, MammaPrint, OncotypeDx, Breast Cancer Index	Each panel designed to predict outcome; prognostic and predictive values of various panels similarly high across several comparing studies; routinely applied in clinics.	No reports so far.	Not assessed.	[60,126,129,130]
	microRNAs	Some panels of prognostic value when measured in primary tumour, but the known panels mostly applied for free-circulating microRNAs.	On-going research to resolve technical issues.	Not assessed.	[128,131–133]
Protein	ER, PR	The most significant prognostic and predictive factors applied in clinics.	Yes, can be robustly assessed.	Prognostic value.	[11,101,103,105–108]
	HER2	One of the key prognostic and predictive factors applied in clinics.	Yes, can be robustly assessed.	Poor prognostic value in terms of PFS in patients with HER2-positive CTCs in comparison to patients with HER2-negative CTCs, no strong prognostic value regarding OS.	[101,105,106,109]
	Ki67	One of the key prognostic and predictive factors applied in clinics.	Yes, but some technical difficulties still to be overcome.	Not assessed.	[134,135]
	EMT pathway molecules	Prognostic role of E-cadherin, vimentin and keratins.	Yes, can be robustly assessed	EMT activation related with reduced PFS and OS in metastatic patients.	[16,136–138]
	Proteomic panels	Prognostic significance of breast cancer subtypes identified by a multi-protein marker set.	Yes, can be assessed.	Not assessed. Used in basic science research.	[139,140]

## 7.2. DNA Level

Analysis of mutations or copy number alterations in CTCs' genomes can inform about oncogene addiction, sensitivity to treatment or mechanism of resistance to therapy. Thus, monitoring of changes in CTCs genotype during therapy administration might provide an invaluable insight into early signs of therapy failure. Since genomic testing is performed most often in single CTCs, reliable protocols and tools need to be applied to minimize the possibility of false positive results due to single-cells DNA amplification errors (reviewed in [141]).

Currently there are no pre-defined multiparametric panels for evaluation of genomic changes in single cells. Single cells analysis is mainly performed after CellSearch enrichment, when cells are flushed out of the cassette and loaded onto DEPArray for single cell recovery. The majority of the analysis is based on Whole Genome Amplification (WGA) of single cells and further Sanger sequencing of selected hot spot regions. In all the studies described below, WGA was performed using Ampli1 WGA assay (Silicon Biosystems), which relies on enzymatic genome digestion with *Mse I*, adapter ligation and further PCR-based fragments amplification. A number of studies testing mutations in CTCs are restricted to the analysis of a single gene in CTCs (e.g., *TP53*, *PIK3CA*, *HER2*, *ESR1*) [13,142–146]. Polzer et al. tested *HER2* amplification (by qPCR) and *PIK3CA* mutations (by Sanger sequencing) in exon 9 and 20 in CTCs isolated from breast cancer patients (stage I-IV) with CellSearch-enrichment and DEPArray single cells recovery. They have found that *HER2* was amplified in CTCs of 16.7% of the patients, with 16 cases (out of 17) showing homogenous *HER2* amplification status, indicative of CTCs clonality [112]. Disparity in the *HER2* status between CTCs and matched primary tumours reached 20%. Mutations in *PIK3CA* gene were found in 37.2% of the patients and showed heterogeneous CTCs mutation status in 9 out of 17 patients [112]. Disparity in the *PIK3CA* mutation status between CTCs and matched primary tumours was higher than that of *HER2* and concerned 66% of the cases. Prior to administration of HER2-targeted therapies, in 28.6% of the patients, *HER2*-amplified CTCs had *PIK3CA* mutations, known to be associated with resistance to anti-HER2 treatment and worse outcome of patients with HER2-positive tumours [111,147,148]. Lower rate of *PIK3CA* mutation in exons 1, 9, and 20 was observed in the study by Neves et al., who have tested MoFlo XDP cell sorter-isolated single CTCs obtained after CellSearch-enrichment of blood samples from metastatic breast cancer patients [117]. Mutated *PIK3CA* (tested by Sanger sequencing) was detected in 16% of the patients and no mutations in *TP53* (exon 5, 7, 8) were found. Copy number alterations, such as frequent amplification of *CCND1* locus (46% of CTCs), tested by aCGH and further validated by qPCR, were characteristic of metastatic breast cancers.

De Luca et al. used targeted sequencing (on IonTorrent PGM system) of a pre-defined set of 50 oncogenes and tumour suppressor genes with a use of Ion AmpliSeq™ Cancer Hotspot Panel v2 in CellSearch-enriched and DEPArray isolated single CTCs from four metastatic breast cancer patients [120]. Overall, 51 sequence variants were found in 25 genes. Observed mutations were mostly not shared among CTCs from the same patient (3–5 CTCs were analysed per patient), apart from mutations in *HER2*, *TP53*, *KIT*, and *PDGFRA* genes, which occurred in at least two CTCs from the same patient. Some of the recorded mutations, such as *TP53* p.R273C, have previously been connected with more aggressive phenotype and found in vitro to be associated with increased cell proliferative and migratory potential as well as drug resistance [149]. Mutation in *HER2* (ErbB2-p.V777L) was described to confer decreased sensitivity to lapatinib in vitro [150]. Sequencing of three available matched primary tumours (for mutations found in CTCs) revealed shared changes in *PDGFRA* (benign change) in one patient and *TP53* mutations in two other patients. The rest of the variants were not present in the primary tumours. In one case, analysis of CTCs performed before and one month after treatment initiation revealed existence of CTCs with on-treatment acquired mutations in *EGFR*, *PIK3CA*, *SMAD4*, *SMARCB1*, and *VHL* genes. However, detected mutations were present only in one out of three CTCs, indicating lack of clonality. Targeted sequencing (on IonTorrent PGM system) with Ion AmpliSeq™ Cancer Hotspot Panel v2 was used also by Shaw et al. in five metastatic breast cancer patients with high CellSearch CTCs count (>100) [10]. Single CTCs were isolated with the DEPArray

system. Mutations (in at least two CTCs from the same patient) were observed in four genes, *PIK3CA*, *TP53*, *ESR1*, and *KRAS*, with the last two present in CTCs, but not in the matched primary tumours. Since *KRAS* mutation is related to worse prognosis of breast cancer patients, its presence in CTCs can potentially be used in therapy selection [151]. Interestingly, serial sampling of blood in one patient and concomitant analysis of ctDNA showed that presence of *KRAS* mutation (p.G12D), not detected in the primary tumour, was found in both CTCs and ctDNA, whereas low frequency mutation in *TP53* (p.P278R) was detected only in ctDNA. As suggested by the authors, discordance between ctDNA and CTCs might reflect the existence of distinct metastatic clones giving rise to either CTCs or ctDNA. If so, identification of the origin of metastasis initiating population (sharing mutations with ctDNA or CTCs) and parallel analysis of CTCs and ctDNA would have significant clinical implications. In another study by Bingham et al., CellSearch-enriched, DEPArray isolated single CTCs or CTCs clusters from metastatic breast cancer were analysed for mutations in *TP53*, *RB1*, *PIK3CA*, and *HER2* using Sanger sequencing or NGS (only mutation in *RB1* gene) [118]. In every case, mutations found in clusters of CTCs were also observed in at least one single CTCs from the same patient. Mutations were chosen based on their occurrence in the matched primary tumours or metastases, thus confirming (or not) the existence of primary/metastatic tumour-derived clones in CTCs. In one patient three genes were mutated in CTCs: *PIK3CA* K111E, *HER2* S310F and V777L, as well as *TP53* C229 fs\*10, in another case two mutations were observed: *TP53* R110 delC fs\*13 and *RB1* K720\*. For other three women, only one mutation was found in CTCs—either *RB1* 607+1 G > C, *TP53* R110 delG fs\*13, or *TP53* P190\_H193 > \*E. In five out of six cases, mutation status results were concordant between CTCs and matched primary/metastatic tumour, however CTCs often showed heterogeneous mutation pattern. In one case, *TP53* mutation found in the chest wall metastases was not detected in five tested CTCs and one CTCs cluster. Since mutations in *TP53* were correlated with the poorer response to anthracycline- or tamoxifen-based treatment as well as sensitivity to paclitaxel, detection of CTCs carrying *TP53* mutations might provide important predictive information [152–155]. Hetero- and homozygous *TP53* mutation R248W (linked with aggressive characteristics and drug resistance) in single CTCs and CTCs clusters from one ER+/PR-/HER2- metastatic breast cancer patient, confirmed in the matched primary tumour, was also reported by Mu et al. [119]. No activating mutations in *ESR1* (exon 8 (c.1607-1621)) were observed in either single CTCs, CTCs clusters (isolated by DEPArray after ScreenCell®Cyto device enrichment) or primary tumour of that patient.

Overall, genomic testing of CTCs holds a great promise as a valid tool to monitor disease progression in breast cancer patients (Table 1). However, assessment of its true clinical utility relies on the follow up data and meaningful statistical analyses, lacking from currently available reports.

### 7.3. RNA Level

Current technology of CTCs enrichment allows for more reliable and easier measurement of RNA than proteins [60,156]. In IBC, information provided by numerous reports on CTCs is limited to single or a small number of genes, but technology for assessment of multiple genetic panels is becoming widely available [73,96,104,126,129,156].

So far, most of the studies have evaluated mRNA expression of key IBC predictive biological genes (*ESR1* (ER), *PGR* (PR), *ERBB2* (HER2)) in CTCs [99,102,107,121,122,157–159]. The major findings were consistent with those for ER/PR/HER2 proteins, with most studies highlighting an intratumoural heterogeneity between IBC CTCs and primary or metastatic tumours [99,102,157–159]. Predictive and prognostic values of *ESR1* or *ERBB2* mRNA levels in CTCs were shown to have closer association with PFS and OS than their protein counterparts [107,109,121,122].

The other group of studies on mRNA in CTCs focused on biological aspect of CTCs, in particular those related to tumour evolution. These dynamic changes encompass several fundamental molecular and phenotypic adaptations, including EMT, acquisition of stem-like features, followed by the EMT reversal, the mesenchymal-to-epithelial transition (MET), required for the formation of a secondary tumour at the ‘destination site’ [160–162]. Several reports showed that expression of EMT markers and

stem-like traits were up-regulated in IBC CTCs [62,97–99,124,163,164]. It must be noted that, similarly to ER/PR/HER2, heterogeneity among IBC CTCs in both stemness and EMT markers levels is also extremely high [12,17,165,166]. Armstrong et al. showed that even 75% of IBC CTCs in metastatic patients simultaneously co-express mesenchymal and epithelial markers. Yu et al. demonstrated dynamic changes in the levels of EMT markers in serial examination and Guan et al. found that expression of ER and PR varied between subpopulations of CTCs expressing different levels of EMT markers [12,159,167].

Because CTCs represent a small but a composite and heterogeneous population of cancer cells, their molecular characterization using small groups of routine markers may not be adequate or sufficient [60,168]. Complex panels that would depict biology of cancer and have a documented clinical value seem essential for the optimal evaluation of CTCs' metastatic abilities [104]. Several studies of molecular gene signatures in IBC CTCs presented encouraging results and showed that analysis of some multigenetic profiles is robustly possible [98,128–130,156,169,170]. However, provided information is insufficient from both the biological and clinical point of view, i.e. the studies analysed small number of patients, did not include all genes from the multigene profiles of known predictive/prognostic value (e.g., PAM50, Nanostring, Mammaprint, OncotypeDx, Endopredict), were limited to technical or innovative aspects of multiparametric panels [60,98,126,128–130,156,168–173]. In addition, small amounts of CTCs RNA and contamination of enriched samples with residual leukocyte RNA often results in non-conclusive results of multiparametric tests, designed originally for larger tissue samples (primary tumour). For example, PAM50 or Prosigna panels could not have been successfully examined in IBC CTCs in studies by Lang et al. and Porras et al. [126,127]. Summarizing, the reports on examination of the clinically and biologically valid gene signatures in CTCs from IBC patients are scarce, e.g., no successful comprehensive analysis of intrinsic molecular subtypes of CTCs in relation to both primary and metastatic foci using a biologically and clinically relevant gene signature has been reported yet (Table 1) [60,126,129,130].

#### 7.4. *microRNA Level*

Free circulating microRNA, a stable, easily isolated, and measurable subtype of RNA, has a unique clinical potential and is the highlight of current translational 'liquid biopsies' research [21–23]. In IBC, plasma microRNA panels of potential biologic, predictive or prognostic value have been developed by a number of research groups [20–23]. In contrast, reports on microRNA profiling in isolated CTCs are far less common. Leong et al. and Gasch et al. described an optimized methodology for microRNA expression measurement in CTCs either by subtracting of plasma microRNA background or by application of in situ hybridization and the CellSearch CTC detection system [131,132]. Sieuwerts et al. demonstrated a panel of 10 microRNAs that were upregulated particularly in IBC CTCs [128]. These few publications show that the measurement of microRNAs level in IBC CTCs is uncommon, mainly due to technological difficulties. These may be overcome by emerging novel single cell-based approaches that should provide a powerful tool for accurate analysis of microRNA profiles in enriched CTCs [133].

### 8. Single Cell Analysis of CTCs—Future or Confusion?

Development of technologies for single cell isolation and genome/transcriptome amplification enabled molecular profiling of single CTCs [133,174,175]. This approach to CTCs analysis, allowing assessment of disease heterogeneity in the limited sample material, is recently gaining wide recognition [120,133,143]. Since molecular characteristics of CTCs are bound to be related to the response to therapy, evaluation of individual CTCs might provide valuable information for tailored treatment. CTCs are known to have different metastatic abilities, which implies that not only the quantity, but also the qualitative features of CTCs are important for estimation of the risk of disease progression [12,16,17,62,176]. Therefore, combination of genomic and transcriptomic profiling techniques from a single cell, such as DRseq [177], G&T-seq [178], as well as proteomic

and transcriptomic [179] single cell interrogation open new avenues for clinically oriented research. Recent reports suggest a direct possibility of application of single cell technologies for personalized treatment guidance for IBC patients [133,180].

The main disadvantages of single cells techniques include the cost, data redundancy, and technical limitations interfering with interpretation of the results. A crucial step in single cell analysis is elimination of low-quality cells/material, as they might bias the results [181]. Loss of material during single cell sample preparation or due to genetic material degradation (especially RNA) at the time of sample collection might give false negative results of gene expression or mutation status. Thus, it is of utmost importance to distinguish inherent heterogeneity of CTCs mutational status from technical errors, such as detection of mutations introduced during sample preparation or allelic drop-out. Moreover, stochastic nature of the transcription process (so called transcriptional burst), together with the dynamic changes in cellular mRNA levels due to degradation, might lead to high cell-to-cell variation, not observed in the analysis of cell pools [182,183]. Therefore, analysis of multiple redundant markers would increase the reliability of the results as would sequencing of multiple cells from the same patients, but this would have to be balanced against heterogeneity of the cells. In NGS technologies, sequencing depth has also to be considered; in single cell RNA profiling it has been estimated that one million reads per cells generates satisfying coverage rates to detect low-level transcripts expression [184].

## 9. Conclusions

CTCs in peripheral blood, the so called ‘liquid biopsy’, are being considered nowadays as a valid counterpart of prognostic and predictive factors in IBC. Molecular characterisation of CTCs is of particular importance in advanced disease, where analysis of metastatic cells should provide information essential for identification of therapeutic targets, otherwise unobtainable from primary tumours. If CTCs are truly representative of metastases in terms of known clinico-pathological factors, characterization of the molecular profile of this easily accessible ‘biopsy’ might be of prime importance for clinical practice. Since CTCs show substantial heterogeneity in clinically relevant markers, single cells analysis seems to be a mandatory approach for accurate characterisation of systemically spread cancer and assessment of clinical utility and validity of CTCs-guided therapies. Although, from the technological point of view, we may be ready for the ‘liquid’ revolution, translational and clinical research, mainly well designed randomized clinical trials, is urgently needed to allow evaluation of the true value of the detailed CTCs examination in relation to known prognostic and predictive disease features.

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