



# Circulating tumor cells (CTCs) for the noninvasive monitoring and personalization of non-small cell lung cancer (NSCLC) therapies

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**Abstract:** Growing evidences for tumor heterogeneity confirm that single-tumor biopsies frequently fail to reveal the widespread mutagenic profile of tumor. Repeated biopsies are in most cases unfeasible, especially in advanced cancers. We describe here how circulating tumor cells (CTCs) isolated from minimally invasive blood sample might inform us about intratumor heterogeneity, tumor evolution and treatment resistance. We also discuss the advances of CTCs research, most notably in molecularly selected non-small cell lung cancer (NSCLC) patients, highlighting challenges and opportunities related to personalized therapy.

**Keywords:** Circulating tumor cells (CTC); non-small cell lung cancer (NSCLC); liquid biopsy

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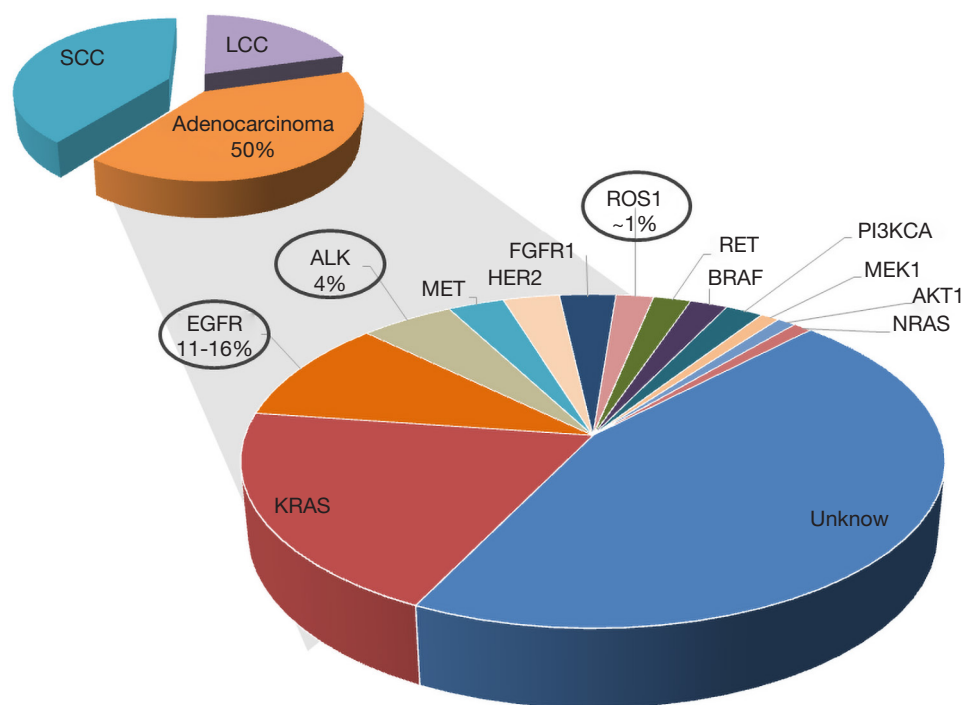
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## Introduction

The development of precision medicine for cancer patients is highly dependent on the identification of the molecular drivers of their disease. Today still therapy decisions are mainly performed on the basis of tumor biopsies. Because of the widespread intratumor heterogeneity, a single biopsy is rarely able to capture the full spectrum of molecular alterations present within a tumor. To identify resistance mutations at disease progression and re-evaluate the changing gene profile of tumors the serial biopsies are required. Subjecting patients to serial biopsies however are complicated, invasive and in most cases not performed. Liquid biopsy offers a non-invasive complement to tumor biopsy for the assessment of the tumor mutational status and monitoring genetic changes in real-time. Nowadays

the analysis of circulating tumor cells (CTCs) and/or circulating tumor DNA (ctDNA) opens new possibilities for cancer patient management. CTCs have the potential to provide complementary information to that delivered by even multiple tumor biopsies. Because they are shed from primary tumor and/or spatially distinct metastatic regions, CTCs provide a global representation of tumor genetic diversity. In contrast to ctDNA, CTCs might be analyzed at single cell level, thus providing a matchless representation of tumor heterogeneity and clonal dominance in a real-time. Moreover, CTCs can be engrafted into mice, in order to obtain preclinical models. CTCs are the main route of metastatic dissemination and contain subpopulations with tumor-initiating potential. Thus, advances in CTC molecular and functional characterization increase our knowledge about the metastasis formation mechanisms,

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**Figure 1** Molecular classification of non-small cell lung cancer adenocarcinoma.

and the combination of CTC and ctDNA assays through routine blood test would provide biomarkers for the real-time patient monitoring and therapy stratification, in different tumor types including non-small cell lung cancer (NSCLC).

### Molecular profiles of patients

At initial diagnosis a majority of NSCLC patients present advanced disease for which no curative therapy exists. NSCLC and in particular adenocarcinoma have been classified into clinically relevant molecular subsets according to multiple oncogenic driver alterations (*Figure 1*) (1). These alterations activate prosurvival oncogenic signaling pathways promoting the tumor growth.

### Epidermal growth factor receptor (EGFR) mutations

Common EGFR alterations (exon 19 deletions and the L858R point mutation) are identified in 11 to 16% of patients with NSCLC in Western countries and in about 50% of Asian patients and confer sensitivity to

EGFR tyrosine kinase inhibitor (TKI) therapy such as erlotinib, gefitinib, or afatinib. The most frequent EGFR mutations affect the EGFR kinase catalytic domain and TKIs are standard first-line therapy that is associated with superior radiographic response and prolonged progression free survival (PFS) compared to standard cytotoxic chemotherapy (2,3). The most frequent EGFR alterations, namely deletions within exon 19 and L858R point mutation account for more than 90% of EGFR mutated patients. The response to EGFR TKI is positively related to constitutional activation of EGFR signaling, which has been shown to be particularly high in non-smoker patients with lung adenocarcinoma. Despite the impressive efficacy of the TKI therapy, most patients will develop clinical evidence of acquired resistance to treatment after a median of 12 months (2,3). Resistance to TKIs is generally classified as either primary (i.e., intrinsic) or secondary (i.e., acquired). Less is known about the basis of intrinsic resistance, which is defined as a de novo lack of treatment response (4). As commonly observed in oncogene-driven malignancies, acquired resistance to EGFR inhibitors is divided into two main groups: on-target mechanisms such as the acquisition of additional genetic alteration in the drug target itself

through secondary mutations or gene amplification, changes in tumor histology or alterations in drug metabolism (4). The principal T790M resistance mutation is detectable in approximately 50% of TKI-resistant patients. In rare cases it has been also identified in tumors before treatment with EGFR TKI. Three additional secondary mutations have been associated with TKI resistance (D761Y, T854A, L747S) and C797S was described as mutation, responsible for acquired resistance to osimertinib (third generation EGFR TKI) (5). Several bypass pathways implicated in EGFR TKI resistance include MET, HER2, FGFR and AXL amplification, and acquired mutations in other oncogenes such as PIK3CA and BRAF. Third generation EGFR TKI—osimertinib, which targets both, activating *EGFR* gene alterations and the T790M mutation is now the standard front-line therapy for EGFR-mutant NSCLC. Up to now the identification of EGFR mutations is mainly performed on tumor biopsies, which carry risks, linked to the surgical intervention itself and could be not feasible, in particular at disease progression. Liquid biopsies comprising CTCs and circulating tumor DNA (ctDNA) enable such mutation analysis for diagnosis and monitoring over the course of the therapy.

### **Anaplastic lymphoma kinase (ALK)- and c-ros oncogene 1 (ROS1)-rearrangements**

A unique molecular subgroup of 4% of NSCLC patients presents the *ALK* gene rearrangements, which involve the *ALK* gene and, most often, the echinoderm microtubule-associated protein-like 4 (*EML4*) locus in opposite directions (6). Several different in-frame fusion variants of *EML4-ALK* have been described with different *EML4* breakpoints. However, all fusion variants harbor identical C-terminal domain conferring a gain of function leading to constitutively active fusion protein with robust transforming activity. In 2012, on the basis of the response rates demonstrated in phase I and phase II studies, crizotinib obtained FDA approval to be used as a second line therapy for advanced ALK-rearranged NSCLC (7,8) patients. It is now the standard therapy in advanced, already treated ALK-rearranged patients, as well in the first line setting (9,10). Another molecular driver event also sensitive to TKI is *ROS1* fusion gene present in about 1% of NSCLC patients that could benefit from crizotinib treatment. Although the TKI show clinical efficacy with significantly prolonged progression-free survival, most of the patients that benefit

from TKI treatment, similarly to EGFR mutated patients, will inevitably develop acquired resistance.

ALK resistance mechanisms have been identified in ~30–40% of crizotinib-resistant patients (11). The “gatekeeper” L1196M and G1296A (12) are the most often identified secondary ALK resistance mutations. Additional secondary mutations detected in crizotinib-resistant ALK-positive tumors are localized throughout the ALK kinase domain, they include: I1151Tins, F1174C/L/V, L1152R, E1210K, C1156Y, I1171T, S1206Y, V1180L (13,14). The study reported that cell lines established from biopsies of patients with crizotinib-resistant NSCLC harboring some of these mutations including the most frequent L1196M and G1296A were sensitive to ceritinib (15). For patients relapsing on crizotinib, more potent second-generation ALK inhibitors such as ceritinib, alectinib and brigatinib, have become standard treatments, re-inducing responses to treatment in the majority of crizotinib-resistant patients. The F1174C/L/V, I1151Tins, L1152P, C1156Y and G1202R mutations have been associated with resistance to ceritinib. Resistance to alectinib was found after acquiring V1180L and I1171T/N/S mutations (16) and double mutations in E1210K and S1206C or D1203N confer resistance to brigatinib (17). Mutations in ALK kinase domain emerging on treatment with second-generation ALK inhibitors are only targetable by lorlatinib (third generation TKI). However recently has been described the re-sensitization to crizotinib in the tumor that acquired both the C1156Y and L1198F mutations during the lorlatinib treatment (18). In approximately one-third of crizotinib-resistant tumors, the activation of by-pass signaling pathways, such as the development of EGFR mutations or activation of wild-type EGFR, KIT or HER2, has been identified as a cause of acquired resistance [4]. In about 30% of patients the resistance mechanism is still unidentified (19).

### **Targeting**

Recent clinical studies are giving growing arguments for the using next-generation TKI as upfront therapeutic option in ALK and EGFR NSCLC. Indeed nowadays according to encouraging results from the clinical studies upfront alectinib (ALK patients) or osimertinib (in EGFR cases) show greater efficacy in the treatment of brain metastases, less toxicity and prolonged PFS than the first generation TKI (20,21). Ongoing are clinical trials with the frontline lorlatinib treatment for ALK rearranged tumors (22,23).

### Importance of non-invasive routine monitoring

Selective TKIs have dramatically transformed the therapeutic landscape for NSCLC. However, acquired resistance to targeted therapies remains a persistent issue, and the development of resistant cell subpopulations within highly heterogeneous cancers still displays an important obstacle to the effective cancer treatment.

Novel biomarkers are, therefore, needed to aid in clinical decision making and ultimately improve patient's outcomes. The prognostic biomarkers are able to state and inform about the cancer features and evolution. Another group of biomarkers has the potential to monitor therapeutic responses and predict recurrences through serial sampling. The CTCs enumeration represents a well-established prognostic value for several tumor types. Recently CTCs are being investigated in parallel to ctDNA and microRNA as a candidate predictive biomarker in different tumors, between them also for NSCLC monitoring.

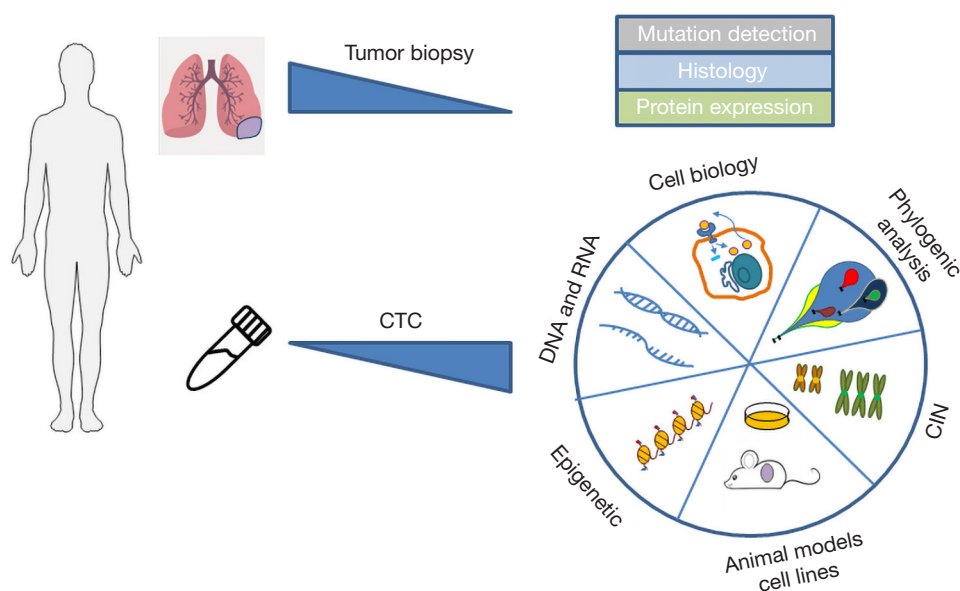
Tumor biopsies may not represent the wide spectrum of genetic aberrations present within a tumor. Because CTCs may be shed from distinct metastatic sites, they are likely more representative of the heterogeneous nature of tumors. The tremendous spatial and temporal heterogeneity in primary and metastatic tumors has been contemporized recently owing to advances in next generation sequencing technologies. However application of such analyses to routine clinical applications is burdened with high technical and medical risks, particularly for cancers like NSCLC that frequently metastasize to the brain, lungs or bones. Finally repeated biopsies from progressing tumors are risky from the medical point of view and the routine sampling of tumors at several time points of treatment remains not only a technical challenge but also a controversial issue. Thus the population of CTCs isolated from the patients' blood provides a much more easily accessible source of tumor material which in addition may be representative of disseminated cancer cells derived from various metastatic sites within individual patient.

### CTC as unique source of information

Noninvasive peripheral blood sampling offers the possibility to obtain a subpopulation of disseminated tumor cells that are the main route of metastatic progression and may contain tumorigenic clones (24). Although the existence of CTCs in blood was first reported in 1869 (25), the CTCs' isolation is still challenging because of their rarity, fragility

and heterogeneity. Only recent advances in technologies, although consuming and technologically difficult gave the possibility to efficiently isolate rare CTCs, potentially enabling the study of tumor heterogeneity and treatment resistance mechanisms at the single CTC level. The enumeration of CTCs is nowadays of strong prognostic value in several cancer types and can be applied clinically as a pharmacodynamic biomarker. Indeed multifaceted CTC-based technologies are expanding into a wide spectrum of tools with a matchless interest in precision oncology, they include: role of predictive biomarkers, understanding the mechanisms and sources of tumor heterogeneity and metastasis but also personalized testing of drug efficacy.

Besides CTCs, the analysis of ctDNA is increasingly used to overcome tumor biopsy limitations. Moreover, circulating microRNAs, extracellular vesicles and tumor-educated platelets are starting to complement the liquid biopsy with potential forthcoming clinical applicability (26,27). Using the ctDNA to monitor the emergence of mutations during cancer progression possesses certain restrictions since the tumor evolution arises not only through genetic changes, but also from phenotypic alterations through epigenetic reprogramming. Such functional modifications require the analysis of living cells. In addition CTCs analysis may constitute a unique approach to properly analyze tumor heterogeneity at the single cell level. Few recent works nicely show the parallel analysis of CTCs and ctDNA as a complement characteristic of tumor evolution and possibility to guide future management of the patients. Although radiographically directed needle biopsies of recurrent or metastatic tumors are still current, clinical standard for EGFR genotyping in the setting of acquired resistance, some laboratories started to use the supplementary analysis of simultaneously collected CTC and ctDNA. Indeed Punnoose *et al.* (28) detected EGFR mutations in CTC and ctDNA, which were strongly concordant with mutation status in matched tumor. Maheswaran *et al.* (29) described the detection of expected EGFR-activating mutations in CTCs from 11 of 12 tested NSCLC patients. Additionally, the T790M resistance mutation has been found in the CTCs of patients who had already received TKI. The CTCs have been isolated for this study using CTC-chip, a microfluidic equipment comprising microparticules coated with anti-EpCAM antibodies. Sundaresan *et al.* (30) show also a successful T790M genotyping in CTCs and ctDNA of NSCLC patients. Discrepancies between a single tumor biopsy and blood-based sampling may result at least in part from the



**Figure 2** The multiple possibilities to exploit CTCs comparing to standard tumor biopsies. CTC, circulating tumor cells.

fact that the latter likely includes material from multiple disease sites. In the light of those promising reports the molecular analysis of CTCs supplemented with ctDNA will certainly contribute to reveal the characteristic of cancer cell dissemination and identify signaling pathways and potentially targetable genes for therapeutic interventions. CTCs offer greater possibilities of analysis (*Figure 2*), behind DNA evaluation, like RNA and proteomic analysis, epigenetic profiling, functional studies and development of CTC derived xenograft (CDX) models, all of those requires integrated and alive cell.

### CTC ongoing and future advantages

#### *No universal technology for CTC detection and evaluation*

CTCs are rare, occurring at a rate of one cell per  $10^6$  or  $10^7$  leucocytes in the human blood. The combination of two successive steps: (I) an initial enrichment and (II) CTC detection by different phenotypical or genetic techniques, are required for reliable CTCs identification. Various technical efforts have been undertaken to properly detect and quantify CTCs, although the elaboration of a universal assay is still pretty debatable. The rarity and molecular and phenotypical heterogeneity of CTCs make their detection highly difficult and although multiple technologies have been developed, there is no consensus on their choice. The

method of CTC analysis should rather be chosen according to the question asked (for example enumeration in a clinical setting or identification of predictive biomarkers or single cell molecular analysis). CellSearch platform uses the cell surface epithelial cell adhesion molecule (EpCAM) to enrich epithelial CTCs. In this platform, enriched cells are identified as CTCs by the costaining with anti-cytokeratin (CK) antibodies, and potentially contaminating PBMC are detected by CD45 counterstain. This system represents the current gold standard for CTC enumeration and is approved by the US Food and Drug Administration (FDA) as an aid to prognosis for patients with metastatic breast, colorectal, and prostate cancer. EpCAM dependence in this method may occur disadvantageous for the CTCs, which have already lost their epithelial characteristic and express epithelial-mesenchymal transition (EMT) markers. Those CTCs undergoing EMT may be missed by the CellSearch (31). The initial report about the feasibility of the enumeration of CTCs in the blood of NSCLC patients using the CellSearch (32) has been followed by the report of Krebs *et al.* (33) that for the first time demonstrated the prevalence and prognostic significance of CTCs in patients with NSCLC. A cutoff value of  $>5$  CTCs in 7.5 mL of blood was highly significant in predicting worse prognosis comparing to patients having fewer than 5 CTCs (33). Few others clinical reports including our own confirmed the prognostic value of CTCs in NSCLC patients at the

baseline (28,34-36).

Using an enrichment method based on blood filtration (ISET, Isolation by Size Epithelial Tumor cells), the prognostic significance of CTCs in NSCLC patients was also stated by Hofman *et al.* (37). We and others demonstrated that the ISET approach identifies a higher proportion of CTC-positive NSCLC patients than CellSearch equally stating high sensitivity for CTC enrichment from blood samples using the ISET technique in the case of NSCLC patients (33,38,39). NSCLC CTCs were identified in higher numbers by ISET compared to the CellSearch platform most likely because CTCs that lost epithelial features are missed by CellSearch (31,40). ISET technique allows conserving and analyzing CTCs posteriori that is particularly useful for longitudinal research. In spite of technical difficulties, the usability of CTC assays as predictive biomarkers has been demonstrated in a few reports. Indeed the differences in the expression of EMT markers may depend on the driver gene/mutation in NSCLCs and potentially confer the predictive factor (41). Two groups including our own have initially reported the feasibility of ALK-rearrangement detection in CTCs enriched by ISET in ALK positive NSCLC patients (42,43). Our group developed a FISH (Fluorescent In Situ Hybridization) technique for CTCs enriched on filters [filter-adapted FISH (FA-FISH)]. Using a FA-FISH, we showed that ALK-rearranged CTCs might be identified in a cohort of 18 ALK-positive NSCLC patients. 100% of tested ALK-positive patients had four or more ALK-rearranged CTCs per 1 mL of blood, while all of ALK-negative patients presented none or one CTC with ALK-rearrangement signal. Interestingly ALK-rearranged CTCs presented a unique ALK rearrangement pattern. Additionally, our group tested markers of EMT in ALK-rearranged CTCs. We establish that CKs and E-cadherin were not detected in ALK-rearranged CTCs, contrary to mesenchymal markers (vimentin, N-cadherin), that were exclusively expressed. Indeed, ALK-rearranged CTCs displayed a mesenchymal phenotype while epithelial and mesenchymal markers are detected in heterogeneous tumors from corresponding patient (41). These data suggest that CTCs may harbor migratory and invasive properties in these patients. Ilie *et al.* showed the detection of ALK rearrangement accompanied with robust ALK protein expression by immunohistochemistry in CTCs from five ALK-rearranged NSCLCs. The ALK-rearrangement status in CTCs at the baseline and after few systemic treatments has been reported also by Provencio

*et al.* (43) and Li *et al.* (44).

The detection of ALK-rearranged CTCs corresponding to tumor biopsies from the same patients was also presented by Tan *et al.* (45). In 2015, we reported that rearrangement in the *ROS1*-tyrosine kinase gene (present in 1% of NSCLC) can be detected in CTCs using FA-FISH (46). We have used FA-FISH combined with fluorescent staining and coupled to the semi-automated microscopy technique. Adapted methodology gave us the possibility to efficiently detect *ROS1* rearrangements in CTCs from *ROS1*-rearranged NSCLC patients.

Following enrichment and identification, the molecular characterization of single CTCs requires technology for their isolation, which can be performed by FACS, DEPArray, microfluidic devices or laser capture microdissection. Several overviews and technology articles of the CTC isolation methods used for the genetic analysis have been already published (47-52).

Except their high heterogeneity, CTCs are also, comparing to the other blood nucleated cells, characterized by increased fragility, which make them even more difficult to isolate. Nowadays many efforts are taking to preserve the viability and enables isolation of intact CTCs with high quality DNA, RNA and proteins. Some promising results have been obtained using EPISPOT method (53), density gradient centrifugation together with telomerase-based assay (54) and recently microfluidic isolation by the CTC-iChip preceded by hypothermic preservation of blood (55). Those approaches might be particularly useful for the characteristic of whole-transcriptome alterations in CTCs. Some laboratories use these methods alone or in combination depending on the type of cancer and the subsequent type of analysis.

### *Molecular and functional characterization of CTCs*

CTCs have the capacity to circulate from one metastasis to another, and may represent cancer genomes issued from multiple metastatic sites (56,57). CTCs may therefore inform on tumor heterogeneity and underlying oncogenic alterations which could serve as predictors of drug sensitivity or resistance. Molecular characterization of CTCs may occur predictive for therapy selection (58).

Our group showed that tumor heterogeneity, evaluated by FA-FISH detection of *ROS1* gene copy number, was significantly higher in CTCs at the baseline comparing with corresponding tumor biopsies in the three patients that presented either disease stability or partial response

to treatment. Progression during crizotinib treatment observed in the two patients increased significantly the copy number in ROS1-rearranged CTCs. We show that non-invasive blood sample containing CTCs might serve for detection of ROS1 rearrangement in NSCLC patients, as mentioned above. Furthermore, our results show that the CTCs from ROS1-rearranged NSCLC patients display substantial heterogeneity of ROS1-gene aberrations accompanied by a high level of chromosomal instability (CIN). Although technically challenging, the experimental process to detect predictive biomarkers such as rearrangements of *ALK*, *ROS1*, *RET* genes in CTCs from NSCLC has been established (42,43,46,59). Even though still more collaborative research is needed to transfer research improvement into the clinic, these studies provide new perspectives for identifying NSCLC patients eligible for TKI therapy. Non-invasive serial molecular analyses of CTCs performed at different time-points upon therapy may potentially be of great utility to inform therapeutic decisions in NSCLC patients treated by sequential TKI.

To fully understand discrepancies between immunohistochemical analyses (IHC) compared with FISH in predicting response and outcome to ALK inhibitors, further evaluation of several biological features such as the variation of the native ALK copy number as well as the c-MET and ROS1 protein expression could be considered (60,61). The utility of CTCs for MET expression assessment in NSCLC patients has been confirmed (62). Additionally, CTCs has been employed in order to assess the expression of predictive biomarkers for immunotherapy, which is of particular interest for NSCLC patients, since the new clinical trials targeting immune checkpoints are ongoing. CTCs and corresponding tumor biopsies of patients with advanced NSCLC were evaluated for PD-L1 expression. Patients that present high expression of PD-L1 on CTCs, even though their tumor biopsies are negative for PD-L1, they could potentially benefit from immunotherapy (63,64). Parallel evaluation of plasma biomarkers such as different cytokines (e.g., IL-8) could also inform about anti-PD1/PD-L1 treatment efficiency (65).

Overall mentioned studies on CTCs concerned their prognostic and pharmacodynamic significance, identification of predictive biomarkers and utility in the real-time monitoring of disease (43,46,59,61,63). Even though that CTCs are considered as the metastasis seeding elements, only a minority of CTCs with the cancer stem cell properties and metastatic capability retain all the necessary features for survive in the circulation,

extravasate and finally form metastases (66). Thus, a key objective right now is the genetic and epigenetic profiling of CTCs and transferring these cells to mouse models to investigate their functional capability, signaling pathways alterations and following drug-screening assays (Figure 2). In metastatic breast cancer, Baccelli *et al.* reported first time a subpopulation of CTCs with a tumor initiating CD45-EpCAM+CD44+CD47+cMet+ phenotype (24). Up to now very few groups, managed to established functional CTC-derived xenograft (CDX) models. The CDX derived from CTC of SCLC patient has been shown to mimic the donor patient's response to chemotherapy (67), which validate these kinds of models in the pharmacologic study. The first report showing the tumorigenicity of CTC issued from metastatic patients with NSCLC was published by Morrow *et al.* (68). Molecular analysis of the CDX confirmed its tumor origin and RNA and protein profiling of CDX showed its mesenchymal phenotype, indicating the functional importance of mesenchymal CTCs (68). The CDX models have been also derived from the melanoma patients (69). CDX-bearing mice developed metastases, which were confirmed to have the human melanoma origin. Our team recently established CDX models derived from CTC of prostate cancer and NSCLC patients (70,71). Additionally research showed the possibility to expand CTCs from early stage lung cancer in a three dimensional co-cultures (72). Extremely low CTC concentration in blood and complicated process of their isolation, together with the limited knowledge about their functional properties, makes the generation and maintenance of CTC in vitro culture remarkably rare. The first CTC cell line has been established from colorectal cancer patient (73). This cell line displayed the phenotypic characteristic of patient's tumor and tumorigenic potential after engraftment to immunodeficient mice. Intensive advances in the establishment of novel in vitro and in vivo models from CTCs are remarkably increasing the knowledge about the CTC's biology and open the opportunity to multiple drug testing. Next to single cell genomic analysis the CTC derived cell lines and CDX would indeed allow a full characterization of molecular mechanisms leading to evolution of tumor disease.

#### ***Perspectives and advances in research to characterize the origins of CTCs' heterogeneity***

Genetic intratumor heterogeneity fostered by genome instability has a central role in cancer evolution.

Indeed, chromosome instability was related with an increased risk of relapse or death of NSCLC patients, and the potential value of chromosome instability as a prognostic predictor has been proposed (74). Some reports show that CIN may provide a source of metastatic potential (75-77). Particularly chromosomal doubling caused by missegregation of chromosomes and cytokinesis failure has been shown to accelerate cancer evolution (78). By analyzing multiple spatially separated tumor and metastasis biopsies increased amount of subclonal copy number alterations (CNA) were associated with disease relapse and death in NSCLC patients. The importance of chromosome instability (CIN) in acquiring resistance has been shown in ALK-rearranged tumors. Our group evaluated by FA-FISH the ALK-rearranged CTC from NSCLC patients under the crizotinib therapy and we correlated the different ALK-FISH patterns with the evolution on crizotinib treatment (59). CTCs were divided into different subgroups according to the existence of ALK-rearrangements and/or ALK copy number gain (ALK-CNG). In line with the earlier report on tumor biopsies, at the baseline of crizotinib treatment no significant report was observed between the amount of ALK-rearranged or ALK-CNG CTCs and PFS. However, we showed that the diminution of the amount of CTCs presenting ALK-CNG upon crizotinib treatment was positively related to longer PFS (59). This research showed that successive analysis of CTCs by FA-FISH may deliver a predictive biomarker for therapy outcome in NSCLC and may stratify patients that present the increased risk of early resistance (59,60). Another example of a predictive role of CIN characterized in CTC has been described for SCLC patients. The minimally invasive CTC-profiling of CNA gave promised results in the classification of chemosensitive versus chemorefractory SCLC patients (79). Even though additional researches are needed to elucidate the clinical utility of CIN profiling in CTCs, these works present a proof-of-concept for the role of genomic instability in the therapy outcome choice. The widespread tumor heterogeneity largely driven by genome doubling and chromosomal instability has been recently described based on the multiple biopsies from lung tumors (80). The important interest relies more often nowadays on liquid biopsies, which not only would be useful for identifying predictive biomarkers of sensitivity or resistance but also increased our knowledge about the heterogeneity driven by genomic instability of CTCs. Recent report presented the phylogenetic ctDNA profiling to characterize the subclonal

dynamics of relapsing NSCLC and predict chemotherapy resistance (81) validating at the same time the importance of using liquid biopsies to follow subclonality of tumor development. Rapidly advancing technology would enable to characterize the subclonal evolution of metastasis-initiating CTCs considering in particular their single cell genomic instability and heterogeneity.

### Summary conclusions

Liquid biopsy in a single non-invasive blood test may deliver valuable source of biomarkers in NSCLC. The presence of CTCs is associated to worse prognosis and the risk of early recurrence in numerous clinical trials of different cancers. Examination of CTCs at the molecular level carries a great potential to decipher the biology of cancer dissemination and inform on potential targets and signaling networks relevant to therapeutic interventions. Shedding the light to the mechanism of tumor heterogeneity in CTCs would allow for a better understanding of cancer progression with potential improvement of therapeutic strategies to overcome resistance to TKI. Additionally, monitoring of acquired resistance mutations in CTCs may allow adapting sequential therapy with TKI. The potential to interrogate the tumor genome sequentially and systematically, by incorporating CTCs, ctDNA and miRNA analysis into clinical trials is highly recommended to provide unique insights into tumor heterogeneity, evolution and emergence of resistance to treatment from the multiple sites.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest



to declare.

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