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Titolo Tesi

Individualized treatment of patients with advanced NSCLC: potential application for Circulating Tumour Cells (CTCs) molecular and phenotypical profiling

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DECLARATION

According to the doctoral degree regulations:

a) I hereby declare that I have written the submitted dissertation by myself and in this process, I have used no other sources or materials than those expressly indicated.

b) I hereby declare that I have not applied this dissertation to be examined at any other institution, nor have I used it in this or any other form at any other institution as an examination paper, nor submitted it to any other faculty as a dissertation.

SUMMARY

NSCLC is a major cause of cancer-related death in both men and women globally [1].

Despite recent advances in early tumour detection, surgical treatment, radio-chemotherapy, and targeted therapy, the NSCLC-related high mortality rate remains a daunting challenge [2]. The therapeutic choice is driven by the molecular/genetic assessment of the primary tumour as determined at diagnosis. This practice needs to be integrated with other methods for real time tumour mutational screening. Indeed, a body of evidence is emerging that the feasibility of new tools for monitoring the malignancy throughout the continuum of care may offer major benefits to the patients.

Since predictive biomarkers are lacking so far, CTC assays have gained interest to assist clinicians in patient management. Recent experiences demonstrated that CTC detection could help improve the diagnosis and predict a prognosis in patients with non–small cell lung cancer (NSCLC). However, EpCAM positive CTCs are less frequently detected in NSCLC patients compared to other epithelial tumours. In NSCLC, CTCs show a different cytokeratin (CK) pattern and a lower expression of full-length Epithelial Cell Adhesion Molecule (EpCAM) compared to other carcinomas [3]. Indeed, 80% of patients were CTC-positive by the EpCAM-independent ISET compared to only 23% by standard CellSearch assay [4].

The **primary aim** of the Project is to determine the percentage of CTC-positive patients, and the total CTC numbers in advanced NSCLC at baseline, before starting treatment. We questioned whether we could detect a higher number of CTCs by implementing the standard CellSearch assay.

Secondary aim is to evaluate if CTCs count modifications are an early predictor of response to treatment.

The **exploratory aim** is set and applying the molecular and phenotypic profiling of CTCs, in order to verify if these data could be relevant to manage patient

therapy. We then investigated in some selected NSCLC patients, the potential of the combined use of CTCs and cfDNA assays as a dynamic indicator of treatment efficacy.

We evaluated 189 patients, enrolled from December 2012 to June 2016 in trial no. NCT02407327 (http://www.clinicaltrials.gov).

To address these aims we determined the percentage of CTC-positive patients and total CTC number in our cohort using standard assay (SA) and an expanded assay (EA) that was set to tailoring the cytokeratin panel for NSCLC. We demonstrated that the presence of tumour cells was associated with a poor prognosis leading to the definition of a cut-off value (≥1 cell) in metastatic NSCLC with standard assay (SA) and with expanded assay (EA), we showed a better predictive value concern STP. The CTCs level variation during the treatment predict a good response to therapy, and the phenotype and molecular characterization of CTCs is feasible using different methods with several degrees of in depth investigation. All data collected provides useful information about disease evolution.

We compared, where possible, the EGFR mutational status of the NSCLC primary tumour and that of the CTC and cfDNA collected at baseline and after the first line of treatment, and found consistent results in all the analysed samples.

Our data suggests a potential complementary use of Circulating Tumour Cells and cfDNA in solid Tumours concerning molecular information. To indicate that in the circulating compartment we could find a window to survey the disease evolution, the relationship between subpopulation of cancer cells, the therapy response and the host relationship.

This evidence documents and confirms the great impact that the detections and the characterization of CTCs, with malignant proprieties, could have in tailored medicine if this biomarker will be inserted in the decisional tree used in the clinical management of patients.

SOMMARIO

Il tumore del polmone non a piccole cellule (NSCLC) è, nel mondo e in ambo i sessi, una delle principali cause di morte per cancro [1]. Nonostante i recenti progressi ottenuti nella diagnosi precoce del tumore, nel trattamento chirurgico, nella radio-chemioterapia e terapia mirata, l'alto tasso di mortalità dei pazienti NSCLC rimane una sfida impari [2]. La scelta terapeutica è ad oggi decisa sulla base dalla valutazione molecolare/genetica del tumore primitivo al momento della diagnosi. Questa pratica deve essere integrata con altri metodi per lo screening in modo da ottenere informazioni sullo status mutazionale del tumore in tempo reale; questo si sta realizzando grazie a nuovi strumenti che rendono possibile il monitoraggio del tumore durante tutto il percorso e periodo di cura, con conseguenti importanti benefici per i pazienti.

Ad oggi, nel NSCLC, mancano dei biomarcatori predittivi utili nel monitorare l'efficacia del trattamento. Le recenti esperienze hanno dimostrato che la rilevazione delle CTCs potrebbe contribuire a migliorare la diagnosi e prevedere la prognosi nei pazienti con carcinoma polmonare non a piccole cellule (NSCLC). Tuttavia, le CTCs EpCAM+ sono rilevate con frequenze minori in pazienti con NSCLC rispetto a pazienti affetti da altri tumori epiteliali. Nel NSCLC, le CTCs mostrano un profilo di espressione delle citocheratine (CK) diverso e una minore espressione di full-length della molecola di adesione delle cellule epiteliali (Epithelial Cell Adhesion Molecule EpCAM) rispetto ad altri carcinomi [3]. Queste differenze si evidenziano dai risultati ottenuti utilizzando metodi diversi per isolare le CTCs. Infatti in studi effettuati utilizzando la piattaforma ISET, che arricchisce le cellule mediante un metodo EpCAM-indipendente, l'80% dei pazienti risultava CTCs-positivo, mentre con il metodo di riferimento CellSearch solo il 23% dei pazienti risultava CTC positivo [4].

L'obiettivo principale del progetto è quello di determinare la percentuale di pazienti CTCs-positivi e il numero totale di CTCs nel NSCLC al basale, prima di iniziare la terapia. Per ovviare al fatto che potremmo non rilevare tutte le CTCs

utilizzando il test CellSearch standard (SA) abbiamo disegnato un test espanso (EA) che comprenda anche le citocheratine maggiormente espresse nel NSCLC. Lo scopo secondario è valutare se le variazioni nel numero di CTCs sono un predittore precoce della risposta alla terapia. Infine, come scopo esplorativo ci siamo prefissi di mettere a punto dei metodi per la caratterizzazione del profilo molecolare e fenotipico delle CTCs per verificare se le informazioni così ottenute possono essere utilizzate nella gestione della terapia del paziente. Abbiamo poi studiato in alcuni pazienti selezionati, il potenziale dell'uso combinato di saggi CTCs e cfDNA come indicatore dinamico dell'efficacia del trattamento.

Abbiamo valutato 189 pazienti, arruolati da dicembre 2012 a giugno 2016 nel trial no. NCT02407327 (http://www.clinicaltrials.gov).

Abbiamo determinato la percentuale di pazienti CTCs-positivi e numero totale CTCs nella nostra coorte utilizzando i test standard ed espanso. Abbiamo dimostrato che la presenza di cellule tumorali è associata ad una prognosi infausta. Abbiamo definito un valore di cut-off pari a ≥1 CTC in NSCLC metastatico utilizzando il test standard (SA) mentre utilizzando il test espanso (EA), abbiamo dimostrato un maggior potere prognostico nell'evidenziare i pazienti che precocemente vanno incontro ad una progressione STP (Subsequent Time to Progression). Inoltre i nostri dati evidenziano come la variazione del livello delle CTC durante il trattamento predice la risposta alla terapia, e che la caratterizzazione fenotipica e/o molecolare delle CTCs è possibile anche utilizzando metodi diversi, con diversi gradi di profondità di indagine. Tutti i dati raccolti in questo studio forniscono informazioni utili per quanto riguarda l'evoluzione della malattia. Abbiamo confrontato, ove possibile, lo stato mutazionale di EGFR nel tumore primario NSCLC con quanto riscontrato nelle CTC e nel cfDNA nei campioni di sangue raccolti al basale e dopo la prima linea di trattamento, ottenendo risultati riproducibili in tutti i campioni analizzati.

Per quanto riguarda le informazioni molecolari fornite dalla biopsia liquida, i nostri dati suggeriscono un potenziale uso complementare delle CTCs e del cfDNA nei tumori solidi; a dimostrazione che il compartimento circolante è una "finestra" da cui possiamo valutare l'evoluzione della malattia, la relazione tra

sottopopolazione di CTCs, la risposta alla terapia e il rapporto cancro-ospite. Questi risultati documentano e confermano il grande impatto che il conteggio e la caratterizzazione di CTC, che presentano caratteristiche tipiche delle cellule maligne, possono avere nella terapia personalizzata qualora questo biomarcatore venga inserito nell'albero decisionale utilizzato nella gestione clinica dei pazienti.

ABBREVIATIONS

ADC: Adenocarcinoma

Abs: Antibodies

Ag: Antigen

APC: Allophycocyanin

ccfDNA: circulating cell free DNA

CK: Citokeratin

CS: CellSearch platform

CT: Computed Tomography

CTC: Circulating Tumour Cell

CTM: Circulating Tumour Microemboli

EA: Expanded Assay

EGFR: Epidermal Grow Factor Receptor

EML-ALK: Echinoderma Microtubule-associated protein-like 4 Anaplastic Lymphoma Kinase

EMT: Epithelial to Mesenchymal transition

EpCAM: Epithelial Cell Adhesion Molecule

FDA: US food and drug administration

FFPE: formalin-fixed paraffin embedded

FISH: Fluorescence In Situ Hybridization

FITC: Fluorescein isothiocyanate

HIC: immunohistochemistry

HR: Hazard Ratio

ISET isolation by size exclusion of tumour cells

IVD: In Vitro Diagnostic

LBP: Liquid Biopsy Platform

MET: Mesenchymal to Epithelial Transition

MBC: Metastatic Breast Cancer

NGS: Next Generation Sequence

NSCLC: Non Small Cell Lung Cancer

OS: Overall Survivall

Pts: patients

PE: Phycoerythrin

PET: Positron Emission Tomography

SA: Standard Assay

SNV: single nucleotide variation

STP: Subsequent Tumour Progression

TKI: Tyrosine Kinase Inhibitor

TROP2: Tumour-associated calcium signal transducer 2

TNM tumour, node, metastasis

WGA: Whole Genome Ampliphication

INDICE

INTRODUCTION

Liquid Biopsy in NSCLC

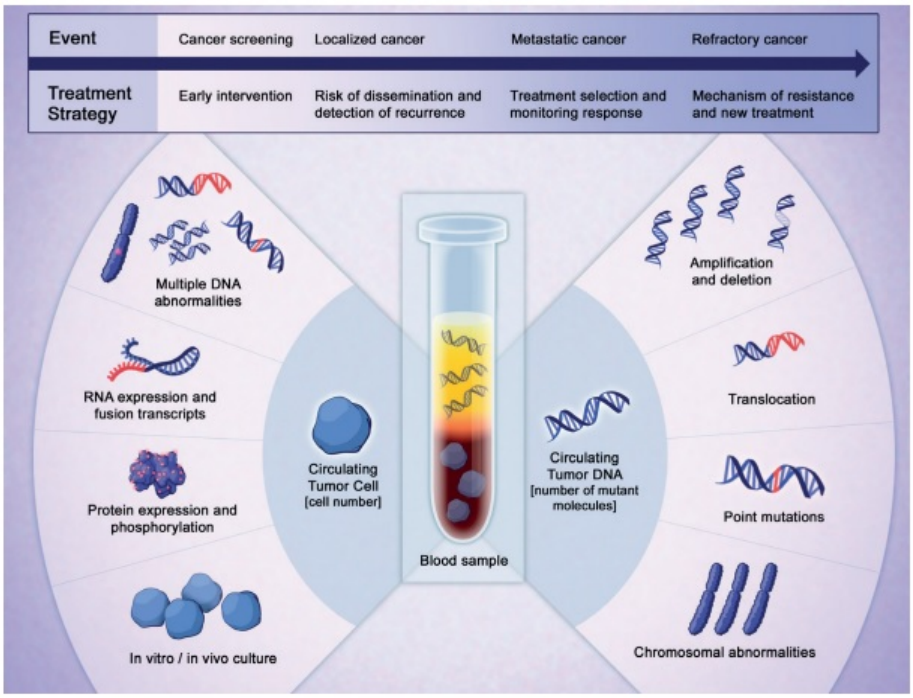


Fig.1. Graphical representation of CTCs and cfDNA and the information they can provide at different times in the history of the disease.

The term "Liquid biopsy" has been used to define a series of non-invasive blood tests devoted to detect circulating tumour cell (CTCs) and fragments of tumour DNA that are shed into the blood from the primary tumour and from metastatic sites.

Blood-based biomarkers are valuable diagnostic tools for the management of lung cancer patients. They support not only differential diagnosis and histological subtyping, but are also applied for the estimation of a prognosis, the stratification for specific therapies, the monitoring of treatment response, the surveillance

monitoring and the early detection of residual or progressive disease and could better reflect tumour heterogeneity. The main components of the liquid biopsy are Circulating Tumour Cells (CTCs) or traces of the cancer's RNA or DNA, circulating nucleic acids that could be protected by extracellular micro-vesicles, mainly exosomes.

To date, the main actor of liquid biopsy in NSCLC are CTCs and cfDNA.

CTCs as Prognostic and Predictive Biomarker

The process by which we are finally able to license any clinical-pathological parameter as a biomarker goes through some mandatory steps, namely analytical validity, clinical validity and, hopefully, clinical utility [5].

In particular, the clinical validity defines a test that is clinically usable [5], on the basis of reliability, accuracy, and needed sensitivity and with a specific predictive value for impacting patient care. On the other hand, with clinical utility we refer to the ability of a test to be used into the medical practice, because of an improved benefit or reduction in costs as well as the best available test.

CTCs could affect the clinical utility in many different cancers in different ways:

1) From changing treatment decision (stopping a therapy that doesn't work or on the contrary, continuing a therapy beneficial for patients);

2) Improving tolerability of a systemic regimen;

3) Improving survival (improving treatment selection and reduction in toxicity);

4) Improving cost effectiveness (with reduction of ineffective drug explosion time) [6]

Circulating Tumour Cells

The main reason for the CTC success as a potential surrogate endpoint comes from afar and depends on strong biological evidence, sustained by the robustness of detection methods.

In 1869 for the first time, CTCs were observed in the blood of a man with metastatic cancer by Thomas Ashworth, who postulated that "cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumours existing in the same person". A thorough comparison of the morphology of the circulating cells to tumour cells from different lesions led Ashworth to conclude that "One thing is certain, that if they [CTCs] came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg". In 1874 De Morgan postulated that cells derived from a primary tumour could escape and travel through environs tissue, invade new areas, using lymphatic or blood vessels. Twenty years after Ashworth, Stephan Paget, a surgeon in UK, proposed the "seed and soil" theory, the theory that suggests that a tumour cell - the seed - either sleeps or thrives within the unique environment of each organ [7].

The first systematic study using smears blood from cancer patients, in 1934, demonstrated the presence of CTCs in 43% of cases .

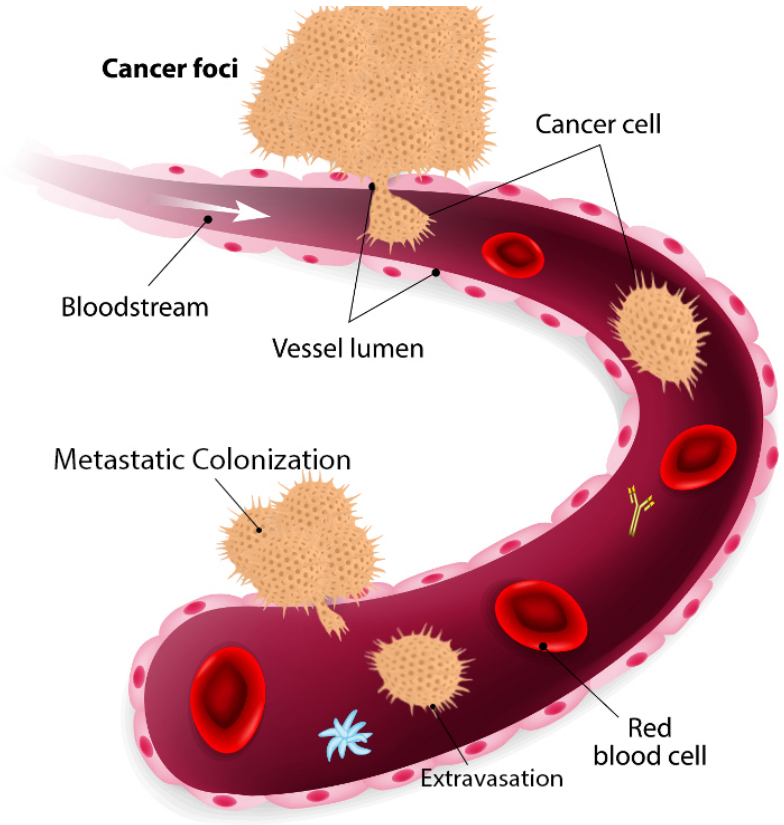


Fig.2. A schematic representation of the CTC intravasation into peripheral blood and subsequent extravasation and metastatic colonization.

Only in 2003 the soil theory was verified, an analysis of CTCs that is a "seed" in the blood, since then has been considered a very important tool in clinical prediction. In 2004, a clinical study reported the importance of CTCs as a prognostic factor. Strong evidence for CTCs as prognostic markers has been documented for breast cancer [8], but CTC detection is also connected to metastatic relapse and progression in other tumour entities, including prostate, lung and colorectal cancer.

The process of metastatic spread from the primary tumour site into distal organs is still not well understood. Recent studies suggest an early spread of tumour cells to lymph nodes or the bone marrow (BM) referred to as "disseminated tumour cells" (DTCs) or as "circulating tumour cells" (CTCs) when present in the peripheral blood (PB) [9, 10].

The rate of tumour cells released by cancer is not known, but several studies estimate that millions of cells are dispersed into the body. Consequently, it is conceivable that only few tumour cells are able to overcome the lack of cell-matrix interaction and escape the immune-surveillance, thus to survive in the blood stream and reach a distant organ and eventually grow into a metastasis.

Only in 2007, for the first time the American Society of Clinical Oncology (ASCO) cited CTCs and DTCs in recommendations on tumour markers. Recently, the American joint Committee on Cancer has proposed a new category, M0 (i+), for TNM staging in breast cancer (BC). This category is defined as "no clinical or radiographic evidence of distant metastases, but deposit either molecularly or microscopically detected tumour cells (no larger than 0.2 mm) in the blood, bone marrow, or other non-regional nodal tissue in a patient without symptoms or signs of metastases".

More recently, Bidard and coll., demonstrated the clinical validity of the CTC assay, as performed by the CellSearch platform, reaching the level I of evidence by the pooled analysis of individual data obtained from close to two thousand European metastatic BC [11].

CTC assay then entered in the ASCO guidelines as a prognosticator for metastatic breast cancer [12] and early breast cancer [13], respectively.

CTC detection methods

CTCs are very rare cells, as only one CTC is contained in about $1x10^8$ or $1x10^9$ of blood cells in cancer patients' blood thus, their detection and characterization requires highly sensitive and specific methods. To date, the only method FDA approved is the CellSearch system. This platform takes advantage of the fact that carcinomas derive from epithelial cells that are not normally found in the blood stream. From 7.5 ml of blood, CTCs are immune-magnetically enriched with a specific antibody for Epithelial Cell Adhesion Molecule (EpCAM) coupled with ferro-fluid. In a second step, the enriched cells were stained with a nucleic acid dye, DAPI, and a monoclonal antibody directed against cytokeratins (CK) 8, 18 and 19; in order to exclude contaminating leukocytes, an antibody that identifies

CD45 is included. An automated microscope collects the images of any fluorescent event and proposes a photo gallery to a trained operator for the manual scoring of CTCs.

Currently, there are many methods in order to isolate and detect CTCs, below you can find an overview of strategies used to capture CTCs and specific examples from every kind (see Table 1).

Methods that use immunoaffinity purification strategy have been proven to be an efficient way to capture CTCs and for this reason, they are the most widely used. These methods typically use anti-EpCAM antibodies, but also other antibodies that recognized tumour - associated antigens, acting as capturing elements for CTCs from human whole blood. The main example is the CellSearch platform but there are also CTC-chip, an array of 78,000 micro spots coated with anti-EpCAM antibodies, Adna-Test and Mag-Sweeper Isoflux, that uses a cocktail of antibodies specific to certain kinds of cancer and the GILUPI CellCollector® that is the first in vivo CTC isolation product world- wide which is CE approved. This device resembles a venous blood withdrawal. The GILUPI CellCollector® is placed directly into the blood stream of a patient via an indwelling catheter (size 20 G, pink), remains in the arm vein for 30 min and thus enables the capture of a large number of CTCs in vivo [14].

It is also known that tumour cells are a heterogeneous population in which EpCAM is not constantly expressed. Furthermore, it has been noted that CTM (Circulating Tumour Micro-emboli) or CTCs with EMT (Epithelial Mesenchymal Transition) which recently have attracted great attention, show no or weak expression of EpCAM, and therefore they are not detectable by the methods above. For this reason, methods to isolate CTC based on their **physical properties, including density, size, deformability and electrical properties, have been developed.**

Some groups use **density gradient centrifugation methods** for separating CTC in mononuclear fraction based on cell density as centrifugation with Ficoll-Paque solution or OncoQuick (combine a porous filter for size-based separation in conjunction with gradient centrifugation). The isolation is in general followed by

a RT-PCR specific for CK. The most promising method is Leukapheresis in which white blood cells are separated from a sample of blood. In this way a large volume of patient blood could be analysed for CTCs, the result is an improvement in the number of CTC isolated and in sensitivity for downstream analysis and characterization.

Microfiltration and Microfluidics are also employed: with microfiltration CTCs are retained on the basis of size, assuming that CTCs are larger than leukocytes. The two main techniques are ISET [15] that uses a polycarbonate filter with 8 μm diameter circular pores for CTC enrichment and ScreenCell that uses circular track-etched filters, the pores range is 7.5-6.5 μm. This method's advantage is that CTCs can be isolated as living cells without fixation. Nowadays, inexpensive and convenient devices are available but they are disadvantageous in that the blood samples have to be isolated in a short time after drawing. Recently De Wit and colleagues [16] were able to isolate CTCs onto a silicon membrane with 5 μm diameter circular pores. Using the microfluidics tool to retain CTCs, the size and deformability of these cells can be explored.

The Dielectrophoresis (DEP) exploits the electrical properties of CTCs to discriminate them from leukocytes by applying a non-uniform electric field. Gupta and coll. developed ApoStream instrument for file flow fractionation [17] and Manaresi and colleagues [18] developed DEPArray, based on a microfluidic cartridge that contains an array of individually controllable electrodes, each with embedded sensors. This circuitry enables the creation of dielectrophoretic (DEP) cages around cells. After imaging, individual cells of interest are gently moved to specific locations on the cartridge, e.g. for cell-cell interaction studies, or into the holding chamber for isolation and recovery.

Functional assay: CTCs could also be enriched by approaches that utilize functional aspects of CTCs as invasiveness and secretion of specific proteins. So far, only two technologies use this strategy, namely EPISPOT and VitaAssay™. With the first one, membrane immune-captures specific proteins secreted near the cells. The second method takes advantage of the propensity of cells to invade into collagenous matrices.

Notably, the numbers of CTCs reported vary widely between different platforms; for this reason, there is a need for a uniform, clear and concordant criteria for defining an events as a CTCs. With regards to CellSearch platform, many studies have been performed and all show a high level of concordance even if the classification is operator dependent [19-21].

However, the same level of evidence has not been obtained yet for other different platforms; studies are few and the great majority of them are lacking in automation in the classification of CTCs.

Hopefully, this step will be overcome in a few years through the results of the CANCER-ID (IMI-JU-11-2013, EoL no. 115749-1, "Cancer treatment and monitoring through identification of circulating tumour cells and tumour related nucleic acids in blood"), an EU founded project that, among other, is working on an Open Source computer program to identify CTCs from images obtained by different platforms. Indeed, the main purpose of the consortium, which so far has collected 37 partners among the academic and industry world, is to construct a consensus regarding the minimum criteria necessary and sufficient to define an event as a CTC (http://www.cancer-id.eu/).

Table.1 Methods to detect and isolate CTC

ASSAY	ENRICHMENT	DETECTION	KEY FEATURES
CellSearch®	EpCAM mAb coupled ferrofluid	Immunofluorescences: CTC is positive for CK8,18,19 and nucleus positive for DAPI, negative for CD45	Semi-automated system with FDA approval for metastatic breast, colon and prostate cancer. CTCs can be enumerated and visualized
Adna Test	antibody cocktail (MUC1, EpCAM) coupled microbeads	Molecular biology: RT-PCR positive for least one following markers: MUC1,Her2, EpCAM	This system doesn't quantify the tumour cell load, false positive results are due to unspecific amplification
MACS	EpCAM mAb coupled beads	Microscope visualization: morphology, high surface area to volume	Possibility to pos/neg enrichment
MagSweeper	EpCAM mAb coupled ferrofluid	Microscope visualization: morphology	High purity, can process WB, 9 ml/h throughput
Ariolsystem	CK antibodies and EpCAM antibodies coupled to microbeads	Positive markers: CKs	Possibility to detect of EpCAM+ and EpCAM-
CTC-Chip	Microdots array: EpCAM mAb coupled microdots	Immunofluorescence: CTC is positive for CK8,18,19 and nucleus positive for DAPI negative for CD45	Microdots are optimized for cell-antibody contact, 1-2 ml/h, high detection rate even in M0 patients
Ephesia	Self-assembly of magnetic beads in columns	Immunofluorescence or immunocytochemistry: CTC is positive for CK8,18,19 and nucleus positive for DAPI negative for CD45	Flexibility with capture antibody
IsoFlux	EpCAM-coated magnetic beads combined with microfluidic processing	Immunocytochemistry for cytokeratin, CD45 and Hoechst	Automated, continuous flow

CTC iChip®	Magnetic bead capture combined with microfluidic inertial focusing	Immunocytochemistry or RT-PCR	Pos/neg enrichment, remove nucleated cells from whole blood by size-based deflection by using a specially designed array of dots performed in CTC-iChip1, inertial focusing to line up cells to prepare for precise magnetic separation and magnetophoresis for sensitive separation of bead-labeled WBCs and unlabeled CTCs
GILUPI cellcollector	Functionalized EpCAM-coated medical wire	Immunocytochemistry for EpCAM, cytokeratin and DAPI	In vivo collection
Ficol-Paque®	Density	Immunocytochemistry	Inexpensive, easy to use
OncoQuick	Density/size	Immunocytochemistry/ RT-PCR	Density gradient centrifugation with OncoQuick results in higher relative Tumour cell enrichment than Ficoll density gradient centrifugation
ISET®	Filtration based on cell size	Immunocytochemistry /FISH	Epithelial and Mesenchymal tumour cells can be isolated
ScreenCell®	Filtration based on cell size	Immunocytochemistry /FISH	Epithelial and Mesenchymal tumour cells can be isolated
VyCAP	Filtration based on cell size	Filtration based on cell size	Epithelial and Mesenchymal tumour cells can be isolated
Dean Flow Fractionation	Size-based selection using centrifugal force	Immunocytochemistry for cytokeratin, EpCAM, CD45 and Hoechst	Non-epithelial cells can be isolated
Dielectrophoretic field-flow fractionation	Membrane capacitance	Immunocytochemistry	CTCs selected are viable

DEPArray™	enables movement of cells within chip by electric field changes	Fluorescence imaging	Requires pre-enrichment step/Isolation of purified single cells for downstream analysis
ApoStream®	Dielectrophoretic technology in a microfluidic flow chamber	Fluorescence imaging	Isolation of purified single cells for downstream analysis
EPISPOT assay	CD45 depletion and short-term culture in plates coated in antibody against MUC-1, PSA or cytokeratin-19	Immunofluorescence secondary antibodies to MUC-1, PSA or cytokeratin-19	Detection of only viable CTCs
Vita-Assay™or Collagen Adhesion Matrix (CAM) technology	Density gradient centrifugation and cells applied to CAM for short-term culture	Immunocytochemistry for cell-surface markers	Detection of only viable CTCs with the invasive phenotype

Circulating Cell Free DNA

The molecular characterization of "liquid biopsy" promises to facilitate the access of cancer patients to targeted therapies. Circulating cell-free DNA (cfDNA) could be released into the blood stream through necrosis, apoptosis or active secretions by nucleated cells such as leukocytes. The large majority of cfDNA was composed by constitutive genomic DNA. For this purpose, as an alternative to investigate CTC's DNA, many authors have proposed to study Circulating Tumour DNA (ctDNA): ctDNA is defined as the fraction of cell free circulating DNA carrying the same genetic and epigenetic alteration as the patient's tumour [22]. The ctDNA seems an appealing and a promising minimally invasive test for the follow-up of malignancies.

Indeed, small DNA fragments have been previously reported in the blood stream of healthy donors at low concentration. The range is from 1.8 up to 44 ng/mL in plasma, although this level can greatly increase following exhaustive exercise, in pregnant women, in elderly patients suffering from acute or chronic disease and in individuals with premalignant lesions, inflammation or trauma [23-25].
To date, several methods have been reported for detecting ctDNA, but none has reached, so far, the FDA approval. The main problem is the pre-analytical phase of the procedure to which some authors attribute the lack of comparable results, while a lack of standardization and appropriate controls is stressed by others researchers [26-29].

However, the concentrations of ctDNA in plasma shows a good correlation with the disease status in gastric cancer. Furthermore, some authors observed a decrease in ctDNA levels after surgical resection. Bettegowda *et al.* [30] analysed 136 metastatic tumours originating from 14 different tissue types; by using patient-specific rearrangements, the authors demonstrated that recurrence of stage II colorectal cancer after surgical resection might be predicted by ctDNA.
Moreover, ctDNA is often used as a DNA source to detect cancer cell-derived mutations [31], promoter methylation [32, 33] and loss of heterozygosity [34].

To date, multiple methods have been developed to enable the assessment of ctDNA, including digital droplet PCR methodology, "BEAMing" (beads, emulsion, amplification, and magnetics) and other approaches based on PCR and next-generation sequencing (NGS) [31, 35, 36]. Early reports using PCR-based methods to identify specific tumour-associated mutations in ctDNA demonstrated that these mutations could be detected in NSCLC.

In a selected patient population with unusually high ctDNA levels, wide coverage exome next-generation sequencing (NGS) detected ctDNA mutations appearing at the time of treatment resistance [37]. However, a significant proportion of mutations detectable in tumour biopsies were undetectable in plasma. This data contrasts with a metastatic ovarian study that reported recovery of most tumour mutations from plasma using more limited, but still multiplexed NGS [38].

In NSCLC, many study reported that cfDNA concentrations in the plasma has an independent prognostic value: a correlation has been reported between an elevated baseline cfDNA concentrations (>10ng/ml) and poor prognosis and it was significantly associated with OS in multivariate analysis [39, 40].

Speaking about ctDNA, in patients undergoing EGFR-tyrosin kinase inhibitors (TKIs) therapy, the monitoring of EGFR mutations could predict [41] tumour response or tumour progression under treatment. Xu and colleagues analysed in advanced NSCLC patients (n=42) ctDNA and tissue; by using a target sequencing approach they showed a concordance of 76% about EGRF, KRAS, PIK3CA and TP53 [42].

Regarding the interpretation of cfDNA mutation results, Yu Zhang and colleagues concluded that the cfDNA concentration must be considered, just as a pathologist must first evaluate tumour tissue before molecular testing. The sensitivity of the cfDNA test was 82.6% in patients with cfDNA inputs of ≥ 5 ng per reaction. However, only 23 of 93 patients (24.7%) had cfDNA inputs of ≥ 5 ng per reaction, while 30 of 93 patients (32.3%) had cfDNA inputs of < 2ng per reaction. They used 2 mL of plasma from each patient for analyses. In real-world settings, 4 mL of plasma might be better for cfDNA testing, because doubling the concentration of cfDNA extracted should double the cfDNA input and thus increase the sensitivity. They showed that the median plasma cfDNA

concentration for specimens collected ≤ 2 years before the analysis was obviously higher than that of specimens collected > 2 years before the analysis (13.83 ng/mL vs. 6.575 ng/mL, P< 0.001). Thus, the plasma cfDNA degraded gradually, despite being stored at −80°C, an inevitable limit of a retrospective study. Prospective studies, or retrospective studies of plasma specimens collected ≤ 2 years before analysis, will be better for further research [43].

As previously reported by J. Uchida, the clinical stage can influence the detection sensitivity. In a study evaluating the diagnostic accuracy of detecting EGFR in lung cancer by deep sequencing of plasma cfDNA, the overall sensitivity was 54.4% for all cases. However, sensitivities for patients with stages IA-IIIA and patients with stages IIIB-IV disease were 22.2% and 72.7%, respectively [44].

Applying captured-based NGS, S. Cui and colleagues demonstrated that it is feasible detecting ALK fusion in patients with advanced-stage NSCLC, who cannot undergo traumatic examinations or have insufficient tissue samples for molecular tests. They showed that the specificity of capture-based NGS for plasma ALK detection was 100%. Furthermore, in a small group of patients who received Crizotinib they found a trend in the median PFS: indeed, PFS of patients with plasma ALK-negativity was longer than that of patients with plasma ALK-positivity [45].

CTC in NSCLC

Lung cancer is the leading cause of cancer-related death worldwide [46].

This is partly due to the advanced stage of the disease at the time of diagnosis, because more than half of the patients have distant metastases at initial presentation.

Non–small-cell lung cancer (NSCLC) can now be subdivided into different molecular groups, for which specific biomarkers can guide treatment selection according to the molecular profile of the tumour [47]. The discovery of epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements revolutionized the treatment of NSCLC by introducing the era of personalized care in this disease. Other actionable genomic alterations include BRAF (B-Raf proto-oncogene, serine/threonine kinase) mutations, MET (MET proto-oncogene, receptor tyrosine kinase) amplification, ROS1 (ROS proto-oncogene 1, receptor tyrosine kinase) and RET (ret proto-oncogene) rearrangements, and HER2 (erb-b2 receptor tyrosine kinase 2) mutations [48]

However, despite the discovery of genetic alterations that drive tumour growth and the development of potent inhibitors that confer high response rates and prolongation of progression-free survival (PFS) in these patients, [49-51] the development of resistance is inevitable. Moreover, most patients are still treated with palliative cytotoxic chemotherapy with dismal results.

In lung cancer the analysis of tumour tissue as challenging because of the nature of specimen type in term of quantity and quality. Typically, in the early stage of lung cancer the primary tumours is characterized by a lower cellularity compared other tumours, instead in advances stages of disease only a small percentage of patients can undergo curative surgical resection. In advanced NSCLC the diagnosis is performed using computerized tomography-guided percutaneous biopsy or ultrasonography-guided endoscopic biopsy with fine needle aspiration. In general, the sample from needle biopsy is limiting (in quantity and quality) for histological and molecular testing.

It we should also consider that the molecular analysis depend to the sensibility and specificity of the method used, and it need or tumour cells alone or a good proportion of tumour and normal cells.

CTCs can be detected at all stages of disease in NSCLC patients. Tanaka et al.in primary lung cancer, using CS, shown that 30.6% patients were CTCs positive. Furthermore, this study suggested that CTCs could be used as a surrogate marker of distant metastasis in early stage of lung cancer [52].

For NSCLC, a general consensus is currently lacking on the role of CTCs to determine survival (a prognostic biomarker) or to predict which patients are likely to respond to a conventional chemotherapy regimen or targeted therapy (a predictive biomarker). This is probably due to the considerable heterogeneity within and between lung cancer patients, regarding epithelial and mesenchymal markers in CTM and CTCs and the methods used to detected CTCs or CTM.

The prognostic significance of enumeration of CTCs in NSCLC has been underlined in several reports by using different methods. In particular, two key reports [4, 53] both used the CellSearch antigen-recognition technology. Allard and colleagues initially reported the proof of principle that CTC identification and enumeration was possible in lung cancer, and then more detail on the benefits and limitations of CellSearch quantification was later revealed in a report showing that 21% of 109 stage III/IV patients had 'positive' CTC counts at baseline (defined as ≥2 CTCs in 7.5 ml blood).

When authors identified CTCs by EpCAM-independent methods, as ISET, they counted 5 times more CTCs than by using CS and identified different subpopulation of CTCs.

By using CS, several authors demonstrated the prognostic significance of CTC enumeration in NSCLC and two meta-analyses were carried out with the aim to clarify this point. The first meta-analysis included data extracted from 27 articles (12 containing survival outcomes) published between the year of 1997 and 2012 [54]. The authors concluded that both pre- and post-treatment CTC detection in peripheral blood were associated with poor prognosis.

Another more recent meta-analysis examined data collected from 20 studies enrolling more than 1500 NSCLC patients [55]. This study showed that CTCs were associated with tumour stage and lymph node metastasis, but not with histology; in addition, the presence of CTCs was associated with a poorer outcome [55].

To date, studies about CTCs level in therapy monitoring, even if in trials with a small number of patients, showed that a decreasing number of CTCs at the end of the first cycle of therapy is associated with a good PFS and OS and is correlated with tumour response as documented by a fludeoxyglucose-positron emission tomography (PET) [56, 57].

A promising prospect of CTCs is the molecular characterization of these cells in order to detect activating mutations as EGFR or fusion gene as EML4-ALK or ALK-ROS1. Using serial CTC samples from lung cancer patients, Maheswaran et al. demonstrated evolution of T790M and other epidermal growth factor receptor (EGFR)-activating mutations during treatment with the tyrosine kinase inhibitor, Gefitinib [58]. To assess the feasibility and specificity of detection of ALK rearrangement in CTCs, [59] evaluated 87 CTC samples from patients with lung adenocarcinoma and estimated ALK status by fluorescence in situ hybridisation (FISH) in both the primary tumour and CTCs isolated by ISET. Five patients showed ALK-gene rearrangement in the CTCs that matched to the corresponding tumour samples. Confirming the specificity of the assay, none of the patients without ALK rearrangement in the tumour showed rearrangement in the CTCs.

By using the same approach Pailler et al. [60] examined the presence of ALK rearrangement in CTCs of 18 ALK-positive and 14 ALK-negative patients. All ALK-positive patients had four or more ALK-rearranged CTCs per 1 mL of blood, whereas no or only one ALK-rearranged CTC was detected in ALK-negative patients. ALK-rearranged CTCs were monitored in five patients during Crizotinib treatment. The levels of CTCs harboring an ALK rearrangement and single ALK native copies decreased in four patients during the treatment. This data suggests a possible role of monitoring quantitative and qualitative changes of CTCs with ALK rearrangements in patients undergoing Crizotinib. Serial determination of

ALK+ CTC was achievably in patients treated with ALK inhibitors and could anticipate the emergent drug resistance [61].

In the systematic review and metanalysis performed by Yafang Liu and Yong Song, the authors highlighted that EGFR mutations, ALK, KRAS and BRAF mutation could be detected on CTCs. Furthermore, Sundaresan TK and colleagues, by using the HbCTC-Chip to isolate CTCs, showed that an allele-specific assay can detect the emergence of T790M during first-line therapy and that the frequency of successful CTC genotyping was 70%.

By using a mRT-PCR based method compared to CellSearch, Hassen and colleagues detected a higher number of CTCs in primary and brain metastatic lung cancer; moreover, by this method they revealed a large fraction of CTCs expressing stemness marker [62].

Epithelial to mesenchymal transition (EMT) phenomenon is a player in the metastatic mechanism, when the cancer invasion in the blood stream is accomplished by loss of cell-cell contacts, a skill of mesenchymal cells [63], meanwhile, on the site of metastasis occurs the reversion to the epithelial phenotype. Several studies document that CTCs can express both epithelial and mesenchymal phenotype.

EMT in lung cancer is less studied in comparison to other epithelial cancers e.g. breast and colorectal cancer [64]. Although EpCAM (a type I transmembrane glycoprotein) and cytokeratin are widely expressed in most epithelial tumours, including NSCLC [65] many authors demonstrated they were expressed heterogeneously in CTCs and CTM in NSCLC (exemplified for NSCLC patient VI) [66].

Farace and colleagues too showed mesenchymal markers in lung cancer CTCs [67]. All these data can explain the different rate of CTCs detected depending on the methods utilized.

Most importantly, all this evidences documented and confirm the great impact that the detections and the characterization of CTCs, with malignant proprieties, could

have in tailored medicine if this biomarker will be insert in the decisional tree used in the clinical management of patients.

AIMS

Non-small-cell lung cancer (NSCLC) represents about 80% of all cases of lung cancer, which is the leading cause of cancer mortality worldwide. The majority of NSCLCs are diagnosed at advanced stage disease. Chemotherapy mainly based on platinum-containing doublets seems to have reached a therapeutic plateau, and the development of more effective strategies in the first-line setting remains challenging. Despite significant improvement in chemotherapy, the outcome for patients with advanced NSCLC remains poor with median survival times approaching 12 months.

However, after significant success in other solid tumour types, tailored therapy has played an important role in the management of patients with NSCLC and it is expected to improve the outcome of molecularly defined subgroups of patients.

Recent randomized studies have shown that patients with advanced NSCLC and tumours harbouring activating EGFR mutations have significant improvement in progression free survival when treated with EGFR tyrosine kinase inhibitors in comparison with chemotherapy [40, 41], whereas EGFR TKIs treatment showed to be less effective than chemotherapy in EGFR wild-type patients [49].

The importance of testing for anaplastic lymphoma kinase (ALK) gene rearrangements has been also recently shown. The phase I-II study of Crizotinib (PF-02341066, Pfizer), an oral inhibitor of ALK and MET (HGF receptor) tyrosine kinases, showed a unprecedented response rate for patients with advanced NSCLC and EML4-ALK gene fusion, despite previous chemotherapy treatment [Bang Y et al., ASCO meting, J Clin Oncol 2012; 28 (18S):946s]. In addition to EGFR mutation and ALK rearrangements, other mutations have been identified (including BRAF and HER2, PIK3CA mutations or copy number gains) with potential clinical benefit.

The information obtained from tumour biopsies becomes more and more essential in clinical oncology for the selection of appropriately targeted cancer therapies. It is also becoming ever clearer that achievement of proper tissue samples for molecular analysis is not always an easy task, mainly in cases as advanced

NSCLC, when the diagnosis is often based on a small biopsy, with a limited amount of tumour tissue or fine-needle aspirations. In this respect, peripheral blood may represent an alternative source of tumour cells that is minimally invasive.

Circulating tumour cells (CTC) refer to cells that detach from a primary tumour or metastatic site, circulate in the peripheral blood and may settle down at secondary sites forming metastasis. In the past decade, technology advances have enabled the detection of these rare cancer cells, shedding light on the disease natural history and showing promising signs as a liquid biopsy and used to tailor treatment for the individual patient. At present, the only validated assay for CTC detection that has been cleared by the U.S. Food and Drug Administration is the CellSearch system (for a review about CTC detection technologies see [68]). Prospective multicentre studies in metastatic breast, prostate, and colon cancer conducted with this system demonstrated that the presence of CTCs was associated with poor survival and failure to eliminate these cells after the first cycles of therapy strongly suggest futile therapy.

In the current practice, cancer tissue is usually taken at diagnosis, and used to assess the presence of treatment targets. This however, is suboptimal since tumour cells evolve due to genomic instability. Assessment of the genotype and phenotype of the CTCs will provide insights into which treatments would be most beneficial for the individual patient. Feasibility to detect treatment targets in CTC has been demonstrated in breast, colorectal and prostate cancer [69-71].

Conversely, very few papers address this topic in lung cancer. In a group of advanced NSCLC patients, the expected EGFR activating mutation was identified in CTCs from 11 out of 12 patients (92%), giving the proof of principle that molecular analysis of CTC may offer the possibility of assessing tumour genotypes in lung cancer [58]. However, the frequency of CTC-positive patients was not determined in this study and the CTCs were enriched with a prototype of a micro fluidic device, not easily exportable in the setting of multicentre clinical trials or for clinical purposes. Recently, it was reported that with the CellSearch system 15% of metastatic lung cancer [53, 66] have 5 or more CTCs in 7.5 ml of blood before initiation of a new line of therapy.

The primary aim of the Project is to determine the percentage of CTC-positive patients and total CTC number in advanced NSCLC at baseline, before starting treatment. We questioned whether we could detect a higher number of CTCs by implementing the standard CellSearch assay. We planned a prospective observational study, to enrol at least 150 patients, regardless of type or line of therapy. Secondary aims is to evaluate if CTCs count modifications are an early predictor of response to treatment than we re-evaluated patients for disease status and CTC count depending on the type and schedule of treatment.

The exploratory aims is set and applying the molecular and phenotypic profiling of CTCs, and to verify if these data could give information useful to manage patient therapy.

For this purpose, we obtained purified CTCs by an automated platform, and we performed DNA sequencing to assess sensitizing mutations in advanced NSCLC patients. We compared the EGFR mutations or EML4-ALK fusion gene detected in CTCs with those reported for the tumour specimen using standard sequencing.

In parallel, we integrated the CTC count assay with mAb targeting EGFR or EML4-ALK fusion gene, to test whether assaying the quote of EGFR-positive and ALK-positive CTCs provides a more sensitive marker for rating pharmacodynamics effects in patients compared with total CTC counts.

Moreover, it was reported that NSCLC cells with EGFR mutations manifest activation of the PI3K (phosphatidylinositol 3-kinase) AKT and MEK, ERK (extracellular signal-regulated kinase) signalling pathways under the control of EGFR, and exposure of such cells to EGFR-TKIs blocks signalling by both pathways and induces apoptosis [72]. To evaluate whether live vs. apoptotic CTC changes under treatment may be used as predictor of tumour response, we quantified M30-positive CTCs as previously [71].

Finally, we correlated data obtained with the CTC assay with common prognostic factors.

The development of molecular profiling of CTCs in advanced NSCLC patients may offer a less invasive source of tumour specimens, amenable to serial sampling. Phenotypical and molecular characterization of CTCs in advanced

NSCLC is expected to provide clinical information on prognosis, therapy choice, and effectiveness, as well as drug resistance.

MATERIALS AND METHODS

Patient Cohort

Between January 2013 and June 2016 peripheral blood was drawn from a total number of 189 NSCLC patients coming from 6 Italian clinical sites, namely CROB (Rionero In Vulture), Ospedale Sant'Andrea di Roma (Roma), Campus Biomedico (Roma), IRST (Meldola), IOV-IRCCS (Padova), Azienda Ospedaliera di Perugia (Perugia). We analysed patients' samples for CTCs in a multicentre prospective observational protocol headed by the CTC-lab of IOV-IRCCS.

Inclusion criteria were as following:

- Age 18-80 years old, males or females;
- Patients with NSCLC confirmed by histopathology or cytology;
- Life expectancy > 6 months.
- Any first line therapy.

Exclusion criteria included any condition that might hamper compliance to the schedule of assessments. Patients are voluntary to participate and sign the informed consent. We collected serial blood samples at baseline, at the end of first cycle of therapy, at radiological assessment and at progression.

Cells Line

The lung cell line H460 (wild type), A549, H2228, and H1975 (mut. Ex 21 L858R and mut. T790M a quote of this cells harbouring EML4-ALK variant 3) were maintained at 37°C and 5% CO2 atmosphere. Cell lines were cultured in RPMI Medium (Life Technologies Corporation, CA, USA) supplemented with 10% Foetal Bovine Serum (FBS, GIBCO, Life Technologies Corporation, CA, USA) and 1.5mM Ultra-glutamine (Lonza, Basel, Switzerland).

Whole blood collection for CTC enumeration

Two blood samples (7.5 mL from each) from each patient were collected into CellSave blood collection tubes (Janssen Diagnostic, Raritan, NJ). Blood samples were maintained and shipped at room temperature, and processed upon arrival within a maximum of 96 h after collection. Circulating tumour cells were captured by the CellSearch System (Janssen Diagnostic, Raritan, NJ) with the CellSearch Circulating Tumour Cell Kit (standard assay). CTC enumeration was performed according to manufacturer's criteria (EpCAM+, CK+, DAPI+ and CD45-) [20]. Images, presented in a gallery format were independently classified by two operators according to predetermined criteria (specified by manufacturer) for the presence of CTCs [8, 73]

CTC Detection by CellSearch

CTCs were enumerated in aliquots of 7.5 mL of blood by the CellSearch system (Janssen Diagnostics). Analysis was performed within 96 hours from the blood draw. Antibodies directed against the epithelial cell adhesion antigen (EpCAM) coupled to ferrofluid were used to enrich CTCs. The enriched cells were fluorescently labelled by using the CellSearch CTC kit (Janssen Diagnostics, Huntingdon Valley, PA, USA). The standard staining protocol (standard assay, SA) includes:

- The nucleic acid dye 4′6-diaminodino-2-phenylindole (DAPI);
- Phycoerythrin (PE) labelled anti-cytokeratin monoclonal antibodies (mAbs) C11 and A53.B/A2;
- Allophycocyan (APC) labelled mAb, directed against CD45 (clone HI30), recognizing leukocytes.

Furthermore, we used the extra marker channel associated with the SA, to investigate drug-induced cell death. Briefly, viable and apoptotic CTCs were detected by integrating CTC standard assay with anti-M30 mAb, targeting a neoepitope disclosed by caspase cleavage at cytokeratin 18 in early apoptosis [69].

Results were expressed as the total number of CTC and M30-positive CTC per 7.5 ml of blood.

In parallel, to investigate the expression of EML4-ALK protein fusion, we added the mAb anti-ALK – PE (clone D5F3 CellSignaling) to the CXC KIT (Janssen Diagnostics, Huntingdon Valley, PA, USA). In this case (named expanded assay, EA), the full profile of CK was covered by the following FITC-labelled mAbs:

- anti-CK7 and CK 14 (respectively clone LP5K and LL002, Millipore, Billerica MA, USA) every mAb at final concentrations 1ug/µl/test;
- anti-C11 (clone C11 ACZON, Bologna, Italy) at final concentration 1ug/µl/test.

Downstream the slides preparation by Autoprep, the CellTracks Analyzer II identified images of fluorescent events in order to score them as CTCs.

To this purpose, each sample was analysed twice, using either PE or FITC as cytokeratin marker, and presented to experienced operators for classification. According to manufacturer's and international guidelines, we assigned fluorescent events as CTCs when the objects were larger than 4 µm, stained with DAPI and cytokeratin, lacked CD45 staining and had morphological features consistent with that of a cell [53]

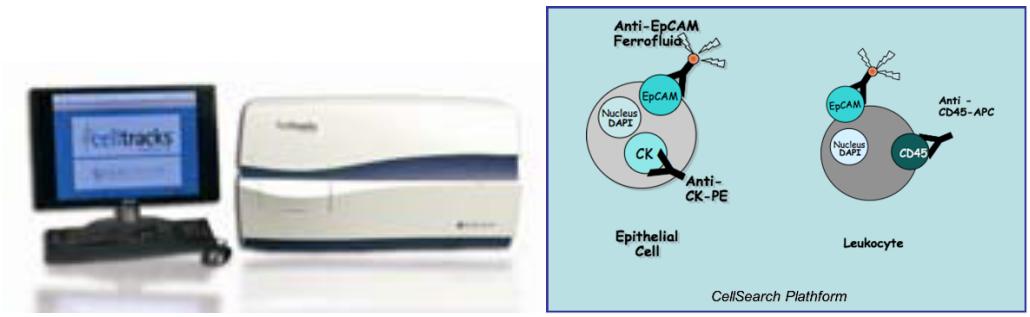


Fig. 3. CellSearch Sistem Platform

Blood waste collection of CellTracks Autoprep

In the standard CellSearch procedure, down stream immunomagnetic selection of EpCAM-positive cells, the CellTracks Autoprep aspirates the blood (that is now void of the selected cells) and transports it to a waste container outside the instrument. To enable the investigation this product for residual tumour cells, a device (named ASCD) was designed and builded to collect the wastes one by one. The device uses a LED and a photo diode to sense the presence of approaching blood in the waste tube; it then diverted the samples into standard 50 mL conical sample tubes by using an actuated 3-way valve. A motorized rotor, holding up to twelve 50 mL tubes, arranges in the right position the correct tube under the valve, at the appropriate time. The ASCD device was controlled using a LabView (National Instruments, Austin TX, USA) program on a laptop computer. Figure 4a shows a schematic representation of the waste collection.

Filtration of CellTracks Autoprep blood waste

The system to filter tumour cells from whole blood or from Autoprep waste comprises a pump unit and a filtration unit, holding a slide with a microsieve (VyCAP, Deventer, The Netherlands). The pump unit maintained a pressure of 100 mbar across the microsieve during the filtration of the blood. A schematic image of this system is presented in Fig. 4b. The microsieve filtration membrane has a thickness of 1 µm supported by 350 µm thick Si and are atomically flat. The total surface area is $8 \times 8\,mm^2$ and contains 111,800 pores of 5 µm in diameter and are spaced 14 µm apart in lanes with a porosity of 10%. The discarded waste of CellSearch samples was transferred to the filtration unit after which the pump was switched on. Completed the filtration, the pump was switched off and the slide containing the microsieve has been removed for staining.

Staining of cells on microsieves

We optimized conditions for staining on microsieves, in order to assure its uniform staining and reduce to a minimum the non-specific binding. After

filtration, the microsieve was removed and washed with PBS-saponin 0.15%. Next, a permeabilization buffer of PBS with 0.15% saponin (Sigma-Aldrich, St. Louis MO, USA) was placed on the sieve and removed after 15 minutes incubation at room temperature.

A cocktail of fluorescently labelled antibodies was used to stain the cells on the sieve for 15 minutes at 37 °C using a heating plate (StatSpin, Westwood MA, USA). The staining solution included the following monoclonal antibodies:

- Two antibodies targeting CK 4–6, 8, 10, 13, 18 & 19 (C11 and A.53B/A2, Janssen Diagnostics) labeled with PE;
- Three antibodies targeting CK 1–8, 10, 14–16, 19 &20 (AE1/AE3, LP5K and Ks20.10) labeled to FITC and one antibody targeting CD45 (HI30) and CD16 labeled with PerCP (Biolegend, San Diego CA, USA).

All antibodies were diluted to a final concentration of 1 µg/mL (HI30, C11, A.53B/A2 and AE1/AE3) or 2 µg/mL (LP5K and Ks20.10) in PBS containing 1% bovine serum albumin (Sigma) and 0.05% saponin. After removal of the staining cocktail, the microsieve was washed 2 times with PBS-BSA 1%. Then the sample was fixed using PBS with 1% formaldehyde (Sigma) for 10 minutes at room temperature. Removal of the fluid during each of the staining and washing steps was done by bringing the bottom of the microsieve in contact with an absorbing material using a staining holder (VyCAP), as illustrated in fig. 4C. The microsieve was subsequently covered with Prolong Diamond Antifade-DAPI (Thermo Fisher Scientific Inc.). A custom cut $0.85 \times 0.85\,cm^2$ glass coverslip (Menzel-Gläser, Saarbrükener, Germany) was placed on top of the microsieve for immediate analysis or storage in the freezer at −30 °C.

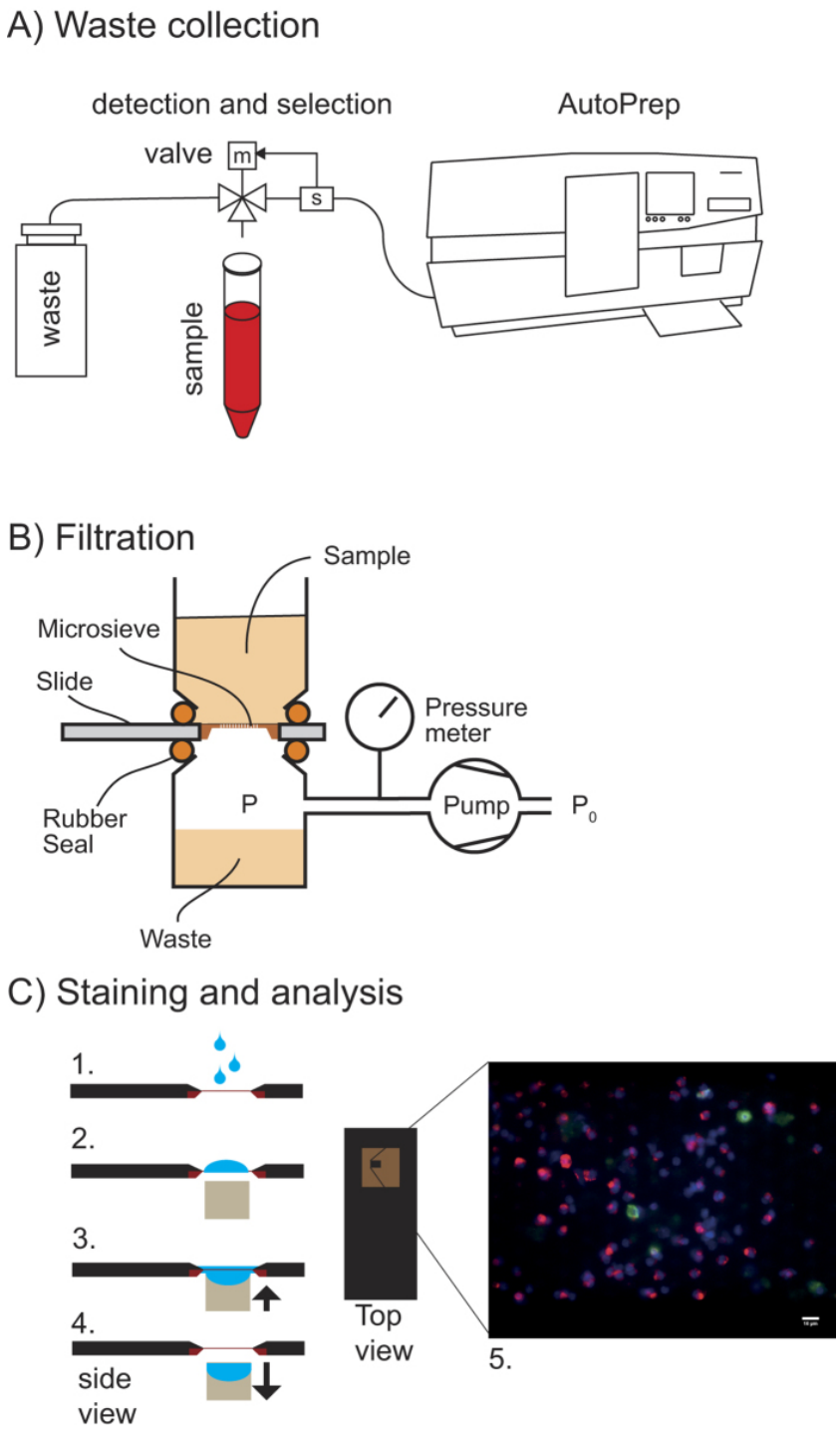


Fig. 4. Method to isolate and characterize CTCs EpCAM low/neg.

Molecular characterization

CTC recovery from CellSearch

To recover CTC-enriched samples from the cartridge, an original protocol was developed. After enumeration, the supernatant was discarded maintaining the cartridge in the Magnest (Janssen Diagnostic, Raritan, NJ) in order not to disturb the captured cells. CTC preparations were then rapidly recovered in 200 µl lysis buffer by gentle scraping of the cartridge surface with a tip, and the suspension was transferred into a tube for digestion at 55°C for 4 h. Nucleic acids were extracted from the lysed cells with the Qiagen's QIAamp DNA Micro Kit.

Whole Genome Amplification (WGA) of DNA from single CTC

After cell sorting or laser micro-dissection single cells were subjected to WGA with AMPLi 1WGA Kit (Menarini silicon biosystem) according to manufacturer' instructions, along with a no cell reaction serving as the negative control. The DNA concentration of the WGA products was measured by picogreen (Quant-iT™ PicoGreen® dsDNA Assay Kit, Life technologies).

cfDNA

Whole blood (7 mL) was collected from the study subjects. The EDTA-treated blood was centrifuged at 1400 × *g* for 10 minutes, and the plasma supernatant was stored at −80°C until analysis. Plasma DNA was purified with the use of a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA), was used along with the QIAVAC system as recommended by the manufacturer (Qiagen, Valencia, CA). The copy number for extracted cfDNA was determined with an RNaseP Copy Number Assay (Life Technologies, Carlsbad, CA). The extracted DNA was stored at 4°C until analysis.

Droplet digital PCR assays

Mutant allele frequency for *EGFR* was measured with the use of the QX100 Droplet Digital PCR System (Bio-Rad, Hercules, CA). The primers and probes for detection of the E746-A750 deletion, L858R, L861Q, and T790M were obtained from Bio-Rad. Primers and probes for G719X were designed to detect G719S, G719C, and G719A: G719X forward primer, 5'-TGAAGGAAACTGAATTCAAAA-3′; G719X reverse primer, 5′-CTTACCTTATACACCGTGC-3'; G719S probe, 5'-/56-FAM/AGTGCTGTC/ZEN/CTCCGG/3IABkFQ/-3'; G719C probe, 5'-/56-FAM/AAAGTGCTG/ZEN/TGCTCCG/3IABkFQ/-3'; G719A probe, 5'-/56-FAM/TGCTGGCCT/ZEN/CCGG/3IABkFQ/-3'; and G719G-WT probe, 5'-/5HEX/AGTGCTGGG/ZEN/CTCCG/3IABkFQ/-3'. The cycling conditions for the PCR reaction included an initial incubation at 95°C for 10 minutes, 40 cycles of 94°C for 30 s and 55°C for 60 s, and enzyme inactivation at 98°C for 10 minutes. After thermal cycling, the plates were transferred to a Droplet reader (Bio-Rad). The digital PCR data were analysed with the Quanta Soft analytical software package (Bio-Rad).

Cynvenio Template enrichment procedures

All sample processing, sequencing and analysis were performed in the Cynvenio Biosystems CAP approved facility (Westlake Village, CA) under CLIA supervision. Whole blood was collected in purple top (K2EDTA) tubes and stabilized using LiquidBiopsy (Cynvenio Biosystems) fixative. A white blood cell control was recovered from 0.1ml of the original sample. Plasma was collected after brief centrifugation to separate cellular components. CTCs were enriched as described [74]. In brief, the cellular component in the starting blood volume was blocked with FcR block and labelled with a biotinylated antibody cocktail consisting of anti-EpCAM alone, or in combination with anti-EGFR, anti-HER2, and anti-TROP2 (Cynvenio Biosystems) followed by iMAG streptavidin beads. The labelled blood was processed in the CTC flow cell on the LiquidBiopsy platform (Cynvenio Biosystems). Captured cells were characterized by evaluating

immunofluorescent staining with anti-Cytokeratin, anti-CD45 and DAPI. Captured cells were recovered by centrifugation to produce an enriched cell pellet. The CTC pellet was digested as described [74] and the resulting digest was diluted to 12µL with TE. The AmpliSeq library specific for Ion AmpliSeq Cancer Hotspot Panel v2 (CHPv2; Thermo Fisher Scientific Inc.) reagents were added directly to the template and further processed as for the germline and ccfDNAsamples. IonAmpliSeq Cancer Hotspot Panel v2 (CHPv2; Thermo Fisher Scientific Inc.) The CHPv2 covers approximately 2800 COSMIC (Catalogue Of Somatic Mutations In Cancer) mutations from 50 oncogenes and tumour suppressor genes (*ABL1, EGFR, GNAS, KRAS, PTPN11, AKT1, ERBB2, GNAQ, MET, RB1, ALK, ERBB4, HNF1A, MLH1, RET, APC, EZH2, HRAS, MPL, SMAD4, ATM, FBXW7, IDH1, NOTCH1, SMARCB1, BRAF, FGFR1, JAK2, NPM1, SMO, CDH1, FGFR2, JAK3, NRAS, SRC, CDKN2A, FGFR3, IDH2, PDGFRA, STK11, CSF1R, FLT3, KDR, PIK3CA, TP53, CTNNB1, GNA11, KIT, PTEN, VHL*).

For enrichment of ccfDNA from the recovered plasma, the QIAamp Circulating Nucleic Acid Kit was used along with the QIAVAC system as recommended by the manufacturer (Qiagen, Valencia, CA). DNA resulting from this purification was quantitated on a Nanodrop (Thermo Fisher Scientific), and directly utilized for sequencing library generation. ccfDNA libraries were produced with a 10 ng input of ccfDNA.

Sequencing data analysis

Primary sequence was demultiplexed and exported from the Torrent Server as FASTQ. The FASTQ files were aligned by reference guided assembly to NCBI GRCh37 p5 using Bowtie 2 [75]. Post-assembly alignments were piled and curated for accuracy using SAM Tools (version 0.1.19) [76] and transferred to Perl using Bio::DB::Sam. ctcDNA and ccfDNA templates were analysed and a mutation was called if ≥20 mutant reads were observed for a limit of detection (LOD) of 1%. ctcDNA and ccfDNA analysis was based upon a case-control model for variant detection, in which total read coverage must be ≥ 2000 reads per

amplicon for ctcDNA and ccfDNA sample and validated calls were required to be absent from the negative control wbcDNA. Sequence FFPE analysis was not case-controlled, and total read coverage threshold was ≥ 500 reads per amplicon.

Statistical analysis

The primary aims of the study was to estimate the percentage of CTC-positive patients in NSCLC. In order to answer at the primary end point of the study, the sample size (n=150 patients), was calculated with COX Proportional Hazard regression, statistical power 91%.

Baseline CTC values, CTC values at two time points, and standard clinical factors including age, stage, performance status (PS), histology, smoking status, sites of metastasis, and treatment received (platinum-based doublet therapy *v* single-agent therapy) were subjected to univariate Cox proportional hazards regression analysis for both subsequent time to progression (STP) and overall survival (OS). Univariate significant parameters were included in a multivariate Cox proportional hazards regression analysis (forward stepwise selection [Wald method]; P =0.05 was selected for entry into the model, and P =0.1 was selected for removal). STP was measured from date of baseline blood sample to date of confirmed clinical progression or death or was censored at last follow-up. OS was measured from date of baseline blood sample to date of death or was censored at last follow-up. Statistical analysis was performed using Statistical Package for the Social Sciences for Windows version 13.0.2004 (SPSS, Chicago, IL), where $P \leq$ 0.05 was considered significant. The association of CTCs with individual clinical characteristics, including stage, PS, histology, smoking status, and sites of metastasis, were compared by Fisher's exact test or Chi square test, where appropriate.

RESULTS

1. Percentage of CTC-positive patients and total CTC numbers at baseline

1.1 Patients' demographics

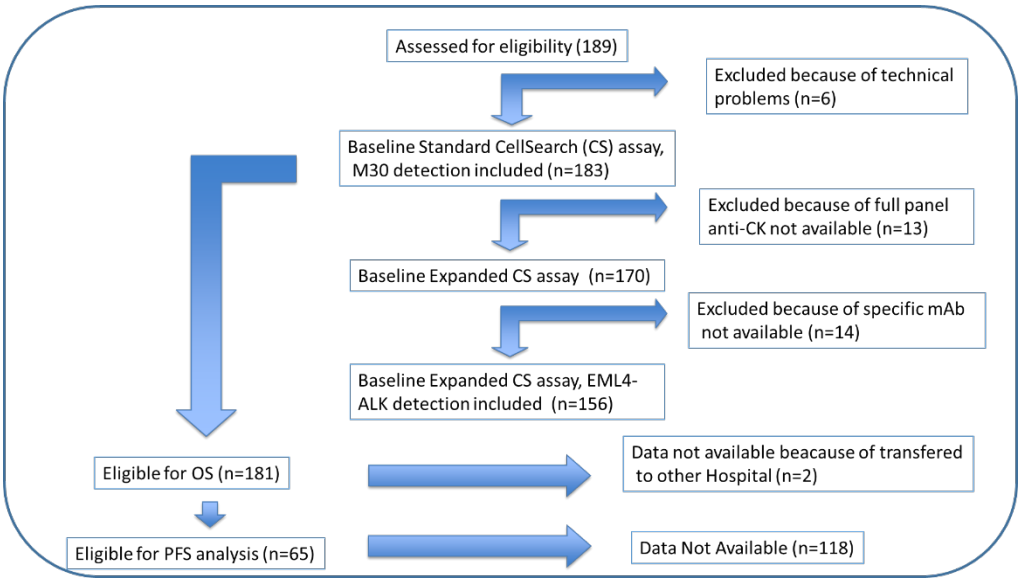
Between December 2012 and June 2016, we consecutively enrolled in the study 189 patients, suffering from stage IV NSCLC. Of them 66 were women (34.9 %) and 123 were men (65.2%); age range was from 28 to 86 years (median 65). In 154 out of 189 patients, the histology was Adenocarcinoma (81.5%), meanwhile in 12 out of 189 was Squamous carcinoma (6.3%). Table 2 summarizes the Demographical characteristics of the cohort.

Table 2. Demographic characteristics of 189 NSCLC patients

		Nr		%
Patients		189		100
age				
	Mean ± SD	64.52 ± 10,72		
	Median (range)	65 (28 - 86)		
Gender				
	Male	123		65,1
	Female	66		34,9
Performance Status at baseline CTC run (ECOG)				
	0	34		18
	1	91		48,1
	2	24		12,7
	3	12		6,3
	4	3		1,6
	Missing	25		13,2
Performance Status at baseline CTC run (ECOG)				
	0	34		18
	1	91		48,1
	2-3-4	39		20,6
	Missing	25		13,2
Stage				
	stage IIIb	4		2,1
	stage IV	142		75,1
	non specified	15		7,9
	Missing	28		14,8
NSCLC Histology				
	adenocarcinoma	154		81,5
	squamous	12		6,3
	non-specified	1		0,5
	other	7		3,7
	unknown	1		0,5
	Missing	14		7,4
Smoking Status				
	never	37		19,6
	ex	65		34,4
	current	39		20,6
	unknown	23		12,2
	Missing	25		13,2
Baseline Cellsearch Total CTC SA (183 paz)				
	Mean ± SD	2.81 ± 9.81		
	Median (range)	1 (0 - 100)		
Baseline Cellsearch Total CTC EA (147 paz)				
	Mean ± SD	2.63 ± 5.32		
	Median (range)	1 (0 - 46)		
TOTAL_CTC_cod				
	Negative	85		45
	Positive	98		51,9
	Missing	6		3,2

The fig. 5 reported the Consort Diagram. We excluded 6 patients from the analysis because of technical problems; meanwhile 13 patients were excluded from Expanded Assay because the full panel of anti-CK antibodies was not available. Speaking about details of EML4-ALK detection, a further group of 14 patients were excluded because the specific antibody was not available at the time of their enrolment.

Fig.5. Consort Diagram



1.2 Prevalence of CTCs using standard assay and expanded assay at baseline

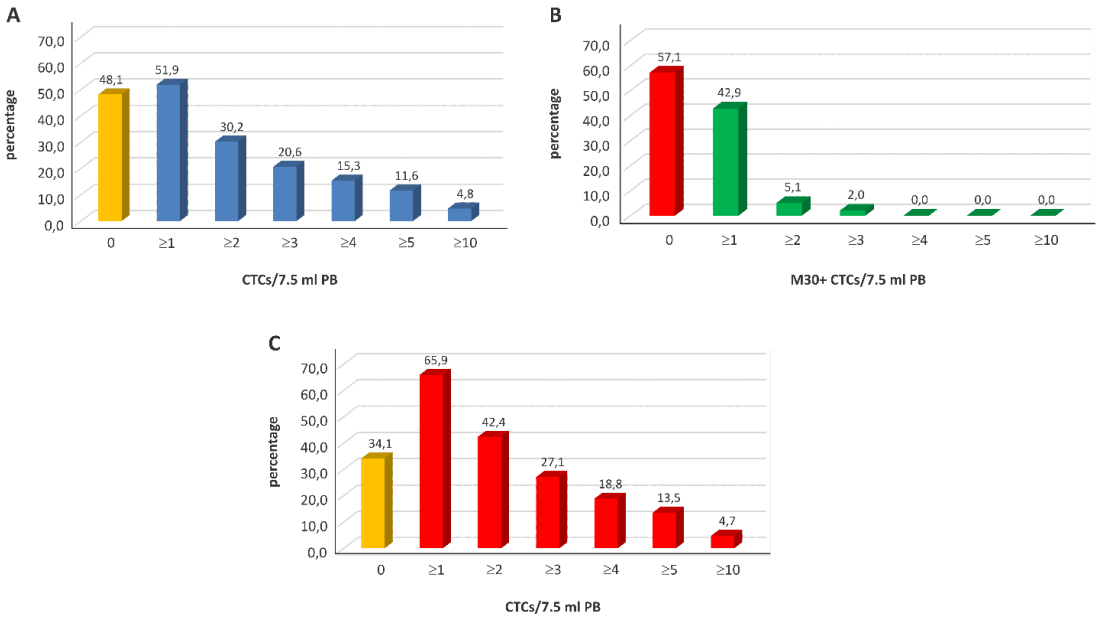


Fig. 6. A) prevalence of CTCs number using SA; B) prevalence of M30+CTCs in CTC positive patients; C) prevalence of CTCs number using EA

Speaking about CTC levels, as determined by the CellSearch standard assay (SA), the number of CTCs ranged from 0 to 100 cells (median 2). More in details, we can describe our cohort as following (see fig. 6A):

- 98 out of 189 NSCLC patients (51.9%) had at least one CTC in 7.5 ml of blood (range 0-100);
- 57 out of 189 (30.2%) had two or more CTCs;
- 39 out of 189 (20.6%) patients had 3 or more CTCs,
- 29 out 189 (15.3%) patients had 4 or more cells,
- 22 out of 189 patients (11.6%) had 5 or more CTCs.

Interestingly, the percentage of patients with 2 or more CTCs resembles the data previously reported by Krebs and colleagues, where only 60 patients with IV stage of NSCLC were studied [4].

The presence of CTCs at baseline did not show any association with the detection of distant metastasis (lung, mediastinal LN, liver or bone) as previously reported by other studies that included NSCLC patients of stages III and IV.

Furthermore, when we evaluated the viability of CTCs by M30 expression in CTC-positive patients, we found that 42 out of 98 CTCs-positive patients (42.9%) had one or more M30–positive CTCs (fig. 6B). The number of M30-positive CTCs ranged from 0 to 3 cells (median 0).

Overall, the percentage of patients that showed at least one CTC and one M30-positive CTC seems to be lower compared to other epithelial cancers [69]. The presence of CTCs and M30-positive CTCs at diagnosis was not associated with any specific clinic-pathologic features.

In NSCLC, CTCs show a different cytokeratin (CK) pattern compared to other carcinomas [3] and CellSearch Standard assay recognizes CK 8,18 and 19 but no other cytokeratin usually expressed in NSCLC.

At baseline, for every patient we analysed the frequency of CTCs with SA, but in the second blood draw we used CS with an expanded CK assay (see Mat. and Meth.).

We noticed that:

- 112 patients out of 170 (65.9%) had at least 1 CTC in 7.5 ml of blood:
- 72 out of 170 (42.4%) patients had 2 or more CTCs;
- 46 out of 170 (27.1%) had 3 or more CTCs;
- 32 (18.8%) patients had 4 or more CTCs.

Expanded Assay documented a slight increase in the number of CTC-positive patients and in patients with 2 or more CTCs compared to Standard Assay (fig. 6C).

Table 3. CTC standard and Expanded Assay Frequency

		CTC SA: Neg	(%)	CTC SA: Pos	(%)	CTC EA: Neg	(%)	CTC EA: Pos	(%)
Geder									
	Male	65	54,6	54	45,4	37	38,1	60	61,9
	Female	19	30,2	44	69,8	15	30,0	35	70,0
Performance Status at baseline CTC run (ECOG)									
	0	13	39,4	20	60,6	10	37,0	17	63,0
	1	43	49,4	44	50,6	25	33,8	49	66,2
	2-3-4	15	39,5	23	60,5	11	33,3	22	66,7
Stage									
	stage IIIb	1	33,3	2	66,7	0	0,0	2	100,0
	stage IV	58	42,3	79	57,7	38	31,9	81	68,1
	non specified	8	57,1	6	42,9	7	58,3	5	41,7
NSCLC Histology									
	adenocarcinoma	65	43,6	84	56,4	41	32,8	84	67,2
	squamous	5	45,5	6	54,5	3	42,9	4	57,1
	other	5	62,5	3	37,5	3	50,0	3	50,0
Smoking Status									
	never	18	50,0	18	50,0	8	25,8	23	74,2
	ex	29	44,6	36	55,4	18	34,6	34	65,4
	current	17	47,2	19	52,8	14	41,2	20	58,8

We found an association between sex and CTCs positivity in women: as a matter of fact, women who have NSCLC, are twice as likely to be CTC-positive compared to men (Pearson Chi-square p-value =0.003).

Furthermore, we investigated the presence of CTCs in patients labelled on the basis of smoking status. In this case, EA detected a number of CTC-positive subjects higher than the SA (table 3), in particular in the never-smoking group of patients, in which we counted 23 CTC-positive patients out of 31 (74.2%) by EA, meanwhile by SA we found only 18 CTC-positive out of 36 patients (50%).

1.3 Subsequent Time to Progression (STP) using standard and expanded assay

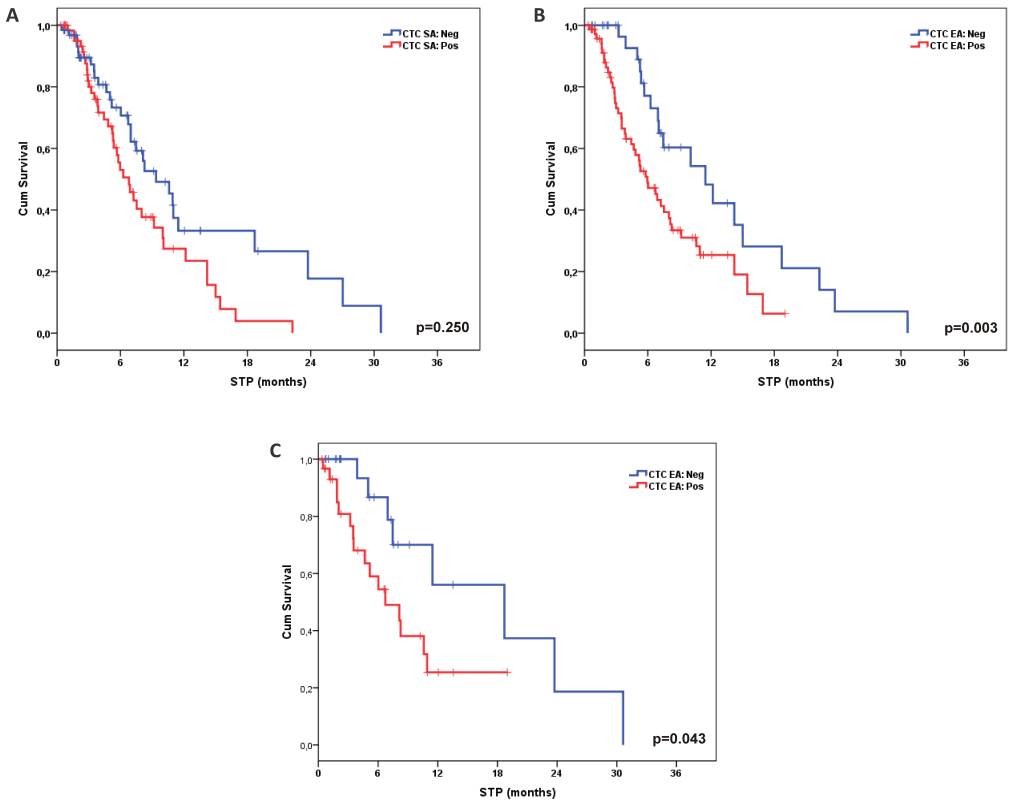


Fig 7. Prediction of STP based on CTC count as determined by SA (A) and EA (B). C) Prediction of STP in CTC-negative patients, as determined by SA, and then analysed with EA

In order to investigate the prognostic value of CTC count by using the SA and EA at baseline, we analysed the STP of our cohort.

CTCs detected with the expanded CK panel were associated with a poorer prognosis compared to Standard Assay. In fact, CTC-positive patients had a significantly lower median STP (133 days) compared to CTC-negative patients (224 days; Kaplan-Meyer, Log-Rank test p = 0.003). Conversely, when we stratified patients according to SA results, we did not found any statistically

significant difference between STP of CTC-positive and CTC-negative patients (159 and 244 days, respectively; Kaplan-Meyer, Log Rank test p = 0.250).
Moreover, because the fork was better opening between CTC-positive and CTC-negative patients in the first 10 months by EA, we could identify, with higher incisiveness, patients at higher risk of progression in this time window, compared to CTC status as determined by SA.

Notably, when we reanalysed the low risk group as determined by SA (54 CTC-negative patients) by using EA, the CK expanded assay identified 31 patients with discordant CTC status compared to the standard panel.

The SA-negative but EA-positive patients showed poorer prognosis if compared with patients that gave negative results by the both assays: the STP was 6.7 (red line) vs 18.7 months, respectively (Kaplan-Meyer, Log Rank test p=0.043).

1.4 Overall Survival using standard and expanded assay at baseline

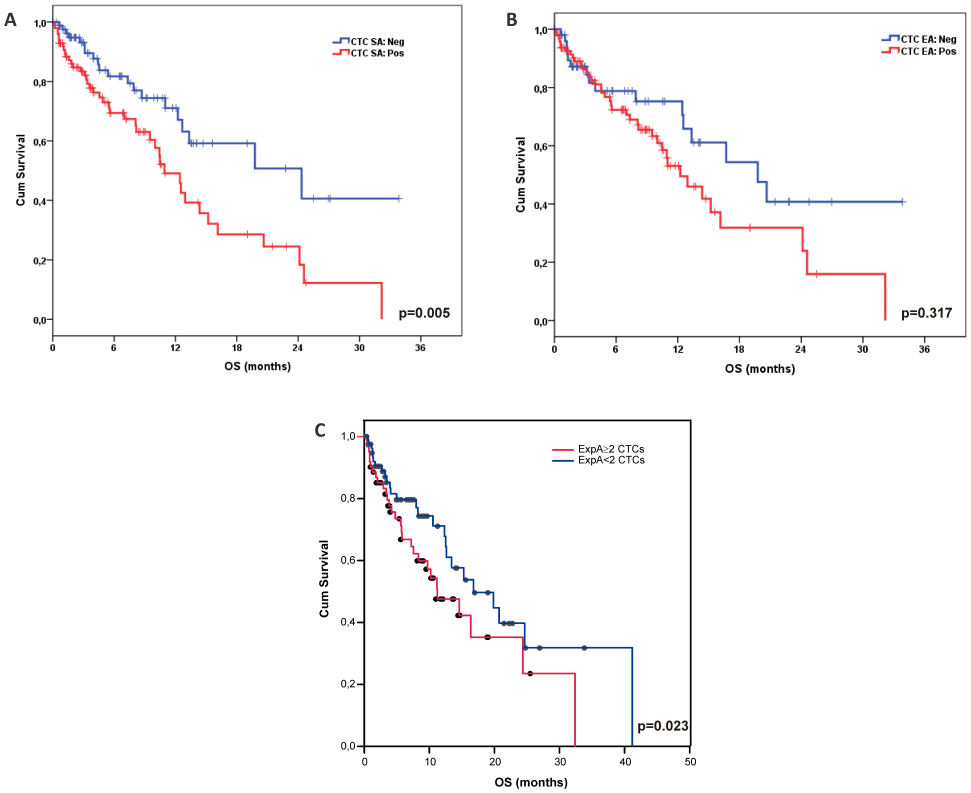


Fig.8. Prediction of OS based CTC count by using SA (A) and EA (B). C) Prediction of OS by using a cut-off value of ≥2 CTCs analysed with EA

Regarding OS, in all 189 patients, the median OS was 198 days. Figure 8A shows the Kaplan-Meyer plot for prediction of OS using baseline CTC count after subtraction of apoptotic CTCs number. CTC count at baseline identified a favourable group, the CTC-negative patients, which showed a median overall survival of 24 months (95% CI 9.9) compared with 10.9 months for the unfavourable group, represented by the CTC-positive patients (95% CI 8.5) (Kaplan-Meier, long rank test, p=0.005).

When we used EA in order to predict OS (fig 8B), we identified two groups of subjects, the CTC-negative patients (favourable group) that showed a median OS of 19.7 months, and a risk of progression lower than CTC-positive patients (median OS 12.3 months; Kaplan-Meier, long-Rank test p=0.317). However, in Kaplan-Mayer plot it is clear that, in the first 6 months, this test was unable to predict a different OS for the CTC-positive and CTC-negative patients.

Furthermore, when we used 2 CTCs as cut-off value in order to predict OS, we identified two groups of patients, i.e. CTCs<2 (favourable group) that showed a median OS of 16.7 months, and a lower risk compared to patients with CTC≥2 which showed a median OS of 11 months (Long-Rank test p=0.023) (fig. 8C).

Table 4. Univariate and multivariate analysis

Variables	B	SE	Wald	df	Sig.	HR	Lower	Upper
Age_baseline (var continued)	-0,005	0,012	0,189	1	0,664	0,995	0,971	1,019
Total CTC SA pos vs. neg	0,758	0,287	7,002	1	**0,008**	2,135	1,217	3,743
Total CTC EA pos_vs_neg	0,442	0,319	1,913	1	0,167	1,555	0,832	2,908
gender M vs F	0,431	0,304	2,010	1	**0,156**	1,539	0,848	2,794
PS 1 vs. 0	0,433	0,388	1,244	1	0,265	1,542	0,720	3,299
PS 2-3-4 vs. 0	1,396	0,409	11,651	1	**0,001**	4,038	1,812	9,000
Smoking Status ex vs. never	0,464	0,365	1,619	1	0,203	1,591	0,778	3,25
Smoking Status current vs. never	0,061	0,41	0,022	1	0,883	1,063	0,475	2,375
EGFR No mut vs. Mut (68 paz)	0,403	0,55	0,536	1	0,464	1,496	0,509	4,397
Previous 1stline stemic treatment	0,456	0,624	0,534	1	0,465	1,578	0,464	5,363
Colonna1	**Colonna2**	**Colonna3**	**Colonna4**	**Colonna5**	**Colonna6**	**Colonna7**	**Colonna8**	**Colonna9**
Multivariate Analysis for OS with Cox regression (adenocarcinoma 153 patients)								
						95,0% CI for HR		
Variables	B	SE	Wald	df	Sig.	HR	Lower	Upper
Total CTC SA pos vs. neg	0,775	0,297	6,82	1	**0,009**	2,17	1,213	3,881
PS 1 vs. 0	0,615	0,4	2,366	1	0,124	1,85	0,845	4,053
PS 2-3-4 vs. 0	1,437	0,418	11,815	1	**0,001**	4,209	1,855	9,552

Univariate and multivariate COX proportional hazards regressions were performed, to assess the association between factors of interest and OS in adenocarcinoma patients (n=153). In univariate analysis, the clinical factor significant for survival was performance status (p=0.001) and the presence of CTCs. For CTC univariate analysis, patients were divided into favourable (CTC-negative patients) and unfavourable patients (CTC-positive patients) prognostic groups, in which we detected CTCs by SA at baseline (p=0.008). The performance status and the CTC level maintained their association in multivariate analysis.

2. Molecular and phenotypic profiling of CTCs and CTCs count modifications as an early predictor of response to treatment.

2.1 CTCs Characterization: EML4-ALK

In order to choose an appropriate first-line treatment regimen, detection of EGFR mutation and ALK rearrangement are recommended as routine genetic profiling for non-squamous NSCLC or non-smoking populations. FISH, RT-PCR and Ventana IHC are all currently accepted methods for the detection of ALK rearrangement in biopsies. Characterizing CTCs could offer new perspectives both for the diagnosis and the monitoring of ALK-positive patients eligible for treatment with ALK inhibitors. We implemented CS assay with an antibody against EML4-ALK protein fusion usually used in IHC (clone 5A4, AbCam).

We set the protocol using three cell lines: H460, a wild type cell line, A549 with a part of cells with EML4-ALK translocation, and H2228 with variant 3 of EML4-ALK translocation; then, we validated this assay in patients CTCs.

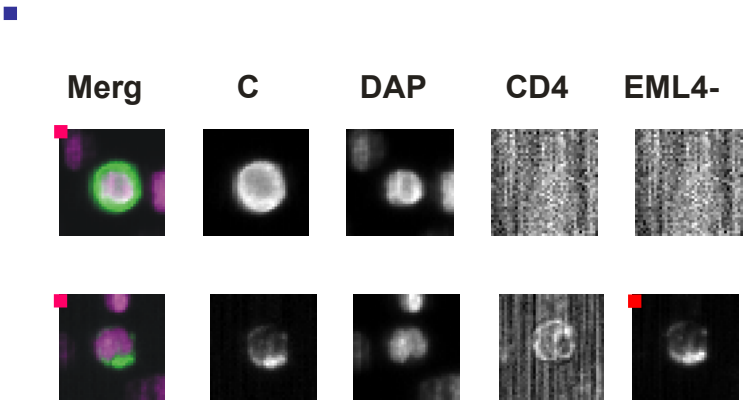


Fig.9. Development of phenotypical CTC assay for EML4-ALK expression. The photo gallery show, in the first row, an EML4-ALK-negative CTC, and, in the second row, an EML4-ALK-positive CTC, respectively.

At baseline we observed the presence of EML4-ALK CTCs+ in 32 out 102 patients (31.4%) (fig.10A). This percentage is very high, considering that only 3–

7% of NSCLC patients were reported to be positive on biopsy. In order to verify the accuracy of this test, we analysed in biopsy specimen the presence of EML4-ALK translocation with FISH analysis (Gold Standard). A biopsy was available for only 11 of these patients and the FISH analysis confirmed the presence of EML4-ALK translocation. Interestingly, not all CTCs detected were EML4-ALK positive, than in the blood stream we identified different populations of CTC.

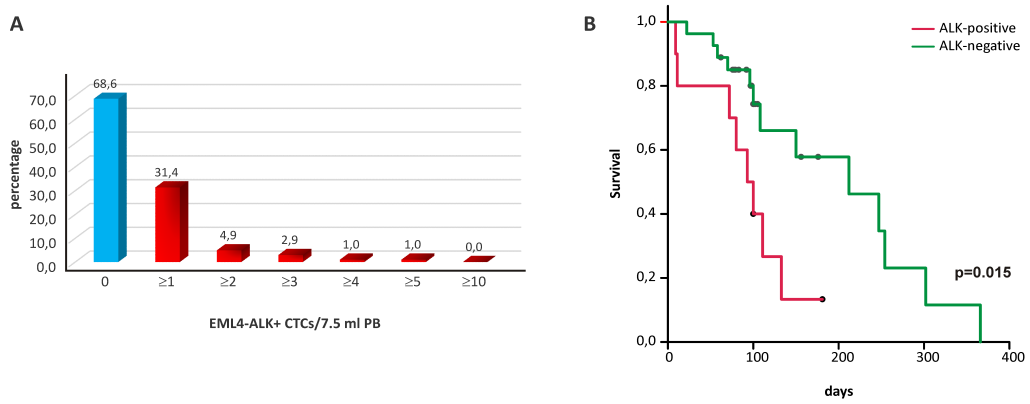


Fig 10. A) Prevalence of EML4-ALK-positive CTCs and in B) Prediction of OS based on EML4-ALK expression on CTC

Patients whose CTCs expressed EML4-ALKpresented a shorter progression free survival (97/days) compared to EML4-ALK CTC- patients (200 days) (fig.10B).

2.2 Dynamic changes in the CTC number after the first cycle of Treatment and OS

After the first cycle of treatment, the CTCs were measured using SA in 119 patients available for evaluation; the global median OS was 16.1 months.

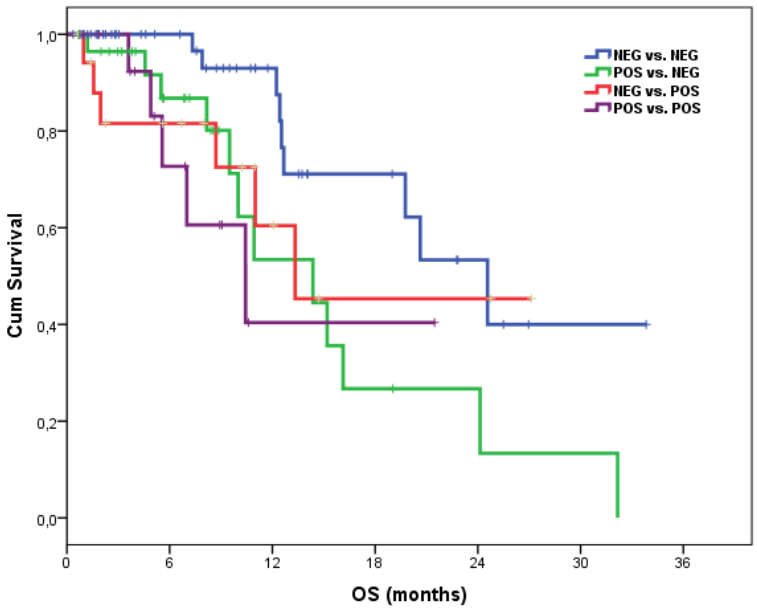


Fig.12. Prediction of OS based on dynamic change CTCs number before and after first cycle of treatment detected using SA.

In figure 12, Kaplan-Meyer shows a significant difference in the OS prediction for these 4 groups (Chi Square 8.378, DF 3, p=0.039). 15 patients were positive at baseline and remained positive after the first cycle of treatment (group 1). This group had a significantly shorter median OS (10 months) compared to the 55 patients that at baseline and after the first cycle of treatment remains CTC-negative (group 2 24.5 months). The median OS of the 31 patients who were CTC-positive at baseline and CTC-negative after the first cycle of treatment (group 3) was 14.3 months. In the patients group who transitioned from CTCs positive to negative, after the first cycle of treatment, we observed an intermediated risk compared to groups of patients always negative for CTCs and always positive. For the last group (group 4) which included 18 patients CTC-negative at baseline that became CTC-positive after the first cycle of treatment, we observed a higher risk compared to group 1 (always CTC positive) and a very similar risk to groups 3 (13.3 months).

Regarding EA, we were able to analyse 104 out of 189 pts at the end of the first cycle of treatment only because of problems with the reagents supply. We didn't

observe any statistical difference concerning changes in the CTCs level as determined before and after the first cycle of treatment.

2.3 Dynamic changes in the CTCs number and phenotype during the follow-up

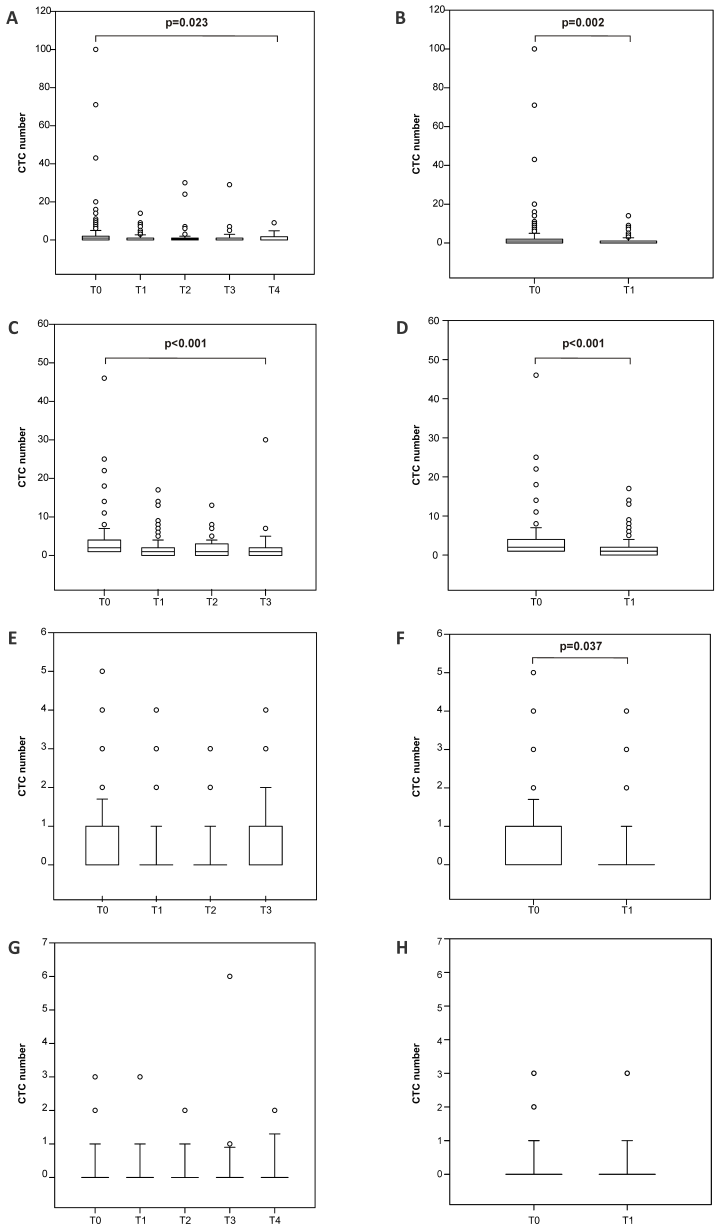


Fig. 11. CTCs count modifications during all period of observations and after the first cycle of treatment using SA (A and B), EA (C and D) EML4-ALK assay (E and F) and M30 assay (G and H).

To investigate whether the integrated test may predict a therapeutic response in NSCLC, we sequentially assessed the frequency of CTCs by SA and EA, and the expression, onto CTCs, of M30 and ALK-EML4, in 119 evaluable patients, during first-line of treatment.

To evaluate the CTCs count modifications as an early predictor of response to treatment, we analysed the CTCs count with SA and EA at baseline, after the first cycle of therapy, at radiological assessment and at progression. As shown in picture 11A-B, using SA the difference in median number of CTCs is statistically significant in the five groups (Kruskall-Wallis one way analysis H=11.3 fd=4 p=0.023). Notably, the major reduction in the CTCs number was between T0 and T1 at the end of the first cycle of therapy (Mann-Withney rank sum test p=0.002).

In figure 11C-D, using EA we observed a decrease in the number of CTCs during the period of observation (median 24 months) but the CTCs did not disappear completely. However, due to the lack of reagents supply, the number of evaluable patients analysed, drastically decreased after the first two check points, therefore, this analysis is not significant. Nevertheless, if we focus our evaluation on the first two points, we observe a significant decrease of the CTCs number after the first cycle of therapy (p=0.001 Mann-Whitney sum Test). Notably, using both assays, we noticed that after the first cycle of treatment the CTCs decreased, but in the following time points, we observed patients with persistence of CTCs.

In order to evaluate if the phenotype and the molecular characterization of CTCs could provide a more sensitive marker for rating pharmacodynamics effects compared with the total CTC count, we contextually analysed ALK-EML4-positive CTC changes.

In our cohort, we evaluated CTCs at baseline, at the first radiological assessment and at progression. We evaluated also the presence of the M30 apoptosis marker (fig. 11G-H) and the expression of EML4-ALK fusion protein (fig. 11D-E). We observed that after the first cycle of treatment and at the radiological assessment, the number of EML4-ALK-positive CTCs was reduced or disappeared, on the

contrary, at the progression the ELM4-ALK-positive CTC reappeared, to what we similarly observed in the first case report (see below).

To address the question of efficacy of treatment in NSCLC, we evaluated the presence of apoptotic CTCs in these patients. Throughout the period of observation, in our cohort, the number of M30-positive CTCs was very low and the changes between the different time points are not statistically significant ($p=0.233$), unlike what we previously reported in other metastatic diseases [69].

Even in the first two observations, the change in the number of M30-positive CTCs was not statistically significant. During the treatment we noticed that the amount of M30-positive CTCs did not increase.

This points to the fact that chemotherapy and/or target therapy hit the cancer cells, but this effect is shown only by a decrease in the CTCs number and not in an increase of apoptotic CTCs, contrary to what we previously demonstrated in other malignancies [69].

2.4 Detection of EGFR Mutation on CTCs

We investigated the presence of EGFR mutations on CTCs in 10 patients that were CTC-positive at baseline. The number of CTCs in NSCLC was very low and therefore, the isolation procedure did not have a sufficient capability of recovery, to obtain pure CTCs in such case; then, in order to obtain mutational information we used the cartridge content rather than a single CTC. Furthermore, we had to apply the WGA procedure and ddPCR to detect EGFR mutations.

Our main findings were that CTCs harbour EGFR mutations, also detected in tumour biopsy, and, secondly, in patients that were progressing during the treatment we detected the EGFR T790M in CTCs before the clinical progression, as documented by imaging (PET or CT).

2.5 Comparison between CTCs and cfDNA

In order to have an overview of the circulating compartment, we compared CTCs and cfDNA in a small group of patients, regarding the presence of the EGFR activating mutations at baseline and during the treatment, by using digital PCR (ddPCR). At the baseline, the level of cfDNA was investigated in 80 patients at baseline and in the follow time point. We analysed cfDNA also 14 healthy donors.

As demonstrated by other authors [47] cfDNA levels in NSCLC patients are markedly higher than in the healthy donors control group ($P<0.001$, Fisher exact test). In 15 NSCLC patients we could identify, by ddPCR, the EGFR mutation histologically detected in the whole group of samples. Moreover, we confirm the role of circulating tumour DNA in reflecting changes in the disease status. In fact, as you can see in the figure 14, at the time of progression there is a rebound in DNA amount for both of these representative patients.

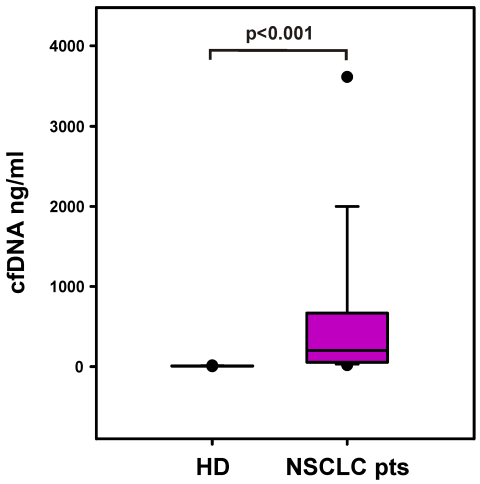


Fig. 13. Box Plot distribution of cfDNA concentration in healthy donor (HD) and NSCLC pts at baseline.

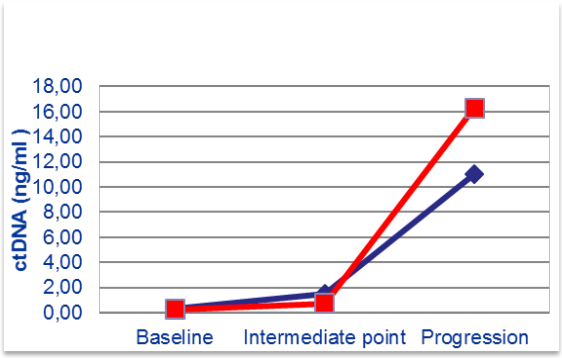


Fig.14. ctDNA trends in two progressing NSCLC patients

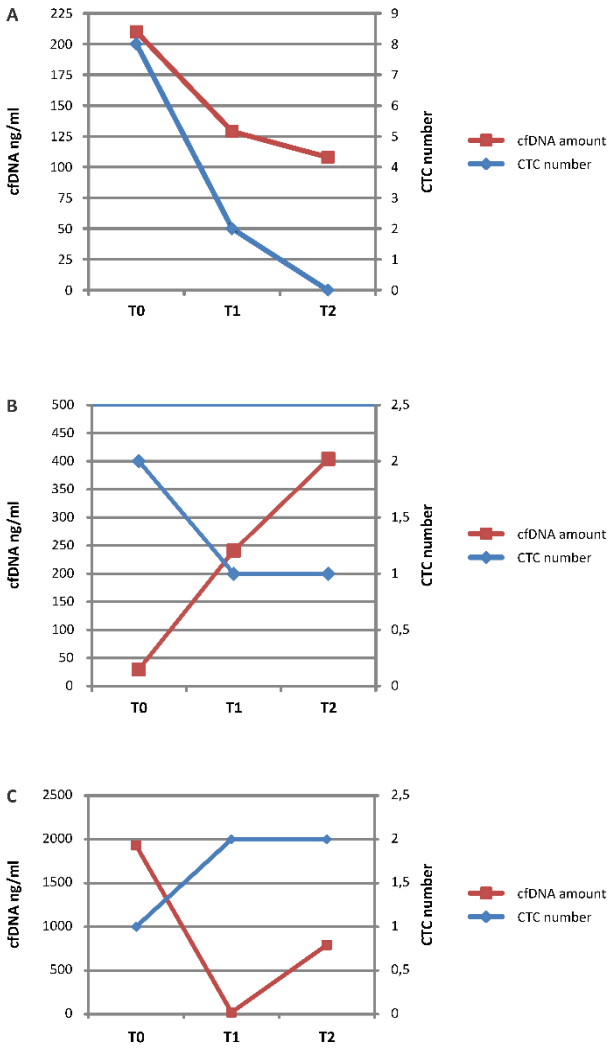


Fig. 15. Anlysis of cfDNA and CTC during the follow up. A) Concordant group with decrease of CTCs number and cfDNA concentrations, B) Concordant group with either CTCs persistence and cfDNA increase, C) Discordant group.

During the target treatment in this small group of patients, we observed the 2 following scenaria:

- A group of patients in which these two tests were concordant, i.e. both showed an increase (or a decrease) in the CTCs number and in the cfDNA mutations;
- A group of patients where these tests were discordant (fig.15).

3.0 Potential use of CTCs information to monitor and to manage patient therapy.

3.1 *First case report: EML4-ALK inhibitor*

In January 2013, a 39 year-old woman never smoker was diagnosed with EML4-ALK metastatic adenocarcinoma of the lung (bone, liver). Table 1 summarizes the timeline of the treatment schedule that she underwent, according to the clinical-instrumental reevaluation of her disease.

At first, the patient received a combination of chemotherapy with carboplatin (AUC 6) + Paclitaxel (200 mg/m^2) + Bevacizumab (15 mg/Kg) once every 3 weeks. She also received radiotherapy and Zoledronic acid for treating discomfort caused by bone metastases.

A computed tomography (CT) scan was performed after two cycles of chemotherapy, and revealed the disease progression. Therefore, because we could document the ELM4-ALK mutation, the patient received Crizotinib, 250 mg orally twice a day as a second line of treatment. The subsequent CT scan (July 2013) showed a partial response.

The Crizotinib treatment was then maintained with substantial disease control until March 2014, when, due to worsening conditions, the patient began a combination therapy with LDK378 (Ceritinib), a second-generation EML4-ALK inhibitor, plus AUY-922, a novel intravenous heat shock protein 90 (Hsp90) inhibitor (ClinicalTrials.gov Identifier: NCT01772797).

In May 2015, due to the progression of the disease, the patient started chemotherapy with Pemetrexed 500 mg/m^2 once every 3 weeks. The treatment was stopped at the end of the first cycle because of severe anemia.

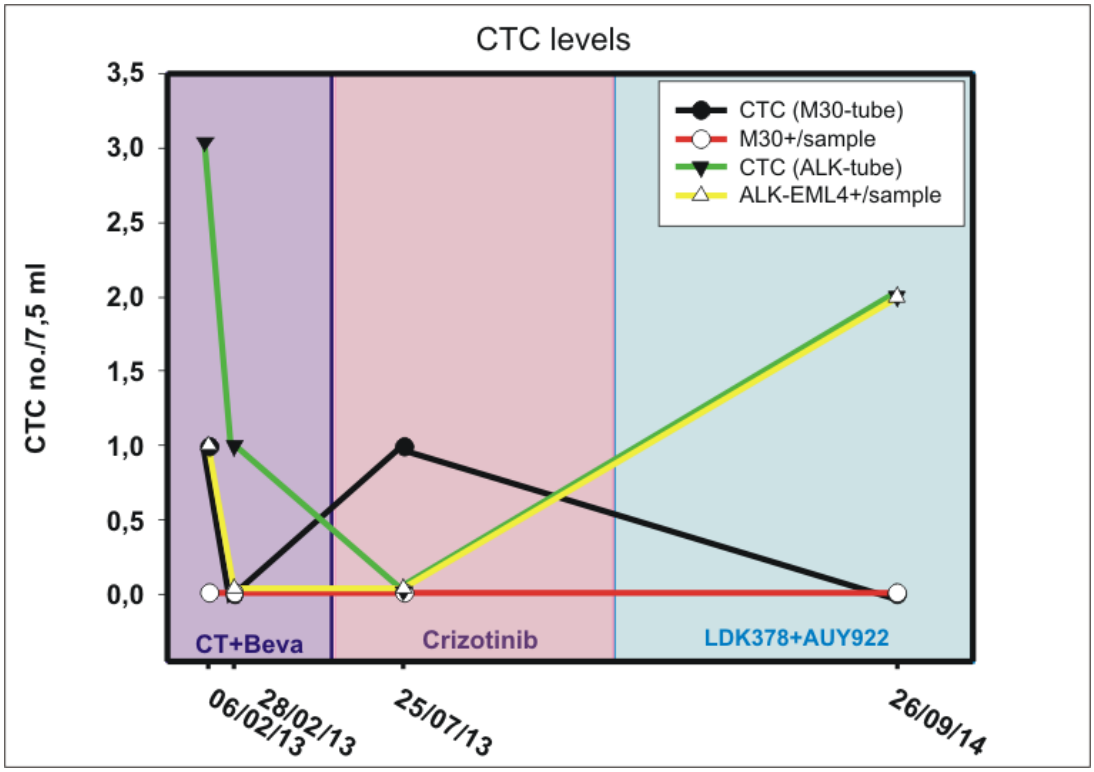
Subsequently, in August 2015, she underwent compassionate use of Alectinib; the patient's death occurred on 5 October 2015.

As shown in figure 16, during the Crizotinib therapy, the patient underwent a sequential monitoring of CTC levels. As previously [69, 71], we analysed two blood draws simultaneously. We used the standard CellSearch assay (IVD) and a customized test with an anti EML4-ALK antibody (as described in material and

methods) in order to evaluate if the EML4-ALK integrated assay reflects the dynamic changes of EML4-ALK+ CTCs under the selective pressure of a specific treatment. For this purpose, we evaluated CTCs at baseline, at the first radiological assessment and at progression.

The CTC analysis revealed the presence of EML4-ALK+ CTCs at baseline (3 CTCs, 1 EML4-ALK+ CTC) and at the relapse (2 CTCs, both cells were EML4-ALK+) suggesting that the disease had developed resistance to the drug. This highlights the fact that when the disease was in response, we could show CTC persistence (1 cell on July 25th 2013), but this CTC was negative for EML4-ALK.

Fig16. The plot shows longitudinal graph of CTC levels from February 2013 to September 2014



By accurately assessing the cumulative changes of live/apoptotic CTCs number under chemotherapy, we disclosed the predictive relevance of apoptotic CTCs in MBC [69]. To address the question of efficacy of treatment in NSCLC, we evaluated the presence of apoptotic CTCs in this patient. Throughout the period of observation, the CTCs detected were M30 negative therefore, CTCs are viable (Fig. 16 red line).

3.2 *Second case report: EGFR inhibitor*

In November 2015 a 65-year-old patient came to see us in a day hospital setting due to exertional dyspnea along with chest pain. He was a former smoker and reported significant passive smoke exposure in childhood.

The physical examination showed decreased breath sounds with prolonged expiration, notably in the right side of the chest.

During the lung examination crackles were present in the lower lobes and scattered whistles and groans were present, too. In relation to blood tests, the white blood cells count was 11x103/µl with 80% of neutrophils, hemoglobin 13.8 g/dl.

The spirometry revealed normal values. The main comorbidity was hypertension.

A CT scan showed a large mass located in the upper left lung of 5.5 cm diameter involving peri-hilar lymph-nodes.

A bronchoscopy was performed with biopsies. The histology revealed an adenocarcinoma P63- and TTF+. The mutation status for EGFR and ALK was negative using Sanger sequencing. Hence, the patient began a neo-adjuvant treatment with Cisplatin 80 mg/m^2 d 1 and Vinorelbine 30 mg /m^2 d 1.8 every 3 weeks. A CT check showed a partial response, the main lesion being reduced by half (2.3 cm) according to the RECIST 1.1 criteria.

Therefore, the patient underwent lung resection by left upper lobectomy and removal of local lymh-nodes. According to the TNM staging system, the initial stage was T2aN1M0.

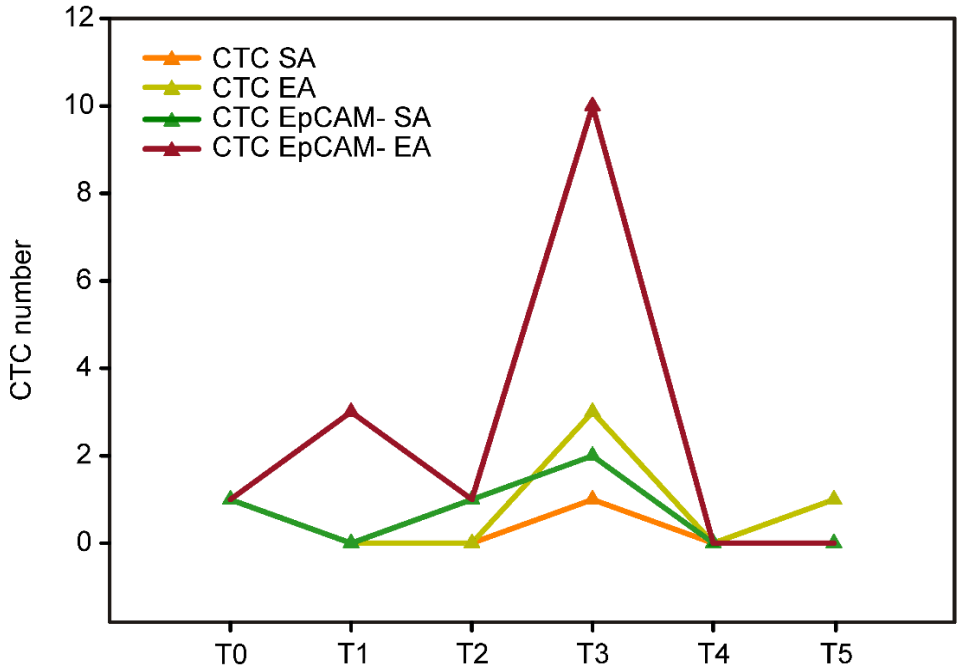


Fig. 17. The plot shows longitudinal graph of levels of EpCAM+ (in light green and orange) and EpCAM low/neg (green and brown) CTCs.

As previously, [69, 71], we analysed two blood draws simultaneously. We used the standard CellSearch assay (IVD) and the expanded assay, in addition we evaluated the fraction discard by CS, with an Autoprep Sample Collection Device (ASCD), in order to identify CTCs with low/negative expression of EpCAM.

The cells recovered by size were stained like EpCAM+ cells [16].

In November 2015, we collected the baseline blood draws in order to analyse the CTC level (fig.17) and cfDNA before starting any treatment. Using SA and EA, we detected 1 CTC per 7.5 ml of peripheral, we applied WGA and analysed the mutational status of CTCs. We observed a mutated EGFR (ex 21 L858R). When we analysed cfDNA, we detected the same mutation.

Following scheduled blood draws collection, after a cycle of adjuvant treatment according to the NCCN guideline, we analysed the second blood draw for CTC level and cfDNA. We observed the absence of EpCAM-positive CTCs, but the presence of 2 EpCAM-negative CTCs. The Exon 21 (L858R) mutation in cfDNA

was present. At radiological assessment, cfDNA did not detect any mutation and CTCs detected harboured EGFR L858R.

In March 2016 we observed the following scenario: 1 CTC by using SA, and 3 CTCs by using EA, 10 CTCs EpCAM-negative; the CTCs characterization showed the presence of mutation L858Rin CTCs detected with the expanded assay. On the contrary, cfDNA revealed only wt cfDNA in the blood stream.

In May 2016 a bio-molecular analysis was performed on a surgical sample from lobectomy that showed an exon 21 (L858R) mutation of EGFR.

The patient received adjuvant treatment according to the NCCN guideline for three courses with Cisplatin and Vinorelbine, which followed the same schedule of the previous treatment.

The patient was only observed subsequently. After three months however, a progression was detected, located on the bone and on the lung.

So the patient began the treatment with a second generation tyrosine kinase inhibitors (TKIs), such as Afatinib 40 mg a day. Despite a good tolerability of the drug, the patient had a new progression to the brain two months after starting therapy. Therefore, the patient underwent radiotherapy, at first with the stereotactic technique and on mediastinum consisting of 20 sessions of 2 Gy dose. A new PET-CT in November showed a good response with thoracic down staging.

In December 2016 we analysed the last blood draw and we observed that cfDNA was persistently wild type, meanwhile CTCs revealed a L858R mutation and a T790M mutation that is a signal of emerging resistance to the TKIs treatment.

3.3 NSCLC circulating compartment and NGS

In NSCLC, oncologists actualize the model of oncogene-driven targeted therapies, given the genomic targets and related target therapies. However, in order to apply this model, we needed a source of tumour as a biopsy, which is the critical point in the case of NSCLC. In fact, patients with advanced disease are largely diagnosed with one of the following procedures: computerised tomography-guided percutaneous biopsy, ultrasonography-guided endoscopic biopsy with 18 gauge needles, with fine needle aspiration. With these methods the sample quantity and quality is limiting for histological and molecular tests. We think that CTCs and cfDNA could offer a source of tumour, which is minimally invasive, to detect target mutations and monitor target therapies.

For this reason, we studied 9 patients, using CS and Liquid biopsy platform (see Mat and Meth) combined with high through-output mutation detection technologies as NGS, in order to evaluate a large number of hot-spot mutation.

From the same blood draws we isolated CTCs and cfDNA. Regarding CTCs we used CS and Lbp with two enrichment cocktails composed of: (cocktails 1) EpCAM, Trop2 (EMT marker), Her2 and (cocktail2) EpCAM, TROP2, EGFR.

A sequencing pipeline was designed to support case control detection of single point variants to 1%.

A

sample	CTC CS	CTC LBP1	CTC LPB2
#1	1	3	10
#2	1	0	0
#3	1	159	63
#4	0	20	44
#5	1	19	51
#6	0	228	1181
#7	3	0	0
#8	2	8	9
#9	4	34	15

B

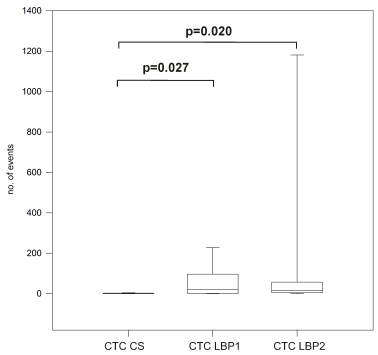


Fig.18. CTCs count and characterization by LiquidBiopsy platform and CellSearch

When we compared CS and Lbp regarding CTCs detection (fig. 18) in 7 patients, the two systems were concordant and detected CTCs; Lbp detected a higher number of CTCs compared to CS, in 4 patients the two systems differed (2 pts CTC+ by Lbp and CTC- in CS and 2 pts CTC- by Lbp and CS+, respectively). The difference in the CTCs number detected was significantly different only when comparing CS and LBP cocktail 1 (P = 0.027) or CS and LPB cocktail 2 (P = 0.020).

sample	CTC	cfDNA	Tissue
#1	wt	wt	wt
#2	wt	KRAS_G12C 6.9% e TP53_R280T_ 3.06	wt
#3	PIK3CA_E545K 2.03%	EGFR_L858R 2.08%	L858R
#4	wt	wt	L858R
#5	wt	wt	del19
#6	wt	EGFR_L858R 2.09%	L858R
#7	wt	wt	del19
#8	wt	wt	del19
#9	wt	TP53_R273L_14.07%	nd

Fig. 19. CTCs and cfDNA and biopsy specimen molecular characterization

Regarding molecular analysis, we observed, as previously described by Strauss W. M. and colleagues [77], that the variance of amplification efficiency manifested in cfDNA, demonstrated that about 50% of the amplicons in cfDNA amplified the target sequence less efficiently than control.

We performed an ultra deep sequencing of CTCs and cfDNA from 9 NSCLC patients. To evaluate SNV-SF from the different components of liquid biopsy we used Ion AmpliSeq™ Cancer Hotspot Panel v2 (CHPv2) target enrichment. Notably, CTCs isolated with the two LpB cocktails, were directly used to prepare library without the WGA step. We enriched for 50 specific cancer genes (see Mat. and Meth.) that covered about 2800 Cosmic mutations in these genes. All variants were filtered through yeld COSMIC validated mutations.

In 5 patients we observed no genomic alterations in CTCs or cfDNA, in 3 patients we observed genomic alterations only in cfDNA, and in 2 patients we observed genomic alterations in both CTCs and cfDNA.

One patient, at baseline, was positive for EGFR_L858R in cfDNA (2.08%) and CTCs presented a PIK3CA_E545K mutation (2.03%). The PIK3CA mutation is considered a potentially useful biomarker of resistance to EGFR-target therapy, and in these patients we could detect it before the start of treatment. We analysed

a blood draw from the same patient after the first cycle of treatment, and we noticed that the number of CTCs had decreased but had not disappeared and no mutations were detected in CTCs or cfDNA (fig.19).

DISCUSSION

Liquid biopsy in NSCLC is an attractive concept, because a simple blood test could offer a lot of information about cancer, prognosis, and efficacy of treatment and anticipates information about drug resistance. In this project, we explore the concept that the CTC count and their characterization could be a useful tool for oncologists. Liquid biopsy' researchers maintain a lively debate about which is better between CTCs and cfDNA, and so, where possible, we compared the data obtained from these two main actors in the circulating compartment.

Cell free DNA was identified in the plasma, for the first time, in 1948. The nucleic acids detected in the plasma derived from both normal and cancer cells. In cancer patients' ctDNA often shows the same genetic alterations present in tumour biopsies, and several different methods have been reported for detecting ctDNA. Cell-free DNA (cfDNA) is released from normal cells and tumours by programmed cell death (apoptosis) and comprises small fragments of nucleic acid that are not associated with cells or cell fragments. QIAGEN's circulating tumour plasma DNA test for EGFR mutations in NSCLC received CE-IVD status in January 2015.

In February 2015, the China Food and Drug Administration (CFDA) approved an update to the Gefitinib label for advanced NSCLC to include blood based diagnostics when tumour tissue is not evaluable. EGFR testing on plasma entered in clinical diagnostics in Europe, following the decision of EMA, allowing the use of cfDNA obtained from a blood sample for the assessment of EGFR mutation status in those patients for whom a tumour sample is not an option (EMA www.ema.europa.eu/docs/en_GB/document_library/EPARProduct_Information/human/001016/WC500036358.pdf.)

Molecular analysis of EGFR, at baseline, in cfDNA, was also a useful tool for prognosis. High concentrations of mutated copies in plasma of advanced NSCLC patients were associated with reduced survival. Interestingly, we observed that

high concentrations of total EGFR copies (mutated plus wild-type copies) were also associated with reduced survival. Even more, EGFR copy burden increased with tumour stage, suggesting a EGFR amplification in tumour that was reflected in cfDNA.

Circulating tumour cells are very rare in the blood stream; it was estimated as 1 cell per 10^6 or 10^7 leukocytes. In the past decade, a lot effort has been made to develop a robust method to detect and count CTCs. The major technical challenges in CTCs detections are obviously due to the rarity of these cells but also to their phenotypical and molecular heterogeneity. To date, the CellSearch platform (CS) based on enrichment by EpCAM, is the only method FDA approved for mBC, mPC and mCRC, and it was inserted as a prognostic biomarker in the ASCO, NCCN, AJCC and AIOM guidelines for early and metastatic breast cancer [12, 13], and adrenal cortical tumour (ACC). In lung cancer (NSCLC and SCLC) CTCs enumeration, as determined by CS, has been demonstrated to be prognostic. The isolation by size of ephitelial cells from the blood, ISET, demonstrated the prognostic value of CTCs in resected NSCLC patients.

As previously stated, in this project, we explore the concept that the CTCs count and their characterization could be a useful tool for oncologist.

Previous studies analysing CTCs in NSCLC using CellSearch detected only 35% CTC-positive metastatic patients [53, 78] and an increase of CTC detection was observed when researchers used EpCAM independent methods [79, 80].

In order to answer our first aim, our study was conducted in a homogeneous cohort of patients affected by stage IV NSCLC, we observed a higher frequency of CTC-positive patients (54.5%) with standard assay. Notably, if we use an expanded assay that includes cytokeratins 5/6, 7 and 14, we document a 65.5% of patients which showed 1 or more CTCs per 7.5 ml of peripheral blood. Unlike the Krebs and Allard studies [4, 53], we demonstrated that the only presence of 1 CTC/7.5ml of blood detected with SA, stratifies patients in two groups that

significantly differ for prognosis (24 months vs 10.9 months, CTC-positive vs. CTC-negative, respectively; Kaplan-Meyer, Log-Rank test p = 0.008).

Moreover, using EA we had to use 2 CTCs/7.5 ml as cut-off value in order to highlight patients with a poor prognosis. Regarding EA, we showed a better predictive value regarding STP, in fact, this test identified patients with a short subsequent time to progression until six months to start therapy (6 months vs 11.4 months; Kaplan-Meyer, Log-Rank test p = 0.008).

The second aim of our study was to characterize CTCs. For this reason, we set an integrated assay to highlight the EML4ALK protein fusion on CTCs and a workflow to test EGFR mutations.

To date, two groups demonstrated the feasibility to detect ALK rearrangement on CTCs, when enriched by filtrations [59, 81]. Our procedure is different and, in our opinion, more promising for clinical application, because it permits to directly quantify both the CTC burden in peripheral blood and the fusion protein expression at single cell level, through a robust automatized platform that is compatible with the multiple measurements required to monitor treatment response in individual patient.

In our hands, with our integrated assay specific for EML4-ALK detection, we could document the presence of EML4-ALK on CTCs and the short overall survival in patients positive for EML4-ALK CTC (3 months vs 7 months). It was intriguing to notice that not all the CTCs detected expressed the EML4-ALK protein and that during the treatment this subpopulation was modulated.

A recent publication postulated that the saw-toothed count pattern suggests that CTC dissemination in the blood is not always continuous but may occur in spurts during therapy. It thus follows that depending on the time of CTC measurement, different CTC counts will be obtained and in turn, different conclusions will be drawn about the nature of the patient's real-time therapeutic response [82].

Throughout our schedule of blood draws, we observed similar profiles in CTC modulation. Furthermore, very often in our cohort in correspondence with the blood draw at the time to progression, or in the time point immediately before, patients increased the CTCs number and/or, if the CTC level did not varied, we documented a change in the phenotype or in the number of mutated positive cells.

Notably, in the first case report in whom we monitored the CTC level and the EML4-ALK presence on CTCs, and their modulations under treatment, we are able to disclose the moment during the treatment when the disease developed drug resistance, earlier than the radiological assessment of disease progression.

In the second case report, we analysed and compared CTC modulations of EpCAM-positive and EpCAM-low/negative cells, the mutational status of CTCs, cfDNA, and the mutational status of the biopsy and surgical specimen.

Also in this patient, we observed a modulation in the CTC number for both EpCAM-positive and EpCAM-low/neg cells during treatment. Interestingly, CTCs reveal the presence of EGFR L858R at its baseline according with cfDNA. On the contrary, in the following time points, when the cfDNA was unable to track this mutation, CTCs revealed EGFR T490M, a mutation that confers resistance to TKIs therapy. Our results are consistent with what He and colleagues reported, i.e. that the secondary mutations, like T490M, occurred earlier in CTCs compared to cfDNA [83].

In the small group of patients, which showed EGFR alterations in biopsy specimen, we observed the same mutations in cfDNA. According the changes of CTC number and cfDNA during treatment, we could distinguish two groups of patients, showing concordant trend or discordant trend between these two biomarkers.

When we observed all patients enrolled, we noticed a decrease of CTC number (by both SA and EA) between baseline and after the first cycle of therapy, and a decrease of EML4-ALK-positive CTCs but we never observed an increase in M30-positive CTCs. This points to the fact that chemotherapy and/or target

therapy hit the cancer cells, but this effect translates only as a decrease in the CTC number without any increase of apoptotic CTCs, unlike to what we previously demonstrated in other malignancies [69].

Taken together, in our opinion, these observations reflect a therapeutic benefit that is merely partial, because of the persistence of viable CTCs in the blood stream that we should consider a sign of aggressive disease.

In order to deep the study of mutational status of circulating compartment, we advantage of next generations sequencing methods. The NGS has several advantages over other sensitive mutation detection techniques, because it allows detecting all types of mutations in a given PCR amplified DNA fragment.

In close to 64% of lung adenocarcinoma it is possible to detect, in biopsy or surgical specimen, a least one somatic oncogenic mutations in EGFR, HER2, KRAS, PIK3CA, BRAF, MEK1, and ALK; in the era of target therapies, it is essential to obtain a full coverage characterization of "druggable" mutations. In this context, few studies have demonstrated the feasibility of NGS in cfDNA and CTCs, therefore, we applied common amplicon based resequencing panel (Hotspot Cancer panel 2) to circulating compartment in 9 NSCLC patients at baseline. CTCs and cfDNA were directly isolated from the same patient blood draw. To date, this is the second study where a population of circulating cancer cells was recovered and directly sequenced.

Unlike to our expectations, we did not observe a large number of mutations in the circulating compartment. At baseline, in 8 out of 9 subjects CTCs were wild type and we detected only in one patient a mutation of PIK3CA gene. Moreover, cfDNA reveal mutations only in 4 cases out of 9. In this small group of patients, we demonstrated that the circulating compartment, even though in 50 genes in the pan-cancer panel, could provide information about rare mutations and/or alterations involved in the target therapy response as PIK3CA_E545K.

Our data stress out, if still necessary, the translational relevance of liquid biopsy, because, as previously reported, about 12% of adenocarcinoma shown PIK3CA mutations [84].

It will be essential and interesting to continue this deep characterization also in the following time points, in order to evaluate the changing of mutational profile and the feasibility to investigate the evolution of disease in this compartment.

It is well establish that the secondary EGFRT790M mutation drives TKIs therapy resistance, so that the detection of T790M is important because of its effect on clinical outcome. In our case series, we are able to detected EGFR mutations both in cfDNA and in CTCs; interesting, we noticed that CTCs showed T790M not only at progression but also before this time point. Indeed, in the subgroup of CTC-positive patients, showing mutation-positive CTCs, we detected the secondary T790M earlier than in cfDNA, as previously observed also by He, Tan and Man [83] which isolated CTCs with a different method.

It should be recognized that the expanded panel regarding STP has a greater predictive power, because it manages to highlight more effectively patients with increased risk of STP within six months of starting therapy, a useful information for the oncologist who could then decide to observe more closely the patients positive for CTCs with this assay. CTCs and cfDNA are complementary templates concerning molecular information. To indicate that in the circulating compartment we could find a window to survey the disease evolution, the relationship between subpopulation of cancer cells, therapy response and host relationship.

On the basis of our data, we propose a decisional tree to observe NSCLC patients. At baseline, we suggest to perform a SA test and if there are no CTCs, researchers should plan to analyse the second blood draw with EA in order to place the patients in the correct risk group, for supporting clinical decisions regarding OS and STP. The second step would be completely characterized CTCs in order to establish the tailored therapy.

In conclusion, we agree with other authors that are hoping for a standardization regarding all methods involved in the deep characterization of these two biomarkers, because then it will be possible to apply them routinely in every lung cancer patient. Moreover, the results obtained in our observational study, get prompt to use the information about CTCs to produce an intervention, as a treatment change, before what established by the current guidelines.

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