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Original Article

Preliminary data on circulating tumor cells in metastatic NSCLC patients candidate to immunotherapy

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Abstract: In the current paper, we aimed to investigate circulating tumor cells (CTCs) in non-small cell lung carcinoma (NSCLC) candidates to immunotherapy and correlate findings with clinical and metabolic parameters. Seventeen metastatic NSCLC patients (12 males, 5 females), were prospectively enrolled. All patients underwent ^{18}F-Fluorodeoxyglucose (FDG) PET/CT and CTCs detection before treatment. CTCs isolation by size was carried out with the ISET method. CTCs were characterized based on cytopathological features and were compared with smoking status, histological subtype, pre-immunotherapy treatment, PDL-1 expression, performance status, and semi-quantitative parameters on PET, including SUVmax, SUVmean, metabolic tumor volume (MTV) and total lesion glycolysis (TLG). We found CTCs in 10 out of 17 patients (59%). Mean number of CTCs was 3 (range 1-7). Only one cell with 3 malignant features was detected in the blood of a healthy control out of 7 (16%). A significantly lower number of CTCs was found in patients previously treated with chemotherapy (P=0.041). No correlation between CTCs and other clinical pathologic characteristics was observed. Patients with an extensive tumor burden, i.e. MTV and TLG, were associated with a higher number of CTCs (P=0.004 and P=0.028, respectively). Likewise, patients with a higher metabolism determined with SUVmean resulted having a higher CTCs count (P=0.048). The presence of CTCs was associated with tumor uptake and metabolic burden on PET/CT, while results were influenced by previous chemotherapy. Whether confirmed in larger series, the combination of the presence of CTCs and FDG PET metabolic parameters might improve prognostic stratification and allow more personalized treatment paradigm.

Keywords: Non-small-cell lung cancer, circulating tumor cells, PET/CT, FDG, immunotherapy, chemotherapy

Introduction

Lung cancer is the leading cause of death worldwide, with non-small-cell lung cancer (NSCLC) representing 80-85% of all cases [1, 2]. Due to lacking symptoms at early onset, almost half of the cases are diagnosed in advanced stage [3]. Surgery, chemotherapy, or radiation therapy have been widely used to treat different sub-types of lung cancer. However, up to 50% of patients, even after curative treatment, show tumor recurrence [4-8], suggesting the need for more sensitive diagnostic strategies and biomarkers able to provide prognostic information. Following the clinically relevant results obtained in the last years with immunotherapy in NSCLC patients, checkpoint inhibitors targeting the programmed death-1 (PD-1) and its ligands (PD-Ls) are gradually replacing or combining to "conventional" chemotherapeutic agents [9-13]. Despite the improvement in survival, immunotherapy is not efficacious in all cases and clinicians are still in need of reliable biomarkers for patient selection and response assessment in this setting.

Positron emission tomography/computed tomography with ^{18}F-fluorodeoxyglucose (^{18}F-FDG PET/CT) represents a consolidated and extensively used image modality in the diagnostic work-up of patients with NSCLC [14-16]. At baseline, before any treatment, it provides important information on disease extent and patient prognosis. Currently, this modality is being investigated also in NSCLC patients during the course of immunotherapy [17, 18].

Table 1. Principal characteristics of the study cohort

		Nr.	(%)
Overall		17	100
Age	mean	72	
	range	51-87	
Sex	female	5	35.7
	male	12	85.7
Tobacco exposure	smoker	3	17.6
	no smoker	3	17.6
	former	10	58.9
	NA	1	5.9
Histology	ADC	12	85.7
	SQC	4	28.6
	other	1	7.1
Performance status	PS 0	9	53
	PS 1	5	29.4
	PS 2	2	11.8
	NA	1	5.8
Treatment before immunotherapy	CHT	6	64.7
	CHT&RT	5	29.4
SUVmax	mean	14	
	range	5-21	
SUVmean	mean	7	
	range	3-10	
TLG	mean	868	
	range	32-3459	
MTV	mean	114	
	range	9-336	
Diameter max (mm)	mean	55	
	range	22.2-78.7	
PD-L1 ≥50		5	29.4
CTCs	mean	3	
	<3	12	
	≥3	5	
	range	1-7	

Notes: NC = not classified; NA = not available.

In the last years, detection of circulating tumor cells (CTCs) in the bloodstream has emerged as a new potential biomarker, able to monitor treatment efficacy in cancer patients, including NSCLC [19-36]. Krebs and colleagues [24], for example, have shown that stage III and IV NSCLC patients with more than 5 CTCs in 7.5 mL of blood have a worst overall survival (OS) and progression-free survival (PFS). With this regards, tumor metabolic parameters obtained from ^{18}F-FDG PET/CT could be able to predict the presence of CTCs, as previously reported in lung cancer [37-41]. These preliminary data suggest the use of CTCs count also for response assessment to immunotherapy with checkpoint inhibitors. Nevertheless, CTCs detection presents some limits. One of the main limits relates to methodological aspects and concerns sensitivity, specificity, and reproducibility of the data [36]. Moreover, CTC count might be influenced by the clinical history of cancer patients and other tumor-related factors.

Following these premises, in the present study we decided to investigate CTCs count in patients affected by metastatic NSCLC candidate to immunotherapy and assess the relationship between these findings and other clinical and metabolic parameters.

Materials and methods

Patients and study design

The current study has been conducted following the approval of the local IRB and the trial has been registered at https://clinicaltrials.gov/ (First posted: 20/06/2018; NCT03563482).

Between March and November 2017, a total of 17 patients (12 males, 5 females) affected by metastatic or relapsed NSCLC and referred to our Institution for immunotherapy with checkpoint inhibitors (nivolumab and pembrolizumab) were prospectively enrolled. In 6 cases (35%), patients were metastatic at presentation, whereas in the other cases indication to immunotherapy was given after first-line treatment failure. Patients underwent ^{18}F-FDG PET/CT before treatment and CTCs detection from peripheral blood sampling at baseline. As negative control, the blood drawn from 7 healthy donors (3 male, 4 female) was collected. Written informed consent was obtained in all cases. **Table 1** summarizes principal characteristics of the patients population.

Imaging protocol

PET scans were acquired approximately 60 min after FDG administration in fasting patients,

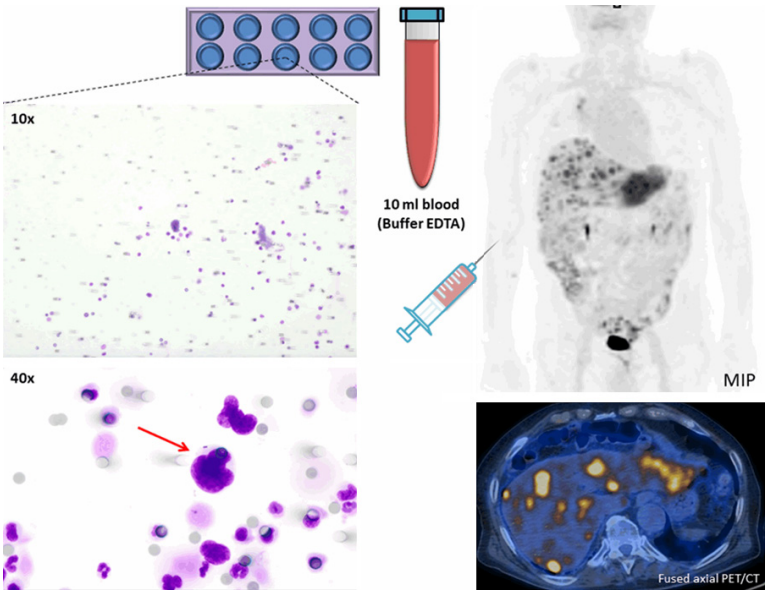

Figure 1. Representative CTC isolated with ISET technique from 10 ml peripheral blood (buffered in EDTA) obtained from a relapsed NSCLC patient with multiple liver metastases. The identified CTC is visualized by MGG staining in 40× magnification (red arrow), and conformed to malignant properties: increased nucleus/cytoplasm ratio, nucleus larger than 3 calibrated pore size of the membrane (>24 µm), irregular nuclear borders, and nuclear hyperchromatism. Round grey spots are 8 µm membrane pores. CTC = circulating tumor cell; MGG = May-Grünwald-Giemsa; ISET = Isolation by Size of Tumor cells; EDTA = Ethylenediaminetetraacetic Acid; MIP = maximal intensity projection.

using an activity ranging from 250 to 500 MBq. Whole body images were obtained from the base of the skull to mid-thigh by means of an integrated PET/CT tomograph: GE Discovery PET/CT 690, with an integrated 64-slice CT. Reconstructed images were then displayed on a GE ADW4.6 workstation (GE Healthcare, Waukesha, WI, USA) and interpreted by experienced nuclear medicine physicians. The scanner used in this study is accredited by the EANM Research Ltd (EARL) program, and image analysis was performed using standardized acquisitions [42]. Tumor masses were identified as areas of increased FDG uptake in relation to normal lung parenchyma or other mediastinal structures. Tumor burden was calculated with three-dimensional volumes of interest (VOIs) drawn on the volume of metabolic tumor-related activity by applying a percentage threshold of 42%: maximal standardized uptake value (SUVmax) was defined as the highest pixel value and SUVmean was defined as mean SUV related to the tumor burden. Volumetric parameters included metabolic tumor volume (MTV), estimated from the isoactivity contours drawn automatically on the GE PETVCAR® (PET Volume Computer Assisted Reading), based on a defined threshold, and total lesion glycolysis (TLG), calculated as the product of SUVmean × MTV. All patients investigated in our cohort had more than one lesion. All lesions have been separately analyzed and quantitative data derived from the sum of all tumor volumes. The highest SUVmax of the hottest lesion was considered as SUVmax for the analysis.

CTC isolation and counting

For CTCs detection, 10 ml of blood were collected in EDTA tubes and processed within 2 hours on the Isolation by Size of Tumor/Trophoblastic Cells (ISET) platform (Rarecells, Paris, France). Peripheral blood was filtered through the ISET polycarbonate membrane containing 10 filter-spots with calibrated 8-µm-diameter cylindrical pores, each spot representing the filtration of 1 mL of blood. The membrane was cut into two parts containing 4 and 6 spots per part. Four spots were stained using a freshly made May-Grünwald-Giemsa (MGG) solution according to the technique described by Hofman and colleagues [21] for 5 minutes with undiluted May-Grünwald and subsequently for 5 minutes with 50% diluted May-Grünwald and 40 minutes in 10% diluted Giemsa, followed by rinsing with water. Membranes were then air-dried and mounted with limonene mounting medium (Sigma) and kept in the dark at room temperature. Stained spots were examined under a light microscopy (Olympus BX51) at 10× and subsequently digitized at 40× magnification (**Figure 1**). All images were analyzed by two cytopathologists blinded to the study data. CTCs were recognized based on four cytopathological features: a) nuclear hyperchromatism, b) increased nuclear volume, c) irregular nuclear borders, and d) increased nucleic ratio/cytoplasm. Cells were defined as CTCs when at least 3 of the above four criteria were fulfilled

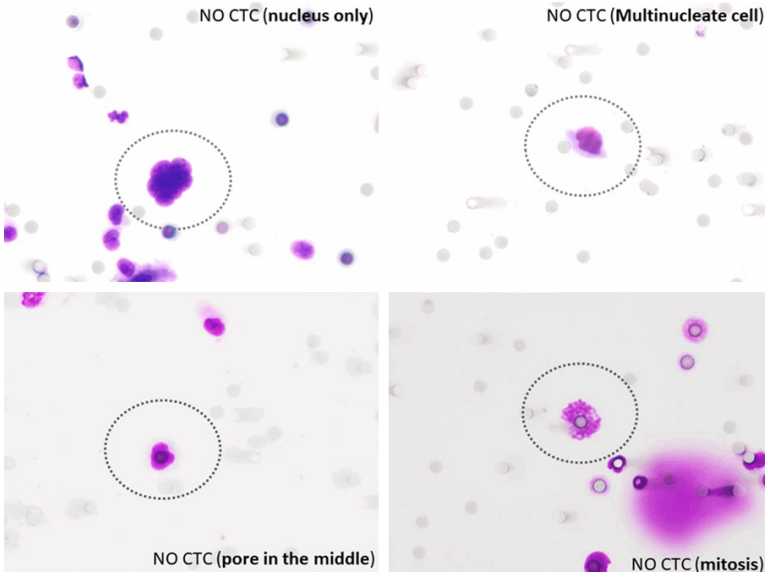

Figure 2. Series of possible findings visualized by MGG staining in 40× magnification that can be confused with CTC and mislead interpretation.

(**Figures 1**, **2**), as previously described by Hofman and colleagues [21].

Comparison with other CTCs isolation techniques

PubMed research for circulating tumor cells and lung cancer was performed. Out of the 2778 records on PubMed corresponding to ("neoplastic cells, circulating" [MeSH Terms] OR ("neoplastic" [All Fields] AND "cells" [All Fields] AND "circulating" [All Fields]) OR "circulating neoplastic cells" [All Fields] OR ("circulating" [All Fields] AND "tumor" [All Fields] AND "cells" [All Fields]) OR "circulating tumor cells" [All Fields]) AND ("lung neoplasms" [MeSH Terms] OR ("lung" [All Fields] AND "neoplasms" [All Fields]) OR "lung neoplasms" [All Fields] OR ("lung" [All Fields] AND "cancer" [All Fields]) OR "lung cancer" [All Fields]), we selected clinical studies for ("carcinoma, non-small-cell lung" [MeSH Terms] OR ("carcinoma" [All Fields] AND "non-small-cell" [All Fields] AND "lung" [All Fields]) OR "non-small-cell lung carcinoma" [All Fields] OR "nsclc" [All Fields]) investigated with the similar methodology based on isolation by size, i.e. ISET (Rarecells, Paris, France) and ScreenCell (Sarcelles, France).

Statistical analysis

The total CTCs count, along with mean and median values was calculated. The presence or absence of CTCs was compared with the patients' baseline epidemiological and clinical-pathologic characteristics, including age, gender, smoking status, histologic subtype, chemotherapy pre-immunotherapy, PDL-1 status, performance status, as well as with metabolic indexes on ^{18}F-FDG PET/CT, comprising SUVmax, SUVmean, MTV and TLG. Associations of CTCs with clinical and metabolic characteristics were studied using Fisher's exact or Student T-test, when appropriate. Chi-square test was applied to score significant difference between CTCs groups. The ANOVA test was used to explore the association between the CTCs number, analyzed as a continuous variable, and clinical parameters. Bonferroni correction was applied to verify significance for multiple testing. All data were indicated as mean ± SD. All statistical analyses were performed with GraphPad Prism (version 7).

Results

CTCs counting and clinical parameters

CTCs were found in 10 out of 17 patients (59%). The mean ± SD number of CTCs was 3±2, 4/4 mL, while median was 2/4 mL, (range 1-7 CTCs/4 ml) in NSCLC patients. Only one cell with 3/4 malignant features was detected in the blood of a healthy volunteer out of 7 donors analyzed (16%). Based on previous research by Hofman [21], patients were categorized as follows according to CTCs number: Group 1, less than 3 CTCs in 4 spots (equivalent to 4 ml of blood sample) and Group 2, 3 or more than 3 CTCs in 4 spots. Before immunotherapy, 11 (64.7%) patients have been treated with chemotherapy only or chemotherapy plus radiation therapy (**Table 1**). A significant association was observed between CTCs and prior chemotherapy status. In particular, patients who had undergone chemotherapy were characterized by a lower number of CTCs than those who had not performed chemotherapy (P=0.041, **Figure 3**). No significant difference in

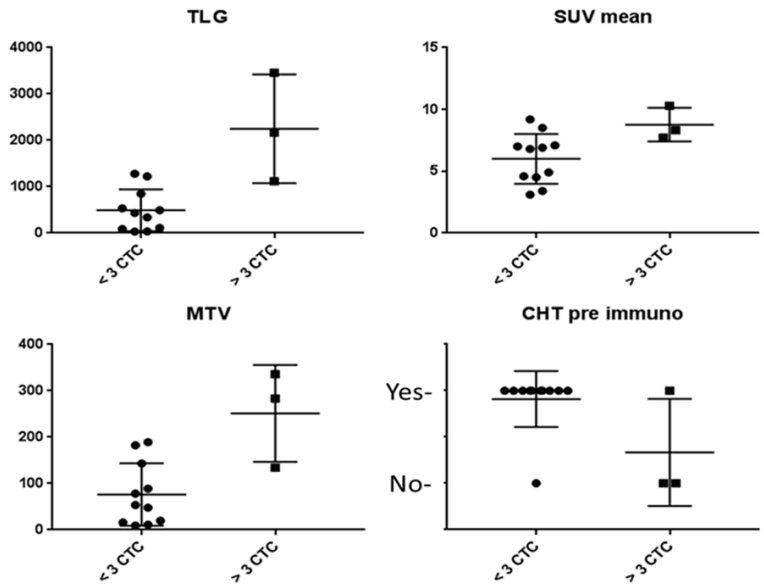

Figure 3. Representative plots comparing the number of CTCs to other clinical and metabolic parameters; Mean number of CTCs resulted significantly associated with increased TLG and MTV (P=0.028, P=0.004 respectively) and concordant with higher SUVmean (P=0.048). The presence of CTCs was significantly reduced in patients who had previously undergone chemotherapy (P=0.041).

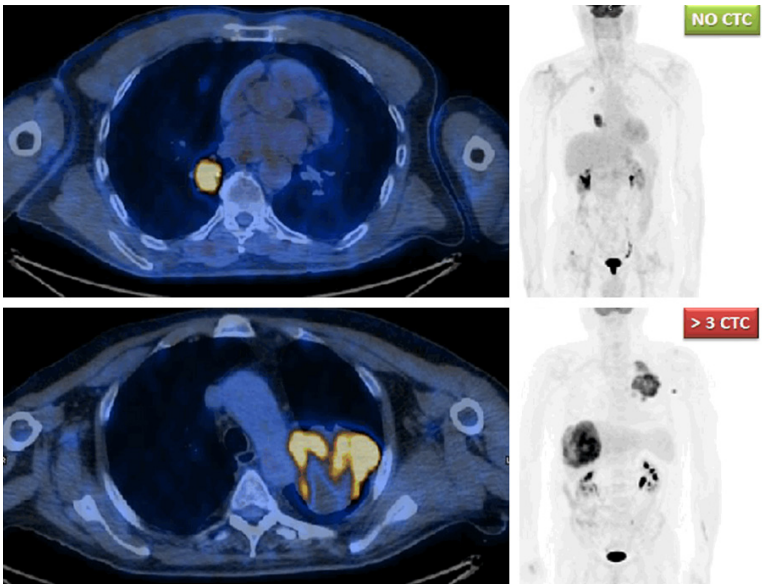

Figure 4. Comparison of two cases with diverse CTCs count and tumor burden at baseline: MIP (maximal intensity projection) images shown on the right side; axial fused PET/CT images at the level of lung lesions are shown on the left. The upper case had no evidence of CTCs at peripheral blood stream, while the lower case had more than 3 CTCs (more precisely, 4 CTCs counted).

terms of cytopathologic characteristics of CTCs between adenocarcinoma and other lung histotypes was also found. Additionally, we did not find any correlation between CTCs and other epidemiologic and clinical-pathologic parameters (age, gender, tobacco history, tumor size, PD-L1 status). Likewise, no significant difference was found between CTCs number and organs interested by metastases.

CTCs counting and PET-derived parameters

A statistically significant association with the presence of CTCs was found for semi-quantitative and tumor burden parameters on PET/CT (**Figure 4**). Patients with an extended tumor burden, expressed by TLG and MTV, were associated with higher number of CTCs. We found a mean TLG of 649.8±187.5 in patients with low CTCs counting (<3) compared to 2510±603.7 in those with a number of CTCs ≥3 (P=0.028, **Figure 3**). Additionally, when compared to patients with low CTCs (<3), patients with ≥3 CTCs had higher MTV values (3541±1560 cm^3 vs 19134±9749 cm^3, respectively P=0.004). Likewise, SUVmean was statistically higher in patients with number of CTCs ≥3 compared to those with less CTCs (8.8±7.9 vs 5.5±8.4, P=0.048) (**Figure 3**). No significant difference of SUVmax was observed in both Groups. After correction for multiple testing, CTCs count was confirmed as significantly correlated to MTV values.

Correlation of metabolism with other parameters

The distribution of PET variables in the study cohort is shown in **Table 1**. We observed a high-

er MTV and TLG in NSCLC patients with positive PD-L1 staining at immunohistochemistry (P=0.002 and 0.003, respectively). No significant difference was observed for SUVmax and SUVmean and tumor PD-L1 expression. Patient who did not performed chemotherapy had in general a significantly higher MTV and TLG compared to those previously treated (P= 0.034 and 0.007, respectively), although there we no statistically significant difference in terms of SUVmax (P=0.574) and SUVmean (P=0.117). Also correlation to other clinical-pathological parameters showed no statistically significant difference with respect to PET-derived variables.

PubMed research results

Overall, 23 papers fulfilled the research criteria. ISET (Rarecells, Paris, France) technology was used in 14 cases, out of which 6 were performed in comparison to CellSearch (EpCam-based). ScreenCell (Sarcelles, France) technology was used in 9 cases. The records obtained from PubMed research and study results are illustrated in **Table 2**.

Discussion

In this work we aimed to investigate CTCs count in NSCLC cancer patients candidate to immunotherapy in order to first assess whether there is an association with metabolic and other clinical parameters. In the last years, many studies have described the detection of CTCs in lung cancer patients with different approaches and objectives (e.g. diagnostic or prognostic). We focused our attention on filter-based isolation techniques, i.e. ISET (Rarecells, Paris, France) and ScreenCell (Sarcelles, France) that in comparative studies have shown an overall higher sensitivity than marker-based approaches, i.e. CellSerach (EpCam-based) [22, 25, 34, 43, 50]. In fact, ISET is capable to identify CTCs in 76% (range: 50-100%) of lung cancer patients, whereas CellSearch can identify CTCs in 36% of the cases (on average, range; 23-45%) [22, 25, 43, 50]: The lack of statistical concordance between the CTCs count detected by the two techniques [25] suggests that they might identify different cancer cell subpopulations.

CTCs isolated by size have been extensively characterized for the expression of common epithelial/mesenchymal markers, such as Vimentin and keratin [29, 44], but also for lung cancer specific biomarkers, such as ALK rearrangement [34, 52], PD-L1, ROS1 and MET expression [35, 50, 52]. In particular, Iliè and colleagues have found a 93% concordance between PD-L1 status in tissue and CTCs and a trend for longer PFS was observed in cases with PD-L1 expression in CTCs or WBCs (P=0.2) [35].

These studies raised the possibility to use CTCs as a monitor of treatment efficacy in NSCLC patients: CTCs count has been investigated during surgical manipulation [49], during stereotactic body radiation therapy (SBRT) [53], after radiofrequency ablation [46] and after cryotherapy [47]. CTCs have emerged as an important tumor biomarker for a wide range of human cancers [54-59] and might find a proper place in treatment regimens based on checkpoint inhibitors.

In this preliminary study, we evaluated at first the relationship between the number of CTCs and a series of epidemiological and clinical characteristics in a cohort of patients with metastatic or relapsed NSCLC candidate to immunotherapy. In our cohort, CTCs were found in 59% of patients with a mean density of 3/4 mL blood, which is consistent with the results of two previous studies that investigated subjects candidate to chemotherapy as first-line regimen [60, 61]. Although the authors used an alternative method for CTC detection, in these same studies, CTCs at baseline and during follow-up resulted a strong independent predictor of survival in advanced NSCLC receiving chemotherapy. No other clinical parameters were associated with the number of CTCs. Also Hofman et al. [21] did not observe any significant correlation between the density of CTCs and disease stage or other clinical parameters (i.e., age, gender, tobacco exposure, tumor size, histologic subtype, and histologic grade). Similar to Krebs et al. [24], we found that the presence and the number of CTCs were influenced by previous cycles of chemotherapy. Indeed, patients with a positive history for previous therapy showed lower levels of CTCs compared to those who had not undergone prior chemotherapeutic treatment (P=0.041). Consequently, CTCs count after chemotherapy in the bloodstream might determine the grade of response and, on the other hand, could be

Table 2. Summary of the available articles analyzing CTCs by morphology in patients with non-small cell lung cancer

Author	Tumor	Nr. of cancer patient	Nr. of patients with CTCs	CTCs/ml mean	CTCs/ml range	Nr. of healthy donors	Nr. of health donors with CTCs	CTC isolation technique	Findings
Farace F et al. 2011 [43]	Lung Cancer	20	20/20 (100%) by ISET; 9/20 (45%) by CellSearch	1.6 by ISET; 90.4 by CellSearch	0.1-13 by ISET; 0-1800 by CellSearch	n.a.	n.a.	CellSearch and ISET	Concordant results between ISET and CellSearch in only 20% of patients.
Hofman V et al. 2011 [21]	Resectable NSCLC	208	76 (36%)	7	0-83	39	0 (0%)	ISET	The number of CNHCs is significantly associated with shorter OS and DFS.
Hofman V et al. 2011 [22]	Resectable NSCLC	210	82 (39%) by CellSearch, 104 (50%) by ISET	1.7 by CellSearch; 4.9 by ISET	1-3, 3	40	0 (0%) by CellSearch and ISET	CellSearch and ISET	The number of CNHCs is significantly associated with shorter DFS.
Hofman V et al. 2011 [23]	NSCLC (among many benign and malignant disease)	394	119 (30%)	n.a.	n.a.	49	0 (0%)	ISET	CNHCs detected on filters were usually higher in patients with malignant diseases than the number observed in patients with nonmalignant diseases (P<.001).
Lecharpentier A. et al. 2011 [43]	NSCLC	6	6 (100%)	8.7	1.6-19	n.a.	n.a.	ISET	Vimentin and keratin double positive CTCs, corrisponding to hybrid epithelial/mesenchymal phenotype, were found in all patients.
Hofman V et al. 2012 [27]	Resectable NSCLC	250	102 (41%)	na	na	59	0 (0%)	ISET	Interobserver variation among 10 pathologists was low for the diagnosis of malignant cells.
Krebs M G et al. 2012 [25]	Stage III and IV NSCLC	40	9 (23%) by CellSearch; 32 (80%) by ISET	0.05±0.5 by CellSearch; 2±8 by ISET	0-139	n.a.	n.a.	CellSearch and ISET	There was no statistical concordance between the numbers of CTCs detected by the two techniques.
Pailler E et al. 2013 [34]	ALK-positive and negative NSCLC	18 ALK+ and 14 ALK-	18/18 (100%) ALK+ CTCs	9	4-46 by ISET; 0-11 by CellSearch	n.a.	n.a.	ISET and CellSearch	ALK rearrangment can be detected in CTCs of patients ALK-positive.
Ilie M et al. 2014 [26]	168 COPD High risk individuals	/	5/168 (3%) COPD	9.4	3-11	77	0 (0%)	ISET	The presence of CTC was significantly correlated to the severity of COPD (P<0.001).
Freidin MB et al. 2014 [32]	Primary lung cancer patients undergoing surgery (32) + pulmonary metastasis (21)	32 + 21	56-65% in primary LC; 47-71% in metastatic patient	n.a.	n.a.	17 Benign Lung lesion	35-29%	Scree Cell	Lung cancer patients have more CTCs that benign lesions.
Pailler E et al. 2015 [45]	ROS1 rearranged and ROS1-negative NSCLC	8	8/8 (100%) by ISET; 4/8 (50%) by CellSearch	8.4 (ISET-ROS1 rearranged); 0.2 (CellSearch)	2.3-18.3 (ISET); 0-1 (CellSearch)	n.a.	n.a.	CellSearch and ISET	ROS1 rearrangement can be detect in CTCs and can predict resistance to ROS1-ihibitor therapy.
Chudasama D et al. 2015 [46]	Lung Cancer before; and after radiofrequency ablation	9	3/9 (33%) before; 7/9 (78%) after cryotherapy	1 before; >10 after cryotherapy	0-17 before; 0->33 after cryotherapy	n.a.	n.a.	ScreenCell	Increased CTC count after radiofrequency ablation (larger increase in metastatic patients).
Chudasama D et al. 2015 [47]	Lung Cancer before; and after cryotherapy	20	5/20 (25%) before; 15/20 (75%) after cryotherapy	1 before; >10 after cryotherapy	0-17 before; 0->33 after cryotherapy	n.a.	n.a.	ScreenCel	Increased CTC count after cryotherapy.

Fiorelli A et al. 2015 [48]	Lung Cancer	60	54 (90%)	17.3±11.6	17.3±11.6	17	1 (6%)	ScreenCell	Moderate correlation between SUV value and CTC count. The presence of >3.3 CTCs/ml is associated with malignant lesion.
Mascalchi M et al. 2016 [33]	Stage III-IV lung cancer before FNA biopsy	28	17/28 (65%)	2.26±1.2	0-15/mL	n.a.	n.a.	ScreeCell	No correlation between number of CTC or CTM and tumor type or stage was observed.
Sawabata N et al. 2016 [49]	Pre and post-operative NSCLC	23	7/23 (30%) before surgery 17/23 (74%) after surgery 1/23 (4%) 6 h after surgery 19/23 (83%) pulmonary vein blood collection	7/23 (30%) before surgery 17/23 (74%) after surgery 1/23 (4%) 6 h after surgery 19/23 (83%) pulmonary vein blood collection	1.6 before surgery 1.6 after surgery 0.04 6 h after surgery 3.13 pulmonary vein blood collection	0-8 before surgery 0-3 after surgery 0-0.3 6 h after surgery 0-4 pulmonary vein blood collection	n.a.	Screen Cell	CTCs detection may increase during surgical manipulation and authors found a correlation with clinical parameters (such as SUVmax).
Coco S et al. 2017 [28]	Stage IIIB and IV NSCLC	73	34>2 CTC/ml (39 hanno ≤2 CTC/mL)	2	0-16.6	n.a.	n.a.	Screen Cell Cyto	The presence of CTC was associated to better OS (opposite to expectation).
Chudasama D et al. 2017 [31]	NSCLC undegoing surgical tratment. 13 adeno, 10 squamous; 18 early stage, 5 stage III-IV	23	80.6% of early stage, 60% of late stage	n.a.	n.a.	n.a.	n.a.	Screen Cell	The presence of CTC correlates to better OS (P<0.0009; opposite to expectation).
Ilie M et al. 2017 [50]	Stage III-IV NSCLC	256 by CellSearch; 106 by ISET	113/256 (44%) and 80/106 (75%)	15 by ISET (among CTC+)	0-35 by CellSearch; 0-64 by ISET	n.a.	n.a.	CellSearch and ISET	ISET approach identifies a higher proportion of CTC+ patient than CellSearch. MET status in ISET-captured CTCs correlate with MET status in tumor tissue.
Mascalchi M et al. 2017 [51]	Lung Cancer	67	47/67 (70%)	0.7	0-4	8	1/8 (12%)	Screen Cell	Due to low sensitivity, the search of CTCs cannot replace CT guided percutaneous FNA or core biopsy.
Kallergi G et al. 2018 [29]	Chemo-naive stage IV NSCLC	30	93.3% (28/30). After chemotherapy 81.8% (9/11)	5	0-23 CTCs/ml	n.a.	n.a.	ISET	Significant correlation between CK-positive (IF) and Giemsa-positive tumor cells (P=0.001).
Ilié M et al. 2018 [35]	NSCLC	106	80 (75%)	15	0.5-64	n.a.	n.a.	ISET	93% concordance between PD-L1 status in tissue and CTCs. A trend for longer PFS was observed in cases with PD-L1 expression in CTCs or WBCs (P=0.2).
Ilié M et al. 2018 [52]	Lung Adenocarcinoma	36 (validation set)	27/36 (75%, EDTA collection) 29/36 (81%, BCT collection)	1.25 (EDTA) 2.75 (BCT)	0.25-19 (EDTA) 0.25-21 (BCT)	10	n.a.	ISET	BCT blood collection tubes preserved morphology of CTCs, when compared to EDTA tubes, and allowed ICC for MET and FISH for ALK rearrangement.

used to characterize any morphological or genetic modification occurring in tumor cells after systemic treatment to determine those at greater risk of disseminated disease.

We also explored potential correlations between the density of CTCs and PET-derived metabolic parameters in NSCLC patients. Our findings revealed a significant relationship between higher densities of CTCs and tumor metabolic activity expressed by high levels of SUVmean. Additionally, the estimated tumor burden, expressed by TLG and MTV, was significantly associated with the number of CTCs (P=0.028 and P=0.048, respectively). These results suggest that the density of CTCs in the peripheral bloodstream can reflect the tumor burden and provide valuable information on the metabolic activity, which may serve as a marker of tumor aggressiveness in metastatic NSCLC. PD-L1 expression on the other side did not correlate to CTCs count, whereas evidence of other therapeutic regimens resulted in our study correlated to both CTCs count and volumetric PET parameters (i.e. MTV and TLG). The fact that FDG uptake correlates to PD-L1 in the tumor [17, 62, 63] does not necessarily mean that also CTCs number should correlate. Differently, we would expect an association between PD-L1 levels on CTCs to the tumor PD-L1 expression [35]. The two aspects, in fact, could provide independent information on tumor prognosis, particularly with respect to immunotherapy outcome. Previously, the relationship between CTCs and ^{18}F-FDG PET/CT has been assessed in different types of malignancies, including lung cancer [37-41, 64-66]. In a cohort of NSCLC chemotherapy naïve patients, Nair et al. [37] and Morbelli et al. [38] have showed that only SUVmax was associated with CTCs, whereas Nygaard et al. [64] demonstrated a worse survival in NSCLC patients with higher MTV and higher levels of plasm cell-free DNA (cfDNA), although no significant correlation between PET parameters and cfDNA was detected. A significant correlation for post-operative CTCs count with SUVmax, pathological stage and surgical approach was demonstrated instead by Bayarri-Lara et al. [67] in 102 stage I-IIIA NSCLC patients. In this cohort, SUVmax resulted the only independent predictor for CTC presence after the operation. Similarly to our findings, Nair et al. [37] have reported no correlation for CTCs and tumor diameter in treatment naïf NSCLC patients. On counterpart, volumetric parameters in our cohort (i.e. MTV and TLG) resulted significantly different in NSCLC patients with more than 3 CTCs compared to those having less. In particular, MTV was confirmed predictive also after correction for multiple testing. In Fiorelli et al. [48], instead, CTCs count resulted significantly correlated to tumor stage and size, and moderately associated to SUV value (**Table 2**). Herein, the presence of more than 3.3 CTCs/ml (25/7.5 ml) in the bloodstream resulted predictive of malignancy in patients with lung lesions. On the other hand, considering patients as responders vs no-responders by PET/CT after erlotinib and pertuzumab, Punnoose et al. [65] highlighted higher levels of CTCs in patients classified as no-responders, suggesting CTCs as an early, non-invasive predictor of response.

Our report is one of the first to examine the association between CTCs and PET parameters in the era of immune checkpoint inhibitors. The present study has anyhow some limitations: at first, it includes a limited number of patients, due to its preliminary nature, making definite conclusions difficult. Secondarily, the lack of extensive follow-up prevents any hypothesis on the predictive and prognostic role of decreasing CTC-levels, and PET-derived parameters during immunotherapy. Thirdly, we did not collect other circulating markers, such as cfDNA, which are known as potential biomarkers in cancer.

Despite the limitations, our study confirms the expectations on CTCs count in NSCLC patients and its correlation with other factors, such as PET-parameters. Future investigations should focus on the use of all these factors to predict response and outcome in patients under immunotherapy.

Conclusions

The presence of CTCs identified by ISET is influenced by previous chemotherapy and may be a reflection of tumor biology and metabolism in metastatic NSCLC prior to checkpoint inhibitors. Future prospective studies will be needed to confirm whether this non-invasive diagnostic tool is of additional value in driving the best treatment and establishing individual prognostic outcomes. An interesting point might be to

evaluate CTCs genetic profile and compare its characteristics with those of primary tumor and other circulating tumor markers.

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Abbreviations

CTCs, circulating tumor cells; NSCLC, non-small cell lung cancer; ^{18}F-FDG PET, 18-fluorodeoxyglucose positron emission tomography; CT, computed tomography; ISET, isolation by size of Tumor/Trophoblastic Cells; PETVCAR®, PET Volume Computer Assisted Reading; SUV, standardized uptake value; MTV, metabolic tumor volume; TLG, total lesion glycolysis.

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