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Circulating tumor DNA reflects tumor metabolism rather than tumor burden in chemotherapy-naïve patients with advanced non-small cell lung cancer (NSCLC): an 18F-FDG PET/CT study

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Abstract

We aimed to evaluate the relationship between Circulating tumor Cells (CTCs) and plasma Cell-free DNA (cfDNA) on one side and a comprehensive range of 18F-fluorodeoxyglucose (FDG)-PET/CT-derived parameters on the other side in chemotherapy-naïve patients with advanced NSCLC.

Methods: Among the seventy-nine patients included in the VeriStrat® trial, evaluating the role of pretreatment circulating tumor markers as predictors of prognosis in chemotherapy-naïve patients with advanced NSCLC, we recruited all subjects submitted to FDG-PET/CT for clinical reasons at our institution before the inclusion in the trial (and thus just before chemotherapy). For each patient a peripheral blood sample was collected at baseline for the evaluation of CTCs and cfDNA. CTCs were isolated by size using a filtration-based device and then morphologically identified and enumerated; cfDNA was isolated from plasma and quantified by a qPCR method using human telomerase reverse transcriptase (hTERT). The following FDG-PET/CT-derived parameters were computed: maximum diameter (dmax) of the primary lesion (T), of the greater lymph node (N) and of the greater metastatic (M) lesion; SUVmax, SUVmean, size-incorporated SUVmax (SIMaxSUV), Metabolic Tumor Volume, Total Lesion Glycolysis. All parameters were independently measured for T, N and M. The associations among CTCs, cfDNA and FDG-PET/CT derived parameters were evaluated by multivariate-analysis. Patients were divided in two groups according to the presence of either limited metastatic involvements (M1a or M1b due to extra-thoracic lymph nodes, $M1b_{Lympho}$) or disseminated metastatic disease ($M1b_{Disseminated}$). Presence (B+) or absence (B-) of metabolically-active bone lesions was also recorded for each patient and patients' subgroups were compared.

Results: Thirty-seven patients recruited in the VeriStrat® trial matched our PET-based criteria (24 males; age 64.5 ± 8.1 years). M-SUVmax was the only variable independently associated with baseline cfDNA levels (p=0.016). Higher levels of cfDNA were detected in the subgroup of patients with metabolically active bone lesions (p=0.02) while no difference was highlighted when comparing patients with more limited metastatic disease with patients with $M1b_{Disseminated}$.

Conclusions: The correlation of cfDNA amount with tumor metabolism, but not with metabolic tumor volume at regional or distant levels, suggests that cfDNA might better reflect tumor biological behavior/aggressiveness rather than tumor burden in metastatic NSCLC.

Keywords: positron emission tomography, circulating tumor markers, non-small cell lung cancer, metabolic tumor volume, maximum standardized uptake value

Introduction

Despite the identification of CTCs and cfDNA as biomarkers potentially able to provide clinically relevant information in cancer patients, at present, their identification is not routinely envisioned in the clinical practice (1). Indeed, incomplete understanding of the specific role of these biomarkers in different tumor types as well as unsolved technical issues still limit their systematic assessment in the clinical setting (1). Several studies have demonstrated a prognostic value of CTC enumeration or cfDNA levels in different malignancies including non-small cells lung cancer (NSCLC) (2-5).

In particular, a significant role of baseline CTCs or cfDNA determinations before first-line therapy has been highlighted in some tumor types, and the presence of increased levels of circulating tumor markers in advanced malignancies has been demonstrated to correlate with poor patients' prognosis (6). However, whether this observation merely reflects their role as tumor burden indicators or might reveal other biological mechanisms associated with tumor aggressiveness is still a matter of debate (1).

In recent years imaging procedures have emerged as meaningful prognostic indicators in oncology and previous studies have investigated the interplay between circulating tumor markers and imaging biomarkers (6). In this framework FDG-PET/CT may be an ideal tool to elucidate the relevance of circulating tumor markers in relation to tumor burden and biology (6). The majority of the available studies investigating the relationship between FDG-PET and circulating tumor markers have been carried out in colon and breast cancer patients (6, 7,8) while data in NSCLC patients are presently limited (9, 10). FDG-PET/CT has a recognized high accuracy in NSCLC patients both in early and advanced stage of disease. Similarly, intensity of FDG uptake and thus tumor metabolism has an established prognostic value as being linked to aggressive

tumor biology (11) and metastatic potential in NSCLC patients (12). To date only one prospective study has been performed by Nygaard and colleagues (10) to assess the relationship between cfDNA and FDG-PET in patients with advanced NSCLC before receiving chemotherapy. This study did not highlight any correlation between tumor DNA and FDG-PET-assessed tumor burden; however, only the whole body metabolic tumor volume (MTV) and average glycolytic volume (TGL) were included in the analysis, while other potentially prognostic variables derived from FDG-PET images were not considered.

Moreover, since different biological and prognostic behaviors can be hypothesized for primary lesions, lymph-node and distant metastases, a detailed investigation of the interplay between circulating tumor markers and PET-derived parameters should take into account these different components, especially in advanced stage patients. Based on these considerations, the present study aimed at evaluating, by a multivariate approach, the relationship between CTCs or cfDNA on one side and a comprehensive range of PET-derived parameters (both at the loco-regional and distant lesion-levels) on the other side in a homogenous population of chemotherapy-naïve patients with advanced NSCLC.

Methods

Patient's enrollment.

The study has been approved by the institutional review board and all subjects signed an informed consent form.

Seventy-nine patients with newly diagnosed advanced NSCLC candidate for first-line chemotherapy were enrolled into a prospective study (13) at the Lung Cancer Unit, IRCCS AOU San Martino-IST, Genova, Italy from October 2012 to October 2015. The trial aimed to test the

value of VeriStrat® (a pretreatment blood-based test of circulating tumor markers) as predictor of prognosis after first line platinum-based combination chemotherapy in advanced NSCLC (ClinicalTrials.gov Identifier: NCT02055144). The inclusion criteria comprised histologically confirmed NSCLC stage IV, no previous treatment, and aged above 18 years. All the patients underwent first-line standard of care treatment for metastatic NSCLC represented by platinum-based combination chemotherapy: cisplatin or carboplatin in association with pemetrexed for adenocarcinoma or with gemcitabine for the squamous histotype, respectively (14).

FDG-PET evaluation was not mandatory; however, a subgroup of patients underwent FDG-PET/CT examinations for standard clinical indications (mainly for staging or restaging completion) at the time of inclusion in the study and before receiving chemotherapy. For each patient a peripheral blood sample was collected at baseline (before treatment) for the evaluation of circulating biomarkers.

The present study has been approved by Ethics committee of IRCCS AOU San Martino - IST (ID#TrPo11.003) and all enrolled subjects provided a written informed consent including the analysis of circulating biomarkers. Among the patients enrolled in this study, only the patients who underwent FDG-PET in our Institution just before the start of chemotherapy were included in the specific analysis of correlation between circulating biomarkers and PET parameters.

CTC isolation

CTC were isolated from 3mL of whole peripheral blood (EDTA tube) by the ScreenCell®Cyto kit (ScreenCell,Paris, France) according to the manufacturer's procedure. Briefly, circulating cells are isolated by size using a polycarbonate filter containing randomly distributed calibrated pores (7.5±0.36 μm) throughout the membrane. After filtration, the filter is then released on the slide

and processed for enumeration and morphology. The isolated non-hematologic circulating cells with malignant features were defined as CTCs and morphologically identified and enumerated under, light microscope, according to the following criteria: Nuclear size greater than or equal to 20μm, high nucleo-cytoplasmic ratio (≥ 0.75), dense hyper chromatic nucleus, and irregular nuclear membrane.

Circulating free DNA (cfDNA) isolation and quantification

Four mL of peripheral blood were collected in EDTA-containing tubes. Such tubes were processed by centrifugation at 1600 rpm for 15 minutes in order to isolate plasma; a further centrifugation was performed to eliminate any cell contamination and the resulting plasma was stored at -80°C. cfDNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the protocol. The purified cfDNA from 400 µl of plasma was eluted in a final volume of 50 µl buffer TE 0,1X and stored at -20°C. The quantification of cfDNA was performed by quantitative real-time quantitative PCR (qPCR), using hTERT (ThermoFisher Scientific, Wilmington, DE, USA) single copy gene. Each qPCR was carried out in a final reaction volume of 10 µl, consisting of 5 µl of TaqMan Universal Mastermix (ThermoFisher Scientific), 1 µl of assay and 4 µl of cfDNA. Each cfDNA sample was run in duplicates, and the plate included a positive and a negative control. The calibration curve was calculated based on a dilution series of a standard DNA (Promega, Madison, WI, USA): 1, 10, 100, 1000, 10.000, 100.000 copy number (3.3 pg of DNA=1 gene copy).

FDG-PET/CT acquisition

All the patients underwent preparation and FDG-PET/CT according to European guidelines (15) and data were acquired using a 16-slices PET/CT hybrid system (Biograph 16, Siemens Medical Solutions, Knoxville TN, USA). Briefly, patients fasted overnight prior to the intravenous administration of 350-450 MBq of FDG, which was performed in a quiet room, with the patient lying in a recumbent position and instructed not to move. Blood glucose was measured before tracer injection, as to ensure blood glucose levels <160 mg/dl. To minimize artifacts caused by the urinary tract, patients were asked to drink 500-1,000 mL of water 1 h prior to image acquisition and to empty the bladder just before the acquisition start. No urinary bladder catheterization was used. Imaging started 60±15 minutes after intravenous tracer administration (patient with longer FDG uptake time were excluded). The technical parameters of the 16-detector row, helical CT scanner included a gantry rotation speed of 0.5 s and table speed of 24 mm per gantry rotation. The PET component of the combined imaging system had an axial view of 16.2 cm per bed position, with an interslice spacing of 3.75 mm. The transaxial field of view and pixel size of the reconstructed PET images were 58.5 cm and 4.57 mm, respectively, with a matrix size of 128×128. Unenhanced low-dose CT was performed at 140 kV and 40 mA for attenuation correction of emissive data and anatomical localization of PET dataset. Emissive scan was performed in in 3D mode, shortly after CT acquisition, with a 3-min acquisition per bed position. PET sinograms were reconstructed by means of ordered-subset expectation maximization (OSEM) iterative reconstruction algorithm (three iterations, eight subsets). Scan was performed starting from the orbital plane on to the mid-thigh, except for the cases where the clinical history demanded a whole body, vertex-to-toes scan.

Images Analysis

For all FDG-PET/CT scans the following parameters were measured: 1. maximum diameter (dmax) of the primary lesion (T); 2. dmax of the greater lymph node (N); 3. dmax of the greater metastatic (M) lesions; 4. Maximum Standardized uptake value (SUVmax); 5. Average SUV (SUVmean); 6. size-incorporated SUVmax (SIMaxSUV); 7. Metabolic Tumor Volume (MTV) 8. Total Lesion Glycolysis (TLG). SUVmax, SUVmean, SIMaxSUV, MTV, and TGL were independently measured for T, N and M. In particular, SIMaxSUV was defined as the product of the greatest diameter (mm) of the primary lesion (for T) or of the greatest lesion (for N and M) and the SUVmax of the same lesion (16, 17); MTV was assessed by means Syngo Siemens workstation and was computed by using SUVmax $\geq$ 2.5 as thresholds (10); TLG for T, N, M was computed as MTVxSUVmean. Finally, the patients were divided in two groups according to the presence of either limited metastatic involvements (patients with M1a or classified as M1b due to extra thoracic lymph nodes only, $M1b_{Lympho}$) or disseminated metastatic disease (all other patients with M1b, $M1b_{Disseminated}$) on FDG-PET/CT scan. Similarly, presence (B+) or absence (B-) of metabolically active bone lesions was recorded for each patient.

Statistical Analysis

The associations among CTCs, cfDNA and FDG-PET/CT derived parameters were evaluated by multivariate analysis (Statistical Package for the Social Sciences, SPSS version 17). T-test was performed to evaluate the difference in CTCs or cfDNA in patients with M1a plus $M1b_{Lympho}$ with respect to patients $M1b_{Disseminate}$ as well as in B+ and B- subgroups respectively. The Mann-Withney unpaired test was used to compare independent variables. $P<0.05$ was regarded as significant.

Results

Patients

Thirthy-seven out of 79 patients recruited in the VeriStrat® trial matched our PET-based criteria. Median age was 64.5 ± 8.1 years (range: 51-80); male/female ratio was 24/13; 19 patients were current smokers, while 16 were former smokers and 2 were never-smokers. Histological subtypes were adenocarcinoma (n=28) and squamous cell carcinoma (n=9). All the patients excluding one showed metabolically active metastatic lesions. In particular, 12 patients were classified as M1a, 23 patients were classified as $M1b_{Disseminated}$, and one patient was classified as M1b due to the presence of extra thoracic lymph nodes ($M1b_{Lympho}$). In addition, 13 out of 37 patients had metabolically active bone lesions. See Table1 for patients' characteristics.

Circulating Tumor Markers

Whole blood withdrawals for cfDNA and CTC evaluations were collected from advanced NSCLC patients at baseline before platinum-based combination chemotherapy. The median baseline CTC count of 6 CTCs/3mL of blood (range: 0-47 CTCs/3mL), and the median cfDNA of 101 hTERT copy number (range: 16-1604) were identified as the most appropriate cut-offs for comparative studies with FDG-PET/CT derived parameters. CTC count was not significantly associated with any PET-derived parameters by Mann-Withney test. The only statistically significant association was observed for cfDNA and M-SUVmax (P=0.003). Indeed patients with cfDNA hTERT copy number above the median level exhibited a higher median M-SUVmax value as compared to those with cfDNA above the median level (Table2).

PET/CT-derived predictors of circulating tumor markers

At multivariate analysis, M-SUVmax was the only variable independently associated with baseline cfDNA levels (p=0.016). No further correlations were highlighted between cfDNA levels as well as CTC number and all the others PET-derived parameters. Table 2 summarizes the results of the multivariate analysis. Notably, higher levels of cfDNA were detected in the subgroup of patients with metabolically active bone lesions (p=0.02) while no difference was highlighted when comparing patients with more limited metastatic disease (M1a + $M1b_{lympho}$) with patients with $M1b_{Disseminated}$ (regardless of the anatomic topography of the lesions) (Figure1). No significant difference was observed in the CTC number when comparing patients with more limited metastatic disease and patients with disseminated lesions ($M1b_{disseminated}$); similarly, no significant difference in the CTC number was observed in patients with or without metastatic bone involvement. Figure 2 shows PET/CT images of two of the analyzed patients with correspondent CTC and cfDNA levels.

Discussion

The present study evaluated the correlation between CTC number or cfDNA level and PET-derived parameters, both at the loco-regional or distant lesion levels in a homogenous population of chemotherapy-naïve patients with advanced NSCLC. Our results reported a positive correlation between high baseline level of cfDNA and tumor metabolic activity. Since neither MTV nor SIMaxSUV (which include a volumetric assessment) showed any relationship with plasma concentration of cfDNA and only SUVmax was significantly associated with this circulating biomarker, it is conceivable that cfDNA might better reflect tumor metabolism and biological behavior rather than tumor burden in metastatic NSCLC.

A previous study by Nygaard and colleagues estimated the tumor burden in terms of MTV and TLG in a similar setting(10). Although higher MTV as well as cfDNA level were associated with a significantly shorter overall survival, no correlation between cfDNA and the PET-derived parameters was found. These controversial results might be explained by the fact that only two PET-derived parameters (MTV and TGL) were taken into account. Indeed, different cut-offs for MTV computation have been proposed but none of them has been validated in this setting and overall consensus about the best PET-based indicator is presently lacking. Previous studies have assessed the relationship of circulating tumor markers and FDG-PET-CT in different cancers (4, 18). In this framework the majority of studies have been carried out in other malignancies, especially in metastatic breast and colon cancer (18). In recent years, a study in NSCLC patients evaluated the change in CTC counts and its relationship with FDG-PET in patients treated with chemotherapy for relapsed disease (4). However, the authors were unable to find a SUVmax cut-off predicting response of CTC to treatment and SUVmax demonstrated a trend in predicting change in CTCs only after allocating patients in responders and non-responders. Similarly, a retrospective evaluation of NSCLC patients who underwent FDG-PET-CT imaging and CTC detection before therapy was previously carried out by Nair et al (9). Again the study demonstrated a weak correlation with SUVmax and no correlation with tumor diameter. However both studies were multi-centers and the results of the analyses might have been influenced by the effect of different PET scanners. Notably, only in the study of Nair and colleagues the FDG-PET inter-scanner calibration was performed (9). Our experience follows the same path but in a different and more homogeneous study population. Indeed while, we only considered chemotherapy naïve advanced NSCLC patients, in the study by Nair and colleagues, early-stage patients were mostly, but not exclusively, included. To summarize, questionable and mostly negative results on this topic

emerged from the few available studies in NSCLC likely uncovering some technical limits such as the different (and often heterogeneous) patients' populations, the use of diverse PET scanners and the lack of gold standard indicators of tumor burden and/or aggressiveness by means of PET. In the present study an independent correlation between SUVmax and cfDNA was disclosed by means of a multivariate analysis which included a wide range of PET/CT derived parameters determining patients' tumor burden and biology (T, N and M). Specifically, the SUVmax of the metastatic lesions was the only independent predictor of cfDNA levels. The presence of a significant correlation between cfDNA and tumor metabolism of the metastatic lymph nodes rather than the primary tumor is in keeping with the hypothesis that circulating tumor markers might be strongly related to the metastatic potential of different tumor types (19). In particular, when we took into account the subset of patients with bone metastases, the relevance of cfDNA in defining the biological behavior of the tumor was further highlighted. Higher levels of cfDNA were observed in the subgroup of patients with metabolically active bone lesions, while no difference was found for the other disseminated metastatic lesions regardless of their anatomical topography. As it has been demonstrated that high FDG uptake may relate to the tumor metastatic potential (12, 20), the identification of the association between cfDNA and the active bone lesions may represent a step forward in the understanding of the mechanisms underlying metastasis and tropism in NSCLC.

Notably, while cfDNA correlated with tumor metabolism, no association was conversely found between CTCs and PET-derived parameters in the present population.

Previous investigations have suggested that cfDNA and CTCs may provide complementary information about tumor biology. In particular, CTCs derive as intact cells shed from the primary or metastatic tumor sites, while cfDNA is released from different sources including apoptosis,

necrosis, phagocytosis and lysis of tumor cells (1). These different biological features and the limited number of patients might be partly responsible for the discordant results, thus preventing to draw definitive conclusions about the role of CTCs in NSCLC metabolism.

The present study has some drawbacks: more specifically, it is a single-center study including a limited number of patients recruited within a clinical trial in which the FDG-PET/CT was not mandatory; as a consequence, only patients referred to FDG-PET/CT for specific clinical reasons before the inclusion in the study were investigated and some of the patients' characteristics might have affected the analysis. Therefore, the present results should be confirmed within a full prospective design including FDG-PET/CT examination as part of the trial.

In conclusion, although additional studies are required to propose a more accurate modeling regarding the interplay among tumor metabolism, circulating tumor markers and tumor aggressiveness, the present preliminary data support the role of cfDNA as an indicator of tumor biology/aggressiveness rather than of tumor burden in advanced NSCLC patients before chemotherapy initiation. The identification and a deeper understanding of clinically reliable noninvasive biomarkers may help identify potential unresponsive NSCLC patients before treatment to allow personalized therapies and limit toxicity.

Disclosure

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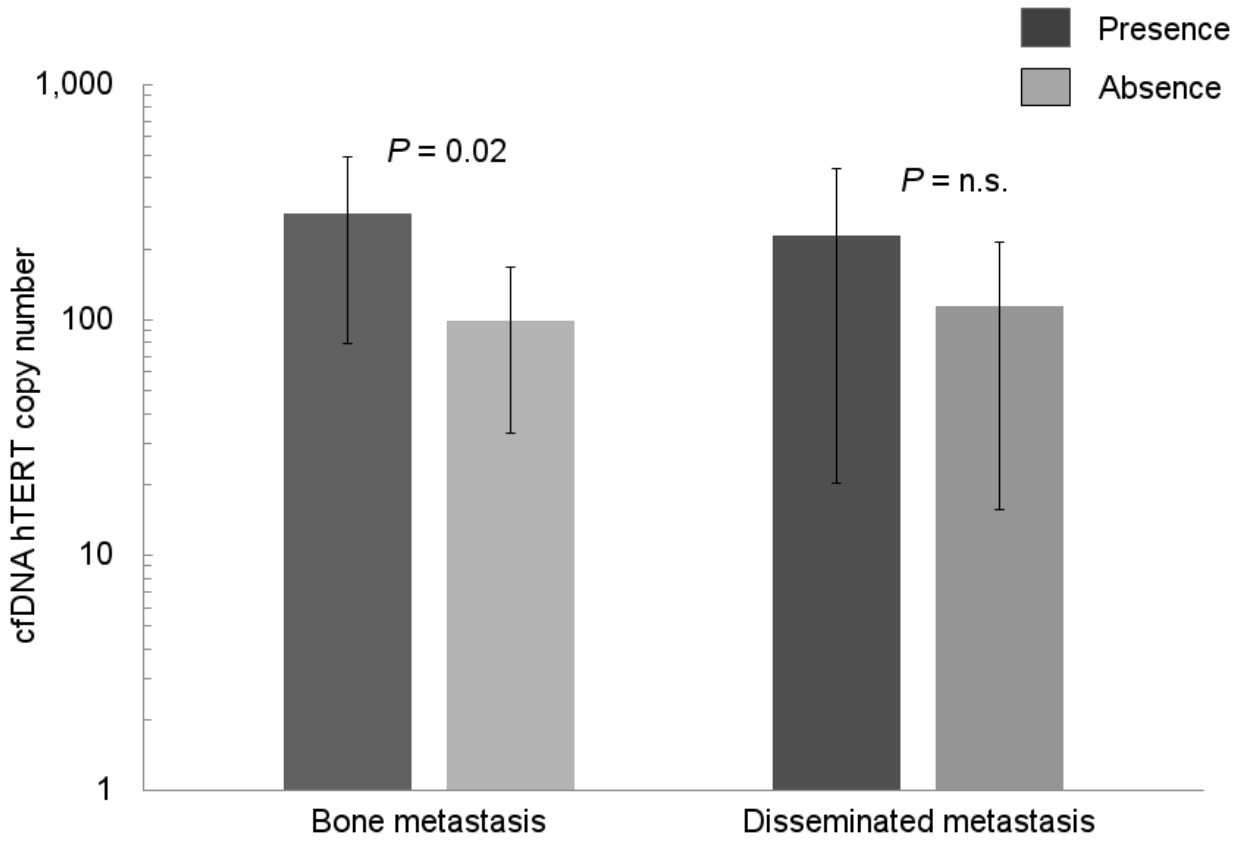

Figure 1.

Histograms showing free circulating tumor DNA (cfDNA) levels in subgroups of patients according to the presence/absence of disseminated metastasis or metabolically active bone lesions. Higher cfDNA levels were detected in the subgroup of patients with metabolically active bone lesions while no difference was highlighted when comparing patients with more limited metastatic disease (M1a + M1b just due to extra-thoracic lymph nodes) with patients with disseminated M1b disease (regardless of the anatomic topography of the lesions).

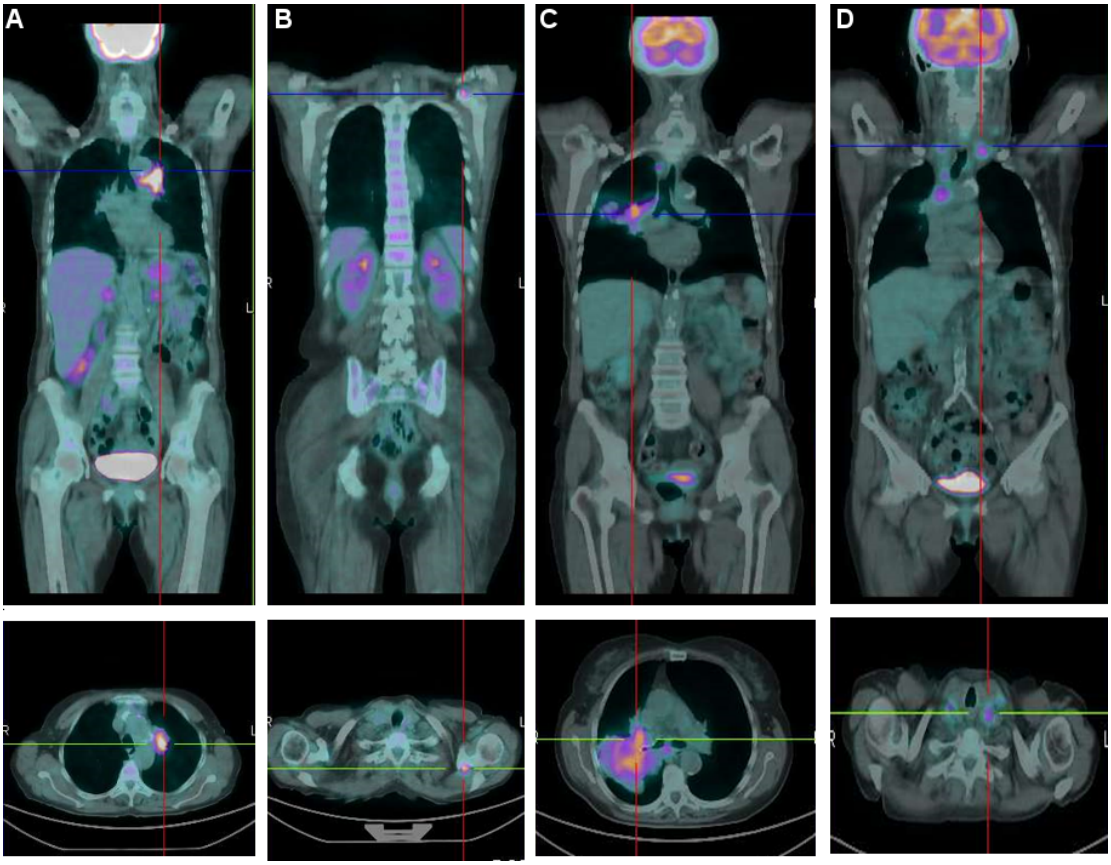

Figure 2

Two representative examples of patients enrolled in present study. Panel A and B show two coronal and trans-axial sections of the FDG-PET/CT scan of a 73 years old female with stage IV NSCLC (adenocarcinoma; cfDNA 462 hTERT copy number ; 3 CTC/3mL). Sections in panel A include the highly FDG-concentrating primary lesion in the left lung (dmax 49 mm; SUV max 7.2; MTV 82.4 ml) while panel B shows a small bone lesion in the left scapula (dmax 10 mm; SUV max 5.7; MTV 5 ml). Panel C and D correspond to the FDG-PET/CT scan of a 70 years old male with stage IV NSCLC (adenocarcinoma; cfDNA 113 hTERT copy number ; 3 CTC/3mL). Sections in panel C include the moderately FDG-concentrating primary lesion in the right lung (dmax 83 mm; SUV max 4.9; MTV 193.2 ml) while panel D shows multiple mediastinal and

cervical lymph nodes (dmax of the greated lymph node 30 mm; SUV max 3.2; MTV 65 ml). Patient displayed in panel A and B showed lower tumor burden (as expressed by the MTV) but higher SUVmax and cfDNA levels with respect to patient displayed in panel C and D. CfDNA, free circulating tumor DNA; SUVmax, Standardized Uptake Value, MTV, Metabolic Tumor Volume).

Table 1. Patients' Demographics and Clinical Characteristics

Demography					
Age (years; mean, range)		64.5 (51–80)			
Gender (m/f)		24/13			
Stage at diagnosis			*Histology*		
IIIb		1	adenocarcinoma		28
IV		36	squamous cell carcinoma		9
TNM			*FDG avid bone lesions*		
T			yes		13
	x	4	no		24
	1	3			
	2	15	*Smoking habit*		
	3	3	yes		19
	4	12	no		2
N			former		16
	x	1			
	0	7	*ECOG PS at baseline*		
	1	0		0	9
	2	22		1	27
	3	7		2	1
M					
	0	1	*Prior Surgery*		
1a Controlater lung		3	yes		5
1a Lung/Pleural disseminated		9	no		32
1b extrathoracic Lymph nodes		1			
1b distant metastasis		23	*Prior Radiotherapy*		
			yes		1
			no		36

Unless otherwise stated, results are reported as numbers of patients
ECOG PS, Eastern Cooperative Oncology Group Performance Status

Table 2. Multivariate Analysis

18F-FDG PET/CT parameters		Mean±standard deviation	p
T	Size	54.4±35 mm	0.175
	SUV max	12.4±4.5 g/ml	0.076
	SUV mean	6.3±2.9 g/ml	0.994
	SIMaxSUV	652±594	0.472
	MTV	179±172 ml	0.463
	TGL	554±595 g	0.313
N	Size	26.4±10.4 mm	0.083
	SUV max	12.7±12.5 g/ml	0.318
	SUV mean	5±2.4 g/ml	0.307
	SIMaxSUV	261±144	0.463
	MTV	22.7±21.10 ml	0.371
	TGL	191±169 g	0.572
M	Size	28.3±23.9 mm	0.313
	SUV max	6.3±4.6 g/ml	**0.016***
	SUV mean	4.1±2.1 g/ml	0.294
	SIMaxSUV	180±148	0.231
	MTV	28.1±31.0 ml	0.201
	TGL	180±31.7 g	0.401

SUV, standardized uptake value

SIMaxSUV was defined as the product of the greatest diameter (mm) of the primary lesion (for T) or of the greatest lesion (for N and M) and the SUVmax of the same lesion

MTV, Metabolic Tumor Volume

TLG, Total Lesion Glycolysis computed as MTVxSUVmean

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