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Use of circulating tumor cells in prospective clinical trials for NSCLC patients – standardization of the pre-analytical conditions

https://doi.org/10.1515/cclm-2017-0764
Received August 26, 2017; accepted December 26, 2017

Abstract

Background: Circulating tumor cells (CTCs) hold potential for noninvasive diagnosis, prognosis and prediction testing in non-small cell lung cancer (NSCLC) patients. Minimizing degradation or loss of CTCs is pivotal for detection and profiling of the low abundance and fragile CTCs, particularly in clinical trials. We prospectively investigated (NCT02372448) whether a new blood collection device performed better compared to commonly used K_3EDTA tubes, when subjected to long-term sample storage.

Methods: Blood samples were drawn into K_3EDTA and blood collection tubes (BCT) (Streck), and filtered by the Isolation by SizE of Tumor/Trophoblastic Cells (ISET® system), for CTC detection in two study populations of NSCLC patients; the training set of 14 patients with stage II/IV NSCLC, and the validation set of 36 patients with stage IV NSCLC). MET expression was evaluated by immunocytochemistry (ICC) and *anaplastic lymphoma kinase* (*ALK*) gene rearrangement by break-apart fluorescence *in situ* hybridization (FISH) on ISET-enriched CTCs.

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Results: Blood processed after 24 h and 48 h in BCT tubes showed stable CTCs counts and integrity, whereas CTCs in K_3EDTA tubes showed an altered morphology in all patients. CTCs recovered in BCT or K_3EDTA tubes at 24 and 48 h were evaluable by ICC for MET expression and by FISH for *ALK* rearrangement.
Conclusions: The BCT tubes gave a high yield and preserved the integrity of CTCs after 24 and 48 h of storage at room temperature, which facilitate their molecular characterization in NSCLC patients entering clinical trials.

Keywords: BCT; circulating tumor cells; Isolation by SizE of Tumor/Trophoblastic Cells (ISET); K_3EDTA; non-small cell lung cancer (NSCLC); stability.

Introduction

Over the past decade, circulating tumor cells (CTCs) have been identified as potential blood-based biomarkers that are able to provide information on patient prognosis and to monitor treatment efficacy in different types of cancers, including non-small cell lung cancer (NSCLC) [1–3]. Moreover, phenotyping of CTCs has the potential for identification of specific protein/gene alterations, generating aberrant signaling and cancer cell proliferation, which may predict response or resistance to specific therapeutic targets [4–6].

CTC counts in NSCLC are indicative of prognosis. CTCs can function as a surrogate noninvasive tumor sample for the detection of predictive biomarkers for targeted therapy, including *EGFR* mutations, MET protein expression or *ALK* rearrangements [3, 7–11]. Recently, "sentinel" CTCs have been detected in patients with chronic obstructive pulmonary disease and may predict the occurrence of NSCLC, thus supporting the potential use in lung cancer screening programs [12]. Thus, CTC testing has become a very active field in oncology in drug development and pharmacodynamic studies. In an extensive literature search in July 2017, there were 554 clinical trials, involving CTC biomarker testing, reported in the "ClinicalTrials.gov" website, with 172 trials in NSCLC alone (ClinicalTrials.gov).

However, while many devices and technologies have been developed to enrich CTCs from patient blood samples, the blood sample is mainly processed on-site and within a few hours of collection to better retain integrity and viability [13, 14]. In this regard, for logistical and practical reasons, it is often not possible to process and store blood samples immediately after phlebotomy to ensure an optimal CTC quality; especially in the context of large multicenter prospective clinical trials, which are essential to definitely establish CTCs as a clinically relevant biomarker. To date, CellSearch® is the only FDA approved and clinically validated CTC assay that isolates CTCs from blood samples collected in CellSave tubes, which allows storage of blood samples for up to 3 days [1, 15]. Nevertheless, as CTCs undergo epithelial-mesenchymal transition into the bloodstream and loose their epithelial biomarkers, the CellSearch system, an EpCAM-based enrichment method, cannot detect all CTC subpopulations [15, 16]. Thus, marker-independent direct technologies for CTC detection based on morphology, which can effectively capture CTCs of varying phenotypes, are very attractive. However, there is still a need for standardization of the pre-analytical conditions that allow longer processing times of blood samples analyzed with direct techniques, which preserve the morphology of CTCs.

It is estimated that $3.2–4.1 \times 10^6$ CTCs are shed daily per gram of tumor tissue, based on a rat mammary model [17], whereas they are thought to have a short half-life of less than 3 h in the bloodstream, as shown in patients with breast cancer [18]. The fragile nature of CTCs may arise due to the anoikis of CTCs, which begins after separation from the tumor of origin and after removal of blood from the patients [18]. Therefore, one of the major technical hurdles for CTC analysis has been efficient recovery of rare and fragile CTCs from a background of approximately 10^9 erythrocytes and 10^7 leukocytes per mL of whole blood [19, 20]. Despite recent technical advances, practical CTC analysis for clinical trials involving multiple sites are particularly challenging, due to strict requirements for pre-analytical conditions of blood samples in transport and storage, and time restrictions to ensure that the CTC integrity and biomarker detectability is maintained. Parameters, such as blood collection devices, sample agitation, storage or shipping temperature and delays in sample processing in a large-throughput laboratory need to be considered before such measurements are conducted for clinical trials. Ideally, CTCs should be protected from apoptosis or cell lysis and, importantly, must preserve relevant tumor biomarkers.

Only a few previous reports on spike-in cell line blood samples suggested that the added preservatives in collection tubes may improve the CTC stability [21–23]. However, the preservative effects on actionable tumor biomarkers in NSCLC, such as MET or *ALK*, on patient-derived CTCs, to our knowledge, have not been tested thoroughly in CTCs isolated from different blood collection tubes.

In this prospective clinical study, we compared the performance over time of two cell stabilizing reagents, the Cell-Free DNA BCT™ tubes and the commonly used tripotassium ethylenediaminetetraacetic acid (K_3EDTA)

tubes, on CTC enrichment and morphological integrity, as well as on detection of MET expression by immunocytochemistry (ICC) and *ALK* rearrangement by fluorescence *in situ* hybridization (FISH) in NSCLC patients.

Materials and methods

Patients and sample collection

Experiments were performed as part of an ancillary study in a prospective clinical trial (STALKLUNG – NCT02372448). Two cohorts were designed to assess the stability over time of CTCs in blood collected in K_3EDTA and BCT tubes (Table 1).

Fourteen patients with lung adenocarcinoma (ADC) and five healthy donors were included in the training set study, and 36 patients with stage IV lung ADC or NSCLC not otherwise specified (NOS) and five healthy volunteers were enrolled in the validation study (Supplemental Table 1).

In the training study, 38 mL of peripheral blood was collected by venipuncture, with 2×9 mL collected in two Monoject lavender top glass K_3EDTA tubes (BD Vacutainer, France), and 2×10 mL in two Streck Cell-Free DNA BCT® tubes (Streck Inc., Omaha, NE, USA). One K_3EDTA blood tube (as a baseline control) and one BCT tube were processed within 2 h of phlebotomy, and the two additional tubes were then stored at room temperature without agitation and processed 24 h after phlebotomy.

In the validation study, 9 mL of peripheral blood collected in a K_3EDTA tube was processed within 2 h post collection (as a baseline control), and 2×10 mL additional blood samples were collected in BCT tubes, stored at room temperature without agitation and processed 24 and 48 h after phlebotomy.

Blood samples were collected using an Institutional Review Board protocol (2014-A00417-40) at the Department of Pulmonary Medicine and Oncology (Pasteur Hospital, Nice). All subjects provided written informed consent. The study complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

CTC enrichment and enumeration

Enrichment of CTCs was performed on the Isolation by SizE of Tumor/Trophoblastic Cells (ISET) platform (Rarecells, Paris, France) as previously described [3, 24]. Briefly, 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 three parts containing, respectively, four spots for May-Grünwald Giemsa (MGG) staining for cytological assessment of the CTC count and integrity and six spots for ancillary studies [25]. The filter-spots were examined for the presence of circulating non-hematological cells with malignant features so-called CTCs, as previously reported [3, 26]. The enumeration was independently assessed by three senior pulmonary pathologists (MI, VH and PH) blinded to the study data.

MET protein staining and *ALK* FISH on ISET filters

MET expression in CTCs was assessed by ICC on two of the remaining unstained filter-spots, as previously described [9]. After 2 min of rehydration with Reaction Buffer 10x (Ventana Medical Systems, Tucson, AZ, USA), ISET filters were placed in the BenchMark ULTRA autostainer (Ventana), and stained with the CONFIRM anti-Total c-MET (SP44) rabbit monoclonal primary antibody (Ventana) and the ultraView Universal DAB Detection Kit (Ventana) [9].

ALK FISH was performed on CTCs isolated using the ISET method on two of the unstained spots by using a break-apart probe for the *ALK* gene (Vysis LSI *ALK* Dual Color, Abbott Molecular Inc., Des Plaines, IL, USA), as previously described [7].

Statistical analysis

Statistical analysis was performed using the SPSS software system (version 16.0, SPSS Inc., Chicago, IL, USA). A two-tailed paired t-test was performed for each sample set. A p-value of less than 0.05 was considered significant.

Results

Level and morphology of CTCs

To test the preservation of CTCs with the two different blood tubes, CTCs were first enriched and enumerated at 2 and 24 h after collection in a training set of 14 patients with lung ADC (Table 1). Four experiments were performed using 38 mL of blood from each patient.

Table 1: Study flow diagram of the patients included in the study.

Cohort	Number of patients	Collection tube	Time before processing		
			2 h	24 h	48 h
Training study	ADC, n = 14	K_3EDTA	☑	☑	×
	Healthy, n = 5	BCT	☑	☑	×
Validation study	ADC, n = 36	K_3EDTA	☑	×	×
	Healthy, n = 5	BCT	×	☑	☑

In the training set, five out of 14 (36%) ADC patients had CTCs (range, 1–5 CTCs/4 mL) when K_3EDTA tubes were processed within 2 h (baseline standard control) (Figure 1, left panel). Likewise, the levels of CTCs (range, 2–8 CTCs/4 mL) enriched in the BCT tubes did not significantly changed in these five (36%) ADC patients (Figure 1, left panel).

After 24 h of storage at room temperature without agitation, we detected CTCs (range, 1–6 CTCs/4 mL) in K_3EDTA tubes from five (36%) patients, with however loss of CTCs in one patient and detection of three CTCs in one patient without initially isolated CTCs at 2 h (Figure 1, middle panel). In contrast, the collection in BCT tubes resulted in higher levels of CTCs (range, 2–16 CTCs/4 mL) in seven (50%) patients ($p < 0.05$; Figure 1, middle panel).

While BCT tubes preserved the morphology of CTCs and white blood cells (WBCs) when compared to K_3EDTA tubes, the CTC and WBC integrity was altered in 100% and 50% ADC patients, respectively, when enriched 24 h after blood collection on K_3EDTA tubes (Figure 2). No CTCs were detected in the blood of the five healthy donors collected with the two types of blood tubes.

In the validation study, we sought to determine if the results on BCT tubes were still reproducible 24 and 48 h after blood collection in 36 ADC patients (Figure 1). At the 2 h baseline, 27 out of 36 (75%) ADC patients had CTCs (range, 1–76 CTCs/4 mL; median = 5) in K_3EDTA tubes. BCT tubes showed stable CTC count when samples were processed at either 24 (range, 1–79 CTCs/4 mL; median = 9 CTCs/4 mL) or 48 h (range, 1–83 CTCs/4 mL; median = 11 CTCs/4 mL) (Figure 1, right panel). Two supplementary patients (overall 29 out of 36 patients, 81%) demonstrated CTCs while having no CTCs isolated at baseline on K_3EDTA tubes. Moreover, the morphology and the integrity of the CTCs were preserved in BCT tubes at 24 and 48 h, when compared to K_3EDTA tubes at 2 h, with no major lysis of WBCs (Figure 3).

Stability of the MET protein expression in CTCs by ICC

In the training set, examination of the stained two-spots ISET filter processed at 2 h on K_3EDTA or BCT identified five out of 14 (36%) samples with at least one CTC defined as MET positive. The MET expression was stable at 24 h in BCT tubes whereas in K_3EDTA tubes the protein was degraded as evidenced from the weak or absent signal (Figure 4). MET expression in CTCs correlated strongly

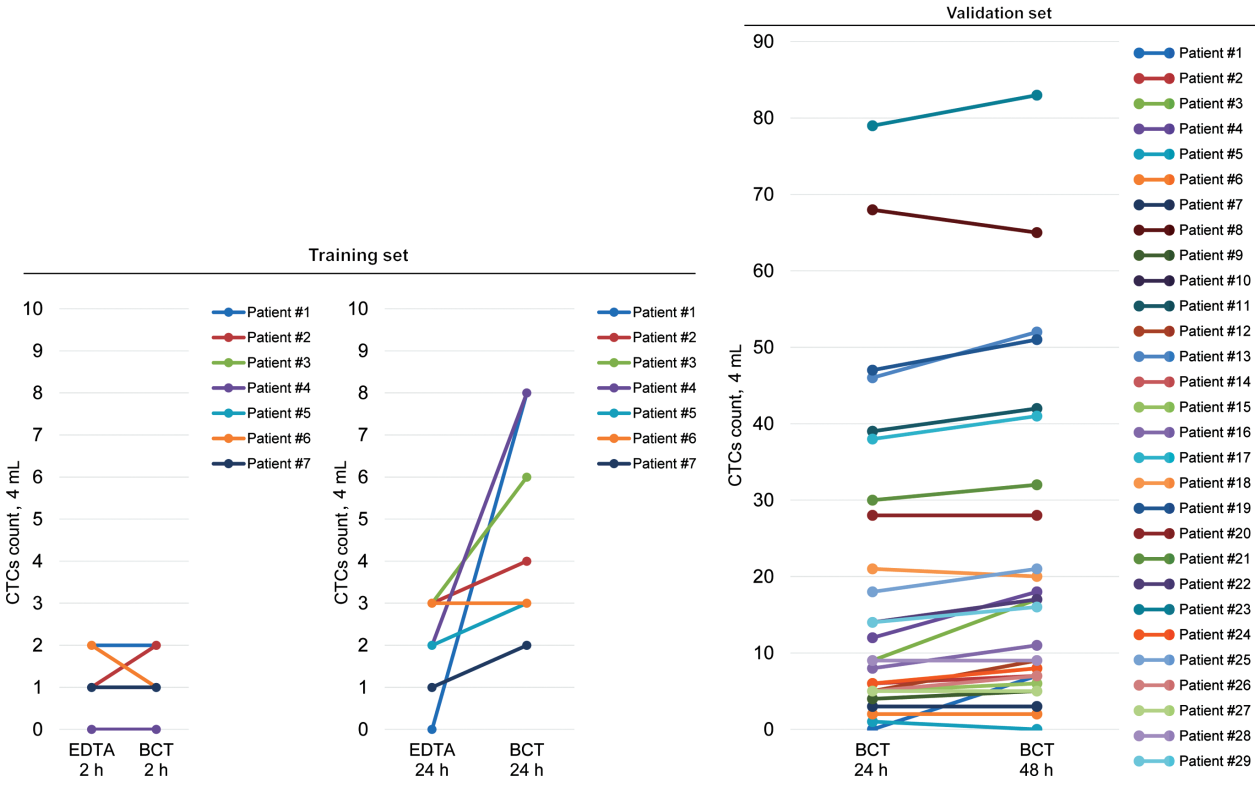

Figure 1: The effect of different blood tube collection storage delays on the levels of enriched CTCs.

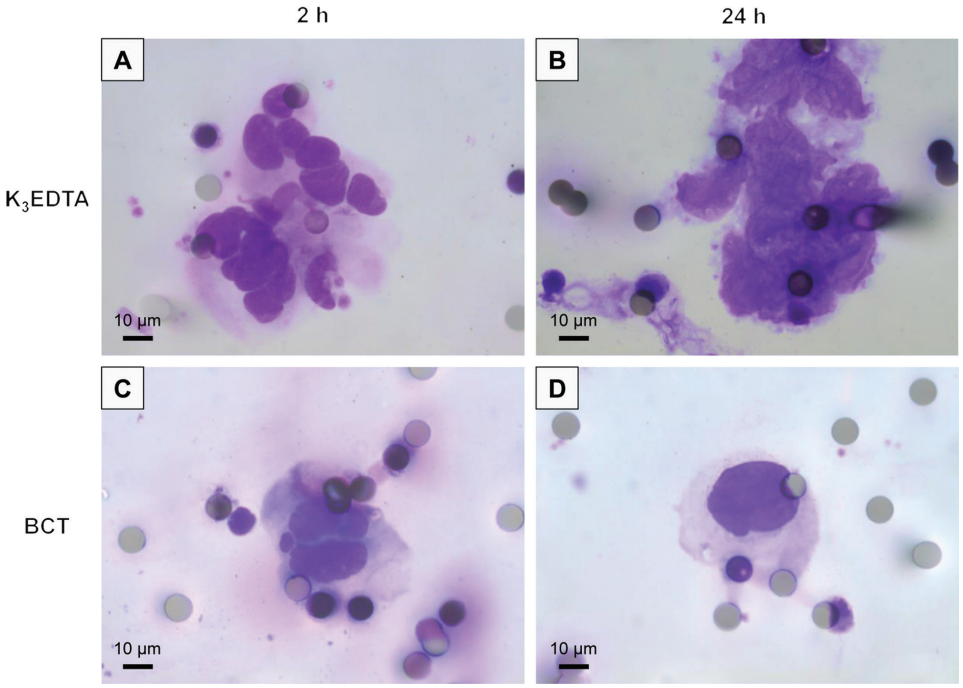

Figure 2: Representative images of morphological analysis of CTCs isolated from NSCLC patients included in the training set. MGG analysis after blood collection in K_3EDTA tubes at (A) 2 h or (B) 24 h, and BCT tubes at (C) 2 h or (D) 24 h.

with the MET status in patient-matched tumor tissue (positive predictive value = 100%, Figure 4).

In the validation set, MET expression was observed in 19 out of 36 (53%) blood samples processed at 2 h in K_3EDTA tubes, and was stable over time in BCT tubes processed at 24 and 48 h (Figure 3). MET expression in CTCs correlated well with the MET status in patient-matched tumor tissue, with only two patients having MET status positive in CTCs, and negative in tumor tissue (Figure 3). No MET expression was detected in the blood of the five healthy donors collected within the two types of blood tubes.

Stability of *ALK* rearrangement in CTCs by FISH

While there was no *ALK* rearrangement detected in the training set, two patients (5%) included in the validation study had CTCs with an *ALK* rearrangement, enriched either in K_3EDTA at 2 h or BCT tubes at 24 and 48 h (Figure 5). The green and red fluorescence had a similar intensity, and no nonspecific FISH signals were identified in BCT tubes at 24 and 48 h, whereas the K_3EDTA tubes processed at 24 h showed a significantly decreased or absent intensity of the green and red probes with high background autofluorescence noise (Figure 5).

Discussion

Long-term storage and shipping of blood samples from the site of phlebotomy to another facility is commonly required for NSCLC patients needing molecular diagnostic testing in clinical practice or multicenter clinical trials [4]. Together with testing for circulating-free DNA (cfDNA) in blood, CTC testing is an interesting field in drug development and pharmacodynamic studies in oncology [4, 8]. Thus, reducing degradation or loss of fragile and rare CTCs is vital for their detection and profiling in NSCLC patients entering clinical trials.

Recently, new collection blood tubes such as BCTs (Streck) have been shown, by ^{13}C-nuclear magnetic resonance to be formaldehyde-free [27], and were previously established to preserve cfDNA after several days of storage at room temperature [28–32]. Moreover, the CE-IVD version of the cfDNA BCTs tubes is now recommended as the preferred collection device for *EGFR* mutation testing with a liquid biopsy from stage IIIB/IV NSCLC patients [33].

In addition, while the CellSave tubes allow long-term storage of blood samples for CTC detection on the CellSearch® system, marker-independent direct technologies for CTC detection, such as ISET, have the potential to effective capture all CTC subpopulations [15, 34].

However, only a few studies have focused on the preanalytical variables that might compromise the accuracy

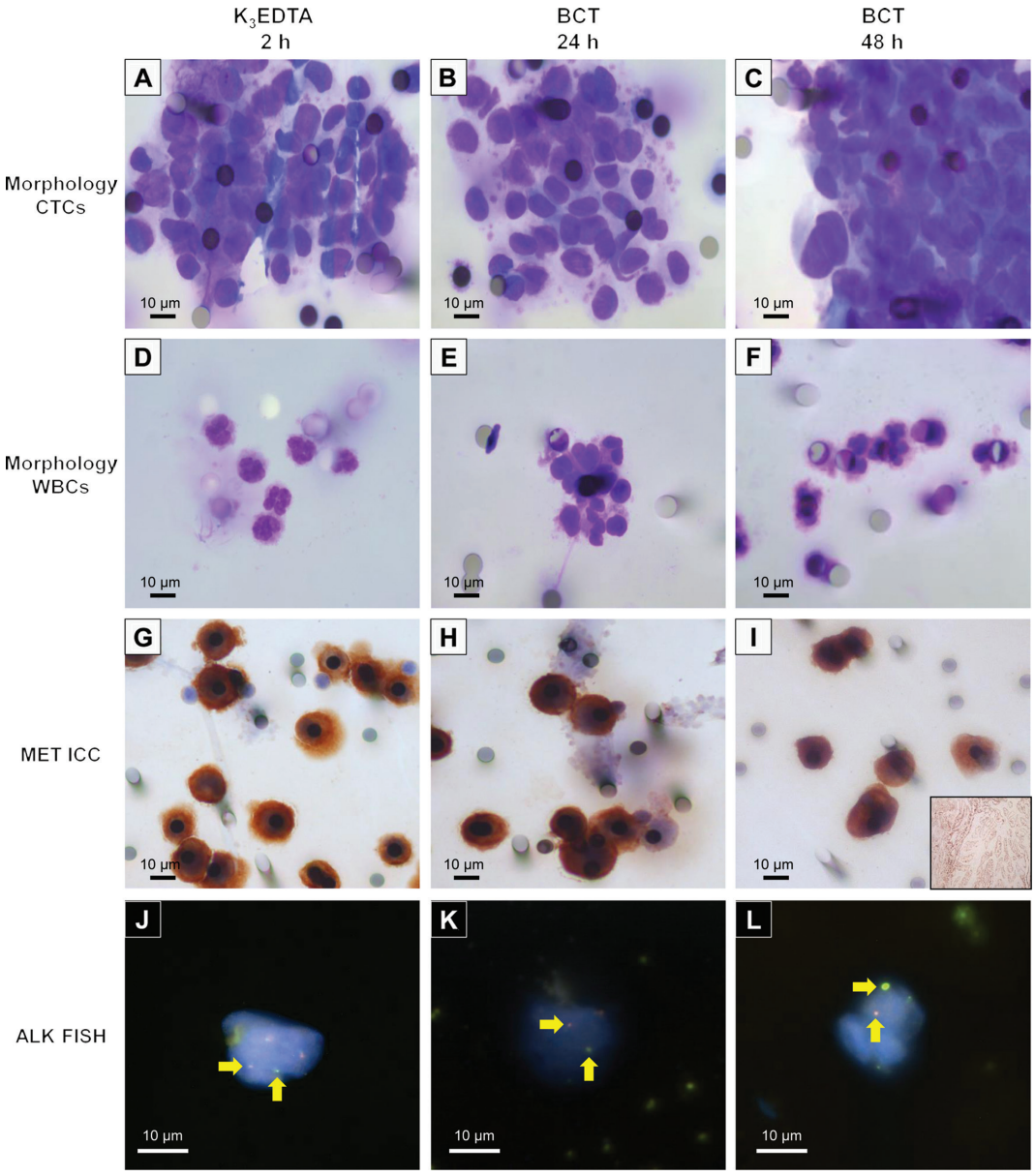

Figure 3: Morphological analysis and detection of MET protein expression and *ALK* rearrangement in CTCs isolated from NSCLC patients included in the validation set after blood collection at 2 h in K_3EDTA tubes (left panel), 24 h in BCT tubes (middle panel) and 48 h in BCT tubes (right panel).
(I) Insert, paired tumor tissue exhibiting strong MET expression in tumor cells (original magnification ×100). (J–L) The two probes (3′, red; 5′, green) show distinct separation of the red and green signals (arrowheads) indicating a rearrangement in the *ALK* gene locus.

of CTC analysis with direct technologies, including the selection of blood collection devices, chemical reagents, sample storage and shipping conditions, but none on their morphological analysis [21–23]. These parameters affect the amount of CTCs recovered as well as the morphology post phlebotomy. CTC lysis and loss leads to false-negative CTC measurement, detection and characterization. Moreover, the effects of preservatives on actionable tumor biomarkers in NSCLC, such as MET or *ALK*, on patient-derived CTCs, to our knowledge, has not been evaluated on CTCs isolated from different blood collection tubes.

Our results show that BCT tubes preserve the CTC integrity and detection of predictive biomarkers, at the protein and DNA level, in blood samples obtained from NSCLC patients and stored at room temperature for at least 24 and 48 h.

While traditional chemicals used in cell stabilization, such as formaldehyde and alcohol-based fixatives, are known to damage DNA and RNA by causing nucleic

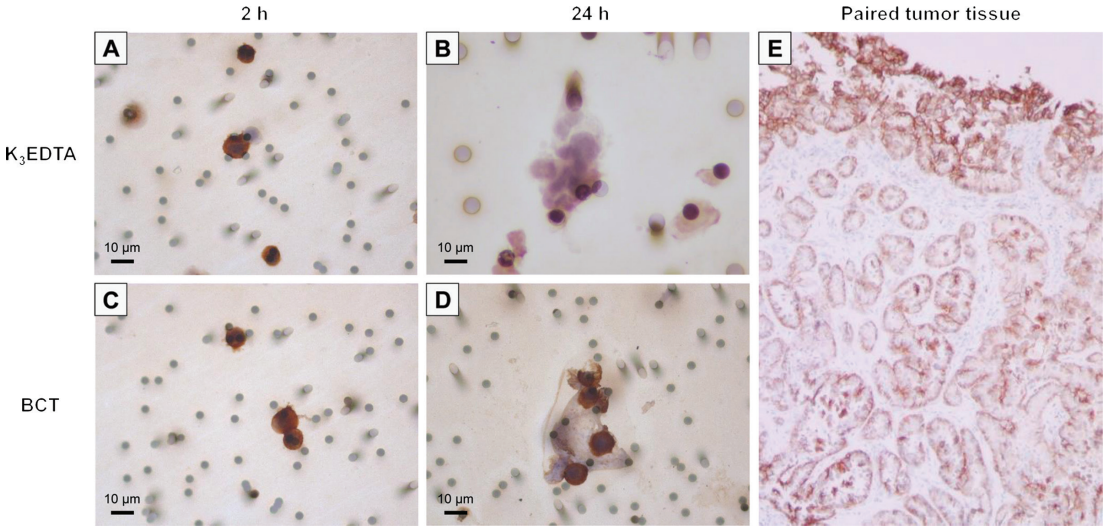

Figure 4: Detection of MET protein expression in CTCs isolated from NSCLC patients included in the training set.
ICC analysis after (A) 2 or (B) 24 h storage in K_3EDTA tubes, and after (C) 2 or (D) 24 h collection in BCT tubes (original magnification ×200; bar: 10 µm). (E) Paired tumor tissue exhibiting strong MET expression in tumor cells (original magnification ×100).

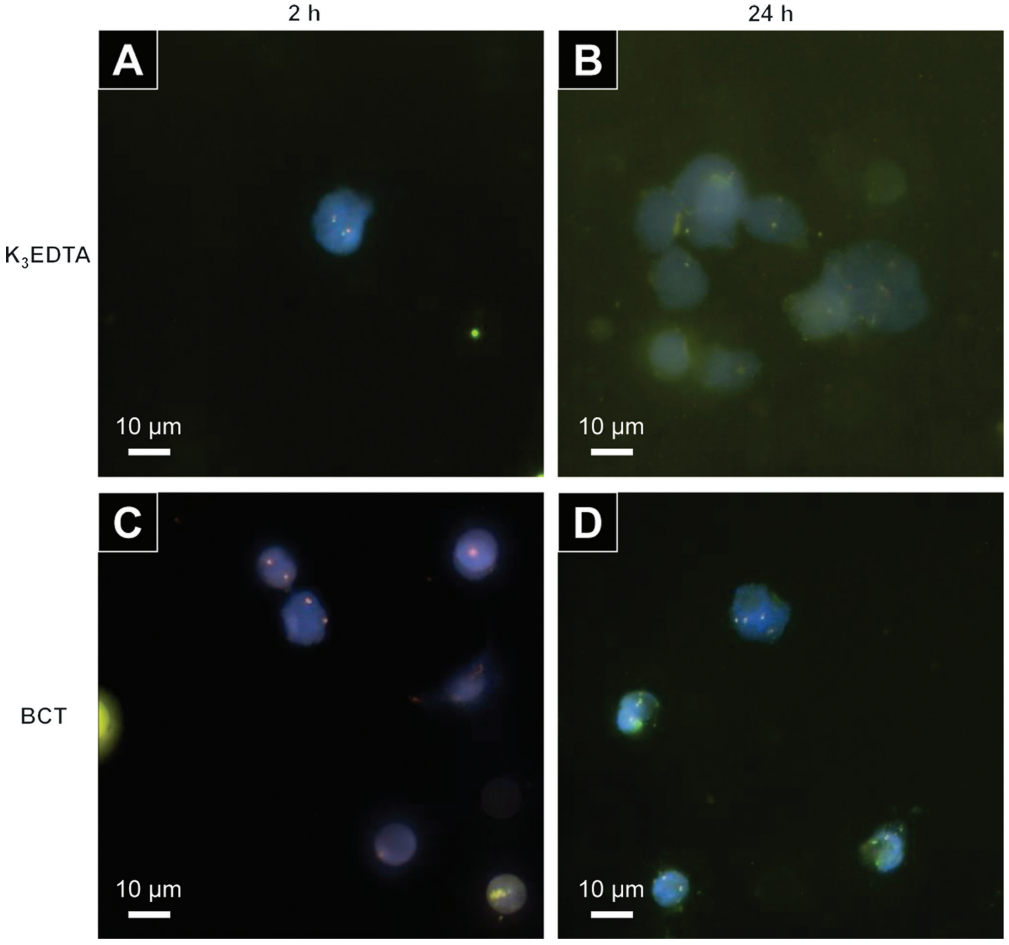

Figure 5: Representative images of *ALK* FISH analysis in CTCs isolated from NSCLC patients included in the training set.
FISH analysis after blood collection in K_3EDTA tubes at (A) 2 h or (B) 24 h, and BCT tubes at (C) 2 h or (D) 24 h. The two probes (3′, red; 5′, green) show overlapping signals in nuclei without an *ALK* gene locus rearrangement (original magnification ×1000; bar: 10 µm).

acid-protein cross-links and make CTC profiling laborious [35], the stabilization cocktail in BCTs tubes has been shown to contain formaldehyde-free fixatives [27].

In our study, intact CTCs were visible under bright field microscopy from BCT tubes after 24 and 48 h, while their morphology was altered in the K_3EDTA tubes in all patients. No increase in the leukocyte levels was observed at 24 and 48 h in BCT tubes.

CTC enrichment was stable over time at 24 and 48 h in BCT tubes. CTCs were isolated from 50% of NSCLC patients after 24 h storage in BCT tubes, compared to 36% in K_3EDTA tubes and from 81% NSCLC patients at 48 h (median = 11 CTCs/4 mL), compared to 75% of patients (median = 5 CTCs/4 mL), in line with the reported sensitivity of the ISET platform for CTC detection in stage IV NSCLC patients [3, 34]. Our results are in agreement with a previous study that found that the yields of spiked-in blood lung cancer cell lines did not significantly decline when processed after 24 and 48 h storage in BCT tubes [21]. Conversely, one study reported that the recovery of spiked prostate cancer cells was not significantly different across different blood tubes within 24 and 48 h of processing [23]. The discrepancy between these results may be attributed to: (i) technological hurdles such as the method of cell enrichment and detection (e.g. direct-marker independent or indirect-marker based systems), types of blood tubes, difficulties of spiking the exact number of cells and (ii) biological constraints related to cultured cell lines being more robust and surviving extended storage in blood samples better that patient-derived CTCs [22, 36]. Our data indicate that BCT tubes are appropriate for CTC detection within 48 h, whereas K_3EDTA tubes should be avoided for long-term processing.

Moreover, analysis of the protein expression and genomic abnormalities from CTCs can provide valuable information for treatment cancer-specific targets, particularly in NSCLC patients, but is notably challenging as delays in blood sample processing may cause alterations in CTCs [5, 7, 9]. Interestingly, Luk et al. [23] did not observe a significant decrease in the levels of cancer cell lines spiked in blood in K_3EDTA or citrate tubes after 48 h, while there was a decrease in RNA levels in three patient-derived CTCs. Our data demonstrate that two important "druggable" NSCLC targets are stable over time at 24 and 48 h after storage in BCT tubes, while they are altered in K_3EDTA tubes. Our findings are supported by recent data showing that spiked breast cancer cells could be retrieved from DNA in BCT tubes after 72 h storage and had accessible DNA, however, whole genome amplification yielded consistently significantly less DNA from K_3EDTA tubes [37].

We have not extended testing after 48 h, as few blood samples (n = 3) prospectively processed after 72 h showed altered levels of CTCs (data not shown). In addition, during transportation, shaking may disrupt nucleated blood cell integrity and compromise CTC integrity. Based on previous observations on cfDNA and for practical reasons (e.g. delivery trucks not equipped with orbital shaker), we did not evaluate sample agitation. For instance, an increase in the cfDNA concentration was observed after 3 h of shaking in BCT tubes [30].

Finally, although the instruction manual for BCT tubes for cfDNA analysis recommends that the tubes be maintained within a broad temperature ranging from 6 °C to 37 °C, several recent studies showed that storage and transport at room temperature is preferable to keeping the tubes at low temperature, to minimize dilution of the mutant DNA fragments by wild-type DNA released from the lysed leukocytes [31, 38]. In this context, for cell morphology analysis on ISET filters, storage of room temperature is recommended [39].

In conclusion, our data demonstrate the usefulness of BCT tubes for patient-derived CTC enrichment and detection by cytomorphological analysis at room temperature for up to 48 h. In addition, if downstream analysis of protein expression by ICC or gene alterations by FISH is intended, they allow long-term flexibility for blood drawn offsite to be sent to centralized laboratories without preliminary preparation.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: STALKLUNG01 (Institut National du Cancer, PHRC National Cancer 2014-A00417-40, Funder Id: 10.13039/501100006364), Cancéropôle PACA, Ligue départementale 06, Conseil départemental 06 Appel à projet santé.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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Supplemental Material: The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2017-0764).