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ARTICLE

Clinical Studies

Leukapheresis increases circulating tumour cell yield in non-small cell lung cancer, counts related to tumour response and survival

Menno Tamminga [1], Kiki C. Andree [2], Hilda van den Bos [3], T. Jeroen N. Hiltermann [1], Anouk Mentink [2], Diana C. J. Spierings [3], Peter Lansdorp [3,4,5], Wim Timens [6], Ed Schuuring [6], Leon W. M. M. Terstappen [2] and Harry J. M. Groen [1] ✉

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BACKGROUND: Circulating tumour cells (CTCs) can be used to monitor cancer longitudinally, but their use in non-small cell lung cancer (NSCLC) is limited due to low numbers in the peripheral blood. Through diagnostic leukapheresis (DLA) CTCs can be obtained from larger blood volumes.
METHODS: Patients with all stages of NSCLC were selected. One total body blood volume was screened by DLA before and after treatment. Peripheral blood was drawn pre- and post DLA for CTC enumeration by CellSearch. CTCs were detected in the DLA product (volume equalling 2×10^8 leucocytes) and after leucocyte depletion (RosetteSep, 9 mL DLA product). Single-cell, whole-genome sequencing was performed on isolated CTCs.
RESULTS: Fifty-six patients were included. Before treatment, CTCs were more often detected in DLA (32/55, 58%) than in the peripheral blood (pre-DLA: 18/55, 33%; post DLA: 13/55, 23%, both at $p < 0.01$). CTCs per 7.5 mL DLA product were median 9.2 times (interquartile range = 5.6–24.0) higher than CTCs in 7.5 mL blood. RosetteSEP did not significantly improve CTC detection (pretreatment: 34/55, 62%, post treatment: 16/34, 47%) and CTCs per mL even decreased compared to DLA ($p = 0.04$)..Patients with advanced-stage disease with DLA-CTC after treatment showed fewer tumour responses and shorter progression-free survival (PFS) than those without DLA-CTC (median PFS, 2.0 vs 12.0 months, $p < 0.01$). DLA-CTC persistence after treatment was independent of clinical factors associated with shorter PFS (hazard ratio (HR) = 5.8, 95% confidence interval (CI), 1.4–35.5, $p = 0.02$). All evaluable CTCs showed aneuploidy.
CONCLUSIONS: DLA detected nine times more CTCs than in the peripheral blood. The sustained presence of CTCs in DLA after treatment was associated with therapy failure and shortened PFS.
TRIAL REGISTRATION: The study was approved by the Medical Ethical Committee (NL55754.042.15) and was registered in the Dutch trial register (NL5423).

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INTRODUCTION

Circulating tumour cells (CTCs) isolated from the peripheral blood are an important biomarker in patients with lung cancer [1, 2]. The Food and Drug Administration cleared CellSearch systems that identify CTCs by their expression of the epithelial cell adhesion molecule (EpCAM), cytokeratins 8, 18, 19 and lack of CD45. CTCs are strongly associated with poor prognosis and worse tumour response to therapy [1, 3–7]. Their clinical use in non-small cell lung cancer (NSCLC) patients is hampered by the low detection rate of CTCs in the peripheral blood. They are detected in merely 30% of NSCLC patients, often only 1–2 CTCs per 7.5 mL peripheral blood [1, 2, 4, 7, 8].

Extrapolation of CTC distributions in 7.5 mL blood of prostate, colon and breast cancer patients showed that all patients were likely to have CTCs in circulation, but that the volume of blood screened for CTCs (7.5 mL) was insufficient for reliable detection [8]. For NSCLC patients, it was necessary to screen 0.75 L of blood in order to detect ten CTCs in 78% of patients [9].

CTCs have the same density as the mononuclear cell (MNC) fraction, allowing separation from red blood cells and plasma by leukapheresis, where centrifugal forces are used to stratify cell components into layers [10–13]. The separated cells are concentrated, allowing screening of a larger volume of blood without

[1]Department of Pulmonary Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. [2]Department of Medical Cell BioPhysics, Faculty of Sciences and Technology, University of Twente, Enschede, The Netherlands. [3]European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. [4]Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC V5Z 1L3, Canada. [5]Department of Medical Genetics, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. [6]Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. ✉email: m.tamminga@umcg.nl

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detrimental consequences to the patients, known as diagnostic leukapheresis (DLA). DLA has been shown to increase CTC counts and detection rates in breast and prostate cancers [9, 10, 12]. Unfortunately, the volume of DLA product that can be screened with the CellSearch is limited, as the enriched leucocytes in the DLA product non-specifically bind the immunomagnetic particles targeting EpCAM, causing the number of cells in the cartridge to be too high for reliable detection of CTCs [10, 11]. A leucocyte count of 2×10^8 was shown to be the maximum that can be analysed in one CellSearch test.

We hypothesised that in NSCLC patients the yield of CTCs can be increased when measured in DLA (as a higher volume of blood can be screened) compared to the peripheral blood. By depletion of leucocytes in the DLA product, an even greater volume of DLA product can be processed with CellSearch to increase CTC counts. By applying this procedure before and after treatment, we hypothesised that the detected changes in CTC counts would more accurately reflect the measured patient outcome in terms of tumour response to treatment and progression-free survival. Furthermore, to provide proof of the malignant origin of the detected cells, CTCs were isolated for single-cell whole-genome sequencing (scWGS) to assess copy number alterations (CNAs).

METHODS

Patient inclusion and clinical data

Consecutive patients with proven NSCLC were prospectively included in an exploratory cohort study. Eligibility criteria were an Eastern Cooperative Oncology Group Performance Status (PS) of 0–2, no use of anticoagulation and no clotting disorders. All patients had to start with a new treatment at the time of inclusion, either as a new patient or as a known patient with tumour progression.

After patient inclusion, the first DLA procedure (T0) was planned before the start of the new line of therapy. A second procedure (T1) was planned after treatment. For patients with early-stage disease, the second procedure would be performed as soon as possible after the end of therapy, up to 3 months later. For patients with advanced-stage disease, the second procedure was planned 6 weeks to 3 months after the start of the new therapy, but could be postponed depending on interruption of therapy or patient performance.

Patients were stratified for stage (early stage: I–IIIA; advanced stage: IIIB and IV) and were treated with surgery, stereotactic ablative radiotherapy, chemotherapy, checkpoint inhibitors or targeted therapy. Baseline clinical characteristics were registered by their treating physician. For patients with advanced-stage disease, response to treatment was measured after three courses of therapy. Tumour response to treatments was measured according to the Revised Response Evaluation Criteria In Solid Tumours version 1.1 (RECIST 1.1) and denoted as progressive disease (PD), stable disease (SD), partial response (PR) and complete response (CR). Patients with PR or CR were classified as responders, while PD and SD as non-responders. All clinical data were gathered blinded to CTC outcomes.

Diagnostic leukapheresis

DLAs were carried out with the Spectra Optia® Apheresis System using an intermediate density layer set and software version 11 (Terumo BCT Inc., Lakewood, CO, USA). The procedure was performed according to the standard continuous MNC protocol with a packing factor of 4.5, the collection pump was set to 1 mL/min, haematocrit minus 3% points and a flexible inlet flow. Acid citrate dextrose formula A was used as an anticoagulant at a concentration of 1:11, but was adjusted depending on the aggregate formation. No pretreatment with granulocyte colony-stimulating factor (often given during a procedure to obtain progenitor cells) was given beforehand.

Prior to each procedure, weight, height and gender were used to estimate the total blood volume (TBV) of the patient according to the formula of Nadler [14]. Blood would be collected in one EDTA tube for full blood count (FBC) and one CellSave tube for CTC enumeration (pre-DLA sample). DLA collection was performed at a haematocrit around 5%.

After the procedure, a sample of the DLA product was drawn in an EDTA tube (1 mL). The DLA aliquot and one blood sample with EDTA were used for an FBC. The CellSave tube was used for CTC enumeration (post-DLA sample). A second EDTA tube was used for the RosetteSEP depletion.

Procedure efficacy was evaluated by calculating the number of lymphocytes in the total DLA product divided by the number of lymphocytes that had passed the machine while the DLA product was collected. Lymphocytes were chosen because of their density, which resembles CTCs of most of the MNC cells and is one of the cells for which the continuous MNC procedure is optimised [15, 16].

DLA product processing for CTC enumeration

The DLA product was divided into two parts and further processed:

1. A DLA aliquot containing 2×10^8 leucocytes was diluted with a buffer (CellSearch Circulating Tumour Cell Kit Dilution Buffer, Menarini Silicon Biosystems, Huntingdon Valley, PA, USA) to 7.5 mL and placed in a CellSave tube immediately after the apheresis procedure. The sample was stored at least overnight at room temperature and was subsequently processed according to the manufacturer's instructions.
2. DLA (9 mL) was mixed with 200 µL Cellsave preservative immediately after DLA and stored at least overnight at room temperature until leucocyte depletion by RosetteSep CD45 depletion Cocktail (Stemcell Technologies, Catalogue# 15162). The RosetteSEP cross-links leucocytes and erythrocytes, increasing the density gradient of the MNC's, allowing their removal by centrifugation.

First, erythrocytes were isolated by centrifugation ($800 \times g$ for 10 min) from the EDTA tube taken after DLA. The erythrocytes were added to the DLA product to reach a final MNC to erythrocyte ratio of 1:40. Fifty microlitres of the RosetteSep depletion cocktail was added for each 1 mL of sample and incubated for 20 min at room temperature. After incubation, the sample was diluted with an equal volume of phosphate-buffered saline (PBS)/2% foetal bovine serum (FBS). The solution was then carefully layered on top of a Ficoll-Paque PLUS density gradient (GE Healthcare, Chalfont, St. Giles, UK) and centrifuged at $800 \times g$ for 30 min at room temperature. The enriched cells were collected and washed by adding 2 volumes of PBS/2% FBS and then centrifuged for 8 min at $800 \times g$. For CTC counting after leucocyte depletion, isolated cells were diluted with CellSearch dilution buffer to a final volume of 14 mL according to the manufacturer's instructions.

CTC detection

CTCs from the DLA product (DLA-CTC and RosetteSEP-CTC) and from the blood samples (blood CTCs pre and post) were enumerated within 72 h after the DLA procedure by CellSearch. Both whole-blood samples, preserved in CellSave tubes, were run with CellTracks Autoprep using the CTC Kit (Menarini) according to standard protocol [12, 13]. DLA product samples were prepared as described above. Black tape was placed where the red blood cell layer would be located when running a blood sample. This black tape allows the CellSearch system to detect a red blood cell layer even when it is missing, allowing the DLA sample to be processed as if it were a blood sample. CellSearch cartridges were scanned using the CellTracks Analyser II (Menarini). The stored CTC images were analysed with CellTracks Analyzer software and subsequently assessed by a trained operator [17].

CTC isolation by puncher

CTCs from patients with ≥4 CTCs enumerated with CellSearch in the DLA product was further isolated by the puncher system of VyCAP (VyCAP, Enschede, The Netherlands) [18]. In this way, the morphology of CTCs could then be linked to single-cell copy number aberrations. The content of the CellSearch cartridges was transferred to a 1.5 mL tube and the emptied cartridge was washed twice with 300 µL PBS to ensure removal of the majority of cells from the cartridge.

Microwell chips (VyCAP, Deventer, The Netherlands) were degassed in a vacuum chamber at −1.0 bar for 15 min. Thereafter, the microwells were placed in a filtration holder, cells were seeded into the microwell chip and transferred to the VyCAP Puncher system. The entire chip was scanned using a × 20 objective. Images were acquired using the following settings: 100 ms 4′,6-diamidino-2-phenylindole (DAPI), 200 ms cytokeratin (PE) and 600 ms CD45 (APC). Cells of interest were automatically selected using the Puncher Software (VyCAP, 64-bit version 5.3). All events with a signal intensity of >2000 DAPI and >1500 PE were reviewed by the operator and CTCs were further manually selected. Those cells were subsequently punched into a 96-well plate containing 95 µL mineral oil (Sigma). After

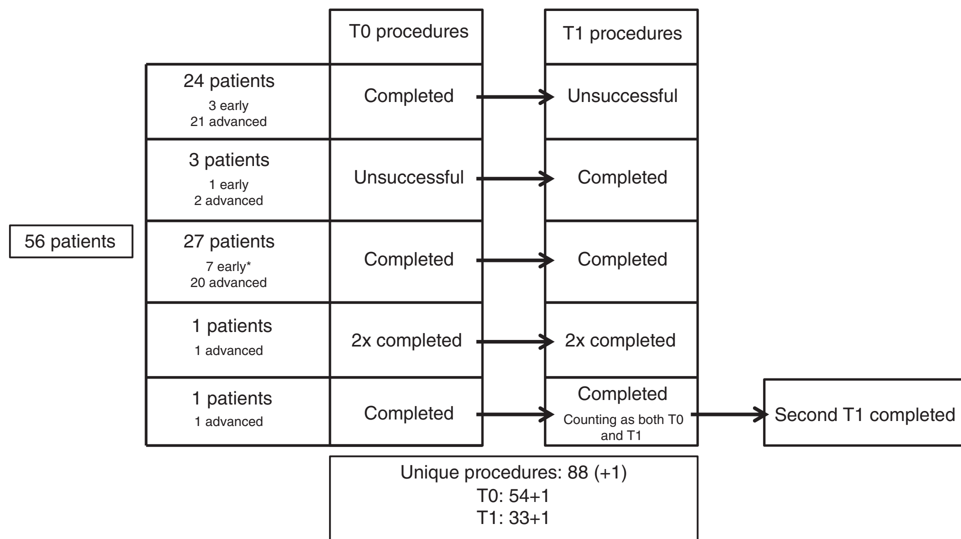

Fig. 1 Patients and apheresis procedures. *One patient with early-stage disease (stage IIIA) was included in the advanced-stage group for the survival analyses as he did not receive treatment with curative intent.

punching, 5 µL of freeze buffer (1 × PBS/42.5% ProFreeze (Lonza)/7.5% dimethyl sulfoxide (Sigma)) was added. Plates were spun down for 5 min at 500 × *g* and subsequently stored at −80 °C until further processing.

Single-cell whole-genome sequencing

Single CTCs and controls with single- and ten-cell white blood cells (WBCs) were stored in the frozen buffer after isolation, followed by scWGS as previously described with some minor modifications [19]. In short, after MNase treatment, decross-linking was performed by incubation at 65 °C for 1 h in the presence of proteinase K (0.025 U) and NaCl (200 mM), followed by AMPure XP bead purification and subsequent end-repair and A-tailing as described before [19]. During PCR indexes are introduced to each DNA fragment allowing multiplexing of the libraries for sequencing. All libraries were sequenced on Illumina NextSeq 500. Data analysis was performed with the AneuFinder software package [20].

Power analysis

We expect that about 30% of all stage NSCLC patients will have baseline CTCs in the peripheral blood. Assuming that the DLA will increase baseline CTC detection to 80% of patients at $\alpha = 0.05$ and $\beta = 0.8$, a sample size of at least 19 patients will be needed. For the dynamic changes of CTCs, we estimated that CTCs will only be detected in 26% (effect size 0.67) after treatment. This difference can be detected with $\alpha = 0.05$ and $\beta = 0.8$ when minimally 20 patients undergo two procedures.

Statistics

Descriptive statistics were used. Patients with early-stage disease undergoing curative treatment (stages I–IIIA) and patients with advanced-stage disease (III and IV) were grouped. Comparisons between the percentage of patients with CTCs detected in DLA product and peripheral blood samples were performed by McNemar's test. CTC counts per 7.5 mL DLA and 7.5 mL of blood were compared by Wilcoxon's matched analysis.

Using the calculated blood volume and the number of lymphocytes in the peripheral blood before DLA, we could estimate CTC counts in the whole-blood volume of the patient. These extrapolated counts were compared with CTC counts measured in the peripheral blood samples by Wilcoxon's matched analysis. CTC counts in DLA were corrected for lymphocyte counts since their sorted weight resembles that of CTCs [15, 16]. CTCs per 1×10^9 lymphocytes were calculated.

Cox's regression analyses were used to determine prognostic effects measured by HRs (>1 detrimental to survival).

Multivariable models were used, with covariables selected in a backward conditional method. In short, all clinical parameters (age, gender, Eastern Cooperative Oncology Group PS, smoking status, stage, tumour type, therapy group, mutations and therapy line) were included in the original model, after which a selection was made. Covariables with $p > 0.157$ (based on the Akaike information criterion) were excluded, starting with the highest p value. An effect is considered significant when $p < 0.05$ in a two-sided test. All analyses are performed using SPSS version 23.

RESULTS

Patient inclusion

In total, there were 56 patients who had 88 DLA procedures, of which one functioned as both a T0 and a T1 measurement (Fig. 1). Twenty-four patients only underwent the T0 and three patients only a T1 procedure. Twenty-nine patients had two procedures. Two patients had disease progression and underwent more procedures, creating two matched pairs for both patients. At the DLA procedure level, 55 were at T0 and 34 at T1. Twenty-four were only at T0, three were only at T1 and 31 matched procedures for both T0 and T1.

Patient characteristics

Eleven patients had early (stage I–IIIA) disease and 45 patients had advanced (stage IIIB–V) disease (Table 1). Early-stage patients had a complete surgical resection ($n = 8$) or had chemoradiotherapy ($n = 3$). Seven of these patients had a second DLA procedure between 1 and 8 weeks (median = 5 weeks) after inclusion. One patient with early-stage disease was not treated with curative intent and therefore included in the late-stage analysis.

Patients with advanced-stage disease were treated with chemotherapy ($n = 6$), tyrosine kinase inhibitors ($n = 11$) and immunotherapy ($n = 27$). Three patients were treated with combined chemotherapy, while one patient with the advanced (oligometastatic) disease was operated on for both the metastatic site (adrenal) and the primary tumour. This patient was included in the early-stage disease group for response and survival analyses. Twenty-four patients with advanced stage had their second procedure between 1 and 4 months (median = 8 weeks) after the first procedure.

CTC detection

Blood CTCs at T0 pre-DLA were detected in 33% (18/55 procedures) and post DLA in 22% (12/55) of patients, and at T1 in 26% (9/34) and 21% (7/34), respectively. There were no large differences between early- and late-stage patients (Fig. 2). We observed no significant decrease in blood CTCs pre and post DLA ($p = 0.11$).

Table 1. Baseline characteristics of 56 NSCLC patients who underwent apheresis.

	Stage I–IIIA, $n = 11$ (20%)	Stage IIIB–IV, $n = 45$ (80%)	Total ($n = 56$)
Age			
Mean (standard deviation)	70 (9)	64 (10)	65 (10)
Gender			
Male	6 (55)	29 (64)	35 (63)
Female	5 (45)	16 (36)	21 (37)
ECOG PS*			
0	8 (73)	23 (51)	31 (55)
1	2 (18)	15 (33)	17 (30)
2	1 (9)	6 (13)	7 (13)
3	0	1 (2)	1 (2)
Smoking status			
Smokers	5 (46)	25 (56)	30 (53)
Previous	4 (36)	7 (15)	11 (20)
Non-smokers	2 (18)	13 (29)	15 (27)
Stage[a]			
I	5 (46)	0	5 (9)
II	3 (27)	0	3 (5)
III	3 (27)	6 (13)	9 (16)
IV	0	39 (87)	39 (70)
Histology			
Adenocarcinoma	7 (64)	35 (78)	42 (75)
Squamous cell carcinoma	1 (9)	9 (20)	10 (16)
Other	3 (27)	1 (2)	4 (9)
Mutations[a]			
None identified or not tested	8 (73)	17 (38)	25 (45)
KRAS	2 (18)	15 (33)	17 (30)
ALK	0	5 (11)	5 (9)
Other	1 (9)	8 (18)	9 (16)
Therapy line[a]			
0	9 (82)	2 (4)	11 (20)
1	2 (18)	19 (42)	21 (37)
2	0	19 (42)	19 (34)
≥3	0	5 (12)	5 (9)
Treatment			
Surgery	8 (73)	1 (2)	9 (16)
Chemo(radio)therapy	3 (27)	6 (13)	9 (16)
Immunotherapy	0	27 (56)	27 (48)
Targeted therapy	0	11 (24)	11 (20)

Within patients with advanced disease, no significant differences between clinical characteristics (except targetable mutations) were observed.
[a]PS, stage and therapy line were significantly lower for patients in the localised therapy group, compared to the advanced disease group. Mutations were also less often detected in the localised treatment group (due to less testing).

DLA-CTCs were detected at T0 in 56% (31/55) and at T1 in 41% (14/34) patients. DLA-CTCs were detected significantly more often and in higher counts per 7.5 mL (both, $p < 0.01$) compared to blood CTCs pre and post DLA (Fig. 2). DLA-CTCs per 7.5 mL DLA were at median 9.2 times (interquartile range (IQR) = 5.6–24.0) higher than blood CTCs in 7.5 mL blood (Fig. 3).

RosetteSEP used to concentrate CTCs did not further improve CTC detection (pretreatment: 34/55, 62%; post treatment: 16/34, 47%) and CTCs per 7.5 mL even decreased compared to DLA ($p = 0.04$), indicating loss of CTCs during the leucocyte depletion by RosetteSEP ($p < 0.01$; Fig. 3).

DLA-CTCs extrapolated to the TBV

Extrapolating enumerated DLA-CTCs corrected for lymphocyte counts to the total DLA product (thus representing CTCs from the total body blood volume) gave an expected median number of 56 CTCs in the DLA product (IQR = 0–233, T0: 113 CTC, IQR = 0–300, T1: 0 CTC, IQR = 0–179). These counts are closely associated with those obtained when extrapolating blood CTCs to the TBV ($\rho = 0.5$, $p < 0.01$) (Fig. 3b, Supplementary Table 1 and Supplementary Fig. 1).

CNAs in CTCs

From nine patients with advanced-stage disease, we were able to extract DLA-CTCs from the CellSearch cartridge by the puncher technique for scWGS (Supplementary Fig. 2 and Supplementary Table 2). Twenty-two out of 24 libraries were of sufficient quality; copy number changes were detected in all these CTCs. They showed intra- and inter-patient heterogeneity (Fig. 4).

Persistence of CTCs after treatment and outcome

In 34 patients a DLA procedure was performed after treatment. All patients with the early disease were followed for at least 6 months, up to a maximum of 18 months. In the early-stage disease group, 2/7 patients had recurrent disease during the study. Both of these patients had persistent DLA-CTCs, but no detectable blood CTCs. Of the patients with advanced-stage disease, 11/27 had persistent DLA-CTCs, which strongly correlated with tumour response (Fig. 5). Of the 11 patients with DLA-CTCs detected, eight had progression within 3 months after treatment.

Patients with advanced-stage disease with DLA-CTCs after treatment showed significantly shorter progression-free survival (PFS) than those without DLA-CTCs (median PFS, 2.0 vs 12.0 months, $p < 0.01$) (Fig. 4c). In a multivariable Cox regression, DLA-CTC persistence remained an independent variable and was significantly associated with shorter PFS (HR = 5.8, 95% CI, 1.4–35.5, $p = 0.02$).

CTCs detected in the peripheral blood before apheresis but after treatment did not show any association with survival (Supplementary Fig. 3).

Treatment response and paired DLA procedures

In 29 patients, 31 matched DLA procedures before and after treatment were performed, six procedures in six patients with early-stage disease and 25 procedures in 23 patients with advanced-stage disease (Supplementary Table 3). Two patients had disease recurrence within 6 months after surgery. Both had an increase in DLA-CTCs after surgery. Blood CTC counts decreased in one patient, while the other had no blood CTCs detected at either time point. Follow-up for all these patients was at least 6 months after treatment. Two patients with advanced disease who progressed during the study agreed to undergo the procedures again. One of these patients progressed 18 months after the start of tyrosine kinase inhibitor (TKI) treatment and received check-point inhibitors. The second patient had disease progression while on TKI treatment and started another TKI (Fig. 6).

Of the 23 patients with advanced-stage disease, 15 (65%) had a PR, 2 (9%) had SD and 6 (26%) patients had PD at first response evaluation according to RECISTv1.1 (Fig. 4a). Two patients had two courses of treatment (Fig. 6 and Supplementary Table 3). Patients without DLA-CTCs detected responded in 5/6 (83%) and patients with decreased CTCs responded in 11/13 (85%), while patients with increased CTCs either had PD (4/5) or SD (1/5, Supplementary Fig. 3). Lymphocyte-corrected DLA-CTCs increased in 6 (24%) patients, decreased in 13 (52%) patients and could not be detected at either time point in 6 (24%) patients. An increase in lymphocyte-corrected DLA-CTCs (increase vs decrease/no CTCs at

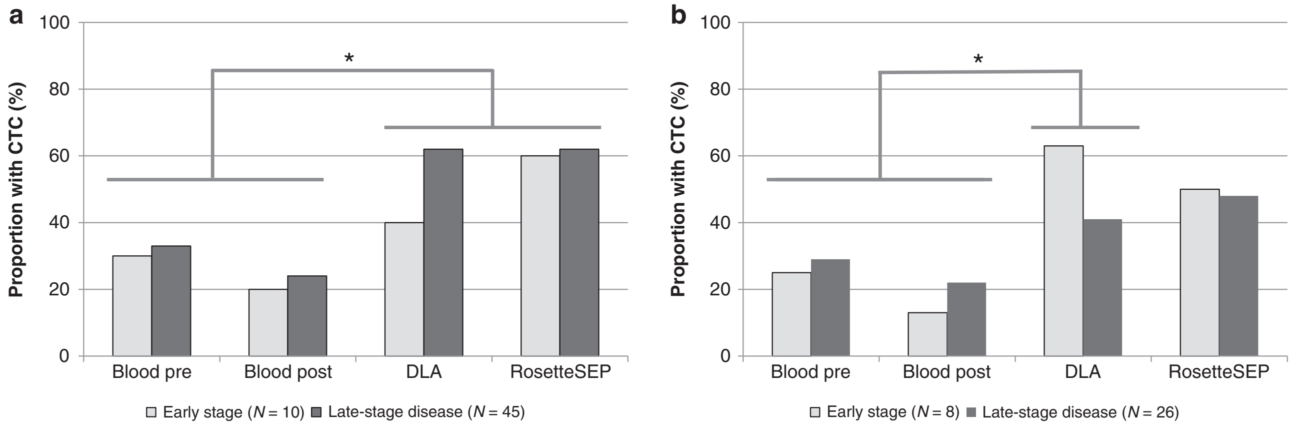

Fig. 2 Proportion of non-small cell lung cancer patients with CTCs detected in either blood or apheresis product before and after treatment. The proportion of patients with CTCs before treatment in patients with early- (stage I–IIIA) and advanced-stage (IIIB and IV) non-small cell lung cancer (**a**) and in patients after treatment (**b**). CTC detection was performed by CellSearch in 7.5 mL of peripheral blood (pre and post apheresis), apheresis product measured directly (2×10^8 white blood cells) and after leucocyte depletion by RosetteSEP (9 mL DLA product). *CTCs were significantly more often detected in the DLA product compared to the peripheral blood (vs pre: $p < 0.01$, vs post < 0.01). RosetteSEP did not increase CTC detection significantly ($p = 0.30$).

Fig. 3 Circulating tumour cells in the blood and diagnostic leukapheresis product of non-small cell lung cancer patients per volume or corrected for the number of lymphocytes. Boxplot of CTCs per 7.5 mL of diagnostic leukapheresis (DLA) product or per 7.5 mL of blood before and after treatment (**a**, **c**) and CTCs extrapolated to the total blood volume (TBV) of the patient after correction for the lymphocyte counts (**b**, **d**). Estimates are stratified for the stage of disease (early versus advanced). CTCs are given for measurements in blood, pre and post DLA, and in DLA product directly and after RosetteSEP concentration. CTC counts per 7.5 mL DLA were significantly higher than those per 7.5 mL of blood ($p < 0.01$). RosetteSEP allowed for the processing of larger volumes of DLA product, but CTCs per 7.5 mL DLA product decreased. The horizontal bar depicts the median (which is 0 for the peripheral blood draws and therefore equal to the *x*-axis), with the first and third quartiles represented by the box, while the whiskers represent the other two quartiles. Outliers (defined as outside the Q3 or Q1 ± 1.5 × interquartile range) are shown as dots.

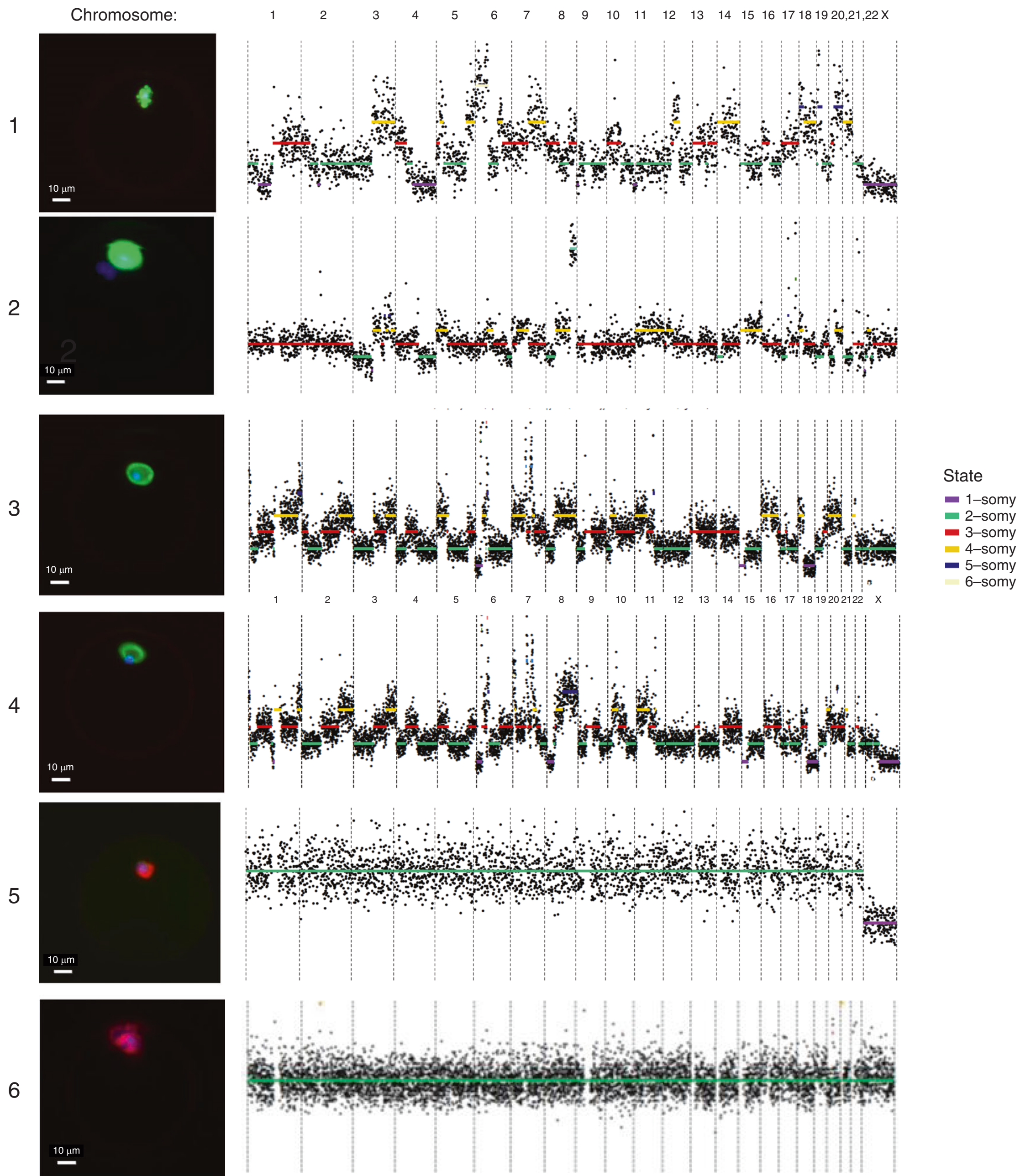

Fig. 4 Single-cell whole-genome sequencing of isolated circulating tumour cells from non-small cell lung cancer patients derived from leukapheresis product. Circulating tumour cells (CTCs) were isolated by the punching system of VyCAP (Enschede, The Netherlands), allowing for direct comparison of copy number changes by scWGS and their immunofluorescence appearance. Depicted are four typical CTCs and two white blood cells, stained for cytokeratin (=green), CD45 (=red) and nuclei (=blue), which were isolated from the apheresis product. In total, 25 CTCs from nine different patients were isolated for scWGS and 22 successful libraries showed all aneuploidy, with inter- and intrapatient heterogeneity. In this image, CTCs 3 and 4 have been derived from the same patient.

either time point) was associated with significantly shorter survival (median PFS = 1.4 vs 19.1, $p < 0.01$) (Fig. 4b). After correction for clinical variables, this association remained significant (HR = 5.9, 95% CI, 1.6–21.7, $p < 0.01$). Change in CTC counts detected in the peripheral blood did not show any significant association with survival.

DISCUSSION

CTCs identified in the peripheral bloodstream can be used to identify targetable mutations [21, 22]. They have also shown a correlation with survival in several malignancies [23, 24]. Unfortunately, the low detection rate of CTCs in peripheral blood precludes functional genomic tests in patients with NSCLC. In this study, we show that DLA can detect CTCs in larger numbers in all stages of NSCLC compared to peripheral blood. DLA-CTCs seem to be more strongly associated with tumour response and survival than blood CTCs, although the numbers are small. Our study also confirms the malignant origin of isolated DLA-CTCs by CNAs detected with scWGS. This shows that apheresis does not cause benign cells to be falsely identified as CTCs by CellSearch. This is an important finding as CTC counts can be falsely increased during surgery [25, 26]. It confirms another study performed with the apheresis product that shows all isolated and analysed CTCs have CNAs and are therefore of malign

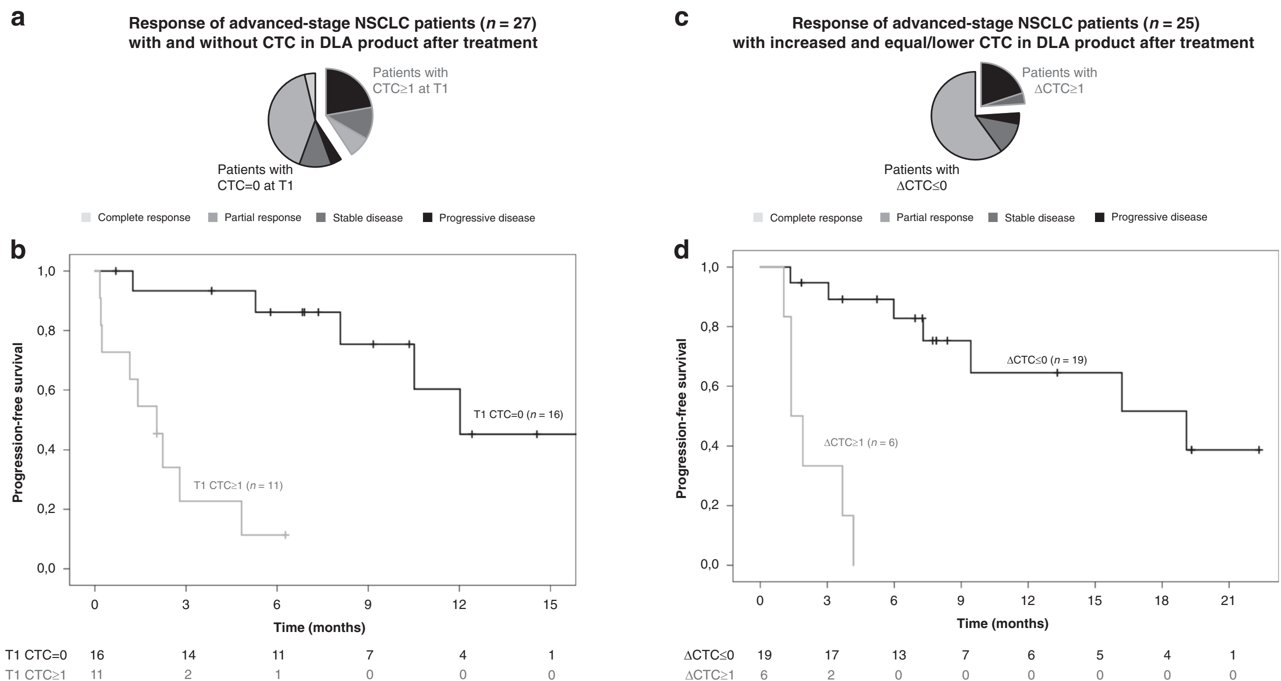

Fig. 5 Persistent CTCs after treatment in advanced-stage non-small cell lung cancer and their relation with tumour response and survival. Tumour response in advanced NSCLC by the presence of CTCs in the diagnostic leukapheresis product after treatment (T1). Out of 23 patients, 15 (65%) had a PR, 2 had SD (9%) and 6 patients had PD (26%) (**a**), and by the change in lymphocyte-corrected CTC counts in the DLA product (**b**). Patients with persistent CTCs after treatment had a significantly shorter progression-free survival time (median PFS measured at T1, 2.0 vs 12.0 months, $p < 0.01$) (**c**), as did patients with increased CTC counts between T0 and T1 (median PFS, 1.4 vs 19.1 months, $p < 0.01$) (**d**).

origin [27]. Apheresis is known to be a tolerable procedure [28–32]. Recently, DLA was shown to increase CTC detection in prostate and breast cancer, even when patients had early-stage disease [10–12, 27]. It does seem that CTCs in NSCLC are less concentrated by apheresis than those in breast and prostate cancer. Possibly this is due to a different density of CTCs in NSCLC patients [12, 15, 32]. Further research would be required for this hypothesis. If this is the case, a different layer could be sequestered in order to optimise the apheresis procedure for the isolation of CTCs of different malignancies. The whole DLA product was expected to contain over 100 DLA-CTCs in the majority of patients, which would suffice for more advanced genomic and functional tests if the whole DLA product could be processed. However, the processing technology is still limited by volume and the number of WBCs. Unfortunately, the complete DLA product cannot be used for CTC enumeration by CellSearch, which is limited to 2×10^8 leucocytes and volumes of about 2 Ml [10, 11]. Using the RosetteSEP, we were able to process a larger volume of DLA products. This method detected CTCs in a larger proportion of patients. However, Rosette-CTC counts per 7.5 mL DLA and per lymphocyte were lower than the DLA-CTCs. This indicates loss of CTCs during this procedure, making it less suitable for processing a larger volume of DLA. While some loss is to be expected during purification steps, the loss of CTCs during RosetteSEP was higher than expected. Likely CTCs were lost during centrifugation, either due to non-specific binding to the used antibodies or entrapment in the cell formations. Possibly, other CTC identification techniques such as filtration techniques or microfluidic chips, which are not reliant on antibodies, may be able to process larger volumes [33]. Filtration methods would have the added benefit of being able to identify CTCs with a more mesenchymal phenotype. CellSearch is dependent on the presentation of EPCAM, but EpCAM expression can be downregulated in CTCs [34–37]. These CTCs with a mesenchymal phenotype have been associated with several characteristics, amongst which is resistance to chemotherapy [34, 36].

In the DLA product after surgery, we observed a significantly larger number of CTCs compared to those in peripheral blood. The larger volume of blood screened for CTCs seems to increase the detection rate in patient's post surgery, which would fit with the findings of Fischer et al. in breast cancer [10]. Another option is that during surgery epithelial cells are dislodged into the bloodstream, which due to their larger size are sequestered into the DLA product [25]. This could explain the small difference observed between early- and late-stage disease patients.

The mean efficacy of DLA, measured as the captured number of lymphocytes compared to the number of lymphocytes passing through during the apheresis machine, was 66% and comparable to those reported previously in other tumour types and continuous MNC procedures in donors [11, 16]. We noticed that DLA-CTCs corrected for lymphocytes approached CTCs measured in the blood, but were not equal. Especially in the few cases when high blood CTC counts were measured in the peripheral blood, there was a difference in DLA-CTCs and blood CTCs after correction. This could be an indication that CTCs are less efficiently captured than lymphocytes. Most likely, this is due to the more heterogeneous density distribution of CTCs compared to lymphocytes. It could also be that when CTCs are measured in high numbers in the peripheral blood of NSCLC patients, they represent a CTC 'spike' and not the real concentration of CTCs in the blood. It is known that CTCs may cluster, which could explain such a CTC 'spike', and it is also known that repeated measurements within patients differ [25, 34, 35, 37]. Apheresis, which allows us to screen a larger volume of blood would therefore also allow for a more accurate estimation of the concentration of CTCs in the circulating blood. However, due to the low number of patients in which these differences were observed, any effect of this on survival or response analyses could not be discerned.

Our study has been performed in a range of NSCLC stages, which allows us to assess the value of DLA in all disease stages. This heterogeneity poses no issue for the tests assessing the

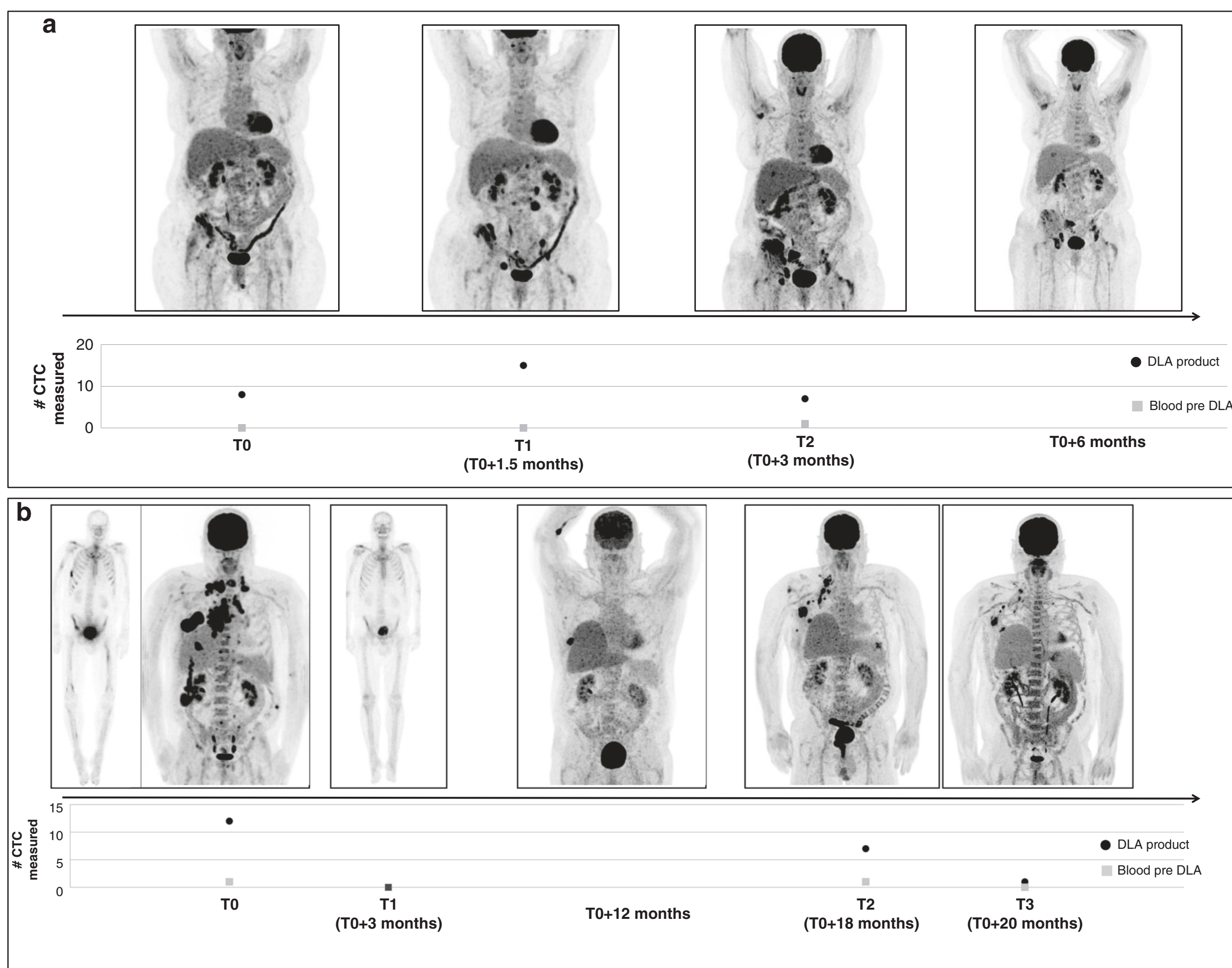

Fig. 6 Two non-small cell lung cancer cases showing an association between measured CTC counts and response by imaging. a Female patient presented at our centre after treatment with cisplatin/pemetrexed, crizotinib and ceritinib for stage IV adenocarcinoma with an ALK translocation. Fourth-line treatment was started with alectinib (T0–T1), but the patient had progressive disease after 1 month so medication was changed to lorlatinib (T1 and T2). Lymphatic metastatic sites responded well as observed by CT (T2); however, 3 months later an increase of bone metastases was seen on PET-CT. CTC counts in DLA (8–15–7) accurately reflected response by imaging. CTC counts in the blood remained stable (0–0–1). **b** Male patient arrived after lobectomy with several bone metastases and multiple affected lymph nodes. The patient had an ALK translocation and a mutation in HRAS (p. R123P). Treatment was started with lorlatinib at T0, with a partial response at T1. One year later, a PET-CT scan showed minimal tumour activity. One and a half years after the start of treatment, the patient developed complaints of pain in his shoulder; a suspect for metastases on PET-CT scan. The biopsy confirmed the presence of the original two mutations. Treatment with immunotherapy in combination with chemotherapy was started (T2). At T3 patient showed a partial response. CTC counts in blood (1–0–1–0) and DLA (12–0–7–1) accurately reflected response measured by PET-CT.

difference between CTC counts in DLA product and peripheral blood as each patient serves as their own control.

A major limitation remains that the complete DLA product cannot be used for CTC enumeration by CellSearch (limitation to 2×10^8 leucocytes). If the whole DLA product could be screened, detection of CTCs will be further increased. Thereby, the DLA product contains enough cells for functional genomic testing. While it might be too expensive to perform DLA in all NSCLC patients, it could provide a welcome alternative in those patients with inaccessible tumours or in those where conventional biopsies have failed to come to a diagnosis.

CONCLUSIONS

DLA can be used to increase CTC detection in NSCLC patients, allowing for higher CTC numbers in a larger proportion of patients. Even in patients with earlier disease stages, CTCs could be detected in the DLA product of the majority of patients. CTCs in DLA products may be associated more strongly with both tumour response and survival than CTCs from the peripheral blood. This needs to be evaluated in larger studies.

DATA AVAILABILITY

Data obtained in this study are available upon reasonable request from the corresponding author.

REFERENCES

1. Wit S de, Rossi E, Weber S, Tamminga M, Manicone M, Swennenhuis JF, et al. Single tube liquid biopsy for advanced non-small cell lung cancer. Int J Cancer. 2019;12:3127–37.
2. Wit Sde, Dalum Gvan, Lenferink ATM, Tibbe AGJ, Hiltermann TJN, Groen HJM. et al. The detection of EpCAM+ and EpCAM− circulating tumor cells. Sci Rep. 2015;5:12270–9. https://doi.org/10.1038/srep12270.
3. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical significance and molecular characteristics of circulating tumor cells and

circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol. 2012;30:525–32. https://doi.org/10.1200/JCO.2010.33.3716.
4. Krebs MG, Hou JM, Sloane R, Lanaschire L, Priest L, Nonaka D, et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol. 2012;7:306–15. https://doi.org/10.1097/JTO.0b013e31823c5c16.
5. Punnoose EA, Atwal S, Liu W, Raja R, Fine BM, Hugher BGM, et al. Evaluation of circulating tumor cells and circulating tumor DNA in non-small cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. Clin Cancer Res. 2012;18:2391–401. https://doi.org/10.1158/1078-0432.CCR-11-3148.
6. Hofman V, Bonnetaud C, Ilie MI, Vielh P, Vielh P, Vignaud JM, et al. Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker. Clin Cancer Res. 2010;17:827–35. https://doi.org/10.1158/1078-0432.ccr-10-0445.
7. Tamminga M, Wit SD, Hiltermann TJN, Timens W, Schuuring E, Terstappen LWWM, et al. Circulating tumor cells in advanced non-small cell lung cancer patients are associated with worse tumor response to checkpoint inhibitors. J Immunother Cancer. 2019;2:1–9. https://doi.org/10.1186/s40425-019-0649-2.
8. Tamminga M, Wit SD, Schuuring E, Timens W, Terstappen LWMM, Hiltermann HJMG, et al. Circulating tumor cells in lung cancer are prognostic and predictive for worse tumor response in both targeted- and chemotherapy. TLCR. 2019;8:854–61. https://doi.org/10.21037/tlcr.2019.11.06.
9. Coumans FAW, Ligthart ST, Uhr JW, Terstappen LWMM. Challenges in the enumeration and phenotyping of CTC. Clin Cancer Res. 2012;18:5711–8. https://doi.org/10.1158/1078-0432.CCR-12-1585.
10. Fischer JC, Niederacher D, Topp SA, Honsich E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci USA. 2013;110:16580–5. https://doi.org/10.1073/pnas.1313594110.
11. Fehm TN, Meier-Stiegen F, Driemel C, Jäger B, Reinhardt F, Naskou J. et al. Diagnostic leukapheresis for CTC analysis in breast cancer patients: CTC frequency, clinical experiences and recommendations for standardized reporting. Cytom Part A. 2018;93:1213–9. https://doi.org/10.1002/cyto.a.23669.
12. Andree KC, Mentink A, Zeune LL, Terstappen LWWM, Stoecklein NH, Neves RP, et al. Toward a real liquid biopsy in metastatic breast and prostate cancer: diagnostic leukApheresis increases CTC yields in a European prospective multicenter study (CTCTrap). Int J Cancer. 2018;143:2584–91. https://doi.org/10.1002/ijc.31752.
13. Andree KC, Van Dalum G, Terstappen LWMM. Challenges in circulating tumor cell detection by the CellSearch system. Mol Oncol. 2016;10:395–407. https://doi.org/10.1016/j.molonc.2015.12.002.
14. Nadler SB, Hidalgo JH, Bloch T. Prediction of blood volume in normal human adults. Surgery. 1962;51;224–32.
15. Stoecklein NH, Fischer JC, Niederacher D, Terstappen LWMM. Challenges for CTC-based liquid biopsies: low CTC frequency and diagnostic leukapheresis as a potential solution. Expert Rev Mol Diagn. 2015;7159. https://doi.org/10.1586/14737159.2016.1123095.
16. Punzel M, Kozlova A, Quade A, Schmidt AH, Smith R. Evolution of MNC and lymphocyte collection settings employing different Spectra Optia ® Leukapheresis systems. Vox Sang. 2017;112:586–94. https://doi.org/10.1111/vox.12540.
17. Zeune LL, de Wit S, Berghuis AMS, IJzerman MJ, Terstappen LWMM, Brune C. How to agree on a CTC: evaluating the consensus in circulating tumor cellscoring. Cytom A. 2018;93:1202–6. https://doi.org/10.1002/cyto.a.23576.
18. Stevens M, Oomens L, Broekmaat J, Weersink J, Abali F, Swennenhuis JF, et al. VyCAP's puncher technology for single cell identification, isolation, and analysis. Cytom Part A. 2018;93:1255–9. https://doi.org/10.1002/cyto.a.23631.
19. van den Bos H, Bakker B, Taudt A, Guryev V, Colomé-Tatché M, Lansdorp PM, et al. Quantification of aneuploidy in mammalian systems. New York: Humana Press; 2019. p. 159–90.
20. Bakker B, Taudt A, Belderbos ME, Porubsky D, Spierings DCJ, De Jong TV, et al. Single-cell sequencing reveals karyotype heterogeneity in murine and human malignancies. Genome Biol. 2016;17:115 https://doi.org/10.1186/s13059-016-0971-7.
21. Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Eng J Med. 2008;359;366–77. https://doi.org/10.1056/NEJMoa0800668.
22. Sundaresan TK, Sequist LV, Heymach JV, Riely GJ, Jänne PA, Koch WH, et al. Detection of T790M, the acquired resistance EGFR mutation, by tumor biopsy versus noninvasive blood-based analyses. Clin Cancer Res. 2015;1–9. https://doi.org/10.1158/1078-0432.CCR-15-1031.
23. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, et al. Circulating tumor cells versus imaging—predicting overall survival in metastatic breast cancer. Clin Cancer Res. 2006;12:6403–9. https://doi.org/10.1158/1078-0432.CCR-05-1769.
24. Hiltermann TJN, Pore MM, van den Berg A, Timens W, Boezen HM, Liesker JJW, et al. Circulating tumor cells in small-cell lung cancer: A predictive and prognostic factor. Ann Oncol. 2012;23:2937–42. https://doi.org/10.1093/annonc/mds138.
25. Tamminga M, de Wit S, van de Wauwer C, Van den Bos H, Swennenhuis JF, Klinkenberg TJ, et al. Release of circulating tumor cells during surgery for non–small cell lung cancer: Are they what they appear to be? Clin Cancer Res. 2019;1–12. https://doi.org/10.1158/1078-0432.ccr-19-2541.
26. Chemi F, Rothwell DG, McGranahan N, Gulati A, Abbosh C, Pearce SP, et al. Pulmonary venous circulating tumor cell dissemination before tumor resection and disease relapse. Nat Med. 2019;25:1534–9. https://doi.org/10.1038/s41591-019-0593-1.
27. Lambros MB, Seed G, Sumanasuriya S, Gil V, Crespo M, Fontes M, et al. Single-cell analyses of prostate cancer liquid biopsies acquired by apheresis. Clin Cancer Res. 2018;24:5635–44. https://doi.org/10.1158/1078-0432.CCR-18-0862.
28. McLeod BC, Sniecinski I, Ciavarella D, Owen H, Price TH, Randels MJ, et al. Frequency of immediate adverse effects associated with therapeutic apheresis. Transfusion. 1999;39:282–8. https://doi.org/10.1046/j.1537-2995.1999.39399219285.
29. Crocco I, Franchini M, Garozzo G, Gandini AR, Gandini G, Bonomo P, et al. Adverse reactions in blood and apheresis donors: experience from two Italian transfusion centres. Blood Transfus. 2009;7:35 https://doi.org/10.2450/2008.0018-08.
30. Kraal KCJM, Timmerman I, Kansen HM, van den Bos C, Zsiros J, van den Berg H, et al. Peripheral stem cell apheresis is feasible post ^{131}Iodine-metaiodobenzylguanidine-therapy in high-risk neuroblastoma, but results in delayed platelet reconstitution. Clin Cancer Res. 2019;25:1012–21. https://doi.org/10.1158/1078-0432.CCR-18-1904.
31. Kiss F, Toth E, Miszti-Blasius K, Nemeth N. The effect of centrifugation at various g force levels on rheological properties of rat, dog, pig and human red blood cells. Clin Hemorheol Microcirc. 2016;62:215–27. https://doi.org/10.3233/CH-151965.
32. Coumans F, van Dalum G, Terstappen LW. CTC technologies and tools. Cytometry. 2018;93:1197–201. https://doi.org/10.1002/cyto.a.23684.
33. Tamminga M, Andree KC, Hiltermann TJN, Jayat M, Schuuring E, van den Bos H, et al. Detection of circulating tumor cells in the diagnostic leukapheresis product of non-small-cell lung cancer patients: comparing CellSearch® and ISET. Cancers. 2020;12:896 https://doi.org/10.3390/cancers12040896.
34. Zhang X, Wei L, Li J, Zheng J, Zhang S, Zou J. Epithelial-mesenchymal transition phenotype of circulating tumor cells is associated with distant metastasis in patients with NSCLC. Mol Med Rep. 2019;19:601–8. https://doi.org/10.3892/mmr.2018.9684.
35. Hosokawa M, Kenmotsu H, Koh Y, Yoshino T, Yoshikawa T, Naito T, et al. Size-based isolation of circulating tumor cells in lung cancer patients using a microcavity array system. PLoS ONE. 2013;8:e67466 https://doi.org/10.1371/journal.pone.0067466.
36. Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol. 2017;14:611–29. https://doi.org/10.1038/nrclinonc.2017.44.
37. Mascalchi M, Falchini M, Maddau C, Salvianti F, Nistri M, Bertelli E, et al. Prevalence and number of circulating tumour cells and microemboli at diagnosis of advanced NSCLC. J Cancer Res Clin Oncol. 2015;142:195–200. https://doi.org/10.1007/s00432-015-2021-3.

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AUTHOR CONTRIBUTIONS

Study concepts: LWMMT and HJMG. Study design: HJMG, MT, TJNH, KCA and ES. Data acquisition: MT and HJMG. Quality control of data and algorithms: MT, ES, HJMG and TJNH. Data analysis and interpretation: MT, TJNH and JMG. Statistical analysis: MT and HJMG. Manuscript preparation: MT. Manuscript editing: KCA, HvdB, TJNH, AM, DCJS, PL, WT, ES, LWMMT and HJMG. Manuscript review: PL, WT, ES, LWMMT and HJMG.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Medical Ethical Committee (NL55754.042.15) and was registered in the Dutch trial register (NL5423). Informed consent was obtained from all patients.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Harry J. M. Groen.

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