Analysis of Released Circulating Tumor Cells During Surgery for Non-Small Cell Lung Cancer

Menno Tamminga[1], Sanne de Wit[2], Caroline van de Wauwer[3], Hilda van den Bos[4], Joost F. Swennenhuis[2], Theo J. Klinkenberg[3], T. Jeroen N. Hiltermann[1], Kiki C. Andree[2], Diana C.J. Spierings[4], Peter M. Lansdorp[4,5,6], Anke van den Berg[7], Wim Timens[7], Leon W.M.M. Terstappen[2], and Harry J.M. Groen[1]

ABSTRACT

Purpose: Tumor cells from patients with lung cancer are expelled from the primary tumor into the blood, but difficult to detect in the peripheral circulation. We studied the release of circulating tumor cells (CTCs) during surgery to test the hypothesis that CTC counts are influenced by hemodynamic changes (caused by surgical approach) and manipulation.

Experimental Design: Patients undergoing video-assisted thoracic surgery (VATS) or open surgery for (suspected) primary lung cancer were included. Blood samples were taken before surgery (T0) from the radial artery (RA), from both the RA and pulmonary vein (PV) when the PV was located (T1) and when either the pulmonary artery (T2 open) or the PV (T2 VATS) was dissected. The CTCs were enumerated using the CellSearch system. Single-cell whole-genome sequencing was performed on isolated CTCs for aneuploidy.

Results: CTCs were detected in 58 of 138 samples (42%) of 31 patients. CTCs were more often detected in the PV (70%) compared with the RA (22%, $P < 0.01$) and in higher counts ($P < 0.01$). After surgery, the RA but not the PV showed less often CTCs ($P = 0.02$). Type of surgery did not influence CTC release. Only six of 496 isolated CTCs showed aneuploidy, despite matched primary tumor tissue being aneuploid. Euploid so-called CTCs had a different morphology than aneuploid.

Conclusions: CTCs defined by CellSearch were identified more often and in higher numbers in the PV compared with the RA, suggesting central clearance. The majority of cells in the PV were normal epithelial cells and outnumbered CTCs. Release of CTCs was not influenced by surgical approach.

Introduction

Circulating tumor cells (CTCs) can be detected in the peripheral blood with the FDA-cleared CellSearch system. The system identifies cells as CTCs when they have a morphology resembling a cell with a nucleus identified by DAPI, expression of epithelial cell adhesion molecule (EpCAM) and cytokeratins (CK), but not CD45. CTCs have been shown an independent prognostic marker of survival for patients with non–small cell lung cancer (NSCLC) (1–6). In early stage NSCLC, the presence of CTCs detected in the peripheral blood after surgery has been associated with a shorter time to recurrence (7–11). Of note, in advanced disease, CTCs are only observed in around 30% of patients with NSCLC. Patients without detectable CTCs by CellSearch in 7.5 mL of blood may still have CTCs in the bloodstream; however, these are not detected due to the low blood volume screened or the lack of expression of EpCAM or the CK identified by the C11 and A53B/A2 clones (12).

During surgery, we have the unique opportunity to draw blood from the pulmonary vein (PV) draining the lobe containing the primary tumor. As the PV is closer to the source, we hypothesized that the blood in this vein would contain more CTCs. The number of CTCs in the PV may be influenced by several factors. For example, the type of surgery [video-assisted thoracic surgery (VATS) or open thoracotomy] and their associated differences in vessel dissection (PV or pulmonary artery) and manipulation of the tumor while the surgery is ongoing may influence CTC counts.

In this study, we investigated the release of CTCs in the PV and the radial artery at the start of and during surgery. As a secondary goal, we evaluated differences in CTC counts between VATS (PV ligated before pulmonary artery) and open thoracotomy (vice versa, pulmonary artery ligated first). To evaluate the malignant origin of CTCs, we performed low-coverage single-cell whole-genome sequencing (scWGS) to detect copy-number alterations (CNAs).

Materials and Methods

Patient inclusion and acquisition of blood samples

We prospectively included consecutive patients who were eligible for surgery due to primary NSCLC or a suspected lung malignancy by fast-growing pulmonary nodules after they gave informed consent. Patients undergoing lobectomy, bilobectomy, or pneumonectomy were eligible for inclusion. An important difference between VATS

[1]Department of Pulmonary Diseases, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. [2]Department of Medical Cell BioPhysics, Faculty of Sciences and Technology, University of Twente, Enschede, the Netherlands. [3]Department of Cardiothoracic Surgery, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. [4]European Research Institute for the Biology of Ageing, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. [5]Terry Fox Laboratory, BC Cancer Agency, Vancouver, British Columbia, Canada. [6]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada. [7]Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands.

Corresponding Authors: Menno Tamminga, University Medical Center Groningen, Hanzeplein 1, Groningen 9713 GZ, the Netherlands. Phone: 0031503616161; Fax: 0031503619320; E-mail: m.tamminga@umcg.nl; and Harry J.M. Groen, h.j.m.groen@umcg.nl

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Translational Relevance

Circulating tumor cells (CTCs) are rarely detected in non–small cell lung cancer (NSCLC). It has been suggested that CTCs are removed from the bloodstream in the microcirculation. Measurement closer to the source, i.e., the primary tumor, would increase CTC yield, useful to identify patients at risk of recurrence after surgery or for creating xenografts. Here, we show that CellSearch detects higher CTC counts in the pulmonary vein compared with the radial artery, implicating CTC clearance before they reach the microcirculation. Furthermore, CTCs measured in the pulmonary vein were often euploid cells, indicating a benign origin. This highlights that the CellSearch system, although well validated and sensitive in the peripheral blood, is prone to misclassify cells as CTCs in the pulmonary vein during surgery. This could be the reason why measurements in the peripheral blood are at least as capable as measurements in the pulmonary vein to identify recurrence of disease as shown in other studies.

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and open surgery is that during open surgery the pulmonary artery is dissected and closed first, whereas during VATS, the PV is the first large vessel to be dissected. For patients undergoing an open thoracotomy, 7.5 mL of blood was drawn from the radial artery at the start of surgery (baseline, T0), followed by blood draws from both the radial artery and the PV that drained the lobe which contained the tumor at two time points: when the draining PV was identified (T1) and after dissection of the pulmonary artery but before the PV was dissected (T2; **Fig. 1**).

For VATS patients, blood samples were obtained at the start of surgery from the radial artery (T0). Blood samples were obtained from both the radial artery and the PV directly after identification of the draining PV (T1) and after closure of the PV (T2). No sample was obtained after the pulmonary artery was closed.

At all time points, the diseased lobe was still inside the patient with blood circulation in the tumor. Only at T2 would, depending on surgery type, either the PV or artery be dissected and would influence intravenous pressures.

The study was performed according to the Helsinki declaration. It was reviewed and approved by the local medical ethical committee (METc 2015/602) and registered at the Dutch study register (NL55754.042.15).

CTC enumeration

Blood samples were processed within 96 hours and analyzed blinded to clinical outcomes, as described previously (1, 2). Cells were CTCs when identified by CellSearch, according to the FDA-approved definition, as cells positive for the EpCAM, CK identified by the clones C11 and A.53B/A2 and nuclear stain DAPI, but negative for leukocyte marker CD45 in the blood. CTC numbers are reported as the number per 7.5 mL of blood. Clusters of CTCs (i.e., $\geq$2 CTCs clustered together) are counted as 1 CTC/7.5 mL. The presence of clusters was noted.

CTC isolation by FACS

CellSearch cartridges were stored at 4°C up to 24 months before further processing. When a sample had CTC counts $\geq$4/7.5 mL, the content of the cartridges was transferred to a 1.5-mL tube and washed twice with 300-μL PBS to ensure removal of the majority of cells from the cartridge. Single CTCs were subsequently isolated by FACS.

Predefined gates were used for isolation of DAPI$^+$/CK$^+$/CD45$^-$ CTCs. White blood cells were sorted based on a DAPI$^+$/CK$^-$/CD45$^+$ phenotype and served as controls for single-cell sequencing. Cells were collected in a 1x ProFreeze solution as single cells or in groups of 10 cells and stored at −80°C before copy-number aberration (CNA) analysis.

CTC isolation by puncher

After the first CNA analysis results, CTCs $\geq$4/7.5 mL from four patients were isolated by the puncher system of Vycap (Vycap; ref. 13). This allowed direct comparison of the morphology of the CTCs with the single-cell sequencing result. Cells were removed from the CellSearch cartridge in the same manner as for FACS isolation and transferred to a 1.5 mL tube.

Microwell chips (VyCAP) were degassed in a vacuum chamber at −1.0 bar for 15 minutes in PBS. After degassing, the microwells were placed in a filtration holder. The cells were then transferred and seeded into a microwell chip according to manufacturer's instructions. After seeding, the microwell chip was transferred to the VyCAP Puncher system. The entire chip was scanned using a 20x objective. Images were acquired using the following settings: 100-ms DAPI, 200-ms PE, and 600-ms APC. Cells of interest were selected using the Puncher Software (VyCAP, 64 bit, version 5.3) by using the automatic selection tool. All events with a signal intensity of >2,000 DAPI and >1,500 PE were reviewed by the operator, and CTCs were manually selected. Cells of interest were subsequently punched into a 96-well plate containing 95 μL mineral oil (Sigma). After punching, 5 μL of freeze buffer (1XPBS/42.5% freeze buffer/7.5% DMSO; Sigma) was added. Plates were spun down for 5 minutes at 500 g and subsequently stored at −80°C until further processing.

Genomic analysis of CTCs

Single cells and mini bulk cells ($n = 10$ cells) were stored in freeze buffer after isolation, followed by scWGS as previously described with some minor modifications (14). In short, DNA was fragmented with MNase, after which decrosslinking was performed by incubation at 65°C for 1 hour in the presence of proteinase K (0.025 U) and NaCl (200 mmol/L) followed by Ampure XP bead purification. Hereafter, End-repair, A-tailing, adapter ligation, and PCR amplification were performed as described before (14). 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 (15).

Matched primary tumor tissue

All patients had tissue of the (suspected) primary tumor frozen (−80°C). Of those patients who had successfully isolated CTCs for CNA analysis, tissue sections were sliced (50 μm) and used to isolate tumor nuclei. These were subjected to scWGS in the same manner as the CTCs and control cells in mini bulk (30 nuclei).

Statistical analysis

Differences in samples with CTC present between open thoracotomy and VATS were evaluated by Fisher exact tests. Paired analyses were performed by McNemar and Wilcoxon tests. Correlations were tested by Spearman's rho, and if significant further investigated with logistic regressions. Differences were considered significant when $P < 0.05$.

Systematic differences in CTC counts between different time points, PV versus RA, and type of surgery were evaluated using a longitudinal marginal mixed model, with a variable slope and intersect using type of
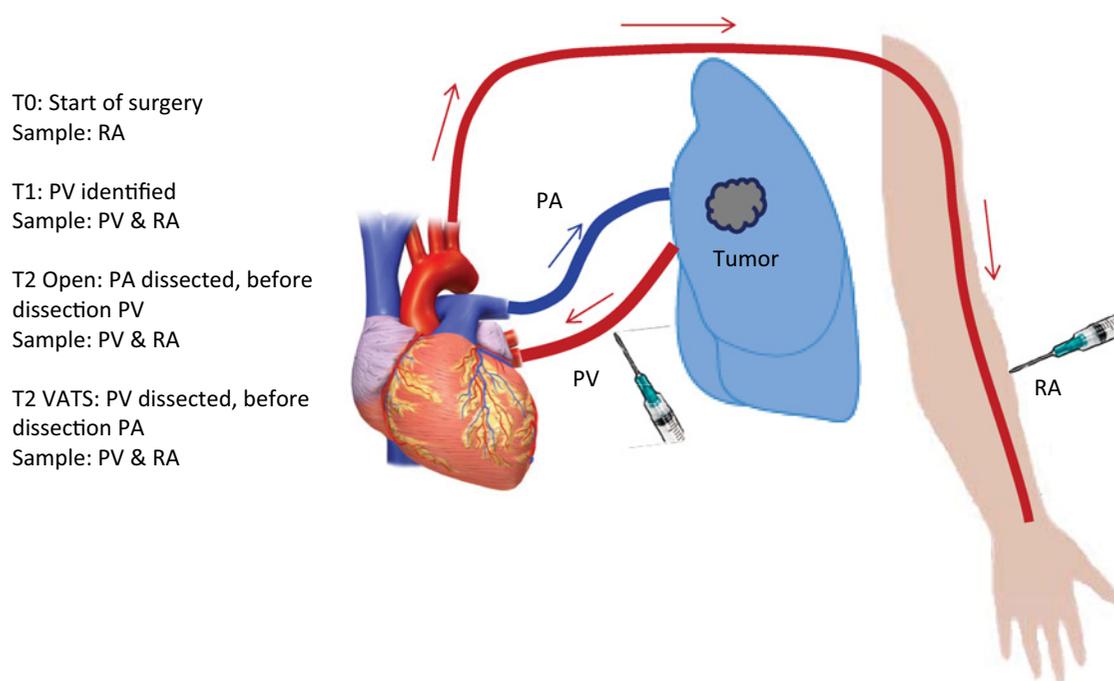
Figure 1.
Order of blood sampling for CTC enumeration from the PV and radial artery (RA) during either open or video-assisted (VATS) surgery for (suspected) NSCLC. PA, pulmonary artery. Surgical approach differed between open surgery and VATS. The main difference being the order of vessel ligation: During open surgery, the pulmonary artery is dissected first, whereas during VATS, the PV is dissected first.

surgery and location of blood draw as covariables with the repeated measurements in an unstructured matrix. Interaction terms were tested for type of surgery, time point, and location of measurement. All analyses were performed using SPSS version 23.

Results

Patient characteristics

Thirty-one patients were included (18 open thoracotomy and 13 VATS, **Table 1**). Patients undergoing VATS were significantly more often female, but showed no other significant differences. Three patients presenting with fast-growing pulmonary nodules had benign disease (two in the open and one in the VATS group), and these patients served as a benign control group. Open surgery patients provided 82 and VATS patients 56 blood samples that were successfully processed by CellSearch. For 28 patients, paired PV and RA T1 samples were collected, and for 25 patients, paired samples were collected at T2.

CTCs were detected in 58 of 138 blood samples (42%). The PV had CTCs in 40 of 57 samples (70%) and the RA 18 of 81 blood samples (22%). Combined, we identified CTCs in any of the two PV samples in 25 of 31 patients (81%) and in any of the three RA samples in 13 of 31 patients (42%). Remarkably, all three patients with benign disease had CTCs (as defined by CellSearch) detected in the PV, but not in the radial artery, indicating a misinterpretation of malign character of the epithelial cells.

Presence of CTCs

Using the matched samples at T1 and T2 ($28 + 25 = 53$), CTCs were more often detected in the PV (70%) than in the RA (22%, $P < 0.01$), and in higher counts ($P < 0.01$). This difference was also significant when testing was performed in a nonmatched manner, or when comparing only NSCLC samples (71% vs 25%, $P < 0.01$). When stratifying by time point, CTCs were present more often in blood samples derived from the PV compared with the RA (T1: 64% vs 23%, $P < 0.01$; T2: 76% vs 7%, $P < 0.01$, **Table 1**, **Fig. 2**). The type of surgery, comparing video-assisted surgery versus open surgery, was not significantly associated with either the presence of CTCs (RA: OR = 1.5, $P = 0.45$; PV: OR = 0.63, $P = 0.43$) or their number (RA: $\rho = -0.01$, $P = 0.91$, PV: $\rho = 0.17$, $P = 0.21$).

CTCs derived from the PV showed morphologic differences from those detected in the radial artery (**Fig. 3A**). In general, cells identified in the PV were larger and expressed CK more strongly than those detected in radial artery (**Fig. 3B**).

CTC clusters

Clusters of CTCs were detected in 19 blood samples. Eighteen of these blood samples were taken from the PV. CTC clusters were more often detected when CTC counts were high ($\rho = 0.48$, $P < 0.01$; OR = 1.02, $P = 0.01$). The presence of clusters was not associated with any other factors (type of surgery, location of tumor, tumor size, performance status, and histology).

CTCs in the radial artery

The proportion of patients with CTC was lower at the end of surgery when blood circulation through the primary tumor was ended [T0: 9/24 (38%), T2: 2/27 (7%), $P = 0.02$], with matched samples having significantly lower counts ($P = 0.03$).

CTCs in the draining PV

There was no difference in the proportion of patients with CTCs before and after clamping the tumor vessels (T1: 64% vs T2: 76%,

Table 1. Baseline characteristics and outcomes of patients with NSCLC or growing pulmonary nodules undergoing open surgery or VATS.

		Open thoracotomy $n = 18$ (58%)	VATS $n = 13$ (42%)	All $n = 31$ (100%)
Age	Median (range)	64 (45–83)	66 (56–81)	65 (45–83)
Gender[a]	Female	5 (28)	10 (77)	15 (48)
	Male	13 (72)	3 (23)	16 (52)
ECOG PS	0	17 (94)	11 (85)	28 (91)
	1	1 (6)	2 (15)	3 (9)
Smoking status	Ex-smokers	15 (83)	12 (92)	27 (87)
	Nonsmokers	3 (17)	1 (8)	4 (13)
Clinical stage	1	3 (18)	5 (42)	8 (26)
	2	7 (41)	5 (42)	12 (39)
	3	4 (29)	2 (16)	6 (19)
	Oligometastatic disease[b]	2 (12)	0 (0)	2 (6)
Histology	Adenocarcinoma	6 (33)	5 (38)	11 (35)
	Squamous cell	7 (39)	6 (46)	13 (42)
	Other	3 (17)	1 (8)	4 (13)
	Granulomatous nodule	2 (11)	1 (8)	3 (10)
Tumor location	Left upper lobe	5 (26)	1 (8)	6 (19)
	Left lower lobe	2 (11)	3 (23)	5 (16)
	Right upper lobe	2[c] (11)	5 (38)	7[c] (22)
	Right middle lobe	3[c] (16)	1 (8)	4[c] (12)
	Right lower lobe	7 (36)	3 (23)	10 (31)
Size tumor	Median (cm)	4.2	2.7	3.6
	Range	0.8–10.5	1.2–5.5	0.8–10.5
Resection borders[d]	R0	16 (89)	12 (92)	28 (91)
	R1	2 (11)	1 (8)	3 (9)
Samples obtained	RA T0	14 (78)	10 (77)	24 (77)
	RA T1	17 (94)	13 (100)	30 (97)
	RA T2	17 (94)	10 (77)	27 (87)
	PV T1	17[c] (89)	11 (85)	28[c] (88)
	PV T2	17[c] (89)	12 (92)	29[c] (91)
Number of patients with CTCs detected	RA T0	5 (36)	4 (40)	9 (38)
	RA T1	3 (18)	4 (31)	7 (23)
	RA T2	2 (12)	0 (0)	2 (7)
	PV T1	12 (71)	6 (55)	18 (64)
	PV T2	11 (65)	11 (92)	22 (76)
CTC median (interquartile range)	RA T0	0 (0–1)	0 (0–1)	0 (0–1)
	RA T1	0 (0–0)	0 (0–1)	0 (0–0)
	RA T2	0 (0–0)	0 (0–0)	0 (0–0)
	PV T1	2 (0–18)	0 (0–23)	2 (0–20)
	PV T2	1 (0–40)	10 (1–51)	4 (1–38)

Note: CTCs were not significantly correlated to any described clinical parameter.

Abbreviations: ND, not done; RA, radial artery; T0 is at baseline, before opening the chest cavity; T1 is during surgery when the PV is identified; T2 is after ligation of the pulmonary artery, right before dissection of the PV (open surgery), or immediately after dissection of the PV when the pulmonary artery is still intact (VATS);.
[a]Patients undergoing VATS compared with thoracotomy were more often female ($P = 0.01$). No other significant differences were observed.
[b]Two patients had oligometastatic disease, with metastatic sites removed before lobectomy. One patient had a brain metastasis, and the other patient a scapula metastasis.
[c]One patient had two pulmonary lobes affected by one tumor mass. We took blood samples from the PV draining both lobes.
[d]Evaluation on completeness of resection: R0, no evidence of tumor remains; R1, microscopic evidence of tumor.

$P = 0.16$), nor was there a difference in the number of CTCs measured ($P = 0.54$).

Mixed model analyses

To evaluate patterns in the change of CTCs influenced by manipulation during surgery (difference in CTCs between time points) and type of surgery, we used a mixed model. Changes in CTCs in the PV were significantly higher than those in the RA ($P = 0.01$). CTC counts were not associated with any clinical parameter. There was no difference between CTCs measured in VATS patients and those undergoing open surgery ($P = 0.48$). No systematic change in CTC counts was observed in the pulmonary or radial artery (**Fig. 2**).

Copy-number aberrations in so-called CTCs and primary tumor cells

Matched primary tumor samples from all patients with NSCLC showed aneuploidy. As expected, normal tissue taken from the controls only showed euploid cells.

From 12 blood samples (11 from the PV and 1 from the RA) obtained from 10 different patients, containing at least four CTCs,
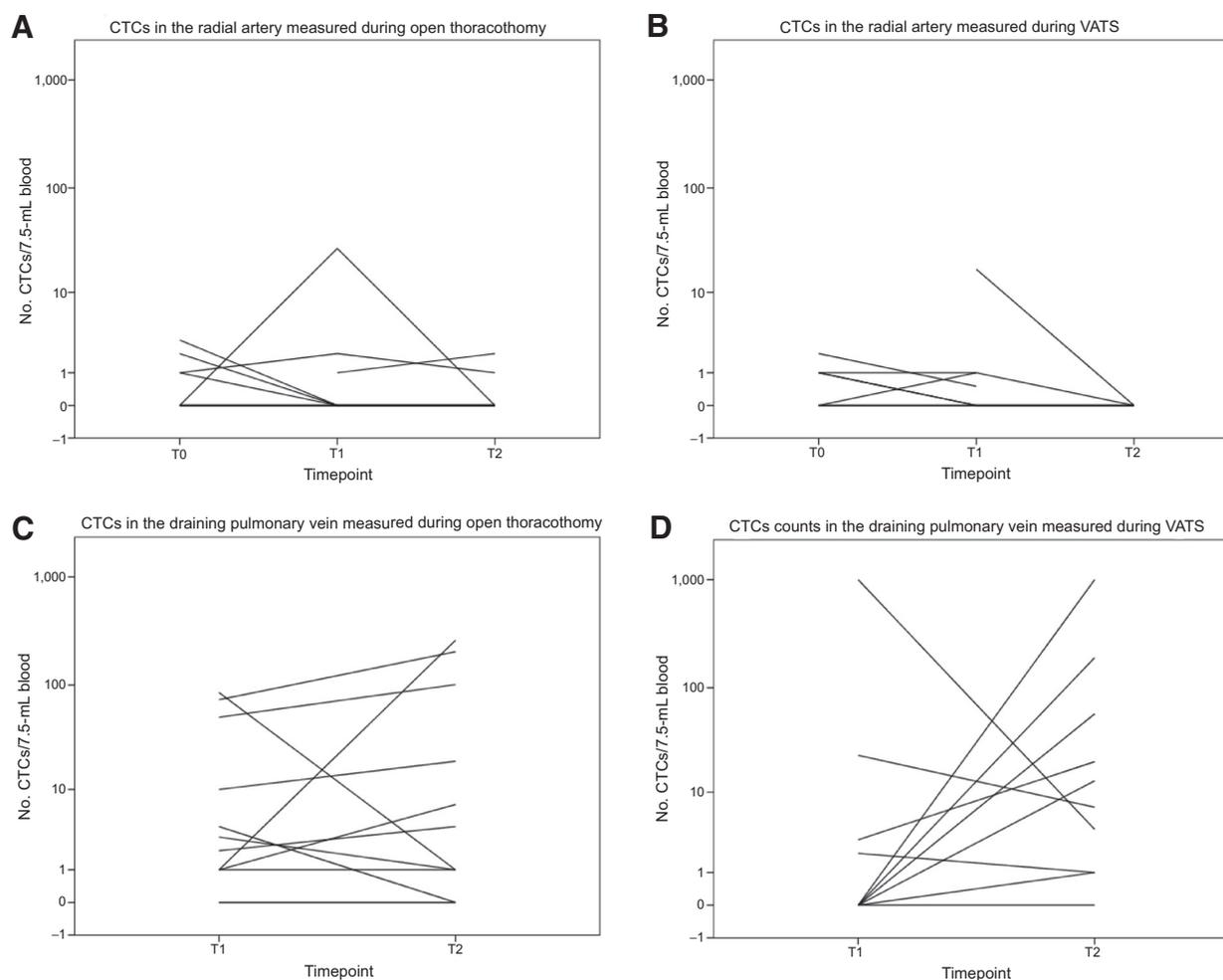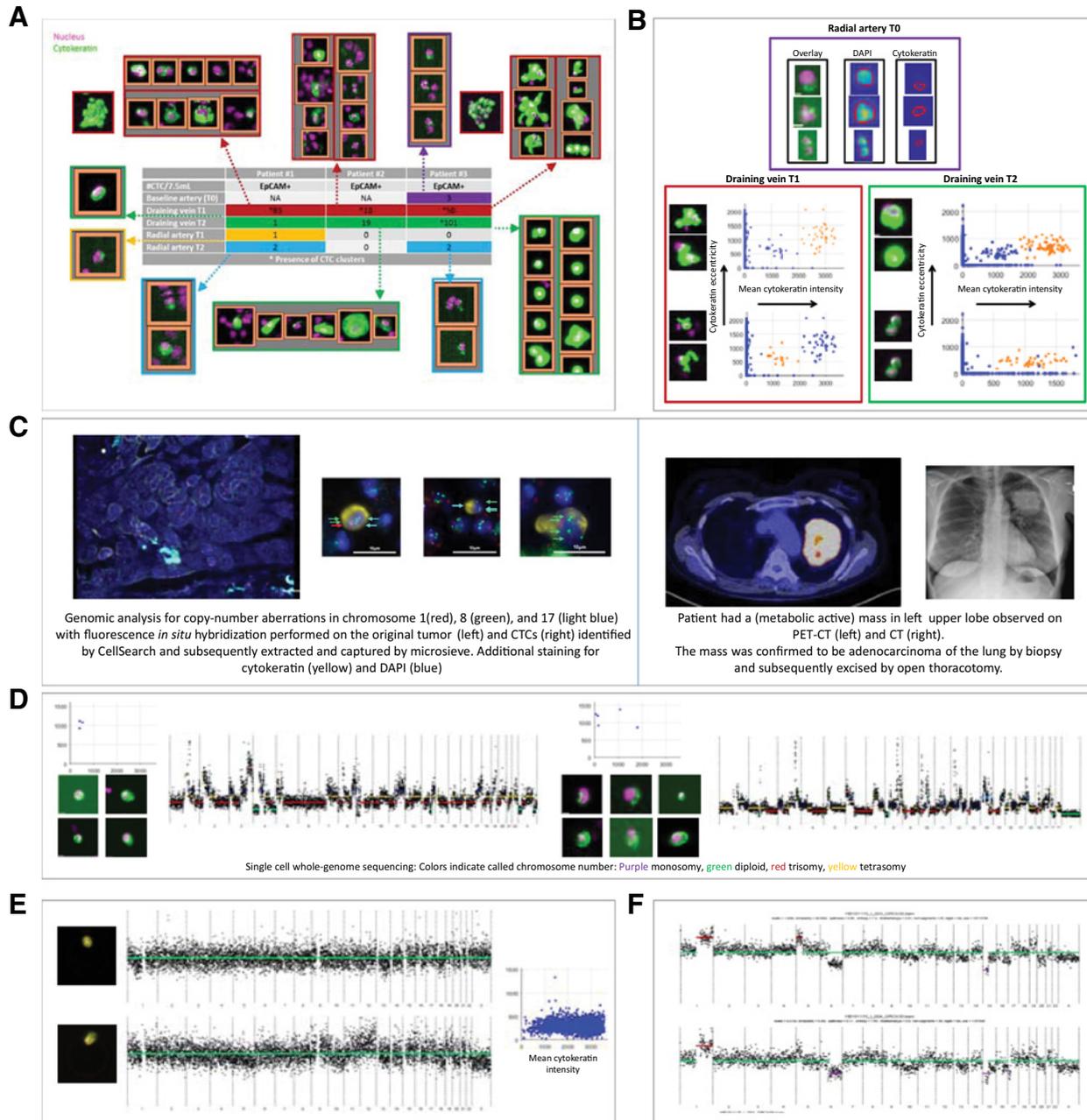
Figure 2.
Change in CTCs of patients with NSCLC ($n = 27$) and 3 control patients with fast-growing granulomatous nodules during surgery. CTCs were identified in samples from the radial artery and from the draining PV in patients undergoing open surgery (**A**, **C**) and VATS (**B**, **D**). Measurements were performed at the start of surgery in the radial artery (T0) and in both the radial artery and the PV during surgery when the draining PV was identified (T1) and either right before clamping the draining vein after dissection of the pulmonary artery (T2 open surgery) or immediately after closure of the PV with the pulmonary artery intact (T2 VATS). CTCs were identified by CellSearch and defined as EpCAM/CK/DAPI-positive cells without the expression of CD45. In the radial artery, 19 patients (12 open and 7 VATS) had no CTCs at any time point. In the PV, six patients (four open and two VATS) had no CTCs at either time points. CTC counts and changes in CTC counts did not differ between surgical approaches. CTC count in the PV was higher than in the radial artery ($P < 0.01$). The proportion of patients with CTCs in the radial artery was decreased at the end of surgery ($P = 0.05$), but CTC presence and count remained the same in the PV.

CTCs were successfully extracted from the CellSearch cartridges and isolated by FACS for scWGS (**Table 2**). In three patients with NSCLC, CTCs were aneuploid. In 2 of these patients, only one circulating cell was successfully extracted and analyzed, which was aneuploid (**Fig. 3D**). The third patient had 81 CTCs that were extracted successfully from three different samples. Of these extracted CTCs, only two showed aneuploidy, both derived from the same sample. The other extracted cells were euploid. The remaining 184 extracted CTCs from the other for patients (including two patients with fast-growing benign nodules) were all euploid. The structural changes of aneuploid CTCs closely resembled those identified in the primary tumor cells (**Fig. 3D**). In addition, of two patients, samples from the PV at T1 were analyzed for CNA by FISH after being passed over a microsieve. The first sample had 85 CTCs detected by CellSearch, yet all were euploid for chromosomes 1, 8, and 17. The second sample contained 19 CTCs as detected by CellSearch. In two CTCs,

aneuploidy was detected with loss of chromosome 1 and one had a trisomy of chromosome 8, changes that were similar to those in the primary tumor (**Fig. 3C**). No other aneuploidy cells were found. In total, four of 12 (33%) patients had CTCs with proven genomic aberrations.

Copy-number aberrations in punched CTCs

Another 125 CTCs from 4 different patients were isolated for genomic analysis by the punching system. We aimed to include both subsets of so-called CTC with different morphology, but were only able to successfully extract two CTCs from the radial artery at T1. The other three samples were from the PV. Two samples were derived from the PV at T1 (42 CTCs and two CTCs, respectively), one from the PV at T2 (79 CTCs). All punched CTCs from the PV showed no structural genomic changes (**Fig. 3E**), indicating that they are circulating epithelial cells rather than tumor cells.

Figure 3.
CTCs detected in the PV and the radial artery during surgery for suspected NSCLC: Morphology and genomic analysis. **A,** CTCs defined by CellSearch show distinct morphologic characteristics when found in the PV draining the primary tumor or the radial artery during surgery for suspected NSCLC. Two clusters found in the vein at the end of surgery are depicted as well. **B,** Morphologic CTC subtypes as defined by the ACCEPT program. CTCs detected in the peripheral blood have very low CK expression, whereas CTCs in the draining PV express CK more strongly, both at the start of surgery (T1) and after either the PV or pulmonary artery has been dissected (T2). **C,** CTCs from two samples were extracted from the CellSearch cartridge and passed over a microsieve. FISH was used to detect copy-number variations (CNVs). Three CTCs out of 10 derived from one sample were suspected to have CNV, which were also seen in the original tumor. All other cells in this sample and the other (85CTC) were euploid. **D,** Two CTCs from 2 different patients with aneuploidy as detected by scWGS. CTCs were isolated by FACS in a sample with four and six CTCs identified by CellSearch, of which the images are depicted. CTCs with structural genomic changes were the minority (4/267 isolated CTCs). **E,** Morphology of two CTCs which were isolated by the Vycap puncher for scWGS. Both were euploid, despite being defined as CTC by CellSearch. A graph of cytokerating and DAPI expression measured by the ACCEPT program is provided. **F,** Primary tumor from the patient of whom the CTCs in **D** were derived was analyzed with WGS in mini bulk (30 cells) and showed aneuploidy that was not found in the CTCs.

Table 2. Number of CTCs enumerated by CellSearch and extracted for copy-number analysis by scWGS in patients undergoing surgical resection for NSCLC or growing lung nodules.

Patient	Blood draw	No. CTCs/7.5-mL enumerated	No. CTCs isolated for CNA (%)	No. CTCs aneuploid	Primary tumor aneuploid	Diagnosis
4	PV T2	4	1 (25)	1	Yes	Squamous
5	PV T2	7	1 (14)	1	Yes	Adeno
6	RA T1	27	14 (52)	None	Yes	Squamous
7	PV T1	>1,000	34 (3)	None	Yes	Adeno
8	PV T2	>1,000	40 (4)	None	No	Control
10[a]	PV T1	204	26 (13)	None	Yes	Squamous
	PV T2	260	38 (15)	2	Yes	
	PV T2	73	17 (23)	None	Yes	
14	PV T1	20	14 (70)	None	No	Control
21	PV T2	20	1 (5)	None	Yes	Adeno
22	PV T2	13	5 (38)	None	Yes	Adeno
23	PV T1	>1,000	76 (8)	None	Yes	Adeno

Abbreviations: RA, radial artery; T1, sample taken at localization of PV during surgery; T2, sample taken after either ligation of pulmonary artery or PV.
[a]Patient had two lobes affected.

Of one of these patients, primary tumor tissue was frozen and used for genomic analysis by minibulk (2 × 30 cells). The primary tumor showed structural abnormalities (**Fig. 3F**).

Discussion

CTCs enumerated with the CellSearch system were present in higher counts in samples obtained from the PV compared with samples from the RA. Most of them are epithelial cells, and few are CTCs with genomic aberrations. Release of these so-called CTCs did not appear to be influenced by manipulation during the course of surgery. The different surgical approaches and their associated differences in vessel ligation also did not influence CTC counts. It appears that large numbers of CTCs disappeared during their travel from the PV through the heart to the RA. CTC numbers in the RA were low and did not differ substantially from those reported in the peripheral venous system. That means that these CTC are lost in the central compartment (heart and large blood vessels) and not so much in the peripheral microcirculation as has been previously theorized (16).

The majority of the CellSearch-identified and -extracted CTCs did not have any structural genomic changes when analyzed by scWGS. This was surprising because all primary lung cancer samples were characterized by a complex karyotype with many structural aberrations, a typical finding in lung cancer. This might be due to the FACS, a method that is less reliable in isolating rare cells (17, 18). Genomic analysis on a limited number of cells isolated by the more reliable cell puncher method showed however similar findings. Given these technical constraints, we concluded that in the PV during surgery many normal epithelial cells are observed and only limited numbers of CTCs with abnormal genomic patterns.

When examining the images from the CellSearch analyzer, two populations of CTCs could be discerned based on their morphology. The one more closely resembling CTCs observed in the peripheral blood by CellSearch is probably a real CTC. The other population analyzed in this study did not have any structural abnormalities. This implies that these cells, though defined as CTCs according to the CellSearch protocol, are more likely to be nonmalignant epithelial cells. Other methods often isolate CTCs by their larger size, sometimes in combination with their expression of CK and EpCAM (19, 20). Therefore, it is likely that they would also have isolated these cells. However, using different markers than CK or EpCAM, e.g., markers used

pathology like p40 or TTF-1, might be able to distinguish malign CTCs more accurately (21).

The exact mechanism by which these nonmalignant epithelial cells enter the bloodstream is as yet unclear and deserves further exploration. Endothelial cells occasionally can express CK and may be released into the bloodstream during surgery influencing measured CTC counts by CellSearch (22). However, endothelial cells should be excluded by the EpCAM-based separation. In addition, high numbers of CTCs were identified even when the blood vessels were intact. Therefore, it seems unlikely that contamination by endothelial cells can explain the high number of identified (euploid) CTCs by itself. Probably, a spectrum of epithelial cells is released into the bloodstream from normal and premalignant to malignant epithelial cells. Their presence could explain the large difference between CTC counts as defined by CellSearch in the PV and the RA. Benign epithelial cells are less able to survive in the circulation because of lower tolerance of shearing forces and the mesenchymal environment, leading to a fast clearance or destruction. Therefore, benign cells are not (often) detected in the peripheral bloodstream. That so-called CTC may be present in the peripheral blood of healthy individuals, even without surgery, has been shown by Allard and colleagues, albeit in very low numbers (23). The large decrease in CTC count between PV and RA cannot be explained by the dilution from the four other PVs as the difference is too large. However, the high numbers in the PV could represent a short-lasting peak that was missed in the RA. Other possible explanations are the destruction of (suspected) CTCs by physical forces from the moment these cells enter the bloodstream. The benign epithelial cells maybe more susceptible to such forces than malignant cells.

That epithelial cells are misidentified as CTCs by CellSearch when measured in the PV during surgery would explain previous findings summarized in **Table 3**. The CTC presence during or shortly after surgery in the peripheral circulation is strongly associated with disease recurrence and recurrence-free survival (7, 9, 10, 24–28). Suspected CTCs identified in the PV were also shown to be associated with survival, but the association is less well defined (16, 29–31). Studies comparing both measurements like Crosbie and colleagues and Li and colleagues show that despite higher cutoff values, the association of suspected CTCs in the PV with disease recurrence is not stronger than the association of CTCs identified in the peripheral system (16, 31). Possibly, CTCs in the

Table 3. Overview of surgical studies where CTCs were measured in pulmonary and/or peripheral veins.

Author/Journal/Year	Surgery/CTC detection	Blood draws	Outcome
Okumura Ann Thorac surg 2009[a] (29)	30 thoracotomy CTC detection by CellSearch	PV: 2.5 mL after resection. Peripheral: 7.5 mL just before surgery	CTCs were more often detected in the PV than in the RA. CTCs in the PV and peripheral samples were not associated with worse outcome.
Hashimoto Int Cardiovasc and Thorac Surgery, 2014[a] (42)	30 thoracotomy CTC detection by CellSearch	PV: 2.5 mL before and after lobectomy. Peripheral: RA before surgery	CTCs were more often detected in the PV than in the RA. CTCs were more often detected in the PV after resection.
Crosbie JTO 2016 (16)	30 thoracotomy CTC detection by CellSearch	PV: 10 mL before dissection. Peripheral: 10 mL venous sample before surgery	CTCs were more often detected in the PV than in the peripheral sample.
Hashimoto JTD 2018	30 thoracotomy CTC detection by CellSearch	PV: 2.5 mL before and after lobectomy Peripheral: ND	Most patients had an increase in CTCs, large increase was associated with more postoperative metastases within 5 years.
Lv Oncol letters 2018 (37)	32 thoracotomy CTC detection by CellSearch	PV: 7.5 mL after lobectomy Peripheral: 7.5 mL during lobectomy	CTC count was associated with size and vessel invasion. CTC count in PV was significantly higher than in peripheral samples.
Chudasama Oncol Letters 2016 (39)	8 thoracotomy, 2 VATS CTC detection by SceenCell	PV: 3 mL before dissection and after lobectomy. Peripheral: 3 mL venous sample before and 3 days after surgery	More CTCs detected in PV at the start of surgery.
Sawabata Surg Today 2016 (41)	23 thoracotomy CTC detection by ScreenCell	PV: 3 mL after lobectomy Peripheral: 3 mL before and after surgery	CTCs were more often detected in the PV than in peripheral samples. CTCs were more often detected in peripheral samples taken during surgery and less often after surgery.
Li Scientific Reports 2017 (31)	25 thoracotomy CTC detection by AutoMACS	PV: 15 mL before dissection. Peripheral: 15 mL before surgery	CTCs were more often detected in the PV than in peripheral samples. Both peripheral and central samples were associated with shorter disease-free survival.
Murlidhar Cancer Res 2017 (40)	35 thoracotomy/VATS CTC detection by OncoBean chip	PV: 3 mL blood before dissection. Peripheral: venous samples before surgery, at PV sample, and 3 days after surgery	PV samples had significantly more CTCs than peripheral samples. In samples with higher CTC count, gene expression of genes related to resistance was increased.
Reddy J Thorac Cardiovasc Surg 2016 (38)	32 thoracotomy/VATS CTC detection by microfluidic chip	PV: 5 mL before resection. Peripheral: 7.5 mL pre, during and post lobectomy	More CTCs in PV compared with peripheral samples.
Hofman IJC 2010[a] (7)	210 thoracotomy CTC detection by CellSearch and ISET	PV: ND. Peripheral: 7 + 10 mL before surgery	CTC presence was associated with shorter disease-free survival.
Hofman Clin Cancer Research 2010[a] (24)	208 thoracotomy detection by ISET	PV: ND Peripheral: 10 mL before surgery	CTC presence was associated with shorter disease-free survival.
Bayarri-Lara Plos one 2016 (10)	36 thoracotomy 20 VATS CTC detection by microscopy after enrichment	PV: ND. Peripheral: 10 mL venous samples before surgery and 1 month after	CTCs were less often detected after surgery. CTC presence after surgery was significantly associated with early recurrence and shorter disease-free survival.
Dandachi Lung Cancer 2017 (9)	50 thoracotomy CTC detection by size- based microfilter	PV: ND. Peripheral: 7.5 mL before surgery	CTC presence was associated with shorter disease-free survival.
Matsutani JTD 2017 (8)	29 thoracotomy CTC detection by ScreenCell	PV: ND. Peripheral: 3 mL RA before and directly after lobectomy	CTCs more often detected after surgery and in more advanced stage tumors.

Abbreviations: ND, not done; RA, radial artery.
[a]Overlap of patients possible.

PV are a mix of true CTCs with other epithelial cells released into the bloodstream, explaining the high counts in the PV, and are therefore not representative of tumor burden. A large part of these cells, primarily the benign cells, are unable to survive in the bloodstream, explaining the low CTC counts peripherally, which are associated with survival (32–36).

In line with previous studies, higher CTC counts in the PV were found compared with the peripheral measurements (16, 29, 31, 37–42).

Also, significantly lower CTCs counts after surgery are observed when measured in the peripheral circulation (10, 38–41, 43). However, together with Hashimoto and colleagues, we are the only ones that have performed sequential measurements in the PV (42). Hashimoto and colleagues found significantly higher numbers in the second sample taken from the PV, but they took the sample after the lobe had been completely removed from the body, allowing stagnation of the blood. Hashimoto and colleagues also explored whether sequence of vessel ligation influenced CTC release and found no difference. They also found no difference between VATS and open surgery, where the order of vessel ligation differs. It is also in line with the fact that the outcome of both surgical techniques is comparable and release of CTCs is associated with worse outcome (44, 45). Moreover, no significant differences in survival or disease recurrence were observed between patients who had the PV or artery clamped and dissected first (42, 46–48).

CellSearch is originally developed for measuring CTCs in peripheral blood samples expressing both EpCAM-positive and CK but lacking CD45 and with morphology features consistent with a cell containing a nucleus (DAPI). CTCs detected in this way have been associated with survival many times over, making them a well-validated biomarker. It is known that the CTCs identified by CellSearch in the peripheral bloodstream do exhibit a large variation of genomic abnormalities when analyzed by FISH, scWGS, or NGS (49–55). Our findings do not contradict these previous reports, but do indicate that care should be taken when implementing the CellSearch in different blood compartments, like the PV.

The definition of a CTC, currently described as an EpCAM^{+}, CK^{+}, DAPI^{+}, CD45^{-} cell identified by CellSearch, may need the addition that the measurement should be performed in peripheral blood. Furthermore, manual classification of CTCs is currently being replaced by automated classification using the ACCEPT imaging analysis program, which could further improve the definition of a CTC (56, 57). CTCs from peripheral blood measured with CellSearch remain, in our studies and many other clinical trials, a strong biomarker and especially a sensitive test for low numbers of CTCs (1–6).

In short, a spectrum of epithelial cells is released into the circulation during surgery. The number of CTCs was higher in the PV compared with the radial artery. Release of CTCs was not influenced by the type of surgical approach and the difference in vessel ligation, or by manipulation during surgery. In the peripheral blood, CTCs were less often detected at the end compared with the start of the surgical procedure. Two morphologically distinct circulating epithelial populations were observed: the majority of these cells were euploid epithelial cells released during surgery, and a small minority were CTCs with structural genomic abnormalities. Normal epithelial cells were likely filtered away during their passage in the central blood compartment. We recommend to include genomic tests to verify the malignant character of circulating cells further than by morphology and fluorescence features, especially when using CellSearch in a manner for which it is not validated.

Disclosure of Potential Conflicts of Interest

H.J.M. Groen is a paid consultancy for Eli Lilly and is an unpaid consultant/advisory board member for Bristol-Myers Squibb and Takeda. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Tamminga, T.J.N. Hiltermann, P.M. Lansdorp, L.W.M.M. Terstappen, H.J.M. Groen
Development of methodology: M. Tamminga, S. de Wit, H. van den Bos, T.J. Klinkenberg, T.J.N. Hiltermann, D.C.J. Spierings, P.M. Lansdorp, L.W.M.M. Terstappen, H.J.M. Groen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Tamminga, S. de Wit, C. van de Wauwer, H. van den Bos, T.J. Klinkenberg, T.J.N. Hiltermann, K.C. Andree, D.C.J. Spierings, A. van den Berg, H.J.M. Groen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Tamminga, S. de Wit, H. van den Bos, T.J.N. Hiltermann, D.C.J. Spierings, W. Timens, L.W.M.M. Terstappen, H.J.M. Groen
Writing, review, and/or revision of the manuscript: M. Tamminga, S. de Wit, C. van de Wauwer, H. van den Bos, T.J. Klinkenberg, T.J.N. Hiltermann, K.C. Andree, D.C.J. Spierings, P.M. Lansdorp, A. van den Berg, W. Timens, L.W.M.M. Terstappen, H.J.M. Groen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Tamminga, S. de Wit, H. van den Bos, J.F. Swennenhuis, D.C.J. Spierings
Study supervision: T.J.N. Hiltermann, H.J.M. Groen

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