



Possible role of circulating tumor cells in early detection of lung cancer

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Abstract: The prognosis of lung cancer varies highly depending on the disease stage at diagnosis, from a 5-year survival rate close to 90% in stage I, to 10% or less in stage IV disease. The enhancement of early diagnosis of this malignancy is mandatory to improve prognosis, because lung cancer patients stay long asymptomatic or few symptomatic after disease onset. Nowadays, liquid biopsy has emerged as a minimally-invasive tool to address the urgent need for real time monitoring, stratification, and personalized treatment of malignancies, including lung cancer. Liquid biopsy refers to a class of biomarkers, including circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA) and tumor-derived extracellular vesicles (tdEV). Since CTCs represent a crucial step in disease progression and metastasis, we reviewed here the scientific literature about the use of CTCs in early diagnosis of lung cancer; different techniques, and different strategies (e.g., source of analysis sample or high-risk groups of patients) were discussed showing the potential of implementing liquid biopsy in the clinical routine of non-metastatic lung cancer.

Keywords: Circulating tumor cells (CTCs); single-cell analysis; NGS; tumor heterogeneity; precision oncology; early lung cancer; CTC detection technologies; lung cancer surgery; pulmonary vein (PV); CTC clusters; CTC dissemination; lung cancer screening

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Introduction

Lung cancer is the first malignancy worldwide for incidence and mortality, with an estimated 2.1 million diagnoses in 2018 (1). About 80% of lung cancers are classified as non-small cell lung cancer (NSCLC), 15% are small cell lung cancer (SCLC), and 5% are other histological variants (2).

In USA, from 2004 to 2015, only 1 out of 5 patients were diagnosed for lung cancer in stage I, whilst more than 3 out of 5 patients were in stage III/IV of disease (3). Similar data were reported in several national and international reports (4,5), and treatment options and prognosis changed depending on stage at diagnosis. In fact, whereas surgery

is the main treatment option of stage I disease with a 5-year survival rate close to 90%, in stage IV disease, chemotherapy, tailored therapy and immunotherapy can be used as single agents or in combination, but the 5-year survival rate does not reach 10% (6). Hence, beyond tobacco smoking prevention, a more comprehensive knowledge of lung cancer biology and its early detection represent the main steps in an ideal road map to improve patient prognosis (4,5).

Nowadays, liquid biopsy has emerged as a minimally-invasive tool to address the urgent need for real time monitoring, stratification, and personalized treatment of malignancies, including lung cancer. In principle, liquid

biopsy from peripheral blood could provide the genetic landscape of all cancerous lesions at any time, detecting genomic alterations responsive to a specific therapy or associated with drug resistance. Moreover, it would allow a prognostic/predictive biomarker evaluation in patients for whom biopsies are inaccessible or difficult to repeat. Liquid biopsy now comprises a class of biomarkers, including circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA) and tumor-derived extracellular vesicles (tdEV). Among these, CTCs are the most suitable to identify malignancies more prone to give metastasis, only because, until proven otherwise, intact cells, and not their DNA, have the potential to cause metastasis.

Indeed, a high number of CTCs measured at any time during treatment has been associated, in several studies, with a shorter time to progression (7,8) in metastatic breast cancer, whilst a decreasing CTC number during treatment indicated therapeutic success (8,9). The prognostic value of CTCs in early breast cancer has then been proven (10). Similar findings were even reported in lung cancer (11,12) using the CellSearch® (CS) platform that first obtained the IVD mark from FDA, and to date, represents the “gold standard” (13). Data from several authors were gathered in meta-analyses (14-16) and confirmed CTCs as an independent prognostic indicator of progression-free survival (PFS) and overall survival (OS) in advanced NSCLC. Moreover, CTCs have been proposed as surrogate markers of distant metastasis in the early stages of lung cancer (17). Actually, based on the frequency distribution of CTCs detected by CS in 7.5 mL of blood, we could extrapolate that almost all cancer patients should have at least 1 CTC in peripheral blood, if enough blood volume could be analyzed (18). This observation impacts all clinical settings where there is a pressing need to disclose high-risk patients or anticipate a diagnosis of malignancy. From the clinical perspective, the exploitation of liquid biopsy in lung cancer promises to have a real effect on patient possibility of care and quality of life (19), in particular if combined with imaging examination (20).

However, we cannot ignore that the huge number of studies and techniques—often with different levels of validation (13)—that have used CTC enrichment, isolation and characterization, produced controversial results and limited transfer from benchtop to bedside (21). To understand this limited transfer, we reviewed the current scientific literature using the keywords ‘CTC and early lung cancer detection’, ‘CTC and lung cancer surgery’, ‘CTC and pulmonary vein’, ‘high-risk lung cancer patients

and CTCs’ and ‘liquid biopsy and lung cancer screening’, in order to include all the references referring to CTC research, independently of the technologies used.

Thus, herein we will highlight different techniques, and different strategies (e.g., source of analysis sample or high-risk groups of patients) that could successfully contribute to implementing liquid biopsy in the clinical routine of non-metastatic lung cancer.

The technological race to detect CTCs in increasingly early stages of lung cancer: the finish line is still far away!

Due to sensitivity constraints in current CTC detection technologies, there is limited published data on CTC prevalence in early-stage lung cancer, or on the correlation of CTCs with disease progression and OS. Most of these studies have been conducted using different enrichment methods and identification techniques, making the results difficult to compare, especially since, for early-stage lung cancer, no reference CTC detection technology is yet available (22-34).

Table 1 shows a list of studies in which CTCs have been recovered from peripheral blood samples of lung cancer patients, even at early stages. Some authors, as expected, have highlighted a greater difficulty in finding positive CTC samples in early-stage patients than in later stage patients (30). This is not always the case, as Lou *et al.*, using CytoploRareH circulating lung cancer cell kit (GenoSaber Biotech Co. Ltd., Shanghai, China) could detect, with the threshold of 8.5 CTC units, 8/10 (80%) of stage I–II, 6/9 (67%) of stage III, and 13/14 (93%) of stage IV initially diagnosed NSCLC (24). Nair *et al.* using the ‘high definition CTC’ (HD-CTC) technology (23,36) detected more than 2 HD-CTCs/mL in 61% of all TNM stage patients, and in 60% of stage I patients (25).

In some studies, the CTC count has been related to clinical stage, with a higher number of CTCs detected in more advanced stages (24,34,35); in others, no significant difference in the percentage of CTCs between primary and metastatic stage (22,23,26,29,30,33) was observed. Some authors related the number of CTCs to other clinic-pathological parameters; for example, Li *et al.* noted that, in those patients with adenocarcinomas, the CTC counts differed significantly with tumor depth. On the other hand, Jin *et al.* did not find any statistically significant correlation between the number of CTCs and tumor size; however, in the same group of patients, they noted that highly abundant

Table 1 Overview of studies where CTCs were detected in early lung cancer patients

Study	Number of cancer patients	Disease stage*	Peripheral blood volume (mL)	CTC enrichment method	CTC identification method	Median follow-up duration (months)	Results	References
Hofman <i>et al.</i>	208	I–IV	10	ISET®	Modified May-Grünwald-Giemsa staining method	24	102/208 (49%) patients showed CNHCs; no correlation was found between the number of CNHCs and the disease stage; ≥50 CNHCs were associated with shorter OS and DFS in all stages	(22)
Wendel <i>et al.</i>	78	I–IV	–	Non-enrichment method	IF (panCK, CD45, DAPI)	–	73% of patients had a positive HD-CTC count; no significant differences were detected in median HD-CTC count between tumor stages	(23)
Lou <i>et al.</i>	72	I–IV	3	CD45 leukocyte magnetic depletion	qPCR	–	With the threshold of 8.5 CTC units, the assay detected 8/10 (80%) of stage I/II, 6/9 (67%) of stage III, and 13/14 (93%) of stage IV initially diagnosed NSCLC; metastatic patients had more CTCs than localized patients	(24)
Nair <i>et al.</i>	71	I–IV	6–10	Non-enrichment method	IF (panCK, CD45, DAPI)	–	43/71 (61%) of all TNM stages and 26/43 (60%) of stage I patients had more than 2 HD-CTCs/mL; HD-CTCs were weakly correlated with partial volume corrected tumor SUV _{max} and not correlated with tumor diameter	(25)
Freidin <i>et al.</i>	57	I–IV	3	ScreenCell® Cyto filter devices	H&E	–	No significant differences were found in the percentage of CTCs for primary and metastatic cancer as well as for cancer stages	(26)
Zhang <i>et al.</i>	19	I–IIIA	1	Microfluidic CTC-capture device	IF (CK7/8, CD45, DAPI)	–	CTCs were identified in 13/19 (68%) patients; CTCs isolated from 14/19 patients were successfully expanded	(27)
Boffa <i>et al.</i>	112	I–IV	7.5	Non-enrichment method	IF (CK, CD45, PD-L1, DAPI)	Up to 36	CCAMs expressing PD-L1 were detected in 26/112 (23%) patients; patients with >1.1 PD-L1(+) cell/mL experienced worse OS than patients with ≤1.1 PD-L1(+) cell/mL	(28)
Jin <i>et al.</i>	45	I	10	CanPatrol™ (CD45 leukocyte magnetic depletion followed by size-based filtration)	RNA-ISH (CK8/18/19, EpCAM, vimentin, twist)	12 (cohorts A and B)	In cohort A CTCs were found in 17/18 (94%) cases; E-CTCs, M-CTCs and E&M-CTCs were detected in 5/18 (27.8%), 10/18 (55.6%) and 14/18 (77.8%) cases, respectively; in cohorts A and B CTC count increased significantly in patients with tumor progression	(29)

Table 1 (continued)

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Study	Number of cancer patients	Disease stage*	Peripheral blood volume (mL)	CTC enrichment method	CTC identification method	Median follow-up duration (months)	Results	References
Togo <i>et al.</i>	123	I–IV	7.5	TelomeScan F35	IF (EpCAM, CD45, CD133, panCK, CK19, vimentin, CEA)	–	CTCs were detected in 59.6% stage I, 40.0% stage II, 85.7% stage III, and 75.0% stage IV patients; EMT-CTCs were detected in 57/123 (46.3%) patients, non-EMT-CTCs were detected in 48/123 (39.0%) patients; EMT-CTC count at baseline showed a negative correlation with PFS	(30)
Jiang <i>et al.</i>	72	I–IV	1	poly (lactic-co-glycolic acid) (PLGA)—nanofiber (PN)-NanoVelcro chip	IF (CK, CD45, DAPI)	–	Most EGFR mutations in tumor tissue could also be detected in CTCs; in one patient T790M mutation was detected in CTCs long before tumor progression.	(31)
Chen <i>et al.</i>	49	I–IV	2	CellMax™ (CMx) microfluidic platform	IF (CK18, CD45, PD-L1, DAPI)	–	CTCs were detected in 42/49 (86%) patients; 28/42 (67%) patients with detectable CTCs had at least one PD-L1(+) CTC	(32)
Li <i>et al.</i>	174	I–IV	3.2	NE	FISH (CEP8, CD45, DAPI)	–	CTCs were detected in 138/174 (79.31%) patients; the diagnostic efficacy of CTC detection in peripheral blood was significantly higher than that of serum cancer markers	(33)
Wei <i>et al.</i>	73	I–IV	7.5	Nano-enrichment method	IF (CK, CD45, DAPI)	12	CTC count in stage IV was significantly higher than that in stages I–III; CTC count in poorly differentiated cases was significantly higher than that in well-differentiated cases; CTC numbers in patients with EGFR mutations were significantly higher than that in patients with no mutations	(35)
Xu <i>et al.</i>	20	I–IV	4	Integrated microfluidic platform consisting of DLD isolating structure and immunomagnetic purifying device	IF (CK7, CD45)	36 (only for 1 patient)	CTCs were detected in 15/20 (75%) patients; whole-exome sequencing revealed six new gene mutations in both single CTC and surgical specimen of a representative early-stage lung cancer patient	(34)

*, clinical or pathological stage. CEA, carcinoembryonic antigen; CEP8, centromere probe for chromosome 8; CK, cytokeratin; CNHC, circulating nonhematologic cells; DAPI, 4',6-diamidino-2-phenylindole; DFS, disease-free survival; E-CTC, epithelial CTC; E&M-CTC, epithelial-mesenchymal CTC; EMT, epithelial-mesenchymal transition; FISH, fluorescence in situ hybridization; HD-CTC, high definition CTC; H&E, hematoxylin and eosin; IF, immunofluorescence; ISET, isolation by size of epithelial tumor cells; M-CTC, mesenchymal CTC; NE, negative enrichment; OS, overall survival; PFS, progression-free survival; qPCR, quantitative polymerase chain reaction; RNA-ISH, RNA-in situ hybridization; SUV_{max}, maximum standardized uptake value.

mesenchymal CTCs were more prone to be in tumors >2 cm (29). Nair *et al.* observed that HD-CTCs were weakly correlated with tumor maximum standardized uptake value corrected for partial volume and not correlated with tumor diameter (25).

Some researchers compared the sensitivity of their CTC detection method with the sensitivity of common serum markers used for early detection or screening of cancer (30,33). Togo *et al.* noted that in early-stage (0–IA) lung adenocarcinoma patients, the TelomeScan F35-based detection sensitivity was 58.2%, whereas the sensitivity of carcinoembryonic antigen (CEA) detection (cut-off value of 5.0 ng/mL) was only 11.7%. However, by combining both assays, they achieved 65.1% sensitivity for the detection of early-stage NSCLC. Notably, 53.5% of early-stage patients tested negative in the CEA assay but positive for TelomeScan CTC detection (30). Li *et al.* observed that the sensitivity of CTC detection through negative enrichment-fluorescence *in situ* hybridization (NE-FISH) (37) and of a combination of tumor markers (CEA, CA 125, CYFRA 21-1, and SCC) for lung cancer diagnosis was 68.29% and 63.41%, respectively. However, combining all of those markers for the diagnosis of lung cancer, sensitivity was 82.93%. For patients with early-stage disease (I–II), sensitivity improved from 63.93% to 78.69% using this combination of all tested markers (33).

A few authors have also attempted to find an association between a specific level of CTCs and the survival of early lung cancer patients (22,27–30,35). In the work of Hofman *et al.*, a level of 50 or more circulating nonhematologic cells (CNHCs) was associated with shorter OS and disease-free survival (DFS), independently of disease stage, and with a high-risk of recurrence and death in stage I–II resectable NSCLC (22). Jin *et al.* noted that a higher CTC level or change range could be found postoperatively in patients with tumor progression, as compared with patients with DFS (29). Togo *et al.* observed that patients testing positive for epithelial-mesenchymal transition CTCs (EMT-CTCs) at baseline had poor response to chemotherapy and decreased PFS in comparison with those testing negative (30).

Lastly, a limited number of researchers performed genetic analysis of CTCs isolated from patients with early lung cancer (27,31,35). Zhang *et al.* demonstrated that 9/15 (60%) patient samples examined for the *TP53* gene by real-time polymerase chain reaction (RT-PCR) had mutations. Five patient samples had matched mutations in primary tumors and CTCs. In 3 patients, *TP53* gene mutations were noted only in the primary tumor and not in the matching

CTCs, whereas in 1 patient, the mutation was found only in the CTCs but not in the primary tumor. NGS further revealed additional matched mutations between primary tumor and CTCs of cancer-related genes and highlighted concordance for key genes involved in lung cancer progression (27). Jiang *et al.* observed that most *EGFR* mutations in tumor tissue could also be detected in CTCs. They concluded that, through the genetic analysis of the CTCs, it is possible to detect the onset of therapy resistance before tumor progression, as they have shown in the case of a patient treated with gefitinib, in which they detected the presence of the T790M mutation in CTCs long before tumor progression (31).

In conclusion, the use of CTCs in the early detection of lung cancer seems to be a promising, but insidious, route. While attempts are being made to increase the sensitivity of detection techniques, it is essential to understand whether the “CTCs” detected in patients with early-stage lung cancer could be clinically useful. Undoubtedly, the great improvements that are being made in the NGS field may reveal the characteristics of these cells and help us to understand their destiny, but a very long road still lies ahead.

Alternative sources for CTC recovery from early lung cancer patients

Surgical resection is considered the best option for treating patients with early-stage lung cancer; however, a proportion of patients develop recurrence, even after complete resection (38,39). The dissemination of CTCs is a relevant event in the process of metastasis and tumor recurrence (40). Intraoperative manipulation can cause the shedding of CTCs in the blood and accelerate metastases after surgery (41,42). Repeated CTC evaluation during the postoperative period might provide a useful tool for monitoring patients after curative resection (43,44). Several studies have attempted to identify the specific characteristics of CTCs in patients undergoing surgery for early-stage lung cancer. However, CTCs are very rare events (few CTCs among millions of blood cells), especially in early-stage lung cancer, thus requiring alternative strategies for their collection (45,46). In some cases, CTCs were recovered near their origin, i.e., from the pulmonary vein (PV) of the affected lobe, which can be accessed at the time of tumor resection (47–62).

The techniques used for the enrichment and the identification of CTCs from the PV are numerous, blood volumes are different, the number of patients is variable

and it is not always possible to obtain significant data on the survival of patients due to too short follow-up periods (48,54,56,62). Despite these initial differences, it is clear that most studies converge towards similar conclusions (Table 2). Indeed, in most cases, a higher number of patients were positive for the presence of CTCs in the PV than in peripheral blood (48,49,52,54,55,57,61), and a higher number of CTCs were detected in the pulmonary vein (pvCTCs) than in peripheral blood (periCTCs) (49,53,55-57,59,61). Moreover, in some cases, a higher number of CTC clusters (55,57,61) or larger clusters (56) were noted in the PV than in peripheral blood.

Some authors observed an increase in the number of samples positive for pvCTCs (58) and periCTCs (52,54) after surgery. Sawabata *et al.* noted that negative CTC patients before surgery became positive immediately after surgery (54). This increase in the number of CTCs has been attributed to the shedding of cells that can occur in concomitance with the manipulation of the tumor during surgery (47,49-52,54,58). Duan *et al.* observed that the increase in the number of CTCs occurred most frequently in patients where the PV was interrupted before the pulmonary artery (PA) (58); Song *et al.* noted an increase of CD44v6 and CK19 expression in patients where the PA was ligated before the PV (51). In other cases the sequence of vessel ligation did not influence CTC counts (49,50,61).

Data in the literature are contradictory about the correlation between the sequence ligation of the vessels before surgery and the increase in CTC number (48-50,58), and the data on the correlation between the number of CTCs detected in the PV and the survival of patients are also discordant (49-51,58,61). In the work of Sienele *et al.*, 7/10 patients with CK+ cells in PV blood died from cancer-related causes (70%), while only 13/39 patients without occult cancer died from cancer-related causes (33%). The multivariate regression analysis demonstrated that cancer cell dissemination into PV blood was an independent significant prognosticator for reduced cancer-related survival in patients with pN0-pN1 lymph nodes. Okumura *et al.* did not find any significant difference in the incidence of recurrence or death according to pvCTC status; however, the median follow-up time from thoracotomy was only 13 months (48). In Hashimoto *et al.*, the increasing pvCTC count (Δ pvCTC) during surgical manipulation was significantly correlated with postoperative distant metastasis. Significant shorter time to distant metastasis (TDM) was observed in high Δ pvCTC group (Δ pvCTC ≥ 119 cells/2.5 mL) than low Δ pvCTC group (Δ pvCTC < 119 cells/2.5 mL). Neither DFS nor OS

was significantly correlated with Δ pvCTC (50). Chudasama *et al.* did not observe any significant association between the development of distant metastatic deposits and increased levels of CTCs (52). Dong *et al.* noted that the PFS in the group with pvCTCs $\geq 16/5$ mL was shorter than that in the group with pvCTCs $< 16/5$ mL (11.1 vs. 21.2 months, respectively). The PFS in the group with peripheral arterial CTCs $\geq 3/5$ mL was shorter than that in the group with peripheral arterial CTCs $< 3/5$ mL (14.8 vs. 20.7 months, respectively). Crosbie *et al.* found an association between the pvCTC 'high' count (≥ 18 pvCTCs/7.5 mL blood) and DFS. The detection of any CTCs (≥ 1 CTC/7.5 mL blood) in peripheral blood was associated with reduced DFS and OS (55). When Chemi *et al.* applied the cut-off point of 18 pvCTCs in the TRACERx cohort, a significant association with poorer DFS was found, and this remained an independent predictor in multivariate analysis when adjusting for tumor stage. Moreover, a pvCTC-high status of ≥ 7 pvCTCs/7.5 mL blood showed a significant association with lung cancer relapse in Kaplan-Meier analysis and remained an independent predictor in multivariate analysis when adjusting for tumor stage (60).

Similarly, the presence of CTC clusters has been related to the survival of early lung cancer patients. Some authors observed that patients without clustered CTCs at the time of surgery had a higher relapse-free survival rate compared with those with clustered CTCs revealed during surgery (52,54). In accordance, Murlidhar *et al.* noted that patients with CTC clusters in the preoperative peripheral blood showed a trend toward lower PFS than those patients without clusters. Crosbie *et al.* identified circulating tumor microemboli (CTMs) in 6/7 high-pvCTC (85.7%) patients but not in the low-pvCTC group. The presence of CTMs was associated with a significant reduction in DFS and OS (55).

It is remarkable to note that the single cell whole genome sequencing analyses performed by Tamminga *et al.* unmasked the purely epithelial and non-malignant nature of several "CTCs" detected in the PV blood of early NSCLC patients. Indeed, only the CTCs isolated by FACS from 4/11 PV blood samples presented genomic aberrations. At this point, it should be asked whether the cells detected in PV during surgery or in peripheral blood immediately after surgery are in fact only non-malignant epithelial cells, released into circulation by surgical procedures, and destined to be rapidly eliminated due to their lower resistance to shear forces and mesenchymal environment (51,61). However, Lv *et al.* demonstrated the tumorigenicity of pvCTCs and pvCTMs by a xenograft experiment in

Table 2 Overview of studies where CTCs were measured in pulmonary vein

Study	Number of patients	Disease stage*	PV blood volume (mL)	Peripheral blood volume (mL)	CTC enrichment method	CTC identification method	Median follow-up duration (months)	Results	References
Sienel <i>et al.</i>	62	I-IV	10	–	Non-enrichment method	ICC (CK8/18/19)	25	11/62 patients (18%) had CK-positive cells in PV blood before tumor manipulation. 7/10 pN0-pN1 patients (70%) died from cancer-related causes	(47)
Okumura <i>et al.</i>	30	I-IV	2.5	7.5	CellSearch® system (EpCAM-based enrichment)	IF (CK8/18/19, CD45, DAPI)	13	29/30 (96.7%) had pvCTCs. 5/30 (16.7%) had periCTCs. No significant correlation between pvCTC count and incidence of recurrence	(48)
Funaki <i>et al.</i>	94	I-IV	1	–	RosetteSep® human CD45 depletion cocktail	Papanicolaou and CK-IHC staining	13	ITCs were detected in 68/94 (72%) patients; cancer recurrence occurred in 16/94 (17%) cases; DSF rate was worse in patients with clustered ITCs	
Hashimoto <i>et al.</i>	30	I-IV	2.5	7.5	CellSearch® system (EpCAM-based enrichment)	IF (CK8/18/19, CD45, DAPI)	64.4	22/30 (73.3%) patients had pvCTCs. 2/30 (6.7%) patients had periCTCs; after surgery pvCTC count increased in 24/30 (80%) patients; increasing pvCTC count during surgical manipulation was significantly correlated with postoperative distant metastasis	(49,50)
Song <i>et al.</i>	30	I-III A	10	–	Non-enrichment method	FQ-RT-PCR (CK19, CD44v6)	–	Significant increase in CK19 and CD44v6 mRNA expression in PV blood during surgery, when PA was ligated before PV	(51)
Chudasama <i>et al.</i>	10	I-III A	3	3	ScreenCell® filtration device technology	H&E	–	periCTCs were detected in 80% of preoperative samples; pvCTCs in 100% of samples; periCTCs in 100% of postoperative samples	(52)
Reddy <i>et al.</i>	32	I-III A	5	6–8	EpCAM-based microfluidic chip	IF (CK7/8, DAPI, CD45)	–	PV blood samples contained a higher number of CTCs compared to peripheral blood samples	(53)
Sawabata <i>et al.</i>	23	I-III A	3	3 (peripheral atrial blood)	ScreenCell® CTC selection kit	H&E	12	pvCTCs were detected in 19/23 (82.6%) samples; periCTCs were detected in 7/23 (30.4%) samples collected before surgery; in 17/23 (73.9%) samples collected immediately after resection; in 1/23 (4.3%) samples collected 6 h from the end of the surgery; 10/16 (62.5%) patients negative for periCTC before surgery, became positive after lung resection	(54)
Crosbie <i>et al.</i>	30	I-III	7.5	7.5	CellSearch® system (EpCAM-based enrichment)	IF (CK8/18/19, CD45, DAPI)	22	pvCTCs were detected in 13/30 (43%) patients; periCTCs in 6/27 (22%) patients; pvCTMs in 6/30 (20%) patients. pvCTC 'high' count (≥18 pvCTCs) was associated with a poorer DFS and OS	(55)
Murlidhar <i>et al.</i>	36	I-III	2–3	4	OncoBean Chip using antibodies against EpCAM, EGFR, and CD133	IF (CK7/8, CD45, DAPI; additional CD44 staining for clusters)	10	CTCs were detected in 83.3% samples collected from PV blood; larger clusters were detected in PV samples	(56)

Table 2 (continued)

Table 2 (continued)

Study	Number of patients	Disease stage*	PV blood volume (mL)	Peripheral blood volume (mL)	CTC enrichment method	CTC identification method	Median follow-up duration (months)	Results	References
Lv <i>et al.</i>	32	I-III	7.5	7.5	CellSearch® system (EpCAM-based enrichment)	IF (CK8/18/19, CD45, DAPI)	–	pvCTCs were detected in 29/32 patients (90.6%); pvCTMs in 12/32 patients (37.5%); periCTCs in 8/32 patients (25%); periCTMs were not detected; tumor size, lymph node metastasis and pathological staging were significantly correlated with increased pvCTC counts	(57)
Duan <i>et al.</i>	33	I-III	4	–	Cell seeding with transfection reagent (oHSV1-hTERT-GFP)	Flow cytometry: negative selection with CD45 labeling and positive selection with GFP expression	–	CTCs were detected in 26/33 (79.0%) patients before surgery; in 33/33 (100%) patients after lobectomy; the post-CTC count was significantly higher in patients in whom the PV was interrupted prior to the PA	(58)
Dong <i>et al.</i>	103	I-IV	5	5 (PPA)	CanPatrol™ (size-based membrane filters)	Multiplex RNA-ISH (capture probe sequences for the PD-L1, EpCAM, CK8/18/19, vimentin, twist, and CD45 genes); DAPI staining	24	Pre-op PPA CTCs were found in 92/103 patients (89.3%); pvCTCs in 101/103 patients (98.1%); CTCs in PPA blood during the surgery were found in 97/103 (94.2%) patients; post-op PPA CTCs were detected in 79/103 (76.6%) patients	(59)
Chemi <i>et al.</i>	100	I-III	7.5	–	CellSearch® system (EpCAM-based enrichment)	IF (CK8/18/19, CD45, DAPI)	33	pvCTCs were detected in 48/100 patients, and were associated with lung-cancer-specific relapse	(60)
Tamminga <i>et al.</i>	31	I-III	7.5	7.5 (RA)	CellSearch® system (EpCAM-based enrichment)	IF (CK8/18/19, CD45, DAPI)	–	CTC were more often detected in PV (70%) than in RA (22%) blood samples, and in higher counts; only few pvCTCs were aneuploid	(61)

*, clinical or pathological stage. CK, cytokeratin; CTMs, circulating tumor microemboli; DAPI, 4',6-diamidino-2-phenylindole; DFS, disease-free survival; FISH, fluorescence in situ hybridization; FQ-RT-PCR, fluorescent quantitative real time polymerase chain reaction; H&E, hematoxylin and eosin; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; ITCs, isolated tumor cells; OS, overall survival; PA, pulmonary artery; periCTCs, CTCs from peripheral blood; PPA, peripheral artery; post-op, postoperative; pre-op, preoperative; RA, radial artery; PV, pulmonary vein; pvCTCs, CTCs from pulmonary vein blood; RNA-ISH, RNA-in situ hybridization.

which an adenocarcinoma developed subcutaneously in 1 out of 3 mice injected with pvCTCs and pvCTMs from 3 different patients.

We can conclude that other studies are needed to determine whether the cells detected in the PV blood of patients undergoing surgery for lung cancer are of tumor origin and whether they are related to a reduced PFS,

DFS, TDM and OS. If, as Tamminga *et al.* sustained, these were largely non-malignant cells, it would be better to direct research towards the use of methods that allow the recovery of a greater number of CTCs from peripheral blood. Among these, methods for example examining a greater volume of blood, as in the case of the diagnostic leukapheresis (DLA) or of the GILUPI device, are discussed

in the following section.

Alternative sample collection strategies for CTC recovery from early lung cancer patients

In lung cancer, as in several other cancers, the small number of CTCs per mL of peripheral blood is the major challenge. Although a simple solution may seem evident, it is becoming increasingly clear that it is necessary to increase the volumes of blood to be analyzed and/or treated. To date, two ways to overcome this obstacle are being studied: (I) *in vivo* CTC collection- and/or count-devices, and (II) apheresis-based procedures. The GILUPI CellCollector® (CC) allows the collection of CTCs *in vivo*, directly from blood. It is a functionalized medical wire covered with anti EpCAM antibodies which, when inserted in the cubital vein of a patient for 30 minutes, could trap CTCs from up to one liter and a half of blood. Through CC, studies reported an increased number of detected CTCs compared to standard methods (63-65). Recently, Didzdar and colleagues investigated the prognostic impact of CTCs rescued through CC in CRC (66), but contrary to other studies, they did not report any prognostic significance of CC-CTC. The authors estimated that recovering CTCs through CC is similar to extracting tumor cells from a blood volume ranging from 0.33–18 mL, a different volume compared to what was suggested in previous studies (volume range, 1–3 L). To date, CC use is feasible (67) and promising; no in-depth studies showing the real advantage of using this device in early lung cancer have been done.

To avoid the bottleneck due to the low number of CTCs, the CTCTrap consortium and Nick Stoecklein's group used and validated DLA to enrich a higher number of CTCs. The CTCTrap efforts demonstrated that the procedure allowed improvement in the number of CTCs isolated in metastatic breast and metastatic prostate cancer and could be safely performed in different clinical sites (68).

The DLA product analyzed with CellSearch® demonstrated a 30-fold increase in median CTC numbers detected (69) and an increase in the number of CTC positive patients in M0 breast cancer patients (70). This high number of enriched CTCs was exploited for single cell molecular characterizations (71) and for CTC culture (70). Despite DLA enrichment, the success of cultured CTCs is limited to a few patients, such as in 2 out of 8 patients when CTCs were cultured from fresh and cryopreserved DLA (70) and, like for other groups, culture success is generally reached in samples with more than 300 CTCs (72).

Statistically, it has been estimated that in order to find at least one CTC, total blood volume (TBV) must be analyzed, and that no less than 7.5 L of blood are necessary to detect at least 10 CTCs in almost every patient (18). Interestingly, before metastasis onset, the presence of about 9 ± 6 CTC/L of blood has been mathematically predicted even in M0 breast cancer patients (73,74). Based on this mathematical prediction, even in low-CTC-number patients, analyzing up to 72 L of blood would result in finding at least 200 to almost 1,000 CTCs or more.

To date, no studies have yet been published using this approach in lung cancer. Tamminga and colleagues recently presented a study where they analyzed CTCs in the DLA product of NSCLC patients before and after treatment. Before treatment, CTCs were more often detected in DLA (32/55, 58%) compared to blood (pre: 18/55, 33%, $P < 0.01$; post: 13/55, 24%, $P < 0.01$). After treatment, the number of patients with CTCs were similar in DLA and peripheral blood (DLA: 14/34, 41%; pre: 9/34, 26%, $P = 0.18$; post: 7/34, 21%, $P = 0.02$) (75).

We look forward to more studies using DLA in early-stage lung cancer, improvements in its early detection and a better stratification of high-risk patients.

High-risk patient monitoring

The diagnosis of many early-stage lung cancers is delayed, due to the fact that most patients are often asymptomatic. Lung cancer screening aims to reduce lung cancer-related mortality by diagnosing the disease at the earliest stage possible in high-risk cohorts (76).

There is an urgent clinical need to develop biomarkers which can distinguish benign from malignant lesions and to identify biomarkers which can supplement computed tomography (CT) when screening for lung cancer, in order to reduce both the number of false positives and false negatives (77).

Despite many published studies on lung cancer screening biomarkers, none has yet been established in clinical practice. Lung cancer is up to five times more likely to occur in smokers with airflow obstruction than in those with normal lung function, and both chronic obstructive pulmonary disease (COPD) and lung cancer are associated with smoking (76,77).

COPD and lung cancer represent the leading causes of lung disease-related mortality worldwide. Among smokers, COPD is an important risk factor which might lead to lung cancer, and predates lung cancer in up to 70–80% of

cases (78). The study by Mannino *et al.*, revealed a distinct linear dose-response relationship between increasing COPD severity and risk of lung cancer (79).

A large retrospective study of patients with lung cancer showed that the coexistence of COPD predicted a significantly worse survival outcome (80-82).

In 2014, Hofman's group compared the presence of CTC and CT-scan in COPD patients without clinically detectable lung cancer, with the aim to identify a biomarker for early lung cancer diagnosis (83). The study enrolled 168 COPD patients and 35 healthy non-smoker donors. Blood was collected at baseline, after 1 and 5 years. Using the ISET[®] method, they detected CTCs in 3% of COPD patients, where the presence of CTCs was correlated with the severity of COPD. At baseline, the CTC-positive COPD patients developed lung cancer; the CT scan performed at the same time point of the CTC analysis confirmed the COPD diagnosis but failed to highlight lung nodules. The CT-scan program revealed the presence of lung nodules with a time delay of about 3.2 years. In these CTC-positive COPD patients, the early cancer diagnosis (IA lung cancer) allowed surgical resection of lung cancer and, in 16 months, the authors documented the absence of both CTCs and tumor recurrence. Recently, by exploiting the Carcinoma Cell Enrichment and Detection Kit (Miltenyi technology), a small study (only 17 COPD patients enrolled), demonstrated the presence of CK+ cells and CD44v6+ markers in the blood; both these markers are present in normal lung tissue but also in lung cancer (84). In COPD patients, due to the chronic inflammation and the relationship between vein and alveolar epithelium, it could be conceivable that lung cells could pass in blood. In our opinion, further study will clarify the origin of these CK+ CD44v6+ cells in the blood and their role as a biomarker (85).

Depending on which study we look at, about 25–50% of early-stage NSCLC patients relapse within 5 years after radical resection; for this reason, such patients are considered high-risk patients. To date, identifying relapsing high-risk patients is a goal we urgently need to meet. Because of this, several studies have analyzed CTCs: (I) their presence in venous or in arterial blood, (II) whether surgical manipulation could increase cancer cell dissemination, and (III) whether CTCs can be used as a prognostic biomarker in the early stages. In 2003, Sielens and colleagues analyzed a cohort of 62 patients with a complete resection of primary NSCLC and without signs of distant metastasis. They obtained PV blood collected at thoracotomy or prior to lung resection. CTC positive patients (18%) showed a reduced survival rate (47). In 2016, Crosbie *et al.* confirmed

that, in the PV, CTCs are more frequent and associated with recurrence after surgical resection (55).

Few papers have analyzed CTCs before and after surgery and unfortunately, all these studies have small cohorts ranging from 30–60 patients. In all these studies, the authors documented a high number of CTCs before surgery compared to after surgery. The patients with persistent CTCs after surgery have a shorter DSF and a higher risk of recurrence.

Tamminga *et al.* recently analyzed in 31 patients the presence of CTCs in blood collected from both the radial artery (RA) and the PV. CTCs were found in 70% of PV compared to 22% of RA samples, regardless of the type of surgery patients underwent. They also documented CTC-clusters in PV samples only. CTCs isolated from 11 patients after scWGS analysis showed that copy number aberrations were present in few CTCs. The authors postulated that normal epithelial cells could be filtered in the central blood compartment from the PV (61).

All these studies highlighted how CTCs could be used as biomarkers in patients with a high-risk of lung cancer or with a high-risk of tumor recurrence in patients who underwent curative surgery.

CTCs to implement screening of NSCLC

The screening of high-risk patients represents the main course to increase the number of patients diagnosed at early stages of malignancies, significantly improving prognosis, as demonstrated by the long experience of breast, cervical and colorectal cancer. The approach to lung cancer does not vary (86), and according to the National Lung Screening Trial (NLST) (87), annual screening for lung cancer has been recommended for adults ages 55 to 80 years who have a 30 pack year smoking history and currently smoke or have quit within the past 15 years (88).

NLST demonstrated that low-dose helical computed tomography (LDCT) screening for lung cancer significantly reduced mortality rates—more than 20% *vs.* chest radiography—among high-risk subjects (88), even if uncertainty still exists regarding the harms of LDCT screening and the feasibility to extend this data to the general population (89,90).

However, a consensus exists that a more refined risk assessment would allow identification of high-risk individuals more likely to benefit from screening and determination of whether a screening detected nodule is or is not malignant.

To this purpose, some authors analyzed the feasibility to

combine LDCT screening with CTC analysis.

In one study, He and colleagues randomly selected 32 cases out of the 88 ground-glass nodules (GGNs) patients, who were identified among 8,313 volunteers screened by LDCT (91). These 32 selected GGNs underwent a CellCollector procedure, and 6 EpCAM/CK-positive CTCs were identified from 5 patients. Notably, NGS data showed that three cancer-related genes contained mutations in 5 CTC-positive patients, including *KIT*, *SMARCB1* and *TP53* genes. Moreover, in 4 patients, 16 mutation genes existed. Therefore, the authors concluded that LDCT combined with CTC analysis in high-risk pulmonary nodule patients was a promising way to screen early-stage lung cancer.

More recently, Manjunath and colleagues (92) compared CTCs and CTC clusters in 29 treatment-naïve NSCLC patients, 31 high-risk LDCT screened patients—with or without lung nodules (lung-RADS 2 and 1, respectively)—and 20 never-smoking healthy controls. The 3 groups differed according to these parameters, since both single CTCs (ranged, 11–40) and CTC clusters were detectable in NSCLC, whilst significantly lower number of single CTCs (ranged, 0–8) and no CTC cluster were reported in LDCT screened patients; as expected the healthy controls resulted negative for both parameters. The findings of this pilot study are promising, even if further confirmation in prospective trials, *ad hoc* designed, is warranted.

Conclusions

The analysis of CTCs represents a useful tool to monitor the evolution of malignancies, especially when tumor biopsy is uncomfortable for the patient or totally impracticable, as it often is in lung cancer.

Moreover, much evidence has been already reported that the CTC analysis has the potential to anticipate the diagnosis of progressing disease in pulmonary high-risk patients or to discriminate the malignant nature of a screened pulmonary nodule. However, more studies are needed to definitively prove the value of CTCs in the early detection of lung cancer, alone or in combination with screening plans, in order to reduce the risk of over-diagnosis and over-treatment, usually determined by the LDCT screening (93).

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