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François-Clément Bidard, François-Régis Ferrand, Florence Huguet, Pascal Hammel, Christophe Louvet, David Malka, Valérie Boige, Michel Ducreux, Thierry Andre, Aimery de Gramont, et al.

► **To cite this version:**

François-Clément Bidard, François-Régis Ferrand, Florence Huguet, Pascal Hammel, Christophe Louvet, et al.. Disseminated and circulating tumor cells in gastrointestinal oncology.. *Critical Reviews in Oncology/Hematology*, 2012, 82 (2), pp.103-15. 10.1016/j.critrevonc.2011.05.008 . hal-00711407

**HAL Id: hal-00711407**

**<https://hal.science/hal-00711407>**

Submitted on 24 Jun 2012

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## **Disseminated and circulating tumor cells in gastrointestinal oncology**

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**Keywords:** gastrointestinal cancer, circulating tumor cells, disseminated tumor cells, micrometastasis, biomarker

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**Short biography of the corresponding author:**

Dr FC Bidard is 31 years old and is currently Assistant-Professor in the Department of Medical Oncology at the Institut Curie, Paris, France. His PhD thesis was about cooperation between heterogeneous cancer cells during the metastatic process in mice models, and his MD thesis concerned circulating tumor cells in breast-cancer patients. He has published more than 20 articles, most of them about disseminated and circulating tumor cells.

**Abstract**

Circulating (CTC) and disseminated tumor cells (DTC) are two different steps in the metastatic process. Several recent techniques have allowed detection of these cells in patients, and have generated many results using different isolation techniques in small cohorts. Herein, we review the detection results and their clinical consequence in esophageal, gastric, pancreatic, colorectal, and liver carcinomas, and discuss their possible applications as new biomarkers.

## **1. Introduction**

Onset of metastasis is a complex yet poorly understood process, which is responsible for most of cancer-related deaths. For several years, cellular dissemination from primary to secondary sites has been a “black-box” of research for clinicians. In non-metastatic (M0) patients, clinical studies have isolated strong prognostic factors associated with the risk of metastatic relapse, and these factors are being used to make decisions on adjuvant treatments. Adjuvant chemotherapy targets cancer cells that may have disseminated throughout the body, but is currently given blinded to the real dissemination status of each patient. With continuous technical improvements, detection methods have been recently set up and validated to isolate cancer cells in the blood (circulating tumor cells (CTC)) and in the bone marrow (disseminated tumor cells (DTC)). The opening of these two windows on the metastatic process in patients has raised three main critical issues: Are detection methods accurate? Is quantitative analysis (i.e., counts) of CTCs/DTCs of clinical relevance? Could qualitative analysis (i.e., molecular characterization) of CTCs/DTCs uncover new cancer features/targets? This review focuses on results obtained by different CTC/DTC detection methods in gastro-intestinal cancers.

## **2. Technical aspects**

### **2.1 CTC and DTC detection sites**

Metastasis is a complex multistep process, which emerged as a biological and clinical research field more than a century ago. In one of the first studies to investigate the metastatic spread of breast cancer, James Paget made the well-known “seed and soil” hypothesis to explain the discrepancy between the blood supply of the different organs and the distribution of breast-cancer metastases[1]. Since this seminal report, several steps in the hematogeneous metastatic process have been described: local invasion, intravasation into blood vessels, interaction with blood-formed elements, arrest on the vessel’s endothelium, extravasation, invasion of the host-organ microenvironment, cellular dormancy, and establishment of a new growth.

Basically, CTC corresponds to the circulation step of cancer cells, after intravasation, whereas DTC corresponds to the post-extravasation steps. Noteworthy, CTCs are generally detected in the peripheral blood, which is supposed to have a homogeneous cancer-cell concentration, but many gastrointestinal (GI) studies have also looked for CTC in the efferent vein of primary GI tumors, i.e., the portal vein. As looking for DTCs in solid organs is technically difficult, DTCs are almost exclusively detected in bone marrow (by a sternum or iliac-crest puncture). These different detection sites have to be kept in mind, especially in GI cancers, in which the liver may act as a physical blood-filter for CTCs released by the primary tumor. Finally, cancer cells are not definitely set into one of these “compartments”: cellular trafficking between the primary tumor site, the blood, the bone-marrow, other target-organs, and metastases are very likely, as has been reported in a few pre-clinical [2] or clinical [3] models.

### **2.2 CTC and DTC detection methods**

In a few solid tumors that exhibit specific gene-fusion transcripts (e.g., EWS/FLI1 in Ewing’s sarcoma), DTC/CTC detection has already been clinically implemented as a strong prognostic marker for use in everyday clinics, either at diagnosis or after the primary treatment, and is often called a “minimal residual disease”. However, since the first CTC description in the late 19<sup>th</sup> century [4], as most carcinomas do not have specific transcripts, DTC and CTC have been detected using different techniques, leading to successive results that can appear heterogeneous [5,6]. These techniques generally consist of isolating cells with epithelial markers out of mesenchymal-derived compartments (blood, bone marrow) [7]; two steps are generally used: primary enrichment, followed by DTC/CTC detection.

Almost every isolation technique relies on an initial CTC/DTC-enrichment step, generally based on positive immunoselection of cells expressing an epithelial membranous marker, such as epithelial-cell adhesion (EpCAM, MUC1...). Other enrichment techniques have been also reported: size-based differential filtering (ISET), CD45-based leukocyte-negative immunoselection... None of these techniques have a 100% yield for CTC/DTC purification, and

the use of multiple-antigen selection (by combining different antibodies) has not yet demonstrated clear superiority over single-antigen selection. Moreover, high discrepancy rates can be observed using different antibodies against the same membranous marker, as is shown in colorectal cancer [8]. This preliminary step, which could be followed by either molecular or cytological detection techniques, is highly heterogeneous among techniques, and is critical to understanding discrepancies between published studies in the same setting.

Molecular detection of CTC/DTC relies on the detection of mRNA (by RT-(q)PCR, mRNA hybridization onto cDNA membrane...), which is related either to epithelial function or to specific cancer hallmarks, such as telomerase activity. Target mRNA (CEA, MUC1, cytokeratins...), and primers used for the amplification step are heterogeneous among studies, which are generally monocentric. For example, in the recent meta-analysis by Rahbari et al [6], on CTC/DTC detection in colorectal cancer, more than 20 different targets were assessed, alone or in combination, in 31 molecular studies conducted in more than 25 different single centers. However, the specificity of these molecular techniques is questioned, as nonspecific expression of epithelial markers has been reported in lymphoid cells activated by cancer-related systemic inflammation [9].

Cytological detection of CTC/DTC, after the initial enrichment step, relies on immunostaining of CTC/DTC, using epithelial antigens thought to be expressed by carcinoma cells (e.g., epithelial cytokeratins). Multiple staining can be performed on isolated cells, generally by immunocytofluorescence. The standardized CellSearch® system [10], which has become the most commonly used CTC detection system over the past few years, enriches the sample (7.5 ml of blood) cells that express the EpCAM molecule, with antibody-coated magnetic beads, and labels the cells with a fluorescent nucleic acid dye (DAPI). Fluorescently labeled monoclonal antibodies specific for leukocytes (CD45-allophycocyan) and epithelial cells (cytokeratin 8, 18, 19-phycoerythrin) are used to distinguish epithelial cells from leukocytes. Beyond epithelial-staining, cytological detection methods allow the optical control of stained cells [11], in order to distinguish cancer cells from activated lymphoid cells. This final validation step of cell morphology is time-consuming, hardly automatable, and relies on expert cytologists and/or technicians who should use a standardized classification consensus [12] whenever available. Our review is mainly focused on the clinical applications of cytology-based CTC/DTC-detection techniques, which are more homogeneous and have, at least for the CellSearch® system, the advantage of demonstrated reproducibility across centers and studies [13,14].

### **3 Esophageal cancers**

#### **3.1 DTCs**

Although bone and bone marrow are not preferential sites for macrometastasis of esophageal cancer, DTCs have been primarily found in 37% of 90 non-metastatic patients after an iliac-crest puncture using a cytological technique based on cytokeratin detection [15]. DTCs were associated, in this study, with poor survival in patients with completely excised tumors. Interestingly, bone-marrow DTCs were more frequently detected (79%) when extracted from a rib contiguous to the tumor than from the iliac crest (8%), but rib-derived DTCs had no clear prognostic significance [16,17]. Biologically, whole-genome analysis of single DTCs isolated by micropipetting in 107 patients showed that most genetic aberrations were different in primary tumors and in bone marrow DTCs [18], supporting the parallel-evolution model, proposed for metastatic processes, by CA Klein [19]. In this study, gain of the *HER2* gene region on 17q was the most common genomic alteration shared between DTCs, and was associated with shorter overall survival.

#### **3.2 CTCs**

CTC detection in esophageal cancers has been mainly reported by molecular techniques in patients undergoing surgery with a curative intent. These studies have focused mainly on CEA mRNA expression [20-24], but also on CK19, CK20 [25,26], SCC [27], deltaNp63 [28], and survivin [29,30]. Detection rates are heterogeneous among studies, even when the same mRNA has been quantified in the same clinical setting, e.g., 77% [29] vs. 47% [30] for survivin, 57% [21] vs. 28% [22] for CEA. Almost all of these exploratory studies show a negative prognostic impact of CTC detection on survival, although these reports are on small heterogeneous cohorts. Reports based on CTC cytological detection are even more limited; a negative prognostic impact and a correlation with pleural dissemination were found in 5 patients out of 23 metastatic esophageal-cancer patients who had  $\geq 2$  CTCs/7.5 ml of blood, as assessed with the CellSearch® system [31,32].

## **4. Gastric adenocarcinoma**

### **4.1 DTCs**

Bone marrow DTCs have been reported by several detection techniques in surgically resected gastric cancers: CK18 [33]; CK2-immunostaining [34]; anti-human epithelial antigen (Ber-EP4) [35]; CK20 RT-PCR [36], CK20/CK19/CEA RT-PCR [37], CK7/CK8 RT-PCR [38], CEA/CK20/TFF1/MUC2 RT-PCR [39]. About half of these studies reported a significant negative prognostic impact on metastases-free and/or overall survival. DTC detection has been associated with increased tumor-microvessel density [40,41]; its association with VEGFR expression by the primary tumor appears controversial [42]. As not every single DTC will later grow into a macrometastase, a German group developed further investigations into DTCs isolated by a cytological technique. They showed that immunostaining of proteins involved in the urokinase plasminogen-activator (uPA) system may distinguish those that have a fully metastatic phenotype and an independent clinical impact [43-45]. Recently, they also reported that the extracellular matrix-metalloprotease inducer (EMMPRIN) may also play a role in DTCs' evolution to later macrometastases [46].

### **4.2 CTCs**

CTCs in gastric cancer have been studied in several small studies, reported in Table 1. No clear conclusion can be drawn from these heterogeneous studies, which are often underpowered. Among the several molecular markers tested (CK18, 19, 20, CEA, hTERT (human telomerase reverse transcriptase)), none has demonstrated a clear and confirmed superiority over the others [47-51]. CTC-detection rate was related to the surgical maneuvers during the surgical removal of excisable cancers, and was initially correlated with poor survival [52]. This CTC release was confirmed in another study ( $n=59$ ), though CTC-positivity after surgery was surprisingly associated with an improved prognosis [53]. A few reports have also focused on the patient's blood stored for autologous transfusion during surgery, by testing CTCs using various techniques after removal from blood by freezing, filtering, and irradiation [54,55]. Decreases in CTC have been also reported in about half of patients receiving neoadjuvant chemotherapy [56]. Biological, preclinical experiments suggest a strong role for CTC arrest in premetastatic niches of target organs, characterized by VEGFR1-positive bone-marrow-derived non-tumoral cells [57]. To date, gastric adenocarcinoma is the only cancer in which the impact of VEGFR1-positive non-tumoral cells has been demonstrated in the presence of CTC, as was reported in 810 Japanese patients [58].

Using the CellSearch® system, CTC detection rates were 14% and 55% in 14 non-metastatic and 27 metastatic patients, respectively ( $\geq 2$  CTCs/7.5ml) [32]. Recently a Japanese group has reported an association between CTC detection and chemotherapy efficacy in 52 metastatic gastric-cancer patients treated with different chemotherapy regimens in advanced



metastatic disease [59]. As for other cancer types, high CTC count under treatment (here,  $\geq 4$  CTCs/7.5 ml at 2–4 weeks after the start of the treatment) was associated with a poorer outcome (progression-free survival (PFS): 1.4 vs. 4.9 months; overall survival (OS): 3.5 . 11.7 months).

## 5. Pancreatic cancer

In the metastatic setting, the CTC detection rate, using the CellSearch® system, has been recently investigated in four small cohorts ( $n=16$  [10];  $n=23$  [60];  $n=14$  [61];  $n=40$  [62]): CTCs were detected in ~50% of patients ( $\geq 1$  CTC/7.5 ml), but the mean count appears lower than in colorectal cancers. Although underpowered, three of these studies addressed the prognostic significance of CTC detection and reported contradictory results (two being rather positive, one rather negative). Using a microfluidic device for CTC sorting (immunoselection and staining being similar to CellSearch®), a group at Massachusetts General Hospital recently reported 100% CTC detection in 15 metastatic pancreatic-cancer patients [63]. However, this detection rate may be overestimated, similar to their prostate cancer results [64]. In 2001, a study from the same hospital reported 105 patients with stage I–IV pancreatic cancer with 26% CTC and 28% DTC detection rates (AE1/AE3 immunostaining)[65]. Both CTC and DTC detection rates were correlated with disease stage and were not prognostic in multivariate analysis. Similarly, an older cytological study (using a cocktail of six antibodies) [66] and two molecular-technique-based studies (CK20 and CEA) [67,68] retrieved an association between DTC and/or CTC detection and disease stage; multivariate analyses of survival were either not reported or were inconclusive

In locally advanced pancreatic cancers, which are unresectable but are still not metastatic, CTC/DTC may indicate how far the dissemination process is advanced, and could guide the physician's choice towards a local (e.g., radiotherapy) or systemic (chemotherapy) treatment. No significant data have been published yet in this setting, but a French companion study of the LAP07 trial (NCT00634725) is currently ongoing, using the CellSearch® system.

In early pancreatic cancer, detection rates are likely to be low with the current cytological techniques. Bone-marrow DTC detection by multiple antibodies has been, however, associated with a shorter time to tumor relapse in 15 patients who underwent complete surgical resection [69]. For CTC detection, a single case report has been published using the CellSearch® system for diagnostic purposes in a patient presenting with a pancreatic mass [70], which found 4 CTCs/7.5 ml, whereas the mass turned out to be a pancreatic carcinoma. However, in this diagnostic setting, both sensitivity and specificity of this technique are too low to fulfill the requirements of a diagnostic test. Molecular techniques have been also tested in this setting [71-73], but their specificity have been discussed when they are used as a single marker, especially for CK20 [74].

Finally, pancreatic and ileal neuroendocrine tumors (NET) strongly express EpCAM, and CTC detection by the CellSearch® system was only reported in one study reported to date: 43% of patients with metastatic ileal NET ( $n=26$ ) and 21% of patients with metastatic pancreatic NET ( $n=16$ ) [75]. CTC detection was associated with tumor progression in this study.

## **6. Colorectal adenocarcinoma: the adjuvant setting**

### **6.1 DTCs**

The first large study to show an association between DTC detection and worse prognosis in non-metastatic colorectal cancer patients ( $n=88$ ) was reported almost 20 years ago [76]. Adjuvant trials that tested the anti-EpCAM monoclonal antibody, edrecolomab, were launched on the bases that most bone-marrow DTCs express EpCAM [77] and that tumor response or stabilization has been observed in metastatic patients in Phase-2 studies [78]. However, adjuvant Phase-3 trials with edrecolomab have shown no clinical benefit [79,80]. Other reports on DTC detection were included in a large meta-analysis published recently (see after 6.2) [6]. Liver DTCs were studied in liver biopsies using molecular techniques, but had no clear clinical impact on resected colorectal cancers [81].

### **6.2 CTCs**

Many studies have been conducted, by different surgical teams, at the time of resecting primary colon tumors. Many have looked for CTCs during surgery in the portal flow, using molecular or cytological [82] techniques, in order to measure circulating tumor load. The focus on portal flow is based on the hypothesis that the portal vein represents a “post-intravasation highway” for CTCs from the primary tumor to the liver, which is the main target organ for the metastatic process in colon cancer. Some studies have also focused on the potential release of CTCs into the portal vein during different surgical procedures using various vascular clamps or “no-touch” techniques [83-85]. Also, in patients diagnosed with an occlusive colorectal cancer, a small study ( $n=58$ ) suggested that endoscopic insertion of a colonic stent resulted in increased levels of CK20 mRNA, compared to staging colonoscopy [86]. Clearance of CTC after surgery has been reported to be a quick event in most patients, possibly associated with the prognosis [87]. A decrease in CTC was also reported after neoadjuvant chemoradiation for rectal cancers [88]. Some other studies have focused on the potential filter role of the liver on CTC dissemination, and compared CTCs in synchronous samples obtained from the portal vein, sus-hepatic veins, and/or the peripheral blood [89].

Globally, cytological techniques, including the CellSearch® technique, have reported very low detection rates in stage II and III colon cancers [90]. With molecular techniques, the different published results were reported as heterogeneous, especially for CTC prognostic impact [91], and no clear conclusion could be drawn. Therefore, a first meta-analysis was published in 2008 based of nine studies that used molecular detection (646 patients) [92]. Two interesting conclusions were reported: (i) CTC detection in the portal flow is correlated with nodal invasion (21% of pN0 patients vs. 50% of pN+ patients); and (ii) CTC status at the time of surgery is correlated with disease-free survival and to further liver metastatic relapse, independently of

tumor stage (I, II, or III). One of the largest studies in stage II patients ( $n=194$ ) was published almost synchronously, and reported detection of CTCs in peripheral blood using four mRNAs (CEA, hTERT, CK19, CK20) [93]. In this pilot study, the combination of tumor-invasion depth, vascular invasion, and mRNA markers as predictors of relapse, showed that patients with any one positive predictor had a hazard ratio, of about 27-fold, of developing a postoperative relapse ( $p<0.001$ ). This result has not been confirmed, but may be of critical importance when selecting stage II cancer patients who may benefit from adjuvant chemotherapy.

A larger meta-analysis has been published recently, which included 36 studies that reported on the detection of DTCs in bone-marrow, on CTCs in the portal vein, and systemic circulation of CTCs, using several molecular and cytological techniques in 3094 patients with non-metastatic colorectal cancers [6]. When pooled together, cancer-cell detection studies were significantly associated with shorter recurrence-free intervals and overall survival rates. When they looked separately at different sampling sites, only peripheral blood CTCs had a prognostic value, whereas bone-marrow DTCs or portal-vein CTCs were not significantly associated with overall survival.

## 7. Colorectal adenocarcinoma: the metastatic setting

### 7.1 DTCs

Bone-marrow DTC detection in the metastatic setting has been reported only in small studies [94,95], and has no clear prognostic significance. This is consistent with similar findings for metastatic breast cancer [96].

### 7.2 CTCs

CTCs have been also detected by cytological or molecular techniques in this setting. Several small studies have been published on molecular techniques, using the same markers as already discussed, alone or in combination with CEA, CK 8/18/19/20, hTERT, MUC1/2... Interestingly, detection of K-RAS mutations has been also investigated [97-99], as have p53 mutations [97,99,100]. Here, again, no molecular marker demonstrated superiority in these small studies.

In metastatic colorectal cancers, surgical teams have focused on patients with resectable liver metastases. In this specific setting, detection of CTCs may reflect an active hematogeneous dissemination process and, therefore, may help to distinguish patients who can experience a short progression-free interval after surgery from those who can have longer complete remission [67,101]. Similar concerns for gastric cancers have been raised regarding autologous blood-transfusion safety during hepatic surgery [54]. CTC release during radiofrequency ablation of liver metastases was also reported to be higher than during surgical resection [102]. A randomized clinical trial has been proposed to compare two surgical procedures for CTC detection during surgery as the primary endpoint [103].

For chemotherapy management, some reports with limited patient numbers, have suggested that variations in CTC counts may be associated with treatment response [104]. For this application, the cytological CellSearch® technique has achieved very strong evidence, with several studies published on metastatic colorectal cancers (Table 2). Based on the study reported by Cohen *et al.* [105], the CellSearch® system gained, in 2007, FDA approval as an aid to monitor metastatic colorectal-cancer patients. This study reported the strong and independent prognostic impact of CTC-positivity ( $\geq 3\text{CTC}/7.5\text{ml}$ ) at baseline, but also of early CTC changes after 3–5 weeks of treatment (chemotherapy with or without bevacizumab). In a large ancillary study to the CAIRO2 trial (chemotherapy + bevacizumab, with or without cetuximab), Tol *et al* [106] reported similar findings at baseline, but a lower CTC-positivity rate after 3–5 weeks of treatment (Table 2).

The correlation with outcome must, therefore, have been inconclusive, and the CTC count after 1–3 weeks of treatment was analyzed instead of its correlation with treatment outcome.

The discrepancy between these studies may be due to the systematic use of bevacizumab in the second trial, which has been reported in breast cancer to lower the CTC-detection rate [107]. Without further research, it is unclear when to perform a CTC count after the start of treatment (i.e., weeks 1–2 vs. weeks 3–5) to detect the best correlation with outcome.

Moreover, these observational studies have focused on patients with good health and have not demonstrated that “managing treatment” according to CTC-count leads to a better outcome. Interventional randomized trials are therefore needed to compare the standard clinical/radiological vs. quantitative CTC-guided management of metastatic colorectal-cancer patients. Moreover, CTC positivity under treatment corresponds to cancers that are spontaneously resistant to a first-line regimen, and there is no evidence to support the idea that introducing a second-line regimen, according to the CTC count under treatment or to radiological evaluation, which occurs 1–2 months later, will help improve the survival of these refractory patients.

Therefore, it is likely that the main management of chemotherapy according to quantitative CTC changes will be the earlier discontinuation of expensive, harmful, and worthless treatments for some patients, and a gain in the cost/effectiveness ratio, rather than an improvement in survival. CTC may be also of interest to patients with potentially resectable liver metastases, i.e., metastases that may be surgically removed if metastasis shrinkage is obtained. These patients usually received intensive polychemotherapy combined with a targeted therapy, and CTC might help to select those patients who will most benefit from this treatment strategy. As an example, Figure 1 shows the design of the CTC companion-study in a recently started large multicenter trial.

## 8. Hepatocarcinoma (HCC)

Non-metastatic hepatocarcinomas are treated whenever possible by local treatments, which may include liver resection, liver allograft, chemoembolization, and alcoholization. Beyond the issue of a possible surgery-induced CTC release [108], any metastasis-associated biomarker could be clinically relevant, as most patients relapse after treatment of the primary tumor. Following initial studies, which have reported that AFP mRNA detection in blood was associated with disease stage [109,110], several CTC molecular-detection studies have been published (**Table 3**): heterogeneous clinical results were reported, whereas AFP mRNA specificity was discussed [111,112]. CTC detection by the same marker has been also reported in small studies [113]. Albumin, human telomerase (hTERT), and melanoma antigen gene-1 (MAGE1) mRNAs detection were also investigated, in blood, in a few small studies [114-117].

Cytological detection methods have also been used in HCC. The ISET technique, which isolates CTC by size on a filter, has been primarily developed in HCC [118]. In 44 non-metastatic HCC patients, CTC-detection rate was about 50% and was associated with shorter survival [119]. Similar detection rates (45%) were reported more recently with the CellSearch® system in 20 patients ( $\geq 1$  CTCs/7.5ml), but these patients either had metastatic or locally advanced HCC [120]. Finally, circulating CD45<sup>-</sup>/CD90<sup>+</sup> cells, described as HCC “stem cells”, have been found by flow-cytometry in 31 out of 34 (90%) non-metastatic HCC patients, but the correlation with clinical outcome was not reported [121].

## 9. Conclusion

Globally, molecular tools used for CTC and DTC detection, based on their epithelial phenotype, are very heterogeneous, and it is hazardous to assess their importance based on them being a small series. Most of the reports in the literature had positive results (i.e., association with outcome), but publication bias may seriously distort any attempts, including those of the published meta-analyses, to estimate the effect of CTC/DTC detection. One may hypothesize that molecular techniques were mostly developed by isolated academic teams, without any strong effort to standardize these techniques. Cytological-detection studies were initially academy-driven, and gave interesting insights into the dissemination process and the genetic evolution from the primary tumor, to DTCs, to overt macrometastasis. This technical heterogeneity among these studies has paved the way for the development of the semi-automated and standardized CellSearch® system, with its development also benefiting large industry-sponsored clinical studies. This system became the 2011 gold standard for CTC detection, and is currently used in exploratory studies for several GI cancer types. The FDA-cleared quantitative approach to manage the chemotherapy of metastatic colorectal cancers is, however, not fully validated, and its clinical interest remains unknown. As recalled in a recent methodological review [122], the validation of CTC changes, as a recognized surrogate end-point for PFS will require data that demonstrate that CTC-changes are prognostic of PFS, but also that the effect of treatment on CTC-changes correlate with that of PFS; this will require the accumulation of data into a large meta-analysis.

The future of DTC and CTC in gastrointestinal cancers could be divided into two main issues. First, the quantitative count obtained by cytological techniques, such as CellSearch®, will be compared to several promising new blood-derived biomarkers, such as circulating tumor DNA or circulating miRNA. However, the future of CTCs as a quantitative biomarker appears somehow compromised by the detection of cancer-specific mutations (e.g., K-RAS mutations) in the blood. The second issue is that CTC may represent, in the near future, a kind of “liquid biopsy”, and provide new insights into tumor biology. The qualitative analysis of CTCs may guide treatment choice at diagnosis, but may also be repeated under treatment to elucidate resistance pathways, without an invasive tumor biopsy. CTCs may become a useful marker to assess many tumor targets by multiple immunostaining, FISH, and/or single-cell RNA/DNA analysis.

**Conflict of interest statement:** none

**Funding source:** Institut Curie incentive and collaborative programs fund (“PIC CTC”). The funding source had no role in the collection, analyses, and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.



**Table 1: CTC detection by molecular techniques in gastric adenocarcinoma**

Studies with  $\leq 20$  patients or without an outcome analysis were not included. Impact on survival was not systematically assessed in multivariate analysis. N/A: not available or not relevant (because M0 and M1 patients were pooled). NS: not significant. DMFS: distant metastasis-free survival; DFS: disease-free survival; OS: overall survival.

Reference	Patients	Amplified mRNA	CTC detection rate	Clinical significance
Soeth, 1997 [123]	N= 30 Stages I–IV	CK20	17%	N/A
Yeh,1998 [48]	N=34 Stage IV	CK19	21%	OS : NS
Noh, 1999 [20]	N=35 Stages I–IV	CEA	M+ : 100% All M- cancer : 33% Resectable cancer : 18%	N/A
Nishida[124]	N=36 Resectable cancer	CEA	22%	N/A
Majima, 2000 [125]	N=52 Stages I–IV	CK19 and CK20	10%	N/A
Miyazono, [52]	N=57 Resectable cancer	CEA - In portal vein, superior vena cava, and the peripheral artery - Before and after surgical removal	37%	- DMFS - Detection rate were similar at the different blood-puncture sites - Surgical maneuvers may cause CTC release.
Obayashi, 2001 [54]	N=25 Resectable liver metastasis (include also colorectal cancer patients)	CEA - Made on patient blood drawn for autologous transfusion during liver surgery	20%	CEA mRNA is no longer detectable after being frozen for 7 days
Sumikura, 2003 [126]	N=106 Resectable cancer	CEA	40%	DFS (non-hematogeneous metastases were included)

Seo 2005 [127]	N=46 Resectable cancer	CEA - at the time of surgery and then every 2 months	48%	Changes after surgery and during follow-up are correlated with DFS
Illert 2005 [128]	N=70 Stage I-III	CK20	40%	OS
Uen, 2006 [129]	N=52 Stage I-IV	MUC1 c-Met	71% 62%	N/A
Wu, 2006 [130]	N=64 Stage I-IV	CK19 CEA MUC1 hTERT	4 markers combined : Sensitivity: 89% Specificity: 91%	N/A
Koga, 2008 [131]	N=101 Stage I-IV	CK19 CK20	In operable patients 12% 15%	OS
Yie, 2008 [132]	N=55	Survivin	44%	DFS
Saad 2010 [133]	N=30 resectable	CK18		- DFS - association with E-cadherin expression in the primary tumor

**Table 2: CTC detection in colorectal cancer using the CellSearch® system**

Reference	Patients	CTC detection rate (threshold)	Clinical significance
Allard, 2004 [10]	N=196 Stage IV	Stage IV: 30% Threshold $\geq 2\text{CTC}/7.5\text{ml}$	N/A
Cohen, 2006 [134]	N=50 Stage IV	Stage IV: 38% Threshold $\geq 2\text{CTC}/7.5\text{ml}$	- CTC positivity was associated with disease status (progression vs. non-progression).
Sastre, 2008 [135] Maestro 2009 [136]	N= 94 Stage I–IV	Stage II : 21% Stage III: 24% Stage IV: 61% Threshold $\geq 2\text{CTC}/7.5\text{ml}$	
Cohen 2008 [105] Cohen 2009 [137]	N=430 Stage IV	Stage IV : 33% before treatment 12% at week 3-5 Threshold $\geq 3\text{CTC}/7.5\text{ml}$	- CTC positivity at baseline was independently associated with worse PFS (5 vs. 8 months) and OS (9 vs. 18 months). - Among initially CTC positive patients, those who become CTC negative at weeks 3–5 had a better PFS (6 vs. 2 months).
Hiraiwa 2008 [32]	N=40	Stage I-III: 18% Stage IV: 41% Threshold $\geq 2\text{CTC}/7.5\text{ml}$	- CTC positivity was not associated with outcome in the 29 stage-IV patients
Jiao 2009 [102]	N=29, with surgically resected liver metastases	N/A 7.5ml of blood analyzed at different sites (peripheral, portal and hepatic veins)	- Surgical resection immediately reduced the number of CTCs compared with radiofrequency ablation, which was associated with an increased number of CTCs - Liver and lungs act as filters for CTCs
Tol 2010 [106]	N=467	Stage IV: 29% before treatment 5% at week 3-5 Threshold $\geq 3\text{CTC}/7.5\text{ml}$	- CTC positivity at baseline was associated with worse PFS (8 vs. 10.5 months) and OS (14 vs. 22 months). - Among initially positive patients, those who became CTC negative at weeks 1–2 had a better PFS (8 vs. 4 months)
Papavasiliou 2010 [138]	N=20, with surgically resected liver metastases	Preoperative :10% Peroperative: 50% Postoperative:5% Threshold $\geq 3\text{CTC}/30\text{ml}$	Postoperative CTC detection was associated with DMFS and OS.

**Table 3: Molecular detection of AFP mRNA in hepatocarcinoma (HCC)**

Studies with ≤20 patients or without an outcome analysis were not included. Impact on survival was not systematically assessed in multivariate analysis.

Periph. Cath: peripheral catheter; Centr. Cath: central catheter

N/A: not available

DMFS: distant metastasis-free survival; DFS: disease-free survival; OS: overall survival.

Reference	Number of HCC patients	Blood-puncture location and timing	CTC detection rate	Clinical significance
Louha M, 1997 [139]	84	Periph. Cath. Before <b>alcoholization??</b> or embolization therapy	33%	DMFS
Matsumura M, 1999 [140]	88	Periph. Cath. Before <b>alcoholization</b> or embolization therapy	62%	DMFS and OS
Wong IH, 1999 [115] + 2000 [141]	62	Periph. Cath. Before, during, and after surgery	25% of samples	DMFS
Kienle P, 2000 [113]	24	Centr. Cath. Before, during, and after surgery	29–45% of patients (time-dependent)	No significance
Miyamoto A, 2001 [142]	23	N/A Before, during, and after surgery	25% of samples	DFS
Witzigmann H, 2002 [143]	85	Periph Cath Before, during, and after surgical or palliative treatment	28%	No significance for surgery-treated patients
Gross-Goupil M, 2003 [144]	52	Before and after embolization therapy	24%	No significance
Cillo U, 2004 [112]	40	Periph. Cath. Before surgery	40%	DFS and OS
Jeng KS, 2004 [145, 146]	81	Periph. Cath. 12 weeks after surgery	23%	DFS
Morimoto O, 2005 [147]	38	Periph. Cath. Before surgery	24%	DMFS and DFS

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Figure 1: CirCe 03, an ancillary study to the Prodigie14-METHEP11-ACCORD21 trial

