

Circulating Tumour Cells in Clinical Practice: Can Lessons Learned From Locally Advanced/Metastatic Breast and Prostate Cancers Guide Their use for other Cancer Types? Lights and Shadows

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THE CELLS PROBABLY INVOLVED IN TUMOUR PROGRESSION

The process of metastatic diffusion of cancer cells is not completely understood. There are a lot of hypotheses and many different, incompletely defined cells are probably involved.

Cancer stem cells (CSC) are characterized by properties conferring them the ability to induce cancer, to survive to the treatment and to produce the regrowth of the tumor both at the primary site and in distant ones^[1,2]

Disseminated tumour cells (DTC) have been identified in the bone marrow and may be a marker of minimal residual disease (a concept defined for hematologic neoplasms and not yet directly demonstrated for other solid tumors)^[3]

Circulating tumour cells (CTC) probably represent an intermediate step between these two cell types. They can be defined as cells shed from the primary tumour and circulating in the bloodstream. They could be responsible, together with other host factors, of the metastatic process. CTCs are very rarely picked up in the blood; they can be identified using different methods^[4,5].

Only one of these is considered as a standardized and repeatable

ABSTRACT

Circulating tumour cells are cells probably involved in different phases of the metastatic process. They can be identified using standardized and repeatable methods and are characterized by EpCAM; Citokeratin 8, 18, 19 and DAPI positivity and CD45 negativity. CTC's identification in patients with metastatic breast and prostate cancer has been demonstrated to have a prognostic and predictive role. It is not still clear if these cells can be used also in patients with non metastatic breast and prostate cancer, or in patients with different diseases (eg. metastatic and/or locally advanced head and neck cancer) or if they can be used to biologically characterize the disease during its natural history. This review, leaving from the report of the knowledge already consolidated, is devoted to identify the new trends in the research and in the clinical application of CTC, both in locally advanced/localized prostate and breast cancer, and in head and neck cancer.

method. This method identifies cells characterized by EpCAM, Cytokeratin 8, 18 and 19, DAPI positivity and CD45 negativity as CTC^[6]. The problem of this method is that it could miss cells that are transforming themselves (from epithelial to mesenchymal). The epithelial to mesenchymal transformation (EMT) has been recognised as one of the steps involved in the metastatic process^[7-9].

Moreover, the group at MSKCC suggested the role of CTC in the self-seeding process by using an animal model (2009). This hypothesis could explain the occurrence of late local relapses^[10,11]. According to this model, a particular type of “aggressive CTC” could be the cause of both local relapse and metastatic disease. This is not really conclusively proven and different authors cast some doubts about this conclusion.

The identification and the recognition of these different types of cells (CSC, DTC and CTC) could give to the physician the possibility to monitor and characterize disease before and during the treatment, as a kind of “liquid biopsy”^[12-15].

CTC’s identification has already been demonstrated to have a prognostic and predictive value in metastatic breast, prostate^[16,17]. However, in the daily clinical activity we are very far from this. There are still a lot of open questions to be solved before CTC determination can be applied in clinical practice.

Among them:

- can CTCs be used to predict survival also in non metastatic, locally advanced tumours (in particular breast, prostate cancer)?
- can CTCs be used in other tumours (e.g. head and neck cancer)?
- can CTCs be used to biologically characterize the disease during its clinical evolution?

BREAST CANCER

Breast cancer is the more frequent tumour affecting females. Nowadays, most of these tumours are diagnosed in an early stage, but a lot of them can progress to metastatic disease. Many different therapeutic options (chemotherapy, hormonal and target therapy) are available to treat and to prolong survival of these patients.

The already available prognostic and predictive factors are patient-related (menopausal status, age) and disease related (hormonal status, disease stage, biological factors as Her2 expression). In metastatic patients the absence/presence of visceral disease has a relevant prognostic value. Tumour markers are often used to monitor the evolution of metastatic disease and its response to the treatment; unfortunately, these factors cannot be used to accurately predict progression free and overall survival during treatment^[18]. In fact, they cannot anticipate the duration of response to therapy and their changes often do not mirror the clinical evolution of disease.

The discovery of DTC and CTC led clinical investigators to hypothesize they could solve the problem of “anticipating response” to treatment in patients with metastatic breast cancer. Similarly, the identification of DTC or CTC could be used to select patients with localized or locally advanced breast cancer requiring a more aggressive treatment.

Clinical relevance of CTC in metastatic breast cancer

CTC have already been identified as a relevant prognostic factor in metastatic breast cancer. The first evidence of their role was published by Cristofanilli in 2005^[19]. In patients submitted to chemotherapy for metastatic breast cancer, he demonstrated the prognostic value of the presence (cut-off value, 5 CTC/7.5 ml) and of the variation in number (increase or reduction) of CTC’s during chemotherapy. In 2006^[16] he concluded that the changes of number of CTC’s during

the treatment can predict survival and response better than and before than conventional staging by imaging. CTC’s prognostic value in metastatic breast cancer was lastly confirmed in a recent retrospective analysis collecting the data of 1944 patients^[20]; 991 of them had ≥ 5 CTC/7.5 ml before chemotherapy and worse progression free and overall survival; similarly, an increase of CTC’s during weeks 3-5 and 6-8 after the beginning of chemotherapy was related to worse progression and overall survival. CTC count during the treatment has a better prognostic value than other tumour markers (CA 15.3 and CEA). Many prospective/retrospective/observational studies confirmed these data. The more recent reports are listed in Table 1.

An additional role of CTC identification in metastatic cancer can be the characterization of the differences between primary disease and metastatic disease in terms of Her2 and ER expression; this could lead to understand if the metastatic process changed the “type” of tumour and consequently to select different treatment options according to these findings^[15].

The data about the difference in HER2 over-expression between primary tumour and the CTC’s detected in metastatic patients point to the same conclusion (<15% in primary tumour vs 15-90% in CTC’s); the different percentages of CTC’s positivity are probably related to the methods used to identify and characterize the CTC itself^[24-28]. Two clinical prospective trials are addressing the possible benefit of using trastuzumab and lapatinib to treat patients with HER2 negative metastatic breast cancer (MBC) but harbouring Her2 positive CTC^[29]. The data about ER expression in CTC’s are scarce; CTC’s ER positivity range between 18 to 57 %, a clearly lower value than in the primary tumour (70%)^[30-31], again, differences between studies can be related to the different identification and determination methods. The predictive role of ER-CTC status is, anyway, not clear, since clinical studies are still lacking.

The possible role of CTC’s in locally advanced/localized breast cancer

The clinical significance of CTC’s in locally advanced, localized breast cancer is far from being clear.

Their use has been investigated in different retrospective and prospective trials based on the hypothesis that CTC’s could be an early signal of residual disease and that they could initiate the metastatic process, consequently changing the natural history of the disease.

In fact, it would be very useful if early CTC’s detection could help in defining the prognosis of patients with early, node negative breast cancer patients, identifying those deserving a more aggressive treatment.

Some trials demonstrated that CTC detection is related to a worse disease free, metastases free, cancer specific and overall survival (Table 2).

Adjuvant chemotherapy and CTC

In his German trial (SUCCESS trials) Rack demonstrated in 2010 and more recently^[32-33] the relevance of CTC detection (cut-off ≥ 1) before and after adjuvant chemotherapy, to predict DFS and OS, also as an independent factor.

Neoadjuvant chemotherapy and CTC

Contrasting results are evident regarding the use of CTC’s before and after neo-adjuvant chemotherapy. In patients submitted to neo-adjuvant chemotherapy CTC’s evolution during treatment is independent from the primary tumour response and it is not associated to any of its clinical and pathological features; some studies relate CTC’s reduction with a peculiar pattern of relapse^[34].

Table 1 Recently published (2013-4) studies: CTC determination is an effective tool to predicting Progression Free Survival and Overall Survival.

Author/Year [ref num]	n° paz	Cut off CTC number value	Timing of detection	Detection system	Statistically significant prognostic value for...
JB Smerage/2014 ^[21]	595	5	Basal and during RT	Standardized	OS (but changing CHT according to CTC number does not increase survival)
M Martin/2013 ^[22]	99	5	Basal and at 21 days during CHT	Standardized	OS (Only CTC value at 21 days resulted an independent prognostic variable at multivariate analysis) OS and PFS
M Wallwiener /2014 ^[23]	393	5	Baseline and after 1 cycle; CTC kinetic	Standardized	Basal CTC value and CTC kinetics prognostic factors for both OS and PFS.

Table 2 Possible role of CTC's detection in locally advanced breast cancer.

Author/Year [ref num]	n° paz	Cut off CTC number value	Timing of detection	Detection system	Statistically significant prognostic value for ...
B Rack/2014 ^[36]	2026 (before adjuvant CHT) and 1492 (after CHT)	5	Before adjuvant CHT, after CHT	Standardized	DFS, distant DFS, DSS, OS; Independent prognostic factor for DFS and OS
FC Bidard/ 2010 ^[37]	115	5	baseline , after neoadj CHT	Standardized	Baseline: distant metastases free survival, OS; Poor metastases free survival and breast cancers specific survival; CTC's identify patients with particularly poor prognosis among low-risk node negative patients who did not receive adjuvant therapy
TJ Molly/2011 ^[38]	733 (early stage)	1	At primary surgery	Not standardized	Distant disease free survival; DSS
B Franken /2012 ^[39]	404	5	Before surgery	Standardized	

An unexpected finding is that in some studies CTC's status after neo-adjuvant chemotherapy does not seem to have any impact on prognosis^[35]; in other reports, it relates with a worse DFS if the CTC's number is ≥ 1 and with a worse OS if their number is ≥ 5 ^[34].

A still unsolved problem is how the information from clinical studies can really help the physician to improve survival also in patients with CTC positive determination. The future should again rely on the biological characterization of these cells in order to guide a tailored treatment.

Can CTC be used to guide treatment in non-metastatic breast cancer?

The problem of understanding if CTC's can be used to change the treatment of patients with localized breast cancer is not a little one. The results from the only study already published seem to show that an early change of treatment in patients with an increasing CTC number does not translate in a better OS: they maintain a prognosis worse than that of patients without CTC or with a reduced number of CTC after 21 days of treatment^[21].

Different prospective clinical trials are now on-going to give an answer to this strategic question.

Table 3 shows only the trials registered at the clinicaltrials.gov website.

PROSTATE CANCER

Prostate cancer is most common tumor in males. It is often discovered occasionally and as a very localized disease (low-risk disease). For these patients the therapeutic options can vary from "watchful waiting" to radiotherapy alone to surgery alone depending also by patient preference.

The patients defined at high risk (locally advanced tumour, high Gleason score and high PSA level)^[40] are usually treated with conservative treatment: radiotherapy (with or without pelvic lymph-nodal irradiation^[41] depending on the risk of nodal involvement and

clinical practice of the different institutions) and hormonal treatment, better if more than two years^[42].

The usual medical treatment of high risk or early metastatic disease is hormonal therapy (LHRH analogue or total androgenic block).

A substantial proportion of high risk patients progresses to metastatic disease and can become resistant to hormonal treatment. There are no clear methods, apart from the already mentioned prognostic factors, to predict the clinical evolution of disease and to identify patients to be consequently treated with the more aggressive treatment options.

The rationale suggesting clinical investigators to search disseminated or, better, circulating tumour cells in the bone marrow or in the blood, respectively, also in prostate cancer is the same already discussed for breast cancer: to identify a simple, more effective method to predict survival and to guide the treatment.

The evidence about CTC prognostic value, however, is weaker for prostate than for breast cancer.

CTC in metastatic hormone sensible (HSPC) or castrate resistant (CRPC) prostate cancer

It has been demonstrated that CTC are detectable in patients with metastatic PC; the number and the evolution of CTC number during treatment is significantly related to survival and response to therapy.

The results of the recently published SWOG-S0421 trial confirmed the prognostic importance, in terms of OS, of CTC enumeration and change in number between baseline and day 21 in a phase III trial of patients treated with docetaxel with or without atrasentan^[43]. Median CTC number at day 0 was 5 cell; a CTC number lower and higher than 5 was associated with PSA levels, bone pain, liver disease, haemoglobin level, alkaline phosphatase and PSA and RECIST response. OS was equal to 26 months for patients with < 5 CTC and 13 months in those with > 5 CTC; a raising count between days 0 and 21 was associated with shorter OS. Less recent phase II studies showed the same results in terms of number of CTC before the treatment^[44-45] and change of the number during the treatment^[46].

These results induced FDA to authorize the use of CTC detection for the evaluation of metastatic CRPC using a standardized method.

The subsequent step will be to verify the utility of CTC count as surrogate of survival in CRPC. The requisite to consider a parameter as a response-indicator biomarker are very restrictive and has to be confirmed and re-confirmed in prospective randomized trials^[47].

In HSPC only a few little studies suggest the utility of CTC. In

2011 Goodman^[48] demonstrated that CTC number is directly related to ALP and LDH levels and not related to PSA and testosterone levels (33 patients); at multivariate analysis only CTC at baseline had independent predictive value in terms of duration and magnitude of response to hormonal therapy. In 2012 Folkersma^[49] compared 30 pts with localized PC, 30 patients with metastatic PC and 30 healthy volunteers. A significant correlation between all the marker of disease

Table 3 Trials still recruiting (8/10/2014) among the 20 listed at clinicaltrials.gov (search criteria: "CTC and breast cancer").

Title of the study	Year	Phase/n° patients	Design of the study	Primary objective
TRastuzumab in HER2-negative Early Breast Cancer as Adjuvant Treatment for Circulating Tumour Cells (CTC) ("TREAT CTC " Trial) (EORTC)	2012	Randomized II/ 2175	Observation vs trastuzumab	CTC detection Compare CTC detection rate at week 18 between trastuzumab treatment arm and observational arm
COMETI Phase 2: Characterization of Circulating Tumour Cells (CTC) From Patients With Metastatic Breast Cancer Using the CTC-Endocrine Therapy Index	2013	Observational and prospective II / 200		Rapid Disease Progression in relation to CTC [3 months after initiation of a new line of endocrine therapy]
Enumeration and Molecular Characterization of Circulating Tumour Cells in Women With Metastatic Breast Cancer	2011	Observational and prospective /150	Determination and enumeration of CTC at different time points during the treatment.	To determine whether numbers of CTC in peripheral blood can give information of prognosis, and to evaluate if the numbers of CTC can give information of the efficacy of given treatment
Randomized Trial to Evaluate the Medico-economic Interest of Taking Into Account Circulating Tumour Cells (CTC) to Determine the Kind of First Line Treatment for Metastatic, Hormone-receptors Positive, Breast Cancer.	2012	Randomized /1000	Hormonal therapy (HT) or chemotherapy (CHT) chosen according to the standard of each center, based on usual parameters VS HT or CHT chosen according to the number of CTC /7.5 ml at baseline: CTC <5 -> HT CTC ≥5 -> CHT	Progression free survival evaluation; Economic impact of the different strategies will be evaluated (standard treatment vs CTC based treatment)
DETECT III - A Multicenter, Randomized, Phase III Study to Compare Standard Therapy Alone Versus Standard Therapy Plus Lapatinib in Patients With Initially HER2-negative Metastatic Breast Cancer and HER2-positive Circulating Tumor Cells	2012	Randomized phase III/228	Standard therapy (chemo- or hormonal) vs standard chemo- or hormonal therapy + lapatinib	Progression free survival Time interval from randomization until PD or death from any cause.
CirCe01 Study: Evaluation of the Use of Circulating Tumour Cells to Guide Chemotherapy From the 3rd Line of Chemotherapy for Metastatic Breast Cancer	2011	Randomized phase III/568	Comparison of CTC level before the first injection of each new line of chemotherapy. Chemotherapy can be continued if CTC response. End of chemotherapy can be decided by the clinician on the basis of clinical or radiological basis.	Overall Survival
Characterization of Circulating Tumour Cells (CTCs) to Direct Pre-Operative and Systemic Therapy in Patients With Locally Advanced or Metastatic Stage IV Breast Cancer	2010	Observational and prospective /280	Identify CTC from patient's with locally advanced or metastatic (stage IV) breast cancer. This research will help to develop better ways to treat potentially very aggressive breast cancer	Targets ER, Her2, and possibly M30, VEGFR2, EGFR positive CTC found in blood stream.
DETECT IV - A Multicenter Single Arm Phase II Study to Investigate the Clinical Efficacy of Everolimus in Combination With Endocrine Therapy in Postmenopausal Patients With Hormone-receptor Positive, HER2-negative Metastatic Breast Cancer and Persisting HER2-negative Circulating Tumor Cells	2014	Prospective non randomized phase II/800	The primary objective: estimate the clinical efficacy of the mTOR inhibitor everolimus in combination with endocrine therapy as assessed by progression-free survival (PFS). Additional research on CTC dynamics and characteristics will provide a better understanding of the prognostic and predictive value of CTCs.	Progression free survival (PFS) Time interval from randomization until progressive disease (PD) or death from any cause.
Validity of HER2-amplified Circulating Tumour Cells to Select Metastatic Breast Cancer Considered HER2-negative for Trastuzumab-emtansine (T-DM1) Treatment.	2013	Phase II/480	Patients with metastatic breast cancer considered HER2 negative are included and screened for HER2-amplified CTC. If at least HER2-amplified CTC is detected, patients are treated by T-DM1 in a single arm phase II with an adaptive design	Tumour response rate to T-DM1 in patients with HER2 amplified circulating tumour cells [Time Frame: Until disease progression]
Isolation of Circulating Tumour Cells Using a Novel EMT-Based Capture Method	2014	Phase II/80		CTC identification in patients with metastatic progressive castration-resistant prostate cancer or metastatic progressive breast cancer with a novel mesenchymal-marker based ferrofluid (N-cadherin or O-cadherin based). Primary objective of each cohort is to compare the detection rate of CTC between standard and novel methods.

extension but Gleason score was demonstrated (PSA, T, N, M class). A prognostic correlation in terms of OS and PFS was evident if CTC count was >4; these data were confirmed at multivariate analysis.

It is still not clear if CTC enumeration can be used as an early marker of the evolution of disease and to early predict the loss of hormonal sensibility. More data are necessary to confirm this data and to allow a practice of change of treatment based on CTC count.

Localized prostate cancer

CTC appear to be less frequently detectable in localized than in metastatic PC. The studies in this patient setting are scarce; recently[50] it has been demonstrated that only 5/36 patients with newly diagnosed high risk PC (age >70, median PSA 14.1, median GS 9) had CTC in their blood. The presence of CTC did not correlate with other already confirmed prognostic factors (PSA, GS, stage T). Same results were evident comparing the percentage of CTC positivity between control subjects (20) and patients (20) with locally advanced PC (median PSA 21 ng/ml, T>cT3a) with a $p=0.66$ ^[51].

The low frequency of CTC positivity in localized prostate cancer forced clinical investigators to search different methods to identify CTC: CTC-chip platform^[52], RT-PCR for prostate specific genes as a surrogate for counting CTC and as a mean to predict PC recurrence after prostatectomy^[53].

Lowes in 2012^[54] investigated the role of CTC in patients treated with radical radiotherapy (RT) for locally advanced PC and did not found a reduction of CTC number after RT; however, PSA responders could have a more pronounced reduction of CTC number than non responders (even if this observation does not reach statistical significance).

Again in this setting of patients the correlation between CTC and DTC in the bone marrow has been looked for, but the correlation was difficult to demonstrate. DTC itself were associated with worse prognosis in terms of probability of metastatic disease (multivariate analysis)^[55-58].

Nowadays it is still not clear if the simple enumeration of CTC with standardized methods could be used in clinical practice for localized prostate cancer, either as a prognostic or a predictive factor.

Molecular characterization of CTC

The future studies on CTC in prostate cancer will probably address the biological characterization of these cells, in order to directly target the treatment to specific and different types of prostate cancer. A lot of different methods can be used to characterize the genomic and proteomic pattern of CTC including immunohistochemistry, immunofluorescence (presence or loss of phosphatase and tensin homolog PTEN; assess the number of copy of the AR gene), gene copy number analysis (detection of DNA mutations and altered copy number), genome sequencing analysis (hotspot mutation of phosphoinositide 3-kinase PI3K/AKT genes in the circulating genomic material) and epigenetic studies^[59].

The interest of these different methods to track biological targets is the opportunity to monitor the disease during its natural history, to identify the evolution of the tumor in response to different treatment, to try to develop specific targeted treatments.

Clinical trials addressing future developments in CTC use

The lack of definitive answers as far as the clinical impact of CTC measurement is concerned is also testified by the ongoing trials reported in Table 4.

The main goals of these trials are: the choice of the better method to identify CTC; the clinical advantages of the use of newer

identification methods; the impact of molecular characterization of CTC on survival.

HOW TO APPLY THE LESSONS LEARNED FROM BREAST AND PROSTATE CANCERS TO OTHER CANCER TYPES: THE CASE OF HEAD AND NECK TUMOURS (HNC)

The interest for a possible relationship between CTC count and outcome in head and neck cancer has been recently growing because of the difficulties in predicting survival with the already known prognostic factors.

Different combinations of surgery, radiotherapy and chemotherapy are the mainstay of the treatment of head and neck cancer. The choice of the actual treatment derives from the tumor site (naso-, meso-, hypo-pharynx, larynx) and, within each site, from T, N and M categories and from their interplay, defining disease stage. Surgery is often the first approach, but radiotherapy is very often chosen to also in locally advanced cases, aiming at the cure of the disease with less functional damage^[60].

Overall and progression free survival times after primary treatment can be predicted using mainly the same parameters (T, N, M).

Only recently some biological factors have been added to the diagnostic armamentarium as prognostic and predictive factors. The most frequently used biological predictors are the EGFR status (since EGFR is the target for some effective drugs, like cetuximab, and even if EGFR positivity is overwhelmingly frequent in HNC)^[61, 62], and the HPV positivity, mainly for meso-pharyngeal cancer, identifying patients with a better outcome even after less aggressive treatments^[63]. The evidence on the predictive efficacy of these markers is growing, but they cannot be considered a standard mean to drive treatment choice.

Locally advanced disease

The interest about CTC determination in head and neck cancer is grown only in the last two-three years.

The research steps were not dissimilar from the case of breast and prostate cancer: 1. Try to identify CTC in the blood; 2. Try to correlate their presence and number to the other already known prognostic and predictive factor; and finally 3. Try to understand if they could more effectively predict response to treatment and prognosis.

Differently from the case of breast and prostate cancers, early studies were focused on locally advanced disease.

The results were not uniform.

Hristozova did not find a statistically significant correlation between CTC identification, T stage and tumour volume; an association with N2b status was however reported^[64]. Bozec did not find any correlation with the already known prognostic factors (T, N, M)^[65]; Buglione found an higher frequency of CTC detection in patients with stage IV disease, as compared with earlier stages, but without statistically significant differences^[66].

The percentage of CTC positivity before primary treatment ranges between 15% and 43% and it is strongly dependent from the search method, being lower with standardized methods (Table 5)

Some authors demonstrated differences between CTC identification in different sites and also different clinical implications for CTC detection according to the tumor site: for example, a favourable prognostic significance in oro-pharyngeal cancer and a negative

prognostic impact for the other disease sites have been reported^[67] Our group detected CTC more frequently in oro- and hypopharyngeal cancer and SNUC cases^[66].

Recently Gröbe^[68] in a selected series of patients treated with surgery for oral cancer demonstrated a correlation between T, N, M category, CTC detection rate (in blood samples) and DTC positivity (in bone marrow). CTC and DTC were present in 12.5% and 20% of the patients, respectively. It is very interesting to note that CTC and DTC were identified together only in two patients. He demonstrated, both at univariate and multivariate analysis a statistically significant correlation between CTC/DTC detection and recurrence free survival. As far as survival is concerned, again, the results reported are not uniform. Buglione^[66] reported significantly better complete/partial response rates in patients without CTC or with a decreasing number

of CTC during the treatment; no statistical evaluation of DFS or OS was done because the follow up period was too short. A decrease in the CTC number or their absence throughout the treatment was related with non-progressive disease, after both complete or incomplete remission, and with the number of patients alive and NED (no evidence of disease) ($p = 0.009$). Tinhofer^[67] demonstrated that CTC presence was not predictive of OS or DFS in the entire series (144 patients); in oro-pharyngeal carcinomas (OPC, 63 patients) CTC detection was associated with improved DFS (CTC+ vs CTC-: 100% vs. 79%; $p=0.059$); the reverse was true for carcinomas of other sites ($n=81$; CTC+ vs CTC-: 29% vs 75%; $p=0.001$). In multivariate analysis, CTC remained an independent prognostic marker for DFS ($p=0.002$) and OS ($p=0.016$) in non-OPC.

Table 4 Trials still recruiting (8/10/2014) among the 20 listed at clinicaltrials.gov (search criteria: "CTC and prostate cancer").

Name of the study	Year	Phase/n° patients	Design of the study	Primary objective
Development of a Novel Method to Detect Prostate Cancer Circulating Tumour Cells (CTCs) Based on Epithelial-mesenchymal Transition Biology	2012	Observational/50	Determine whether CTC can be captured using the novel mesenchymal-marker based Near Infrared-Emissive Polymerosomes (NIR-EPs), the PSMA-based NIR-EP, and the epithelial EpCAM-based NIR-EP.	Change in Non-detection rate of CTC's in men with CRPC Non-detection rate of CTC's in men with CRPC will be measured at baseline, month 3, and at progression Relationship between CTC quantity and pathologic stage Relationship between pre-operative CTC quantity and pathologic stage in men with early stage prostate cancer undergoing prostatectomy.
Circulating Tumour Cell Analysis in Patients With Localized Prostate Cancer Undergoing Prostatectomy	2010	Observational prospective /200	Determination of PSA levels and CTC analysis. Time points: Screening; One day after surgery; 7-14 days after surgery; 3-6 months after surgery; at PSA recurrence or at two years	Persistent CTC and biochemical recurrence: To examine the relationship between persistent CTC and biochemical recurrence after radical prostatectomy for localized prostate cancer
Isolation of Circulating Tumour Cell Using a Novel EMT-Based Capture Method	2011	Non randomized; single group assignment/ 80		Feasibility as measured by successfully detecting at least one CTC in at least 2 out of 10 subjects, comparing the non-detection rate over time. The change in non-detection rate will be measured
Cultured Circulating Tumour Cell - Development of a Novel Platform for Drug Discovery and in Vitro Chemo-sensitivity Testing in Prostate and Other Cancers	2014	Observational /220		Evaluate novel CTC assay method as a predictive tool for prostate, breast and colorectal cancers. Colony counts will be assessed using a novel 3-D assay and a commercial CTC count method (CellSearch™). Samples will be collected prior to and post initiation of treatment. Results will be evaluated for changes in CTC counts.
Characterization of Circulating Tumour Cell Captured by c-MET (CTC-MET)	2014	Observational /60	The investigator will also describe the detection rates of both the c-MET CTC capture and the EpCAM CTC capture techniques in each patient, also separated by disease site.	Feasibility; Feasibility as measured by successfully detecting at least one CTC in at least 2 out of 10 subjects within each disease site.
Collection of Blood Specimens for Circulating Tumour Cell Analysis	2013	Observational / 55	The number of CTCs per milliliter of whole blood. Reported unit of measure will be the number of CTCs/milliliter. Blood samples will be collected from study subjects and analyzed for the number of circulating tumor cells (CTCs) within 96 hours of blood collection.	To test the sensitivity of a proprietary novel filtration device designed to capture and concentrate CTCs.
Immune Profiles and Circulating Tumour Cell Status Following Prostate Cryotherapy	2011	Observational /160		Immune Profiles and CTC Status Following Prostate Cryotherapy
Culture and Characterization of Circulating Tumour Cell (CTC) From Patients With Malignant Melanoma and Other Cancers	2012	Observational case control /1000	Blood samples will be collected from participants with melanoma and prostate and healthy volunteers for purposes of comparison.	This study is designed to evaluate the sensitivity and specificity of the TrueCells assay, a novel approach to isolate and preserve cells for further research.
Biomarkers of Response to Taxotere in Hormone-Refractory Prostate Cancer	2009	Observational /50	All patients are treated with Docetaxel on a weekly, biweekly or three weekly schedule. Blood samples: baseline, every 3-4 weeks during study, and at disease progression or every 12 weeks after completion of study. Samples are analyzed for mRNA via RT-PCR	Presence of mRNA in circulating tumor cells as a predictor of response

Table 5 Main studies exploring the possible clinical uses of CTC determination in head and neck cancer. SCCHN= Head and Neck Squamous Cell Carcinoma.

Author/year [ref num]	N° of patients	Stage and site	% Positive	Timing of detection	Method	Statistically significant clinical correlations
T. Hristozova /2011 ^[64]	42	Locally advanced SCCHN; Oropharynx, Oral cavity, Hypopharynx Larynx Nasopharynx	43%	During RT+CHT	Not standardized	No significant correlation between CTCs and T stage or tumor volume. N2b or more advanced nodal disease was associated with an higher frequency of CTCs. Concurrent radio-chemotherapy reduced CTC positivity; CTC persisted during treatment in 20%
I. Tinhofer /2014 ^[67]	144	Locally advanced SCCHN; oropharynx, oral cavity, larynx, hypopharynx or unknown primary site	29%	After tumor resection before adjuvant treatment	Not standardized (RT-PCR of EGFR mRNA)	Presence of CTC was not predictive of OS or DFS; at multivariate analysis, CTC is an independent prognostic marker for DFS and OS in non-OPC.
A Bozec /2013 ^[65]	49	Locally advanced: oral cavity or oropharynx	16%	Prior to the initiation of therapy	Standardized	No association with already known prognostic factors as TNM;
M. Buglione /2012 ^[66]	73	Locally advanced; oropharynx, nasopharynx, larynx, sinonasal undiff. carcinomas (SNUC), hypopharynx, oral cavity	15%	Before and during radio-chemotherapy	Standardized	CTC mostly identified in oro- and hypopharyngeal cancer and in SNUC. Stage IV: high % of CTC positivity. Absence or disappearance of CTC during treatment related to favourable response to treatment. CTC decrease related with non-progressive disease.
I Tinhofer /2012 ^[69]	31	Locally advanced stage IVA/B disease	29%	Before and during concurrent radio-chemotherapy or induction chemotherapy followed by RT with cetuximab	Not standardized: Flow cytometry, absolute CTC number/ blood volume; expression of EGFR and pEGFR	The number of CTC positive samples significantly increased after radiotherapy. Radiation-induced CTC increase was less pronounced if RT was combined with Cetuximab. Cetuximab was also more effective in reducing pEGFR expression in CTCs.
AC. Nichols /2011 ^[72]	15	Advanced-stage SCCHN (stage III/IV)	40%	Before the treatment	Standardized	CTCs were significantly associated with lung nodules >1 cm (p .04). There was also a suggestion of improved survival in the CTC-negative.
S. He /2013 ^[73]	9	Advanced-stage SCCHN (stage III/IV)(oro-, hypo-pharynx, larynx	33%	Before treatment	Standardized	CTC can be identified
A. Gröbe /2014 ^[68]	80	Squamous cell carcinoma (all stages); oral cavity Metastatic	12.5%	Before surgery	Standardized	Significant correlations found between CTC presence and tumor size (p=0.04), nodal status and DTC (p=0.02), and distant metastasis with CTC (p 0.004) and DTC (p=0.005). Univariate and multivariate analyses revealed that CTC and DTC are significant and independent predictors of recurrence-free survival (p < 0.001) Multivariate analysis: ≥1 CTC was associated with a poor PFS and OS. Disease control was obtained in 8% CTC-positive vs 45% in CTC-negative (p 0.03). The epidermal growth factor receptor (EGFR) expression was identified in 45% of CTC-positive patients.
S. Grisanti/ 2014 ^[71]	53		26% baseline;41% at any time point	Before and during chemotherapy	Standardized	

Table 6 Trials still recruiting (8/10/2014) among the 20 listed at clinicaltrials.gov (search criteria: "CTC and head and neck cancer").

Name of the study	Year	Phase/N° patients	Design of the study	Primary objective
Prognostic Value of Circulating Tumor Cell in Patients With Locally Advanced and Metastatic/ Recurrent Head and Neck Cancer	2011	Observational prospective/300	Blood samples were collected within 7 days before the first dose of chemotherapy	Progression-free survival; relate the response or progression evaluated with standard imaging with CTCs number and time from CTCs checkpoint to disease progression will be analyzed

Only one study tried to relate CTC presence to its biological characterization and to treatment response^[69]. The number of CTC-positive samples significantly increased after radiotherapy. This radiation-induced increase in CTC numbers was less pronounced when radiotherapy was combined with cetuximab compared to the combination with cisplatin/5-fluorouracil. The Cetuximab regimen was also effective in reducing phosphorylate EGFR (pEGFR) expression in CTCs.

Metastatic disease

More than 50% of the cases with stage III-IV head and neck cancer recur with metastatic disease. One third of all treated patients will respond to systemic treatment with complete or – more often- partial remission, even if cure is almost never obtained. It is difficult to predict both which patients will recur with metastatic disease and which patients will respond to the treatment. Only one (not validated) prognostic score has been published to try to predict progression free and overall survival in metastatic disease. This score considers factors like performance status, weight loss, primary site, response to radiotherapy and tumour cell differentiation^[70].

As for breast and prostate cancers, it is possible to use CTC to predict the outcome of patients treated with chemotherapy.

Our group explored also the use of CTC in metastatic patients. Grisanti^[71] confirmed the presence of CTC before chemotherapy (26%) and during treatment. He demonstrated that at univariate analysis ≥ 2 CTCs are related with a prognosis poorer than 0-1 CTC; the presence of ≥ 1 CTC was related to worse PFS and OS. At multivariate analysis, disease control after systemic therapy was obtained in 8% of CTC-positive patients as opposed to 45% of the CTC-negative ones (p 0.03).

An important research effort will be needed do better define the possible clinical use of CTC in this clinical setting; the data collected, even if interesting, are in fact far from being sound enough to find a clear clinical application.

Also for HNC, the final goal resides in the biological characterization of these cells to try to guide the treatment directly according to specific targets.

Probably because the rarity of this kind of tumours the ongoing prospective trials devoted to understand the role of CTC are unfortunately very rare (Table 6).

CONCLUSIONS

A lot of scientific and economic energies have been devoted to understand the role of CTC to enhance the knowledge of the natural history of some cancer types.

Metastatic breast cancer was the first disease for which FDA in USA approved the use of CTCs as a prognostic/predictive factor. Metastatic prostate cancer was the second. Despite this important acknowledgement, until now, the use of this new biologic instrument did not impact significantly on the choice of the treatment.

CTC determination in metastatic breast and prostate cancer can be effective when used as prognostic and predictive factor. We know that the presence of CTC before the beginning of the treatment correlates with worse progression free and overall survival, but neither the knowledge of its change during the treatment neither the early change of treatment guided by CTC determination seem to modify the outcome.

Results are even scarcer in locally advanced and early breast and prostate cancer. For early disease, questions far outweigh answers. Correlations between CTC determination and the other already used

prognostic factors remain unclear and it is impossible to use CTC determination to guide treatment choice.

The reason of these difficulties probably depends from the methods of determination (probably not all the cells are correctly identified) and from the lack of biologic characterization. In locally advanced and early stage disease ineffective detection methods probably are not able to identify the few, possibly present, CTC.

In addition, disease biological features change during its natural history; the treatment should be probably targeted on these new targets rather than on those of disease at presentation.

The ongoing trials in breast and prostate cancer are trying to answer these open questions in order to confirm or discard, also from an health economics point of view, the utility of CTC determination and characterization.

The problem is obviously much more complex for head and neck cancer.

Difficulties with HNC are amplified by the heterogeneity of the disease(s) in terms of staging and prognosis (epi-pharyngeal tumor are very different from meso-pharyngeal or hypo-pharyngeal or laryngeal tumors and all they are different from oral cavity tumors) and treatment (different combinations of surgery, radiotherapy and chemotherapy or biological treatment).

Results on correlations between CTC determination and stage, site of primary are therefore very different in the different trials. The percentage of CTC positivity varies very much between the different trials and according to the the different methods of determination.

The results of the only prospective trial on metastatic disease confirm the prognostic role of CTC before the beginning of chemotherapy and the significance of CTC reduction during the treatment, in terms of disease control.

CTC determination has been applied also to other neoplasms, not covered by this review. Among them at least lung cancer should be quoted^[74-76].

However, in an era of reduced resources for health systems it is easily understandable that very costly predictive and prognostic tests should be fully validated before being adopted in the routine clinical practice.

The study of CTC has important biological implications; it could advance further our knowledge of the neoplastic phenotype and of its variations after therapy. However, clinical applications are in their infancy and should be considered very cautiously. This does not mean that research in this field should be abandoned; rather, more sound biological bases should probably be built before attempting extensive clinical testing.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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