Atlas of Liquid Biopsy

Circulating Tumor Cells and Other Rare Cells in Cancer Patients' Blood

Ludmilla Thomé Domingos Chinen
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Foreword

Cancer represents a big challenge for all who deal with it, mostly patients with neoplasms and those who assist them. The difficulty in managing patients is due to the incessant search for a cure, related to the exact dimension of the treatment offered, either to avoid under- and over-treatment. For the therapy selection, understanding the entire evolution of the neoplastic cycle is necessary, and circulating tumour cells (CTCs) can be fundamental in this process.

The first time CTCs were identified and described was at the end of the nineteenth century, as an autopsy finding. After almost a hundred years, they could be truly isolated. Only in the last decade of the twentieth century, specific methodologies for systematic detection of CTCs emerged. Despite such a development, as several technologies have been present, they often lead to doubtful interpretation and results.

In the beginning of the twenty-first century, strong data validated the role of CTCs as prognostic factor in metastatic breast and prostate disease, and through less robust studies, in metastatic colon and lung cancer. In breast cancer, studies have already corroborated the use of CTC counts as a prognostic factor before neoadjuvant and adjuvant chemotherapy, in addition to assisting in monitoring the treatment of advanced disease. In colorectal cancer, an increasing amount of evidence supports the count of CTCs as a prognostic factor in the scenario of neoadjuvant and adjuvant treatment.

The relationship between CTCs and the comprehension of cancer evolution and progression process is very direct. If we think of these cells as rare cells, difficult to find, we can understand the wealth of information that a relatively small universe can reveal to us. The process of epithelium-mesenchymal transition, the ability of such cells to survive in blood circulation and the inverse process of mesenchymal-epithelium transition, can also be studied. In addition, CTCs constitute a tumour component that can be unveiled in order to understand and even treat minimal residual disease and dormant cells, possibly responsible for tumour recurrences.

In seeking a better understanding of the evolutionary phenomenon of cancer, we need to remember that this is a polyclonal disease, subject to evolutionary changes, imposed not only by the tumour microenvironment, but also by interactions with the

host and the drugs used for treatment. The translation for this temporal analysis, of the evolutionary film of the tumour throughout its development process, is what we call liquid biopsy. In this scenario, CTCs have a very relevant role. There are many advantages to use liquid biopsy:

- Minimally invasive procedure (a blood sample)
- Can be repeated frequently without imposing risks on the patient
- Allows genetic and molecular analysis in real time
- Ability to predict whether the therapy used will provide the expected results

Given the above, the importance of CTCs in the future scenario of oncology is paramount, not only for understanding the entire evolutionary process of cancer but also for the evolution of current therapy.

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About the Editor

Ludmilla Thomé Domingos Chinen began work with CTCs in 2012 at A.C. Camargo Cancer Center, São Paulo, Brazil. Her mission was to initiate an integration project between the daily clinical activity and the research area of the hospital. After careful planning, she realized that the study of CTCs would be a promising field.

In view of the limitations of financial and consequently technological resources, there was no margin for error in the project. International partnerships were established in order to shorten the learning curve. Institutional projects created the foundation for the first publications and allowed educational projects at the medical residency (3) and postgraduate level (around 10).

Today, we can say that thanks to the initiative and effort of Dr. Ludmilla, in addition to the support of people who will never stop being present at the AC Camargo Cancer Center, such as Professor Ricardo Renzo Brentani, the biography of many people gained more shine.

Certainly, this book will contribute to the future work of this fascinating universe of the evolutionary process of cancer and more accurate cancer treatments.

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Chapter 1
Circulating Tumor Cells: Brief Overview of Methods for Detection

Ludmilla Thomé Domingos Chinen

Images of this book were taken at × 400 or x 600 magnification using a light microscope (Research System Microscope BX61 - Olympus, Tokyo, Japan) coupled to a digital camera (SC100 - Olympus, Tokyo, Japan). All images showed in this book-Atlas were checked by Dr. Mauro Ajaj Saieg, the head of cytopathology depatment of ACCamargo Cancer Center.We thank Dr. Mauro for all his support.

In May 2018, I received the invitation to write about circulating tumor cells (CTCs) and to add an Atlas to the book. I accepted without thinking about the huge challenge that lay ahead. CTCs, even today, with so many published studies and so much relevant clinical data, is still a topic with many doubts and unsolved questions. We know that they are rare cells among millions of hematopoietic cells, which come out of the tumor and form metastases, circulating isolated or in the form of circulating tumor microemboli (CTM)), which are more prone to form metastases and probably linked to the formation of thrombi. We also know that CTM leave the primary tumor in this aggregate form and that is not formed in the circulation. We know that CTCs can circulate with extracellular vesicles (EVs), and there are authors who believe that EVs are involved in targeting CTCs. CTCs also interact directly with immune system, silencing or activating them according to "their" needs.

In this book, we discuss a little about data that exists in the literature, about clinical findings in different tumors, and about biological roles of CTCs. Mainly, we share a little of our experience, using an independent marking CTC separation system, ISET (Isolation by SizE of Tumors, Rarecells, France) with the which we have been working since 2012.

We have made several studies with ISET, in different tumors and received different sponsorships (FAPESP 2012/01273-8; FAPESP 2013/08125-7; FAPESP 2014/26897-0; FAPESP 2016/18786-9 (Brazil); MP-TAC PAJ n°000968.2012.10.000/0 (Brazil); IAEA 20541 (Austria); INCT 465682/2014-6 (Brazil); Faber-Castel (Brazil), PRONON 25000.055121/2015-12- (Brazil), Libbs (Brazil), to whom we thank with all gratitude.

We are also very grateful to all patients that kindly gave us samples to analyze and who shared a little of their life experience with us, with generosity. In these last 9 years, we have contact with around 700 patients, to whom we lovingly thank. Here, in Atlas, we share with you, our reader, some CTCs and CTM pictures from

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some patients, without any identification, just to let you know how to identify CTCs/CTMs and how important we believe these cells are in the biology and comprehension of the tumor. And for last, I can not forget to thank Rarecells, which provide me with scientific support so that I could get the best out of the system, and the ISET developer and CTC´s deep researcher, Dr. Patrizia Paterlini-Brechót, human being who deserves my admiration.

1.1 Brief Historic Review

Circulating tumor cells (CTCs) are released from primary tumors or metastases during tumor formation and progression, and are considered as "liquid biopsy" in real time, reflecting the disease complexity [28]. Studies with CTCs have been focusing on their prognostic value, their utility in monitoring treatment, and identification of new targets for therapy and for resistance, leading to a better comprehension of the metastatic process [28]. CTCs can also be considered as pharmacological biomarkers, and their analysis can help clinicians/researchers to: have proof of action mechanisms of drugs; select doses of anti-neoplastic drugs; gain comprehension of therapeutic and resistance mechanisms of anti-cancer drugs; better combine different therapies; and predict treatment outcomes [10].

CTCs were first reported in literature in 1829 [24] (RÉCAMIER), but the most known citation was made in 1869, by Thomas Ashworth, an Australian resident medical doctor. When performing necropsis of a patient with chest sarcoma, he observed cells in the patient's saphenous vein identical of those observed in the chest. Then, the researchers came back to this subject in 2004, when a large study, including 20 centers, was published in *New England Journal of Medicine*. Cristofanilli and his collaborators designed very well a longitudinal study, with the analysis of CTCs, using a system called CellSearch System (at that time, owned by Johnson & Johnson). They evaluated 177 women with metastatic breast cancer, and made CTC counts before and after the start of treatment for metastatic disease. They also included patients with benign breast diseases and health volunteers. They observed that health volunteers and patients with benign breast diseases had less than 2 CTCs in 7.5 ml of blood. In contrast, for patients with metastatic disease, the authors found a cut-off of 5.0 CTCs/7.5 mL, meaning that those with levels above the cut-off, had poor progression free-survival (PFS) and overall survival (OS). The CellSearch System was cleared by FDA in 2007, to be used in patients with metastatic breast, prostate, and colon cancers [7, 8, 25]. It separates CTCs by immunomagnetic biomarkers, enriching for cells that express epithelial cell adhesion molecules (EpCAMs) and depleting those with the leukocyte common antigen, CD45. The bias with this system and all others created since 2004, which separate CTCs by antibodies are as follows: a) not all CTCs express EpCAM, because many CTCs pass through epithelial-mesenchymal transition (EMT), losing epithelial markers and gaining mesenchymal ones (we will discuss in depth in a chapter about mesenchymal tumors); b) by capturing the cells that express EpCAM without

morphological verification of the neoplastic nature of the cells, these systems can erroneously identify circulating non-malignant epithelial cells as CTCs; and c) leukocytes, mainly neutrophils, also express cytokeratins [21–23, 30].

Due these problems, CellSearch enumeration of CTCs has not become a widely adopted test for any tumor entity, as it has not demonstrated to have clinical utility in making treatment decisions [14]. As the majority of clinical trials (clinicaltrials.gov) worldwide were designed to use CellSearch, with its known failures, now, an association of CellSearch with DeepArray was made (Menarini Silicon Biosystems), in an attempt to improve the test and make single cell analysis. In addition, other methodologies have been including in clinical trials.

All these endeavors in trying to find the best methodology to isolate and identify CTCs motivated us to write this book. As system based on size and morphology have gain relevance, as microfluids, per example, having a book that shows the cytopathological features of CTCs will be of a great scientific and practical value.

Nowadays, some international efforts have been made in an attempt to validate the different methods and the optimal intervals between the tests, for different tumor types, to analyze CTC and circulating tumor DNA (ctDNA) as also, to choose the best technique to isolate these tumor compartments.

Despite their well-known weaknesses, many discoveries about the utility of CTCs in prognosis were made with CellSearch, the majority of them with breast cancer. The abundance of studies focused on this disease is reasonable, as about 30% of patients with negative axillary lymph nodes and about 50% of those with positive axillary lymph nodes will relapse within 5 years. So far, there are no sensitive markers recommended for follow-up of patients surgically treated [16]. There is no method useful to monitor micrometastases, predict relapse, and guide drug selection [15]. For patients with no symptoms and no particular findings in clinical examinations, CA15-3 (Cancer antigen 15-3) and CA 27-29 (Cancer antigen 27-29) are not recommended [16]. That is the reason why it is vital to look for new prognostic and predictive biomarkers for breast cancer.

Some studies with CTCs in early-stage breast cancer observed that positivity rates from 9.4 to 48.6% and the presence of one or more CTC/7.5 mL of blood were related to early recurrence and poor overall survival [2, 13, 20].

By getting all results from all trials with diverse techniques to evaluate CTCs, in diverse solid tumors, there is one conclusion: CTC enumeration represents an established prognostic, but not a predictive biomarker. It is a useful finding, considering that conventional serum tumor markers, such as CA-125 (cancer antigen-125), PSA (prostate-specific antigen), and CEA (carcinoembryonic antigen), for example, lack sensitivity and specificity for monitoring and early diagnosis [26]. However, we and other researchers believe that these cells can be predictive markers [4], and efforts have been made in this sense.

It is important to emphasize that CTCs, cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA), extracellular vesicles, circulating tumor RNA (tRNA), tumor proteins and tumor-educated platelets (TEP) are all derived from tumor cells, and, therefore, are considered liquid biopsy. They bring complementary information to each other. Here, we discuss only CTCs, a tumor compartment that can elucidate the

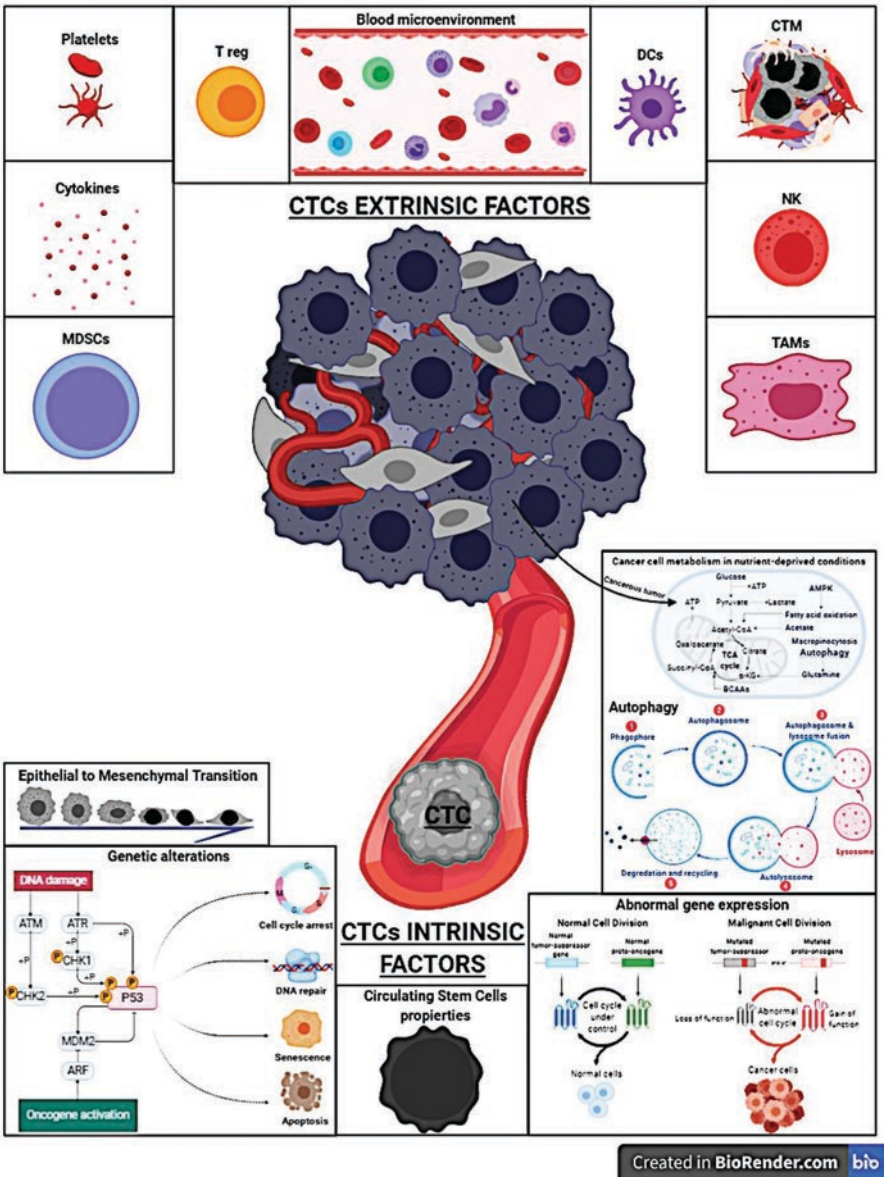

Fig. 1.1 Extrinsic and intrinsic factors related to CTCs/CTM survival

mechanism of metastasis (Fig. 1.1) and to be used to test drugs in vitro. Our intention is to describe the main discoveries about these cells, as well to focus on the cytopathologic aspects of them, as a way to share our experience with other researchers.

Extrinsic factors that can contribute to CTC survival in bloodstream include platelets and TGF-β produced by them, which allows EMT, protects CTCs from anoikis, from NK cell attack and help CTC to intravasate, together with factors produced by neuthophils, such as NETs (neutrophils extracellular traps). Cytokines produced by CTCs as also by Tregs (regulatory T cells) and dendritic cells (DCs) contribute to CTC survival and myeloid derived suppressor cells (MDSCs) recruitment, which will corroborate, inhibiting the inflammatory system. Tumor-associated macrophages (TAMs) help CTCs in the blood traffic, by releasing cytokines and by fusion with CTCs, making an immune "camouflage." Intrinsic factors include genetic alterations that can lead to EMT, tumor senescence, tumor DNA repair, apoptosis, necrosis, and tumor cell cycle arrest. Intrinsic factors include also altered cellular metabolism and abnormal gene expression. All these intrinsic factors together can contribute to the formation of CTCs with tumor stem cells feature, which need to better evaluate in clinical studies.

1.2 Brief Overview of CTC Capture Technologies

In the last few decades, the number of treatment options for patients with metastatic cancer has significantly increased, creating a need for biomarkers to determine whether the tumor(s) will respond to the proposed therapy, monitor, and anticipate resistance and response to treatment. Ideally, these biomarkers would be obtained by minimal invasive means to allow sampling in series for a long period. The identification and characterization of CTCs, for molecular analyzes of tumor heterogeneity, as well as the responsiveness to drugs, can satisfy this need.

Currently, there are two major strategies for enrichment of CTCs, those based on biological properties with marking cell surface, and those based on physical characteristics such as density, size, electric charge, combined with detection techniques, such as immunofluorescence, immunohistochemistry, for identification of CTCs. Among the bio-based technologies is the CellSearch system (applied clinically, but lacking CTCs, which have undergone epithelial-mesenchymal transition) [12] and RosetteSep technique that enhances CTCs without phenotypic excluding CD45+ and CD36+ cells and eliminating them by gradient centrifugation on a Ficoll-Paque plus density.

Recently, a review was published showing that EpCAM-based methods can be useful, and maybe, pivotal, for isolating CTCs from breast, prostate, and small cell lung cancer. It seems that EpCAM can also be involved in EMT process in CTCs from those type of cancers. It corroborates the many findings published on literature showing the utility of CTC counts by CellSearch in separating patients with breast and prostate cancer with good versus poor prognosis (overall and progression free survival) [6].

Employing the strategy of isolating single live CTCs without fixation, there is the DEPArray™ method, a microfluidic system that classifies single live CTCs based on dielectrophoresis, which is capable of detecting rare cells and in minimal

quantities of blood [3, 11, 27]. To analyze the expression of various cell surface markers in CTCs, and the establishment of xenografts, the FAC technique (fluorescence-activated cell classification) was adapted for molecular characterization of CTCs [1]. However, none of the methodologies can fully correspond to the heterogeneity of CTCs. Certainly, each technology has its advantages and limitations. New ideas in CTC biology must be integrated with current techniques enrichment, detection, and isolation to optimize the process and improve its reliability. The RosetteSep and FACS were used for in vivo models (transplantation of CTCs in mouse to verify if they form tumors) establishment. Enrichment using RosetteSep can be advantageous due to the lack of phenotypes in tumorigenic CTCs and a higher recovery rate [1].

Methods based on physical properties with filtering systems have been developed to capture CTCs based on size compared with leukocytes, especially ISET® (*Isolation by Size of Tumor Cells*), CellSieve™ (Creatv MicroTech), Flexible Micro Spring Array (FMSA), Metacell™, and ScreenCell®, capable of detecting CTCs and CTM using micropore polycarbonate filters [5, 9, 29].

The size-based methods are promising approaches to isolate CTCs. These methods usually implicate on blood filtration after erythrocyte lysis and cell fixation, followed by cytomorphological analysis. The principle of these track-etched microfilters is retaining cells according to their sizes, since it is well reported that the majority of CTCs are larger than normal and mature immune cells. Based on this assumption, leukocytes pass through pores and are eliminated. It is known that some types of tumors, such as small cell lung cancer, contain small CTCs that could be lost in the sample processing. However, the rationale between the variation of CTC size and clinical relevance is not clear. In addition, these methods bring an advantage of evaluation of blood components by light or fluorescent microscope that usually are observed together with CTCs/CTM, such as neutrophils with altered adhesive capacity, TAMs, blasts, fibrin, and platelet. The clinical meaning of these components needs to be studied.

Another promising method is one that combines filtration (high-density microporous chip filter) with antibody-based separation of CTCs [17]. A study published by Lee et al. [19] used this technique to evaluate CTCs from 11 breast cancer patients, histological grades II and III (Smart Biopsy™ System Isolation kit; Cytogen, Inc., Seoul, Korea). After isolating CTCs by this antibody-independent method, they divided the sample in two: one half undergone immunofluorescent staining with anti-EpCAM and the CTCs from the other undergone cancer gene panel analysis. Mutations were found in CTCs from all 11 patients. Curiously, in one patient whose CTCs did not stain for EpCAM, mutations in CDKN2A and IDH2 were found, and another one, tested negative for all tested mutations, despite having the highest number of EpCAM-positive cells. These findings show that although EpCAM is considered nowadays an essential protein for detection of CTCs from breast cancer, some cells can be lost using this marker or over detected (as discussed exhaustively in this book).

The use of microfluidic platforms is quite recent. These platforms enrich CTC and CTM according to their physical properties; however, improvements have been

made combining 3D microfluidics structures and specific antigens, such as geometrically enhanced differential immunocapture (GEDI) microfluidic device, using anti-PMSA (anti-prostate specific membrane antigen) [18].

So, after this brief presentation, we hope you, our reader, enjoy this book – Atlas of Liquid Biopsy, that we prepared carefully and lovingly for you. You will note that a lot needs to be done in this area of circulating tumor cells and we invite you to join us in this journey!

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Chapter 2
CTCs in Solid Tumors. Clinical Applications of Circulating Tumor Cells in Breast Cancer

Douglas Guedes de Castro and Felipe Ko Chen

2.1 Introduction

Breast cancer (BC) is one of the most studied types of cancer since the last century. For this reason, numerous studies have investigated the correlation between circulating tumor cells (CTCs) and BC [1].

When we consider using CTCs as a biomarker, it becomes necessary to differentiate early BC (eBC) from metastatic BC (mBC). About 70% of patients with mBC stage IV have >1CTC in 7.5 ml of blood, using CellSearch system to isolate and quantify CTCs. However, in eBC, using this system, we rarely detect CTCs, prompting doubts about its clinical use as a biomarker.

The objective of this chapter is to evaluate the validity and clinical applicability of CTCs in early and advanced BC [2].

2.2 Micrometastasis Biomarkers in BC

Before the use of CTC as a biomarker of micrometastasis in BC, various studies tried to use bone marrow tumor cells (BMTCs) as a viable biomarker.

In 4 of 8 studies analyzed by Bidard et al., in 2016 [1], there was a correlation between BMTCs and CTCs that reached up to 94%. This same study concluded that

Figures separated by Ludmilla T.D. Chinen and revised by Mauro Saieg (Cytopathologist from AC Camargo Cancer Center, São Paulo, SP – Brasil)

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the dissemination of tumor cells in the patients' blood indicated an initial phase disease, while the detection of BMTCs indicated a more advanced disease [1].

In most mBC studies, the preferred method used to identify CTCs is the CellSearch system. This system relies on a semi-automated enrichment and immunostaining device that has been, to this day, the only validated method approved by the US Food and Drug Administration (FDA) to detect CTCs and for prognostication in metastatic colorectal, prostate, and breast carcinomas. This specificity was reliably documented in normal individuals and in patients with benign tumors [8]. CTCs were defined by the CellSearch system as those co-expressing EpCAM and CKs without expressing leukocyte common antigen CD45, and positive for 4″,6-diamidino-2-phenylindole (DAPI) with a nucleus inside the cytoplasm and cell size '4 μm. It is important to emphasize that CTC detection using the CellSearch system does not rely on any true morphological criteria, but rather on the magnitude of antibody fluorescent signal for CK, DAPI, and CD45. The CellSearch system is an epithelium-associated marker-dependent method; therefore, it faces technical problems similar to the PCR-based molecular method; its inability to identify epithelial–mesenchymal transition (EMT)-induced CTCs can give false-negative results [3–5].

Another well-cited method of detecting CTCs is the ISET (isolation by size of epithelial tumor cells) method. ISET methodology is a direct method for CTC and circulating tumor microemboli (CTM) identification, in which CTCs are isolated by filtration without use of tumor-associated markers, as a consequence of their large size relative to circulating blood leukocytes. This method is easy to perform, rapid, and inexpensive and makes it possible to directly isolate and count tumor cells in patients with different types of carcinomas, by cytopathological analysis [6].

A study commanded by Farace in 2011 [7] comparing CellSearch and ISET methods, using different metastatic carcinomas, demonstrated quite considerable discrepancies between the number of CTCs enumerated by the CellSearch and the ISET systems. In total, 30% of patients were negative according to CellSearch, while only 5% were negative using ISET. Interestingly, these discrepancies depended mostly on the patients' tumor type. Specifically, in patients with mBC, CTC counts were generally higher by CellSearch than by ISET. However, CTCs identified by CellSearch may not be true CTCs, because CTCs detected by CellSearch on the basis of the expression of an epithelial marker (EpCAM), which does not formally establish the malignant nature of circulating cells in the blood retained as CTC. Thus, the lower CTC counts obtained by ISET compared with CellSearch, most likely results from cell loss during the ISET procedure. It is important to state that this study did not compare the clinical relevance of both methods.

Although well-designed clinical trials are essential to further understand the clinical applications of ISET, this system could indeed represent a more accurate clinical tool for predicting patient's outcome in certain tumor types, and provide a significant advantage for performing molecular analyses in the era of personalized medicine.

A review conducted by Ma in 2013 [9], confirmed these results. They concluded that, overall, more CTCs were detected by ISET than by the CellSearch system, for two reasons: (1) the CellSearch system may not detect cells if they have undergone EMT (i.e., lack expression of CK and/or EpCAM), while ISET can be much more

efficient in isolating all rare cells of interest; (2) while ISET can isolate CTMs from metastatic cancer patients, the CellSearch cannot [10, 11]. Therefore, the detection of blood samples that only have CTMs will be underestimated by the CellSearch systems that use epithelial-marker-positive selection. However, the CellSearch system may overestimate CTCs in peripheral blood samples if they are contaminated with normal epidermal cells. In addition, the CTC detection efficiency varies in all relevant studies, whether by ISET or by CellSearch system. One of the main advantages of the CellSearch system is that it has the capacity to detect smaller CTCs than does ISET. On the other hand, the use of ISET for detection and identification of CTCs is more reliable than the CellSearch system and requires no expensive or special laboratory equipment. However, ISET is not sufficiently standardized in its current form to be routinely applicable in clinical practice (please see some pictures of CTCs isolated from metastatic breast cancer patients in Figs. 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, and 2.13).

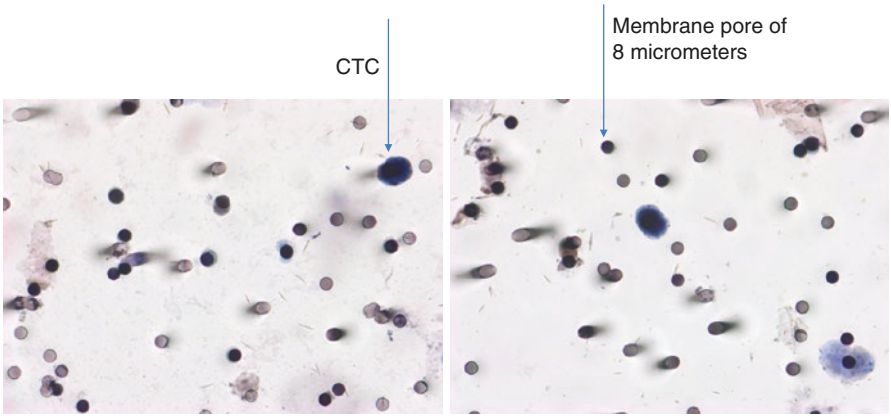

Fig. 2.1 CTCs from a woman, 53 years old, whose primary tumor was HER-2 positive. She had brain metastasis. The CTC count was 5 CTCs/mL. Her CTCs did not stain for HER-2

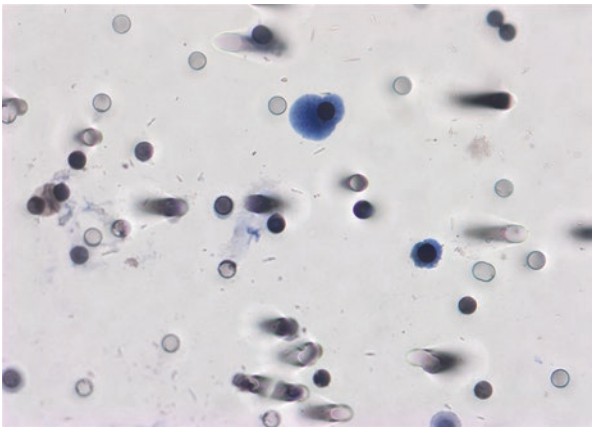

Fig. 2.2 CTCs from the same patient of Fig. 2.1. CTCs were collected around 4–5 weeks after radiotherapy for brain metastatis. CTC count: 3.0 CTCs/mL

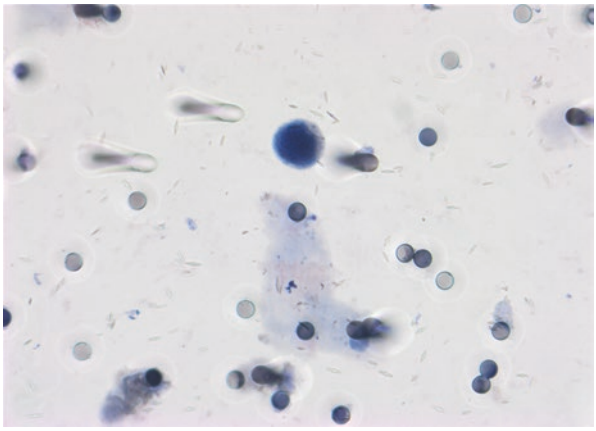

Fig. 2.3 Patient with 44 years old. CTCs were collected before the beginning of radiotherapy for brain metastatis. Her primary tumor was HER-2 positive. CTC count: 1.5 CTCs/mL, without HER-2 staining

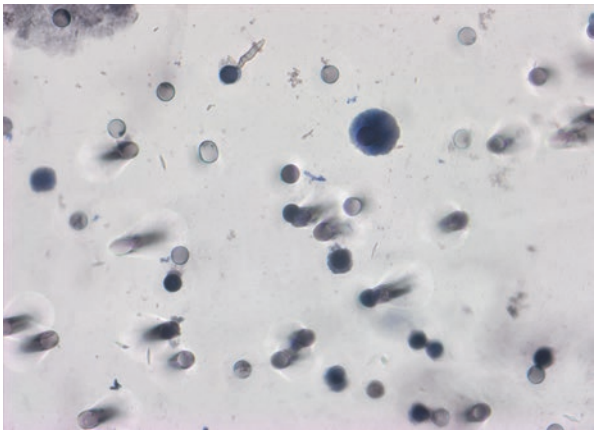

Fig. 2.4 CTCs from patient of Fig. 2.3. CTCs were collected around 4–5 weeks after radiotherapy for brain metastatis. CTC count: 1.5 CTCs/mL. We can observe the presence of a hyperchromic nucleus, irregular, with irregular chromatin. Also note the abundant cytoplasm, not commonly seen in hematopoietic cells. In brown: positive staining with DAB for STGAL

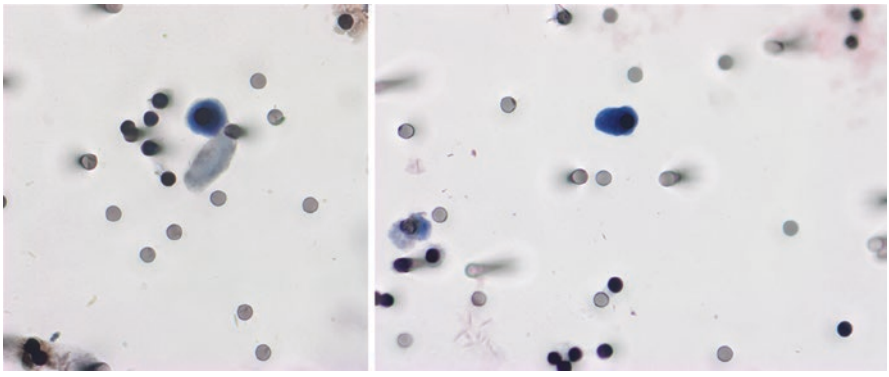

Fig. 2.5 Patient with 57 years old. CTCs were collected before the beginning of radiotherapy for brain metastatis. Her primary tumor was HER-2 positive. CTC count: 0.75 CTCs/mL. On the right, we can observe the presence of a hyperchromic nucleus, irregular, with irregular chromatin. Also note the abundant cytoplasm, not commonly seen in hematopoietic cells. CTC stained with HER-2

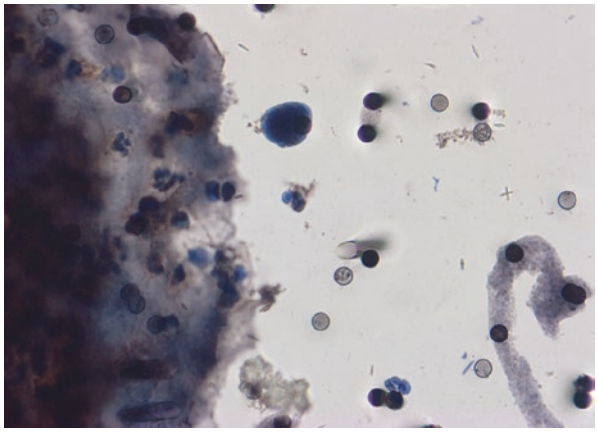

Fig. 2.6 Patient with 56 years old. CTCs were collected around 4–5 weeks after radiotherapy for brain metastatis. Her primary tumor was HER-2 positive. CTC count: 2.0 CTCs/mL. CTCs did not stain for HER-2

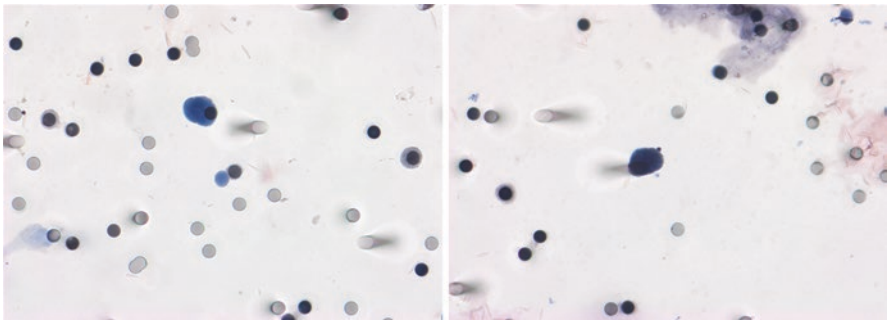

Fig. 2.7 Patient with 40 years old. CTCs were collected before the beginning of radiotherapy for brain metastatis. Her primary tumor was Luminal B. CTC count: 3.5 CTCs/mL (microscope: 20×)

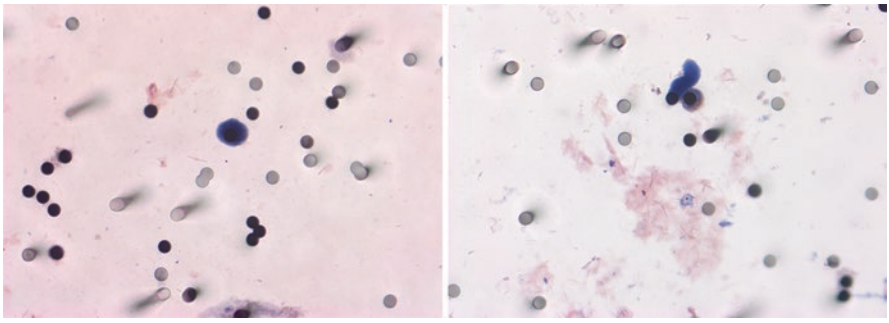

Fig. 2.8 Patient with 46 years old. CTCs were collected around 4–5 weeks after radiotherapy for brain metastatis. Her primary tumor was Luminal B. CTC count: 3.5 CTCs/mL (microscope: 20×)

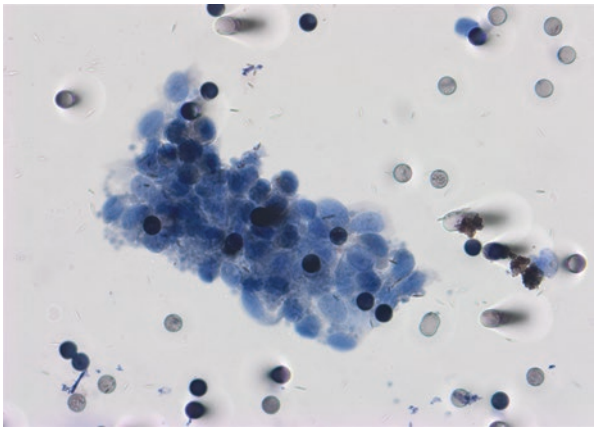

Fig. 2.9 Photo from same patient Fig. 2.8 showing a cohesive group of neoplastic cells, with planetary aggregation, forming neoplastic impaction. Individually, isolated neoplastic cells are noted with alteration of the nuclear/cytoplasmic ratio and irregularity of chromatin (microscope 40×)

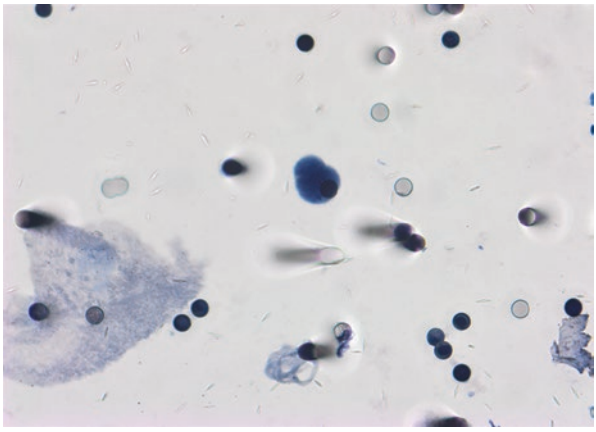

Fig. 2.10 CTM from a patient with 42 years old. CTCs were collected before the beginning of radiotherapy for brain metastatis. Her primary tumor was Luminal B. CTC count: 1.75 CTCs/mL

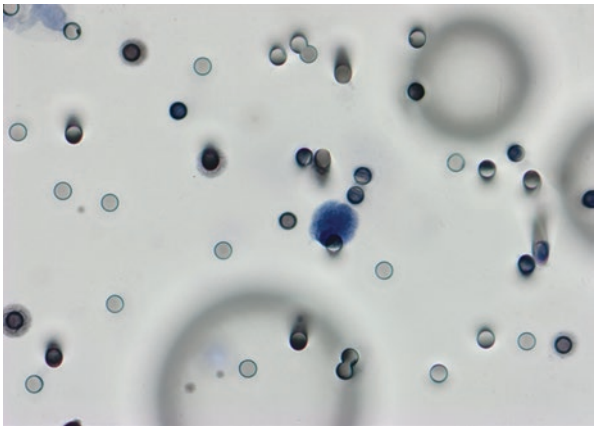

Fig. 2.11 Patient with 61 years old. CTCs were collected around 4–5 weeks after radiotherapy for brain metastatis. Her primary tumor was Luminal B. CTC count: 8.75 CTCs/mL

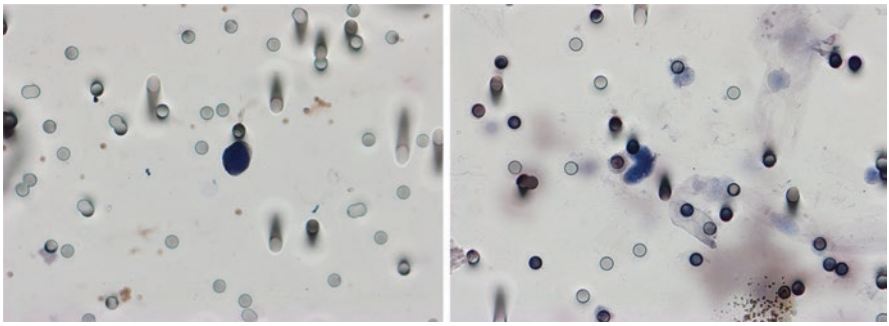

Fig. 2.12 Same patient of Fig. 2.11 in brown : immunocytochemistry with anti-Notch antibody visualized with DAB. Here, we can see a CTC without any staining

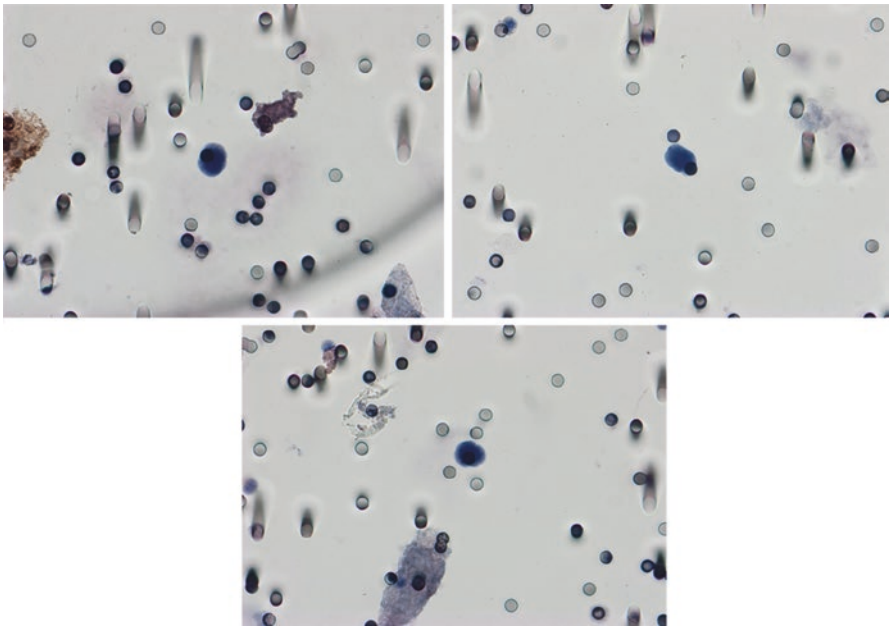

Fig. 2.13 Same patient of Fig. 2.11

2.3 Metastatic BC

2.3.1 Clinical Validity of CTCs in mBC

In contrast to that observed in eBC, there is enough evidence to utilize CTCs as a biomarker in mBC.

A study conducted by Cristofanilli in 2004 [12], utilizing the CellSearch® system to detect CTCs, analyzed the number of CTCs in patients with mBC. Before

initiating a new treatment, patients underwent an evaluation of metastatic sites by means of standard imaging studies and the collection of a blood sample to be used for the enumeration of circulating tumor cells. A different blood sample was collected at the first follow-up visit, approximately 3 to 4 weeks after the initiation of the new therapy. Disease status follow-ups were made every 9 to 12 weeks, utilizing the same techniques used at baseline. This disease status was assessed without knowledge of the levels of CTCs. An alternate control group made up of 72 premenopausal healthy women and 73 postmenopausal healthy women without known illnesses and no oncologic history, 99 women with benign breast diseases, and 101 women with other nonmalignant diseases. The respective testing laboratories were aware that the samples were from a control group, but were unaware to the difference between no known illness and benign conditions.

A worse prognostic relation was established in patients with a high number of CTCs in both instances, when compared to those with a low number of CTCs pre-CT and after one cycle. Interestingly, patients with a high CTC count pre-CT, but with a low count after one cycle, had a similar prognostic value to those with a low pre-CT count. These results were corroborated by Hayes in 2006 [13].

Finally, an analysis of 1944 individuals indisputably established the superiority of using CTC count in comparison to traditional tumor markers, such as CEA and CA15, as a treatment response biomarker in patients with mBC [14].

2.4 Clinical Applicability of CTC in mBC

In a retrospective study conducted by Cristofanilli in 2018 [15], 2436 patients with mBC from 18 cohort studies were analyzed. These patients were arranged in accordance to their tumor's biomolecular type, location, and previous treatments. A cut-off point of 5 CTCs per 7.5 ml of blood was established. Thus, a > 5CTC/7.5 mL count was determined as IV aggressive (IVa) and <5CTC/7.5 mL count as IV indolent (IVi).

Patients IVi had a higher median overall survival, when compared to those stage IVa (36.3 months vs. 16.0 months, $p < 0{,}0001$). Furthermore, patients IVi had a higher overall survival in all tumor subtypes when compared to IVa: positive hormone receptor (44 months vs. 17.3 months, $P < 0.0001$), HER2-positive (36.7 months vs. 20.4 months, $P < 0.0001$), and triple-negative (23.8 months vs. 9.0 months, $P < 0.0001$). Similar results were obtained independent of previous treatment or tumor location [15].

2.5 Early BC

2.5.1 CTCs as a Micrometastasis Marker in Patients with eBC Treated with Neoadjuvant Therapy

Measuring CTCs in patients, submitted to neoadjuvant chemotherapy (CT), intents on evaluating if the micrometastasis process has started and possibly evaluating its response to QT.

The IMENEO meta-analysis observed a significant association between T staging and CTCs ($P < .001$), using CellSearch system. Excluding tumors T4d from analysis, they observed that a positive CTC result was detached from clinical or pathological characteristics of the initial tumor. The positivity was 21.4% and 24.2% in patients with negative and positive lymph nodes, respectively. This study also showed that there was a statistically significant drop of CTC count at the end of neoadjuvant QT ($p < 0.001$). Furthermore, the CTC count pre-QT presented itself as a strong independent indicator of distant metastasis (hazard ratio [HR]: 3.73, 95% confidence interval [CI] = 2.82–4.90), overall survival (HR: 3.93, 95% CI = 2.81–5.45) and local relapse (HR: 3.02, 95% CI = 1.88–4.75) [16]. Curiously, the survival impact was directly related to the number of CTCs detected, suggesting the use of CTCs as a quantitative biomarker in BC (see some examples in Table 2.1).

2.5.2 CTC as a Micrometastasis Marker in Patients with eBC Treated with Adjuvant Therapy

In the context of adjuvant therapy in eBC, a multicentric randomized German study, SUCCESS-A, which tested CTCs in patients eligible to receive adjuvant CT, correlated the positivity of CTC to the lymph node status. This study confirmed that CTCs are an independent factor for disease-free survival (HR: 2.11, 95%CI = 1.49–2.99) and overall survival (HR:2.18, 95%CI = 1.32–3.59). Finally, a high CTC count was associated with worse prognosis, validating the use of CTCs as a quantitative biomarker [17]. The recently published 2-year follow-up of this study showed that those patients that had a positive CTC count after 2 years of treatment had a risk 3.9 times higher of death and 2.3 times higher of relapse in the multivariate models, when compared to those that had a negative result; all these results were true in those patients with HER2-negative BC [18].

In 2018, Sparano et al. [19] conducted a study that analyzed the recurrence of CTC detection after 4.5–7.5 years of follow-up in patients with HER2-negative BC

Table 2.1 Studies with CTCs in breast cancer

Tumor	Stage	No. Patients	Method	Markers	Authors	Year	Main results	Abbreviations
Breast	IV	177	CellSearch™ method (Menarini Silicon Biosystems, LLC)	CD45 and expressing cytokeratin	Cristofanilli et al.	2004	CTCs =/> 5 per 7.5 ml of whole blood, compared with the group with <5 circulating tumor cells per 7.5 ml, had a shorter median progression-free survival ($P < 0.001$) and shorter overall survival ($P < 0.001$). At the first FU visit after the initiation of therapy, this difference between the groups persisted ($P < 0.001$). The levels of CTCs at baseline and at the first FU visit were the most significant predictors of progression-free and overall survival	CTC: circulating tumor cells
Breast	IV	177	CellSearch™ method (Menarini Silicon Biosystems, LLC)	CD45 and expressing cytokeratin	Hayes et al.	2006	Median PFS times for patients with <5 CTC were 7.0, 6.1, 5.6, 7.0, and 6.0 months, respectively. For patients with ≥5 CTC, median PFS was significantly shorter: 2.7, 1.3, 1.4, 3.0, and 3.6 months, respectively. Median OS for patients with <5 CTC was all >18.5 months. For patients with ≥5 CTC, median OS was significantly shorter: 10.9, 6.3, 6.3, 6.6, and 6.7 months, respectively. Median PFS and OS times at baseline and up to 9 to 14 weeks after the initiation of therapy were statistically significantly different	FU: follow-up
Breast	IV	1944	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HER2 and HR	Bidard et al.	2014	46·9% patients had a CTC count of 5 per 7·5 mL or higher at baseline, which was associated with decreased progression-free survival ($p < 0·0001$) and overall survival ($p < 0·0001$) compared with patients with a CTC count of less than 5 per 7·5 mL at baseline. Increased CTC counts after start of treatment, adjusted for CTC count at baseline, was associated with shortened progression-free survival ($p < 0·0001$) and overall survival (HR 2·26)	PFS: progression-free survival

Breast	Early	2026	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HR	Rack et al.	2014	Before chemotherapy, CTCs were detected in 21.5% of patients, with 19.6% of node-negative and 22.4% of node-positive patients showing CTCs ($P < .001$). No association was found with tumor size, grading, or hormone receptor status. After chemotherapy, 22.1% of patients were CTC positive. The presence of CTCs was associated with poor disease-free survival (DFS; $P < .0001$), distant DFS ($P < .001$), breast cancer-specific survival ($P = .008$), and overall survival (OS; $P = .0002$). CTCs were confirmed as independent prognostic markers in multivariable analysis for DFS ($P < .0001$) and OS ($P = .002$). The prognosis was worst in patients with at least five CTCs per 30 mL blood (DFS: HR = 4.51, OS: HR = 3.60)	OS: overall survival
Breast	N1 or High-risk node-negative HER2–negative	4994	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HR	Sparano et al.	2018	A positive CTC assay result was associated with a 13.1-fold higher risk of recurrence (hazard ratio, 13.1). 30.4% patients with recurrence had a positive CTC assay result at a median of 2.8 years before clinical recurrence. The CTC assay result was also positive for 4.1% patients with hormone receptor-negative disease, although only 1 patient experienced disease recurrence (this patient was CTC negative).	
Breast	IV (indolent and aggressive)	2436	CellSearch™ method (Menarini Silicon Biosystems, LLC)	Estrogen receptor, progesterone receptor, and HER2	Cristofanilli et al.	2018	Stage IVi patients had longer median OS than those with Stage IVa ($P < 0.0001$) and similarly for de novo MBC patients ($p < 0.0001$). Moreover, patients with Stage IVi disease had significantly longer OS across all disease subtypes compared to the aggressive cohort: hormone receptor-positive ($P < 0.0001$), HER2-positive ($P < 0.0001$), and triple negative ($P < 0.0001$)	IVi = indolent // IVa = aggressive

(continued)

Table 2.1 (continued)

Tumor	Stage	No. Patients	Method	Markers	Authors	Year	Main results	Abbreviations
Breast	I, II, III	2774	CellSearch™ method (Menarini Silicon Biosystems, LLC)	Hormone receptor +/ [HER2−], HER2+, triple-negative	Bidard et al.	2018	CTC detection revealed one or more CTCs in 25.2% of patients before NCT; this was associated with tumor size ($P < .001$). The number of CTCs detected had a detrimental and decremental impact on OS ($P < .001$), distant disease-free survival ($P < .001$), and locoregional relapse-free interval ($P < .001$), but not on pathological complete response. Patients with one, two, three to four, and five or more CTCs before NCT displayed hazard ratios of death of 1.09 (95% confidence interval [CI] = 0.65 to 1.69), 2.63 (95% CI = 1.42 to 4.54), 3.83 (95% CI = 2.08 to 6.66), and 6.25 (95% CI = 4.34 to 9.09), respectively. In 861 patients with full data available, adding CTC detection before NCT increased the prognostic ability of multivariable prognostic models for OS ($P < .001$), distant disease-free survival ($P < .001$), and locoregional relapse-free interval ($P = .008$)	Neoadjuvant chemotherapy (NCT)
Breast	Early stage, high risk	1087	CellSearch™ method (Menarini Silicon Biosystems, LLC)		Trapp et al.	2019	Two years after chemotherapy, 198 (18.2%) patients were CTC-positive. The median follow-up after this timepoint was 37 months. Cox regressions that included CTC status at baseline revealed that CTC status 2 years after chemotherapy had statistically significant and independent prognostic relevance for OS (hazard ratio [HR] = 3.91, 95% confidence interval [CI] = 2.04 to 7.52, $P < .001$) and DFS (HR = 2.31, 95% CI = 1.50 to 3.55, $P < .001$)	HR: hormone receptor

Breast	IV	254	AdnaTest BreastCancer and CellSearch	HER2	Fehm et al. DETECT III	2010	Using the CellSearch assay, 50% of patients had ≥5 CTCs, and HER2-positive CTCs were observed in 41% of these patients. 39% patients were CTC positive using AdnaTest BreastCancer, and HER2 positivity rate was 47%. The rate of breast cancer patients with HER2-negative primary tumors but HER2-positive CTCs was 32% and 49% using the CellSearch assay and AdnaTest BreastCancer, respectively. Concordant results regarding HER2 positivity were obtained in 50% of the patients (31/62) ($P = 0.96$, $\kappa = -0.006$)	RFS: recurrence-free survival
Breast	High risk early breast cancer	206	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HR	Janni et al. SUCCESS A	2018	The CTC status was positive in (7.8%) patients. Overall, 13 late recurrences were observed; 11 in 153 hormone receptor-positive patients and 2 in 53 hormone receptor-negative patients. In hormone receptor-positive patients, CTC status was a significant prognostic factor for RFS in univariable (hazard ratio [HR] 5.14, 95% confidence interval [CI] 1.47–18.03, $p = 0.011$) and in multivariable cox regressions adjusted for age, tumor stage, nodal stage, grade, histological type, and HER2 status (HR 5.95, 95%CI 1.14–31.16, $p = 0.035$)	NT: neoadjuvant treatment

(continued)

Table 2.1 (continued)

Tumor	Stage	No. Patients	Method	Markers	Authors	Year	Main results	Abbreviations
Breast	III	287	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HER2	Riethdorf et al. GeparQuattro Trial	2010	We detected ≥1 CTC/7.5 mL in 21.6% of patients before NT and in 10.6% after NT ($P = 0.002$). 15.0% initially CTC-positive cases were CTC-negative after NT, whereas 8.3% cases were CTC-positive after NT. CTC detection did not correlate with primary tumor characteristics. Furthermore, there was no association between tumor response to NT and CTC detection. HER2-overexpressing CTC were observed in 24,1% CTC-positive patients, including 8 patients with HER2-negative primary tumors and 3 patients after trastuzumab treatment. CTC scored HER2-negative or weakly HER2-positive before or after NT were present in 11 of 21 patients with HER2-positive primary tumors. HER2 overexpression on CTC was restricted to ductal carcinomas and associated with high tumor stage ($P = 0.002$).	
Breast, Prostate and Lung Carcinomas	IV	60	CellSearch™ method (Menarini Silicon Biosystems, LLC) and ISET (Rarecells, France)		Farace et al.	2011	Concordant results between CellSearch and ISET were obtained in 55% (11 out of 20) of the patients with breast cancer, in 60% (12 out of 20) of the patients with prostate cancer and in only 20% (4 out of 20) of lung cancer patients	

that received primary surgical treatment, followed by adjuvant CT. In the multivariate models, a positive CTC was associated with a risk 13.1 times higher of recurrence in patients with positive hormone receptors (HR: 13.1, 95% CI = 4.7–36.3). No patients with negative hormone receptors and positive assay had a recurrence of CTC (0%, 95% CI = 0% to 37%).

The TREAT-CTC trial was the first attempt to try to demonstrate the clinical applicability of CTCs in patients with eBC. This study also tried to evaluate if the addition of a new adjuvant therapy (Trastuzumab) would help to elongate the relapse-free interval in patients with a positive CTC count. This study, therefore, concluded the following: (1) CTC-based screening is feasible in the adjuvant setting of early breast cancer. (2) CTC-positive patients do have a higher risk of relapse. (3) Trastuzumab has no effect on CTCs in HER2-negative BC [20–23].

Therefore, the use of CTCs as an evaluating tool of metastatic risk in eBC still needs further scientific comprobation. However, it is highly probable that the number of CTCs will have a significant impact as a prognostic and metastatic biomarker in eBC [1].

2.6 Conclusion

The use of CTCs as a prognostic factor in early and mBC has been shown to be quite significant. Despite the detection of CTCs in eBC being a rare event, its clinical validity as a prognosis marker has reached the highest level of scientific evidence. However, its clinical applicability is still a subject to be studied.

Focusing on adjuvant treatments such as radiotherapy, QT, and hormonal therapy, and associating these with new detecting techniques and with new biomarkers such as circulation tumor DNA, will possibly reveal new treatments and early micrometastasis diagnosis [24, 25].

And finally, when we are talking about patients with mBC, the quantitative and qualitative CTC analysis must be considered an important tool with prognostic and therapeutic implications.

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Chapter 3
Circulating Tumor Cells in Head and Neck Cancer

Thiago Bueno de Oliveira

3.1 Introduction

Head and neck cancer is a broad term that encompasses epithelial malignancies originating from the paranasal sinuses, nasal cavity, oral cavity, pharynx, and larynx. Most of these cancers are squamous cell carcinoma for which the main risk factors are tobacco and alcohol [1]. Other established risk factor is HPV infection, especially for oropharynx tumors [2–4]. It is the seventh most common neoplasm worldwide, accounting for 700000 cases and 350000 deaths annually [5].

The majority of patients are diagnosed with locoregional advanced disease and are treated in a multidisciplinary approach. Despite this, however, around 50% of these patients will present disease recurrence [6, 7]. The multidisciplinary approach includes upfront surgery followed by chemoradiation [8–10], upfront cisplatin-based chemoradiation [7, 11], upfront cetuximab-based bio-radiation [12, 13], or induction chemotherapy (ICT) followed by radiation-based local treatment [14–19]. Unfortunately, there are no predictive biomarkers to guide the choice of therapy. In this scenario, the utilization of circulating tumor cells (CTCs) yields a great perspective.

One of the first trials to investigate the role of CTCs in head and neck cancer was done by Pajonk et al. [20] and analyzed 77 patients with locoregional advanced head and neck squamous cell carcinoma (LA-HNSCC) with a RT-PCR based technique for detection of CK19 positive CTCs. The detection rate was only 6.5% (5/77), and presence of CTCs was related to relapse, although without statistical

Figures separated by Ludmilla T.D. Chinen and revised by Mauro Saieg (Cytopathologist from AC Camargo Cancer Center, São Paulo, SP – Brasil)

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significance. Some years later, Jatana et al. [21] analyzed 48 patients with LA-HNSCC that underwent upfront surgery with a immunomagnetic cytokeratin-based approach and found a significant correlation of absence of CTCs and better disease-free survival (DFS) and CTC counts higher than 25/mL with worse DFS ($p = 0.01$ and $p = 0.04$, respectively). Similar results were found by Toyoshima et al. [22] analyzing 48 patients with oral cavity primary cancer submitted to surgical treatment, using a mRNA RT-PCR technique. The detection rate was 37.5%, and the absence of CTCs was related to a better DFS ($p = 0.01$).

The CellSearch System® was also utilized in head and neck cancer studies. Nichols et al. [23] found a detection rate of 40% (6/15) and a relation of CTC presence and lung nodules bigger than 1 cm ($p = 0.01$), suggesting micrometastatic dissemination. Bozec et al. [24] found lower detection rates, of 12% (6/49) in patients with oral cavity and oropharynx tumors before treatment, with no correlation with clinical endpoints.

The largest data on CellSearch® in head and neck cancer were provided by three prospective trials [25–27]. Grisanti et al. [25] evaluated 53 patients with recurrent or metastatic disease, with a detection rate of 26% (14/53) at baseline and 41% (22/53) at any time point. The presence of one or more CTCs correlated with a worse PFS (HR = 3.068; CI95%: 1.53–6.13; $p = 0.002$) and OS (HR = 3.0; CI95%: 1.48–6.00; $p = 0.002$). Disease control with systemic therapy was achieved by 8% of CTC+ as opposed by 45% of CTC− patients ($p = 0.03$). Buglione et al. [26] analyzed 73 patients with LA-HNSCC and reported a detection rate of 15% (11/73) and a correlation of response rate and absence or disappearance of CTCs during treatment ($p = 0.017$). Grobe et al. [27] reported a 12.5% detection rate (10/80) in patients with oral cavity tumors, and a correlation of CTC presence and worse recurrence-free survival (RFS; $p < 0.001$).

Two studies analyzed more than 100 patients [28, 29]. Tinhofer et al. [29] evaluated 144 patients with LA-HNSCC from oral cavity, oropharynx, larynx, hypopharynx, and cervical occult primary that underwent upfront surgical resection. CTCs were analyzed after surgery and before adjuvant radiation-based treatment, with a mRNA RT-PCR technique for EGFR positive CTC detection. The detection rate was 29% (42/144) and the prognosis impact analysis yielded mixed results. Overall, the presence of CTC was not predictive for OS or DFS. However, while in oropharyngeal carcinomas ($n = 63$), the detection of CTC was associated with a trend for improved DFS (2-year DFS: 100% for CTC+ versus 79% for CTC−; $p = 0.059$) the reverse was observed for carcinomas from other sites ($n = 81$), with 2-year DFS of 29% for CTC+ versus 75% for CTC−; $p = 0.001$. In multivariate analysis, CTC remained an independent prognostic marker for DFS (HR = 4.3; 95%CI: 1.7–10.9; $p = 0.002$) and OS (HR = 2.7; 95%CI: 1.2–6.3; $p = 0.016$) in non-oropharyngeal tumors. Liu et al. [28] analyzed 178 patients, with nasopharyngeal ($n = 135$) and hypopharyngeal ($n = 45$) squamous cell carcinoma (SCC), that underwent blood collection for CTC detection before and after treatment. CTCs were isolated using negative immunomagnetic bead enrichment and were identified by fluorescence in situ hybridization. The number of CTCs was associated with distant metastasis

($p = 0.026$) and patients with undetectable CTCs and decreasing or negative CTCs post-treatment had a better prognosis ($p < 0.05$).

What is shown in these trials is a potential role of CTCs as prognostic markers in head and neck cancer, although with some conflicting results. These mixed results were evidenced by three metanalysis exploring this potential prognostic impact of CTCs in head and neck cancer [30–32]. Wang et al. [30] analyzed 433 patients from 8 studies and showed that disease progression (recurrence/metastasis) rate in the CTC-positive patients was significantly higher (OR = 3.44; 95%CI: 1.87–6.33; $p = 0.01$). However, there was no significant correlation of CTCs and TNM (III–IV versus I–II; OR = 1.54; 95%CI: 0.87–2.72; $p > 0.05$) or nodal involvement (OR = 1.20; 95%CI: 0.67–1.90; $p > 0.05$). Wu et al. [31] analyzed 857 patients from 22 studies, but only 5 had data on survival endpoints (DFS, PFS or OS). A significant impact CTC-positivity was demonstrated for DFS (HR = 4.62; 96%CI: 2.51–8.52), but not for PFS or OS. Finally, Cho et al. [32] analyzed 429 patients from 6 studies and found that the presence of CTCs was significantly associated shorter PFS (HR = 4.88; 95%CI: 1.93–12.35; $p < 0.001$) but it was not prognostic for OS (HR = 1.92; 95%CI: 0.93–3.96; $p = 0.078$).

Another common point about these trials is that most of them rely on techniques that are dependent mainly of cytokeratin marking of the CTCs, which could translate into low detection rates, as observed in some trials. For example, the CellSearch System®, which depends on the immunomagnetic capture of EpCAM positive cells, could ignore CTCs that no longer express this marker, as occurs during the epithelial to mesenchymal transition (EMT) process. Other trials depend on complex and costly techniques, like mRNA RT-PCR. In both cases, the integrity and preservation of cellular functions is harmed, which could difficult further analysis in the cells.

Another line of research in CTC isolation utilizes microfiltration techniques, which separates the cells based on size and deformability, like the ClearCell FX System®, utilized by Kulasinghe et al. [33] to evaluate 23 patients with head and neck cancer. The detection rate was 47.5% (11/23) and CTC-positive patients had shorter PFS (HR = 4.946; 95%CI:1.571–15.57; $p = 0.0063$), while PD-L1-positive CTCs were found to be significantly associated with worse outcome (HR = 5.159; 95%CI: 1.011–26.33; $p = 0.0485$).

In general, the microfiltration assays show higher detection rates, probably related to the separation by size of the CTCs, independently of antibodies. Taking this rationale into consideration, our research group demonstrated the potential clinical applicability of the ISET (Isolation by SizE of Tumor cells, Rarecell, France) method in the management of head and neck cancer patients, both as a prognostic factor and as a predictive of treatment response. In a preliminary analysis [34] of 53 LA-HNSCC patients, analyzed for CTCs at baseline and after treatment (first follow-up), we found a detection rate of 92.5% (49/53) at baseline and 93.8% (30/32) at first follow-up. Circulating tumor microemboli (CTM), defined as a cluster of 3 or more CTCs, were found in 28.3% ($n = 15$) at baseline (CTM1) and 23.3% ($n = 7$) in the first follow-up (CTM2). Comparing CTM1 with CTM2, patients with unfavorable evolution (CTM1 negative/CTM2-positive) had PFS of 17.5 months,

patients always CTM-negative showed PFS of 22.4 months, and those always positive, 4.7 months ($P < .001$). The TGF-βRI (transforming growth factor beta type I receptor) expression in the first follow-up correlated with poor PFS (12 x 26 months; $p = 0.007$), being an independent prognostic factor (HR = 6.088; $p = 0.033$). These data showed the importance of CTCs and CTM kinetics, the variation between pre- and post-treatment results, as also the possibility of investigating prognostic and predictive biomarkers expression in the CTCs, once the cells were well preserved for this (please see some pictures of CTCs isolated from patients with localized head and neck cancer in Figs. 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 3.10, 3.11, 3.12, 3.13, 3.14, 3.15, 3.16, 3.17, 3.18, 3.19, 3.20, 3.21, 3.22, 3.23, 3.24, 3.25, and 3.26).

Later, our group presented the final analysis of this trial [35], including 83 LA-HNSCC patients, demonstrating a detection rate of 94% (79/83) and a significant correlation of CTC counts and survival. For each increase of 1 CTC at baseline there was a relative increase of 18% in the risk of death (HR = 1.18; CI95%:

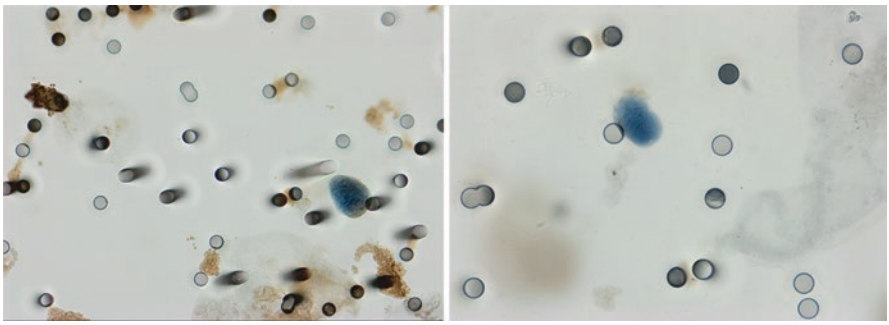

Fig. 3.1 CTCs from a men, 64 years old, with squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage III disease. Patient had 3.85 CTCs/mL. In brown: DAB (anti-EGFR). In blue: hematoxylin (microscope 40×)

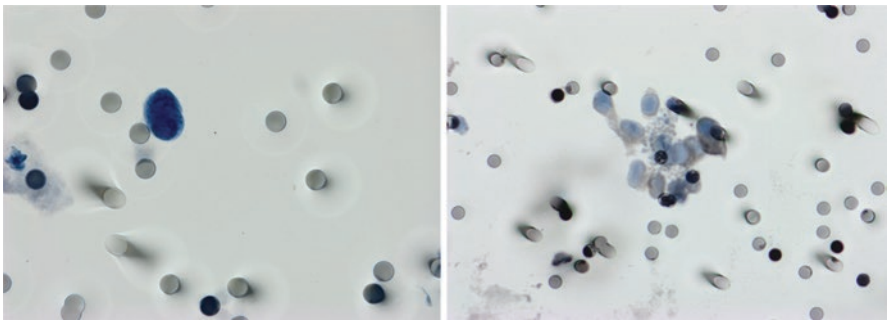

Fig. 3.2 CTCs from the same patient Fig. 3.1. Second blood collection, after until 3 months of treatment. Patient had 1.80 CTCs/mL. In the right figure: Membrane and cytoplasm staining with DAB for anti-MRP-7 antibody (microscope 40×)

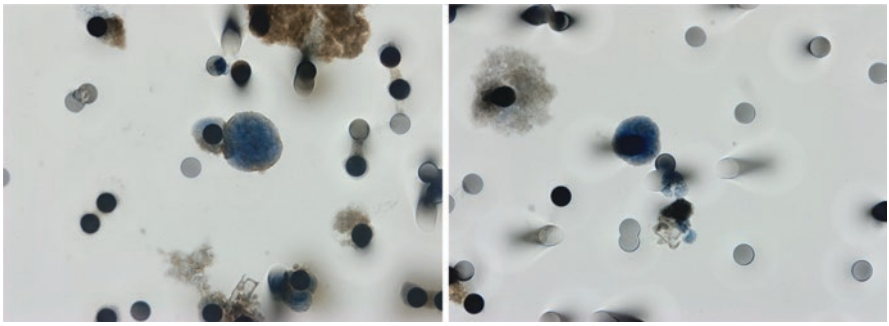

Fig. 3.3 CTCs from a men, 60 years old, with poorly differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVa disease. Patient had 3.42 CTCs/mL and developed lung metastasis; on the left we can see a cell stained with anti-EGFR. (microscope 40×)

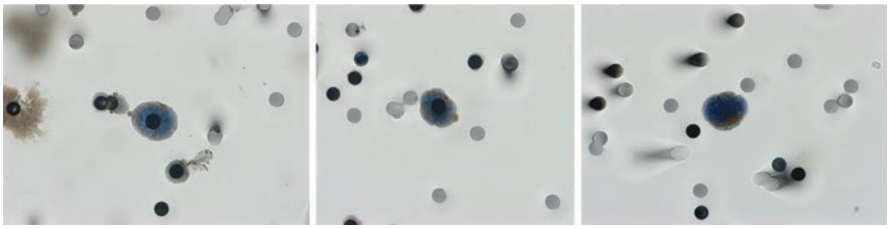

Fig. 3.4 CTCs from the same patient Fig. 3.3. Cell staining for EGFR (microscope 40×)

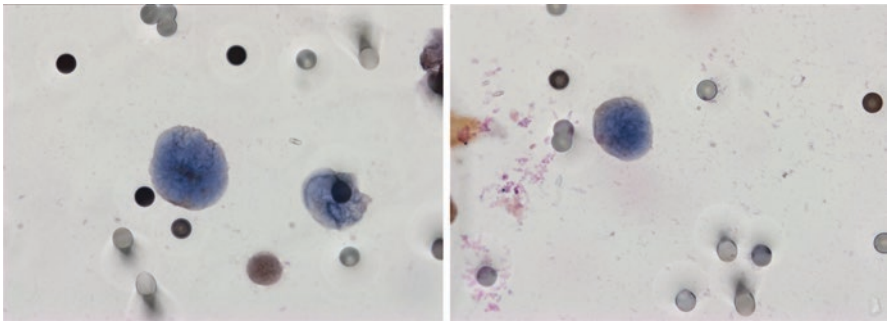

Fig. 3.5 CTCs from a men, 75 years old, with moderately differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVB disease. Patient had lymph node metastasis and 4.0 CTCs/mL (microscope 40×)

1.06–1.31; $p < 0.001$), 16% in the risk of progression (HR = 1.16; CI95%: 1.04–1.28; $p = 0.004$), and a reduction of 26% in the odds of complete response to treatment (nonsurgical group only – OR = 0.74; CI95%: 0.58–0.95; $p = 0.022$). We also established cut-off points of baseline CTCs for OS and PFS, patients with CTCs < 6.5/ml had an estimated 2-year OS of 85.6% versus 22.9% for CTCs ≥ 6.5/ml (HR = 0.18; CI95%: 0.06–0.49; $P < 0.0001$) and patients with CTCs ≤ 3.8/ml had an estimated

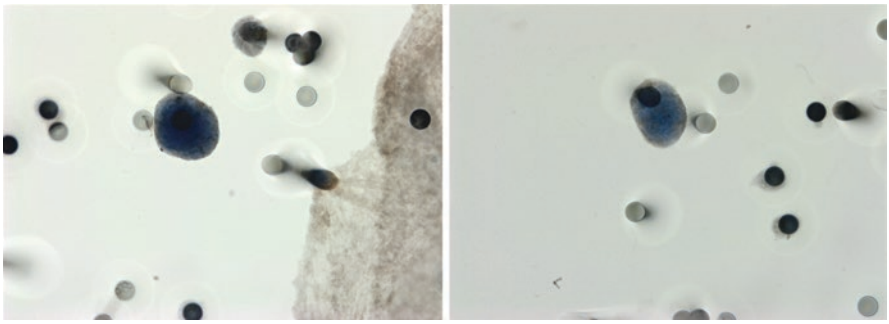

Fig. 3.6 CTCs from a men, 59 years old, with poorly differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had 4.0 CTCs/mL at first collection. At second collection, no response to treatment, 10 CTCs/mL, and recurrence in the bone, liver and lungs. In brown: antiEGFR staining (microscope 40×)

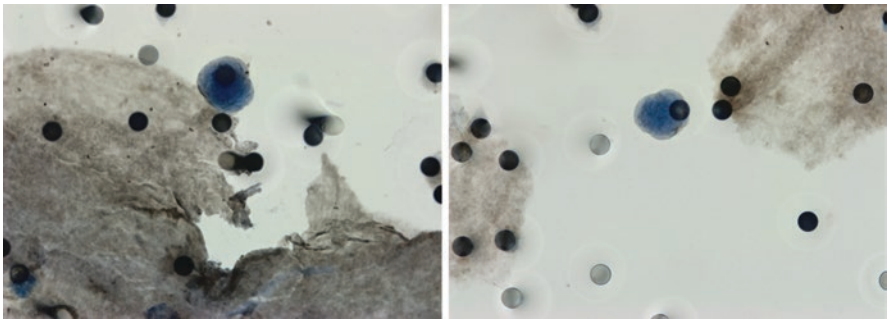

Fig. 3.7 CTCs from the same patient Fig. 3.6 (microscope 40×)

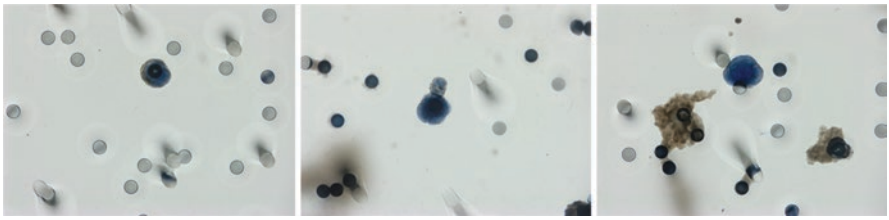

Fig. 3.8 CTCs from a men, 59 years old, with squamous cell carcinoma. Blood was collected after 3 months of treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had 11.6 CTCs/mL at this point. In brown: anti-EGFR staining

2y PFS of 71.8% versus 37% for CTCs > 3.8/ml (HR = 0.32; CI95%:0.15–0.67; $p = 0.001$). In a subgroup analysis of 67 patients treated with a curative nonsurgical approach [36], the presence of CTM was correlated with worse OS (HR = 3.01; IC95%: 1.06–8.52; $p = 0.029$) and PFS (HR = 3.84; IC95%: 1.62–9.11; $p < 0.001$). High CTC counts (cut-off 3.8/mL) and CTM were potential predictors of benefit of

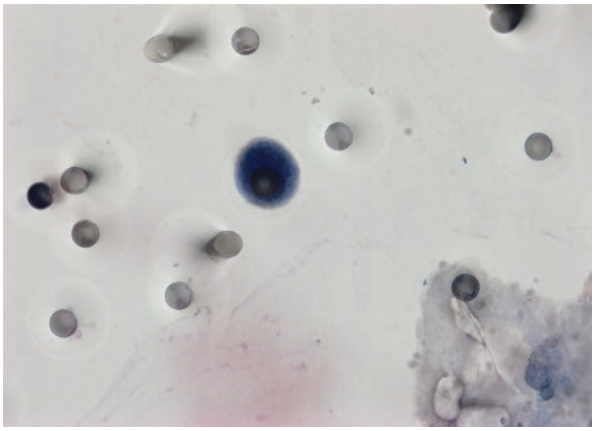

Fig. 3.9 CTCs from a men, 72 years old, with moderately differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage III disease. Patient had 2.75 CTCs/mL (microscope 40×).

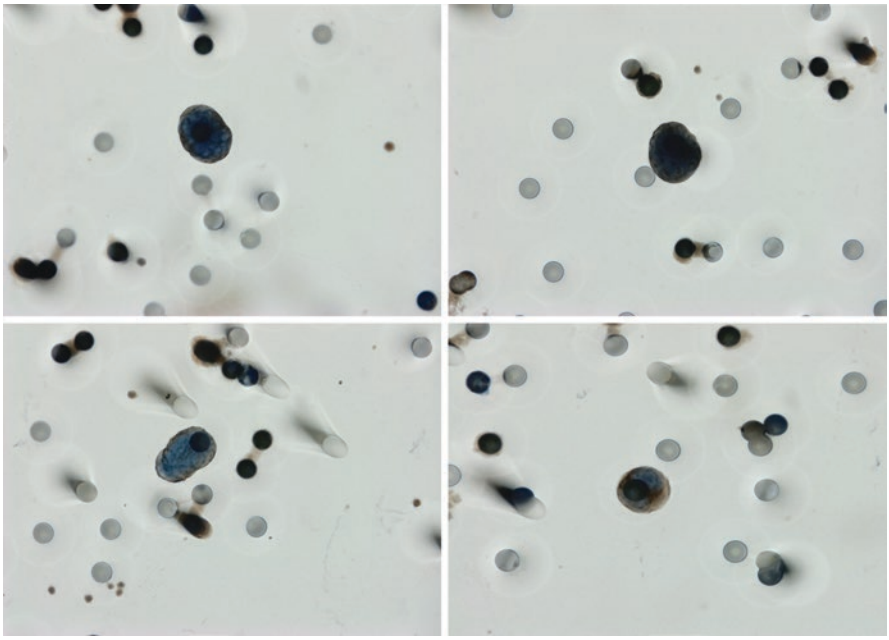

Fig. 3.10 CTCs from a woman, 68 years old, with poorly differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had lymph node metastasis and 2.0 CTCs/mL at this point. In brown: anti-EGFR staining (microscope 40×)

ICT. In patients with CTCs ≤ 3.8 CTCs/mL 2-year OS was 88% for ICT versus 80% for initial radiotherapy (RT) (HR = 0.55; IC95%: 0.10–1.84; p = 0.470), while in patients with CTCs > 3.8/mL 2-year OS was 79% for ICT versus 30% for initial RT (HR = 0.32; IC95%: 0.07–1.38; p = 0.112). The same was observed with CTM, with

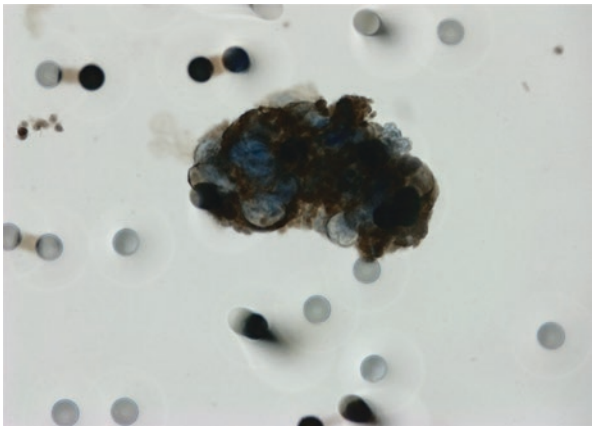

Fig. 3.11 CTCs from the same patient Fig. 3.10. Microemboli staining for MRP-7 (microscope 40×)

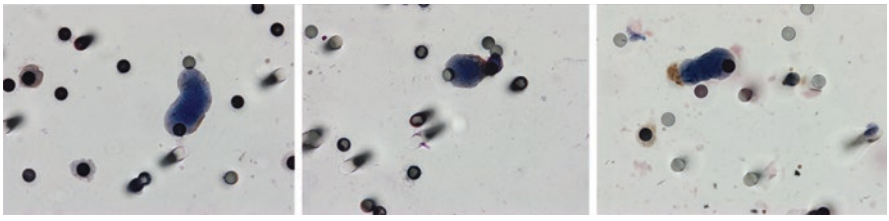

Fig. 3.12 Cell staining for β-tubulin. CTCs from a woman, 48 years old, with squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had lymph node metastasis and 2.0 CTCs/mL (microscope 40×)

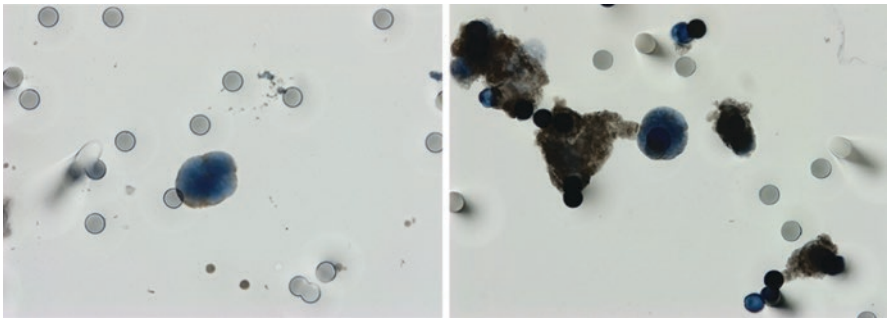

Fig. 3.13 CTCs from a men, 73 years old, with squamous cell carcinoma. Blood was collected before treatment with induction chemotherapy with carboplatin plus paclitaxel, for stage IV disease. Patient had 2.0 CTCs/mL. Cell staining for MMP-2 (metalloproteinase 2). We can observe in both pictures an irregular nuclei and high proportion nuclei/cytoplasm (microscope 40×).

worse outcomes for initial RT in CTM-positive patients, in comparison to the other groups (CTM positive undergoing ICT or CTM negative) both for OS ($p = 0.020$) and PFS ($p < 0.001$).

In summary, CTCs have potential prognostic impact in head and neck patients, both for baseline counts and presence of CTM, as well as for kinetics evolution

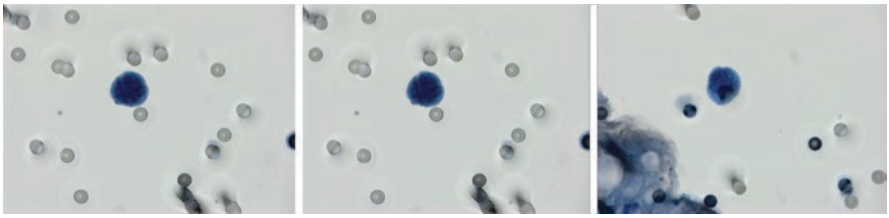

Fig. 3.14 CTCs from a men, 51 years old, with squamous cell carcinoma. Blood was collected before treatment with induction chemotherapy with carboplatin plus paclitaxel, for stage IVA disease. Patient had 2.7 CTCs/mL (microscope 40×)

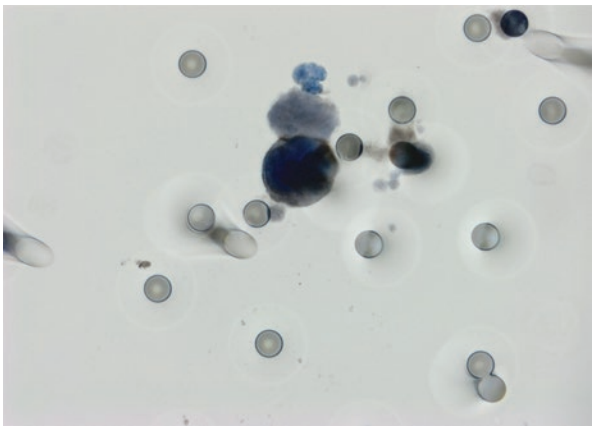

Fig. 3.15 CTC from the same patient Fig. 3.14. We can observe a large cell with irregular and lobular hyperchromic nuclei. In brown: membrane and cytoplasmic staining with anti-MMP-2 (microscope 40×)

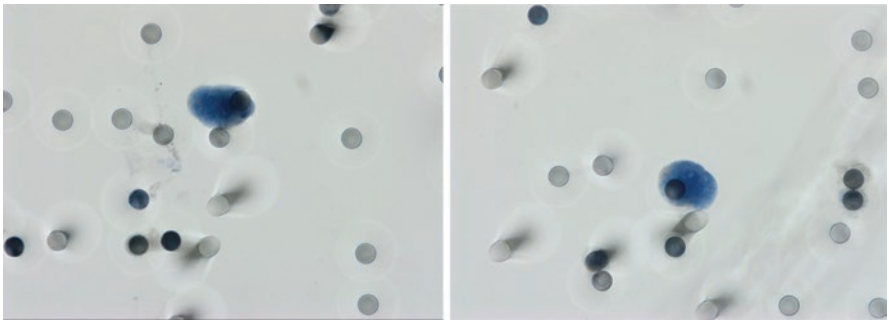

Fig. 3.16 CTCs from a men, 78 years old, with squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had lung metastasis and 4.71 CTCs/mL (microscope 40×).

during treatment (see Table 3.1). It also has a predictive role, especially with techniques that preserves cell integrity allowing biomarker evaluation in the CTCs and CTM. The results utilizing the ISET method are compelling, given the high detection rates, the undisputable prognostic, and the potential predictive role in this scenario.

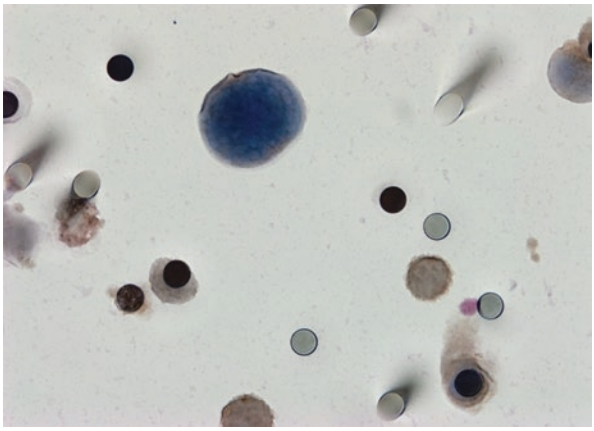

Fig. 3.17 CTCs from a men, 81 years old, with squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cetuximab upfront, for stage IVA disease. Patient had 4.75 CTCs/mL. In brown: staining with anti-EGFR (microscope 60×)

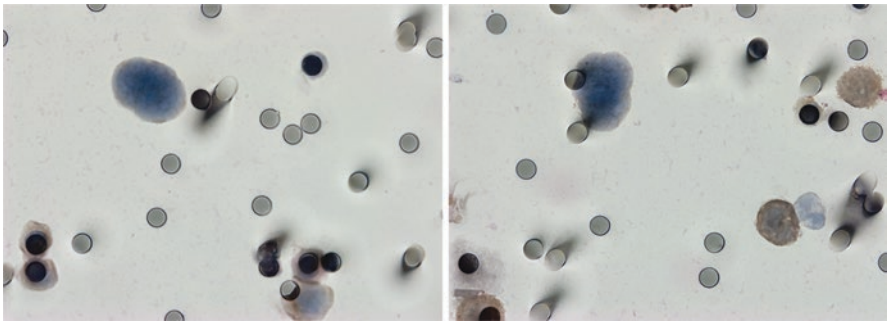

Fig. 3.18 CTCs from the same patient Fig. 3.17. Cell staining for anti-MMP-2 (microscope 40×)

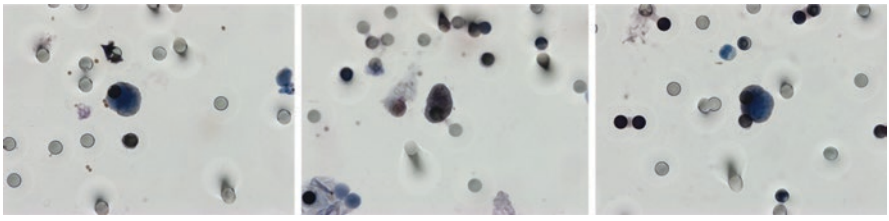

Fig. 3.19 CTCs from a men, 79 years old, with moderately differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage III disease. Patient had 3.0 CTCs/mL. Cell staining for anti-TGF-β receptor I (microscope 40×)

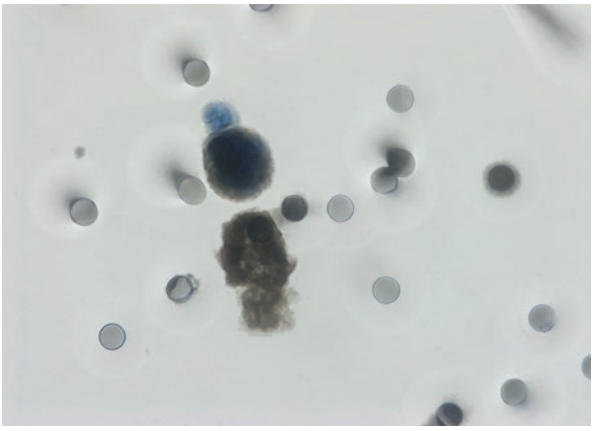

Fig. 3.20 CTCs from a men, 76 years old, with moderately differentiated squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cetuximab, for stage IVA disease. Patient had 3.57 CTCs/mL. Cell staining for anti-MMP-2 (microscope 60×)

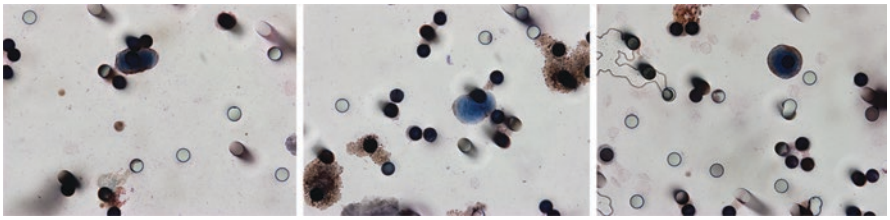

Fig. 3.21 CTCs from a men, 65 years old, with squamous cell carcinoma. Blood was collected before treatment with induction chemotherapy followed by radiotherapy and carboplatin, for stage IVB disease. Patient had 0.5 CTCs/mL. Cell staining for anti-MRP-7 (multidrug resistance protein-7) (microscope 40×)

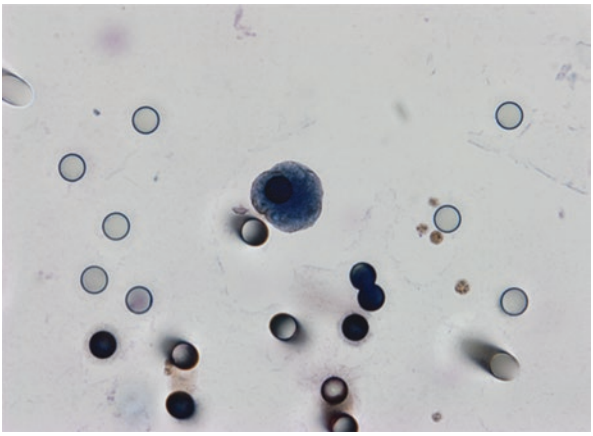

Fig. 3.22 CTCs from a men, 46 years old, with well-differentiated squamous cell carcinoma. Blood was collected after until three months of treatment with radiotherapy and cetuximab, for stage III disease. Patient had 4.0 CTCs/mL. We can observe a large cell with irregular nuclei and membrane staining for EGFR (microscope 40×)

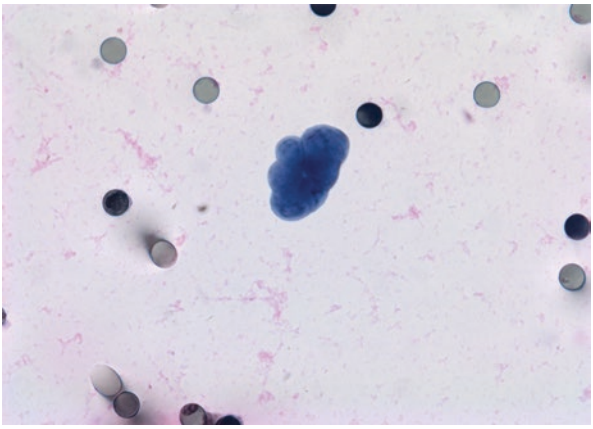

Fig. 3.23 CTCs from a men, 80 years old, with squamous cell carcinoma. Blood was collected after until 3 months of treatment with radiotherapy and cetuximab upfront, for stage IVA disease. Patient had lymph node metastasis and 2.4 CTCs/mL (microscope 40×)

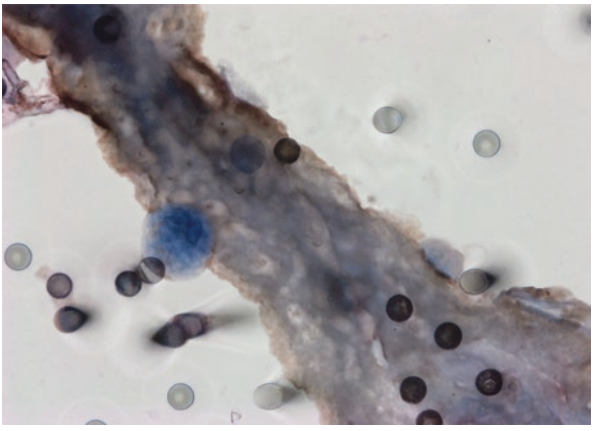

Fig. 3.24 CTCs from a men, 48 years old, with squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had 4.25 CTCs/mL. We can observe a cell with irregular nuclei (microscope 60×)

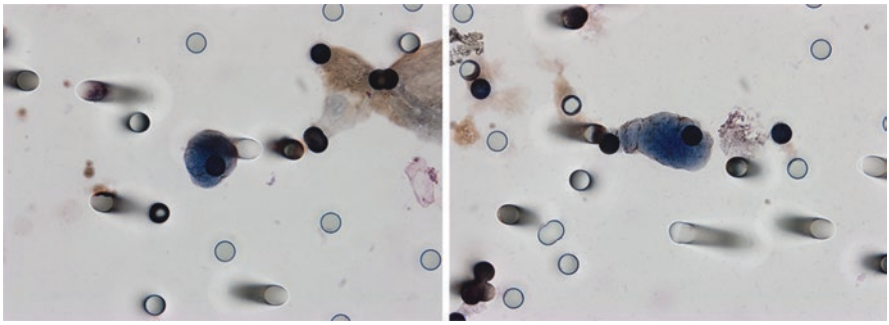

Fig. 3.25 CTCs from a men, 52 years old, with squamous cell carcinoma. Blood was collected after until 3 months of treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had lymph node metastasis and 1.2 CTCs/mL. In brown: cell staining for EGFR (microscope 40×)

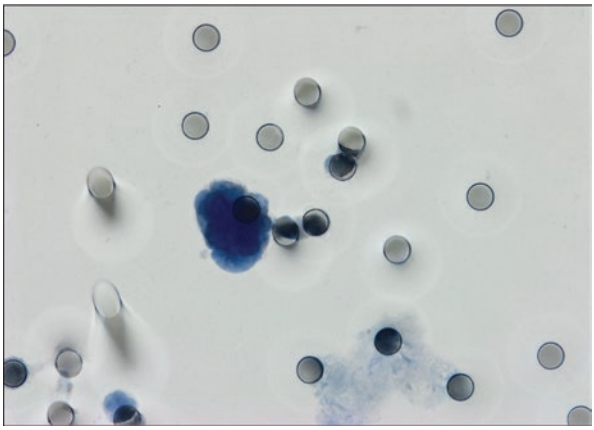

Fig. 3.26 CTCs from a men, 60 years old, with squamous cell carcinoma. Blood was collected before treatment with radiotherapy and cisplatin, for stage IVA disease. Patient had 1.6 CTCs/mL

Table 3.1 Main studies with CTCs in head and neck cancer

Tumor	Stage	*n*	Method	Markers	Authors	Year	Main results
Head and neck	III/IVA/ IVB	49	CellSearch	EpCAM, CK	Bozec A et al.	2013	Detection rate of 16% (8/49). No correlation of CTCs and N stage (p = 0,12)
Head and neck	III/IVA/ IVB	73	CellSearch	EpCAM, CK	Buglione A et al.	2012	Partial or complete response (CR) was related with the absence or disappearance of CTC during treatment (p = 0.017). A decrease in the CTC number or theirabsence throughout the treatment related with nonprogressive disease (p = 0,009)
Head and neck	Recurrent and metastatic	30	Immunomagnetic followed by RT-qPCR	CK19, EpCAM, EGFR, c-Met, PD L1, PD L2	Chikamatsu K et al.	2019	Positivity for any marker was 80% (24/30). PD-L1 expression in the tumor tissue did not correspond completely with that in the CTCs
Head and neck	II/III/IVA/ IVB	22	RT-qPCR assay for HPV mRNA expression in EpCAM(+)CTCs	HPV16 E6/E7	Economopoulou P et al	2019	Significantly higher risk for disease relapse (p = 0.001) and death (p = 0.005) in patients with HPV16 E6/E7(+) baseline CTCs
Head and neck	Recurrent and metastatic	65	EPISPOT, CellSearch, and flow cytometry	EGFR, EpCAM, CK8, CK18,CK19	Garrel R	2019	Median PFS time was significantly lower in patients with increasing or stable CTC counts from D0 to D7 with EPISPOT EGFR (p = 0.0103)
Head and neck	Recurrent and metastatic	53	CellSearch	EGFR	Grisanti S et al.	2014	One CTC or more was correlated with worse PFS (p = 0.002) and OS (p = 0.002). Disease control after systemic therapy was obtained in 8% of CTC+ as opposed to 45% in CTC− (p 0.03)
Head and neck	I-IVC	80	CellSearch	EpCAM, CK	Grobe A et al.	2014	Significant correlations for CTCs and tumor size (p = 0.04),nodal status (p = 0.02), distant metastasis (p = 0.004), and recurrence-free survival (p < 0.001)

Head and neck	III/IVA/IVB	42	Flow cytometry + RT-PCR EGFR	EGFR	Hristozova T et al.	2011	CTCs were detected in 18 of 42 SCCHN patients (43%). No correlation between CTCs with T stage or tumor volume
Head and neck	II-IVC	53	PowerMag System	Podoplanin, EpCAM	Hsieh JC et al.	2015	PDPN+/EpCAM+ CTCs ratio > 20% was a significant prognostic factor for 6 months death ($p = 0.011$) and was correlated with poor PFS ($p = 0.016$) and OS ($p = 0.015$)
Head and neck (oral cavity)	III/IVA/IVB	40	Flow cytometry	EpCAM	Inhestern J et al.	2015	CTCs at baseline >median associated to risk of recurrence ($p = 0.014$). Maximal CTC values >median during the complete course of therapy had a significantly lower OS than patients with values <median ($p = 0.049$)
Head and neck	I-IV	48	Immunomagnetic CK+	Cytokeratin 8, 18, and 19	Jatana KR et al.	2010	Patients with no detectable CTCs had a significantly higher probability of disease-free survival ($p = 0.01$)
Head and neck	I-IV	23	Clear Cell FX System	PD L1	Kulasinghe A et al.	2018	CTC+ patients had shorter PFS ($p = 0.0063$), and PD-L1-positive CTCs were significantly associated with worse outcome ($p = 0.0485$)
Head and neck	I-IV	178	Immunomagnetic + FISH	None	Liu K et al.	2020	CTCs were associated with distant metastasis ($P = 0.026$). Patients with undetectable CTCs and decreasing or negative CTCs post-treatment tended to have a good prognosis ($P < 0.05$)
Head and neck	I-IVC	82	SERS (surface-enhanced Raman scattering nanotechnology)	None	Morgan TM et al.	2019	CTC counts correlated with DFS ($p = 0.047$)

(continued)

Table 3.1 (continued)

Tumor	Stage	*n*	Method	Markers	Authors	Year	Main results
Head and neck	I-IV	94	RT-qPCR	PD L1, EpCAM	Strati A et al.	2017	Patients with CTCs overexpressing PD-L1 at end of treatment had shorter PFS ($p = 0.001$) and OS ($p < 0.001$)
Head and neck	I-IVC	44	RT-qPCR	EPCAM, MET, KRT19, and EGFR. CTC+ only: PIK3CA, CCND1, SNAI1, VIM, CD44, NANOG, ALDH1A1, CD47, CD274, and PDCD1LG2	Tada H et al.	2020	CTC-positivity was significantly correlated with treatment resistance ($p = 0.0363$), locoregional recurrence ($p = 0.0151$), and a shorter PFS ($p = 0.0107$)
Head and neck	III/IVA/IVB	144	RT-PCR	EGFR mRNA	Tinhofer I et al.	2014	CTC+ is an independent prognostic marker for worse DFS ($p = 0.002$) and OS ($p = 0.016$) in non-OPC
Head and neck	II-IVB	53	Flow cytometry	EpCAM	Wang HM et al.	2019	CTC decline was an independent prognostic factor for PFS ($p = 0.03$) and OS ($p = 0.05$)
Head and neck	III/IVA/IVB	53	ISET	TGF-bRI	Fanelli MF et al.	2017	Patients always CTM-negative showed PFS of 22.4 months, those always positive, 4.7 months ($p < 0.001$). The TGF-βRI expression in the first follow-up correlated with poor PFS (12 3 26 months; $p = 0.007$)
Head and neck	III/IVA/IVB	83	ISET	None	Oliveira TB et al.	2019	For each increase of 1 CTC at baseline, there was a relative increase of 18% in the risk of death (HR = 1.18; 95%CI: 1.06–1.31; $p < 0.001$), 16% in the risk of progression (HR = 1.16; 95%CI: 1.04–1.28; $p = 0.004$), and a reduction of 26% in the odds of complete response to treatment (nonsurgical group only – OR = 0.74; 95%CI: 0.58–0.95; $p = 0.022$)

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Chapter 4
Circulating Tumor Cells in Colorectal Cancer

Virgilio Souza e Silva, Angelo Borsarelli Carvalho de Brito, and Daniela Costa

4.1 Introduction

Colorectal cancer (CCR) is the second most common cancer diagnosed in women and third most in men, accounting for approximately 10% of all annually diagnosed cancers and cancer-related deaths worldwide [8]. These rates also vary geographically, with the highest rates seen in the most developed countries. It is a prevalent disease in older patients, but the incidence is rising in younger ones, especially rectal cancer and left-sided colon cancer [19].

CCR is largely an asymptomatic disease until it reaches an advanced stage; in these cases, symptoms such as rectal bleeding, change in bowel habits, anemia, or abdominal pain should alert patients to look for a doctor. In asymptomatic patients, screening methods are important. Colonoscopy, occult blood in feces, and sigmoidoscopy are the most common used methods, but each one has its own limitations [12]. Thus, new and less invasive methods need to be investigated.

For metastatic CCR, systemic therapy typically includes chemotherapy backbone paired with a biological treatment. Fluoropyrimidines combined with oxaliplatin (FOLFOX) and irinotecan (FOLFIRI) chemotherapies are the most commonly used regimens [12]. In terms of response rate and survival, the addiction of a biologic (anti-VEGF or anti-EGFR) antibody in the chemotherapy regimen, depending on the tumor-specific factor, must be considered.

Figures separated by Ludmilla T.D. Chinen and revised by Mauro Saieg (Cytopathologist from AC Camargo Cancer Center, São Paulo, SP – Brasil)

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It is known that genetic intratumor heterogeneity contributes to treatment failure and drug resistance [14]. Several studies comparing mutational profiles of primary tumors and associated metastatic lesions [13, 36] and local recurrences [29] have provided evidence of intratumor heterogeneity.

Early during the formation and growth of a primary tumor, cells are shed from the primary tumor and then circulate through the bloodstream. These circulating tumor cells (CTCs) can be enriched and detected by different technologies, which take advantage of their physical and biological properties. CTC analysis is considered a real-time "liquid biopsy" for patients with cancer [3].

Compared with conventional biopsy, the "liquid biopsy" has some advantages: requires only a small amount of blood [23], is minimally invasive [24], allows early detection of cancer [17] and-real time monitoring for treatment responses and resistance, by repeated analysis [6]. Some disadvantages are the lack of standardization techniques [9] and insufficient clinical and technical validation [4].

In CCR, CTCs can be used for screening (early detection of invasive cancers), in localized cancer (risk stratification), prognosis and monitoring after treatment, and metastatic cancer (selection of therapy, monitoring of response, and resistance mechanisms).

4.2 CTCs for Colorectal Cancer Screening

Although the prognostic value of CTCs in the early stages of CCR has already been evaluated in several clinical studies, its role in screening and early detection remains controversial, but it is a very promising topic [22, 30].

The main study on CTCs with the screening approach was recently presented at ASCO 2018 with 620 participants (182 healthy controls, 111 participants with precancerous lesions, and 327 patients with stage I-IV CRC). The results were compared to a standard clinical protocol, including colonoscopy and biopsy results, revealing an overall accuracy of 88% for all stages of the disease, including precancerous lesions. It is the first study to show high sensitivity in the detection of precancerous colorectal lesions [33].

The simple collection of blood for liquid biopsy can be easily integrated into the routine physical examination of the patient, increasing adherence to the test and, thus, allowing an increase in early diagnosis without the need for invasive tests; however, we still need more studies to support this tracking strategy in colorectal cancer.

4.3 CTCs for Evaluation of Minimal Residual Disease in metastatic CCR

Treatment for patients with localized CRC consists of surgery, and in some cases, stages II and III, adjuvant treatment with chemotherapy in addition to surgery is indicated. Identifying patients at high risk of recurrence and treating them with adjuvant

therapy remains an important clinical issue. In current practice, we used tumor markers such as carcinoembryonic antigen and clinical-pathological factors to define the risk of recurrence and prognosis, with limitations in identifying minimal residual disease (MRD). Therefore, the monitoring of CTCs during post-surgical follow-up evaluations may allow the patient to better stratify in relation to the risk of recurrence.

In a study with 141 patients (stages II and III), the presence of CTCs after curative surgery was associated with worse progression-free survival and overall survival. In this study, recurrence occurred in 72.5% of patients with positive CTCs after surgery, on the other hand, recurrence occurred in only 12.2% of patients with negative CTCs [20].

A research with 138 patients showed that postoperative patients with positive CTC and negative CTC before surgery is an independent indicator of poor prognosis for CRC patients treated with curative resection [38].

A study with 130 patients with stage II-III CRC demonstrated that the postoperative CTC counts were earlier than the preoperative CTCs in predicting tumor recurrence survival in patients with non-metastatic CRC undergoing surgery. In addition, the authors developed CTC-based prognostic models to predict tumor recurrence in stage II-III CRC, which can be used to identify patients at high risk for recurrence and guide aggressive treatment to improve the clinical outcomes of these patients [35]. Please see some pictures of CTCs isolated from localized colon cancer by ISET in Figs. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11, 4.12, 4.13, 4.14, 4.15, 4.16, 4.17, 4.18, 4.19, and 4.20.

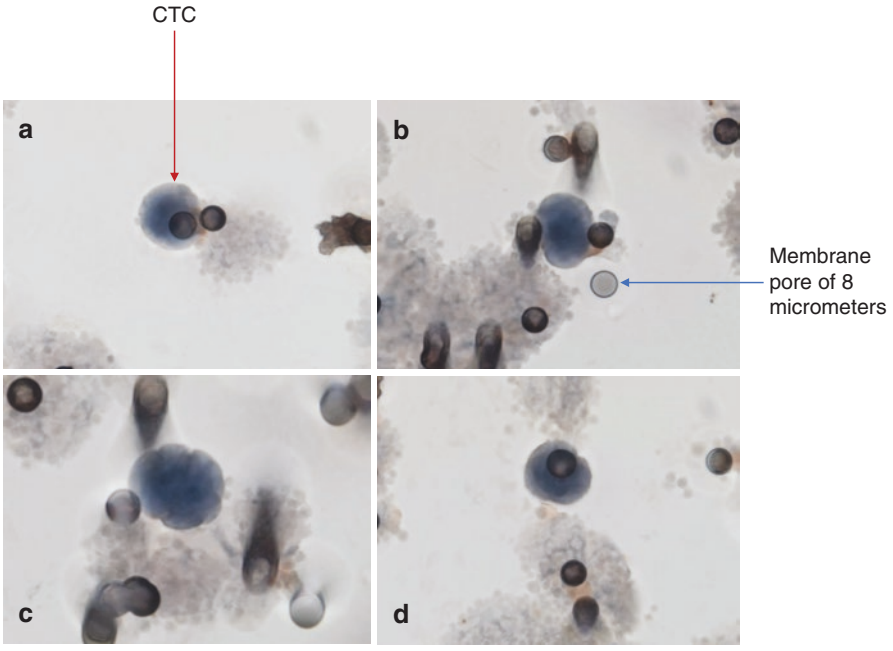

Fig. 4.1 Patient with 58 years old, male, with stage IIIC (1st collection, at diagnosis). CTC count was 4.60 CTCs/mL. The CTC count was 0.33 CTCs/mL after surgery and 4.33 CTCs/mL after adjuvancy. On letter C, we can better visualize nuclear irregularity and lobular nuclei. In boxes (**a–d**) we can oberve CTCs with different shapes

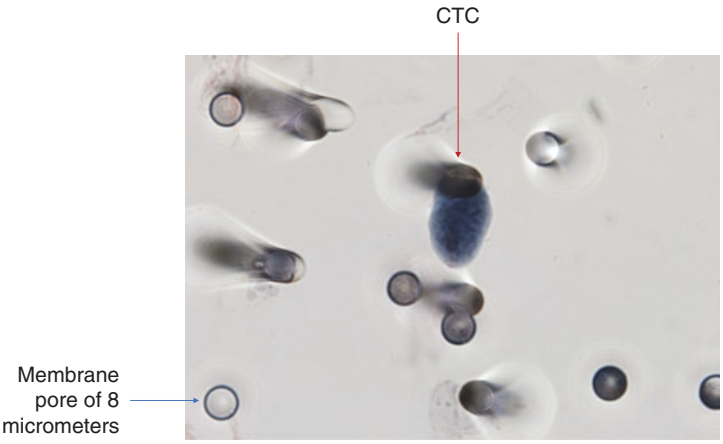

Fig. 4.2 Patient with 61 years old, male, stage IIC. Here, we can observe irregular nuclei. The CTC count was 3.80 CTCs/mL in baseline (blood collection at diagnosis)

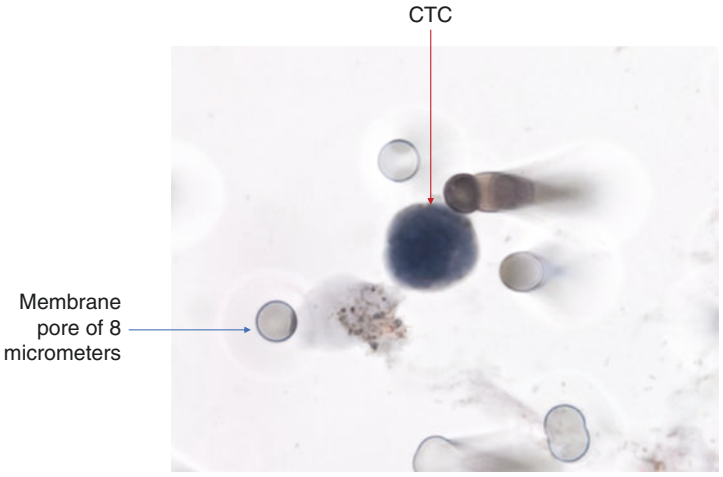

Fig. 4.3 Patient with 51 years old, male, stage IIA. The CTC count was 6.0 CTCs/mL in the 1st collection (cell with irregular nuclei and abundant cytoplasm). After surgery (second blood collection), it was 5.33 CTCs/mL

Finally, a study with 438 patients, with the objective to evaluate the presence of CTCs in the pre- and postoperative scenario in patients with colorectal cancer in stages I-III undergoing curative resection and, thus, identifying a subgroup of patients at high risk of relapse, suggested that the persistent presence of CTCs in the postoperative period can be a crucial prognostic factor, in addition to conventional tumor markers in patients with CRC undergoing curative resection. The identification of these high-risk patients with persistent positive CTCs is important and, therefore, can help to define patients for adjuvant therapy with this tumor entity [34].

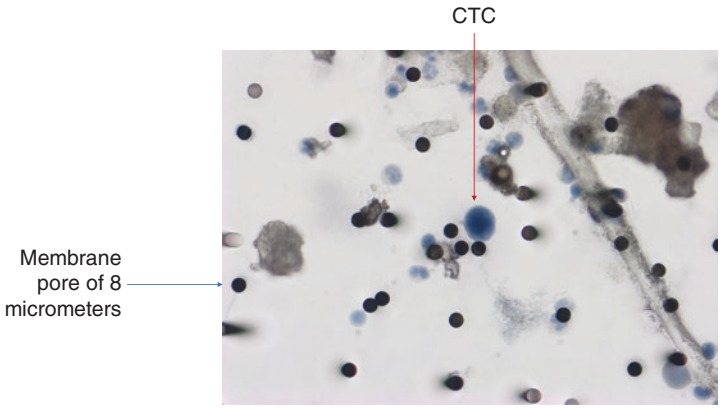

Fig. 4.4 CTCs from the same patient of Fig. 4.3. Isolated CTC of 3rd collection (after adjuvancy). The CTC count was 5.66 CTCs/mL

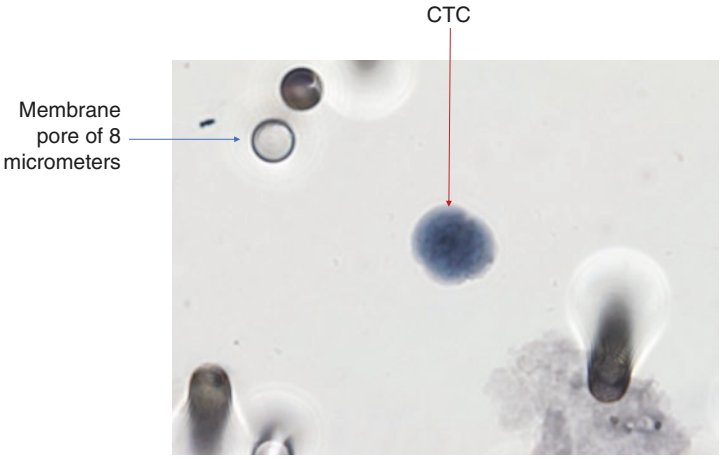

Fig. 4.5 Patient with 37 years old, woman, stage IIIB. The CTC count was 4.80 CTCs/mL in the 1st collection

4.4 CTCs for Prognostic Evaluation in Metastatic Disease

The role of CTCs in the prognostic stratification of patients with metastatic CRC has been demonstrated in several studies emphasizing that the presence of CTCs can predict future metastasis (disease progression) and unfavorable outcome as demonstrated in Table 4.1.

In a previous publication of our group, with 54 mCRC patients, we demonstrated that in addition to the initial CTC count, kinetics was also important for prognostic definition [27]. Evaluating CTC kinetics, when we compared the baseline (pretreatment) CTC level (CTC1) with the level at first follow-up (CTC2), we observed that

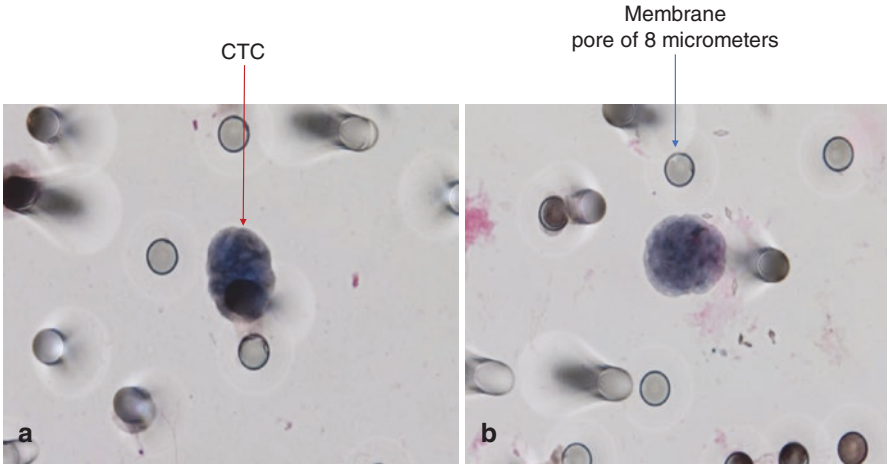

Fig. 4.6 Patient with 70 years old, male, stage I disease. The CTC count was 1.0 CTCs/mL in the 1st collection. This figure is of 2nd collection (on letter (**a**): cytoplasm staining with ERCC1). In letters (**a**, **b**), we can observe chromatin irregularity. The CTC count was 4.67 CTCs/mL

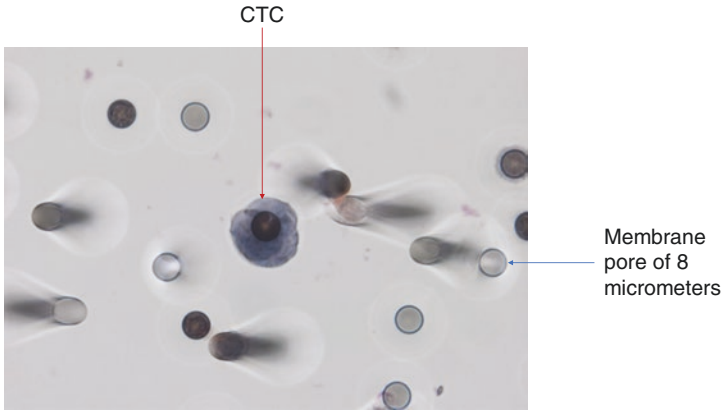

Fig. 4.7 Patient with 85 years old, woman, with stage IIA. The CTC count was 2.25 CTCs/mL at baseline. This figure is of 2nd collection (after surgery), the count was 1.33 CTCs/ml

CTC1-positive patients (CTCs above the median), who became negative (CTCs below the median) had a favorable evolution ($n = 14$), with a median progression-free survival (PFS) of 14.7 months. This was higher than that for patients with an unfavorable evolution ($CTC1^-$ that became $CTC2^+$; $n = 13$, 6.9 months; $p = 0.06$). Patients with WT KRAS with favorable kinetics had higher PFS (14.7 months) in comparison to those with WT KRAS with unfavorable kinetics (9.4 months; $p = 0.02$). Moreover, patients whose imaging studies showed radiological progression had an increased quantification of CTCs at CTC2 compared to those without progression ($p = 0.04$). This study made possible the presentation of ISET as a

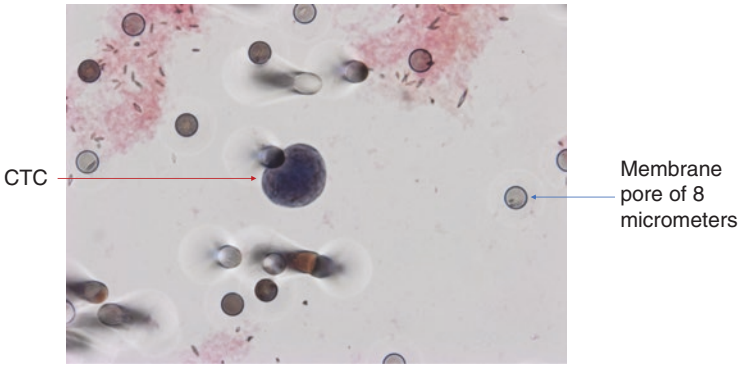

Fig. 4.8 Patient with 56 years old, woman, with stage I. The CTC count was 6 CTCs/mL at baseline. This figure is of 2nd collection (after surgery), the count was 5 CTCs/ml (cytoplasm staining for ERCC1). We can observe a classical CTC and an ISET pore

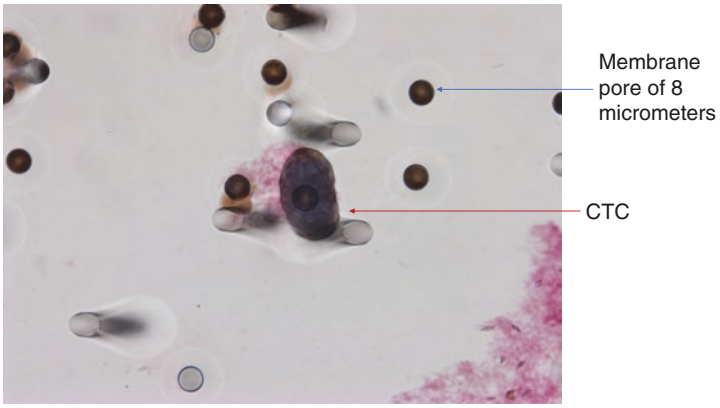

Fig. 4.9 Patient with 59 years old, woman, with stage IIIC. The CTC count was 2.50 CTCs/mL at baseline (cytoplasm staining with TIMP1)

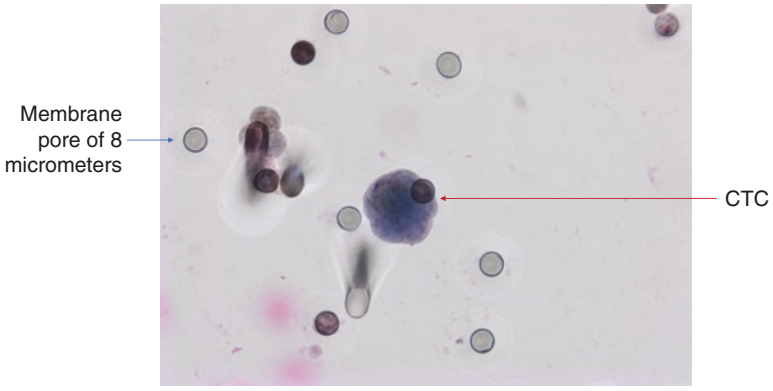

Fig. 4.10 CTCs from the same patient of Fig. 4.9. This picture is of the 2nd collection. The count was 3 CTCs/ml (cytoplasm staining for ERCC1)

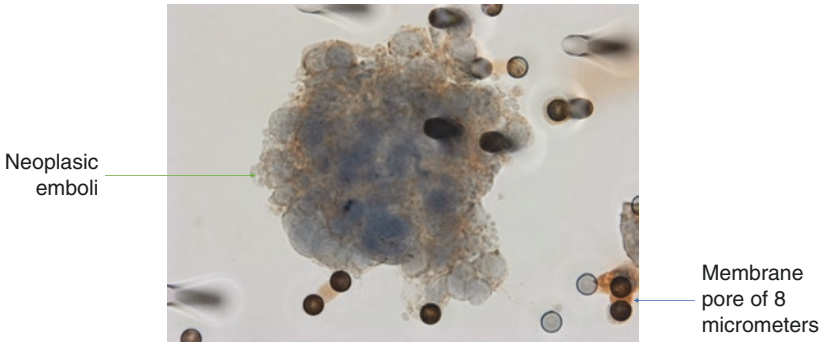

Fig. 4.11 Patient with 71 years old, woman, with stage IIIB. The CTC count was 7 CTCs/mL at baseline (cytoplasm staining with TYMS). Here, we can observe a neoplastic emboli with three-dimensional arrangement of epithelial cells

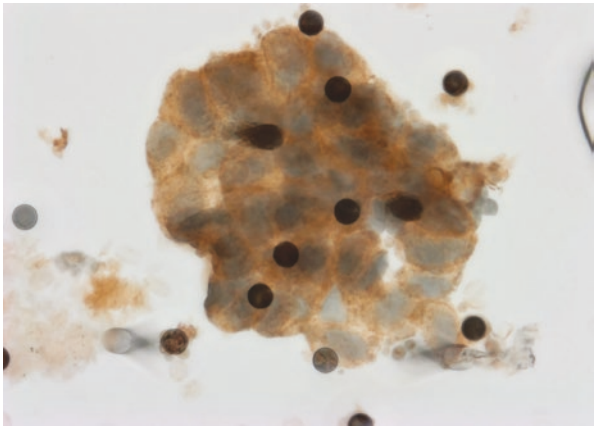

Fig. 4.12 Patient with 69 years old, man, with stage IIA. The CTC count was 3.6 CTCs/mL at baseline (microemboli staining for TYMS)

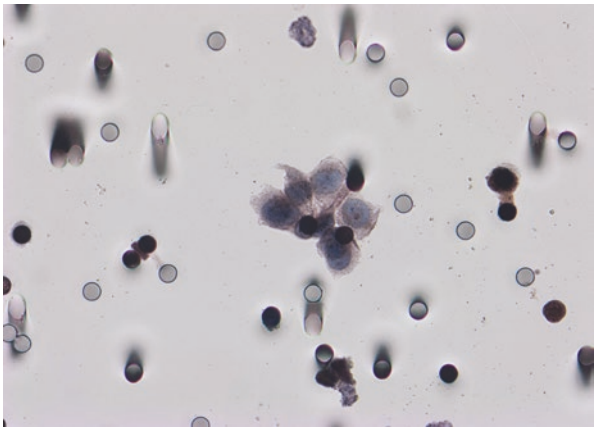

Fig. 4.13 Patient with 63 years old, man, with stage IIIB. The CTC count was 7.0 CTCs/mL at baseline (microemboli staining for β-GAL)

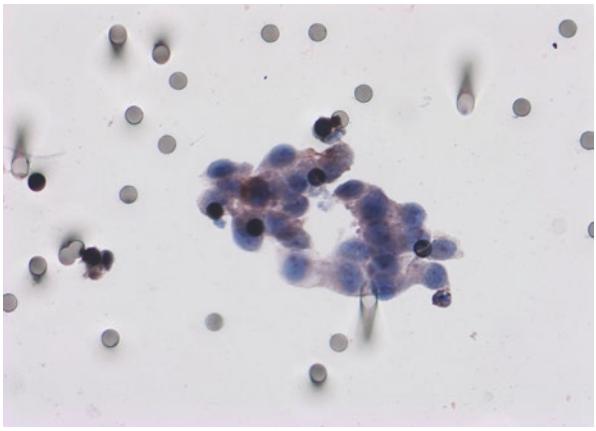

Fig. 4.14 Same patient of picture Fig. 4.13. Here, we can observe a proliferation of epithelial cells with three-dimensional arrangements and columnar-looking cells. Staining for TGF-βRI

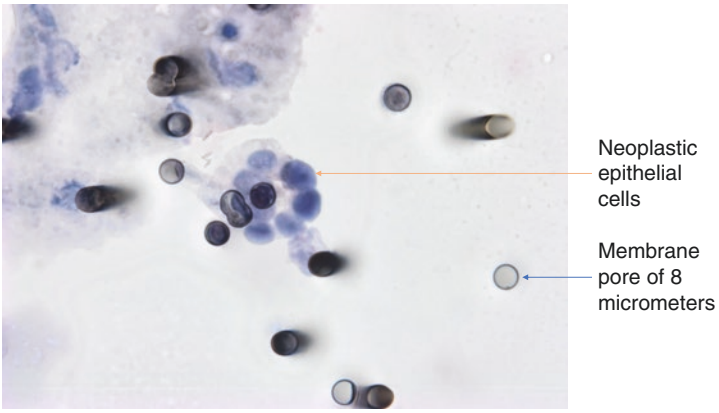

Fig. 4.15 Patient with 71 years old, woman, with stage IIIC. The CTC count was 7.0 CTCs/mL at baseline. We can see neoplastic epithelial cells sketching acinar arrangement

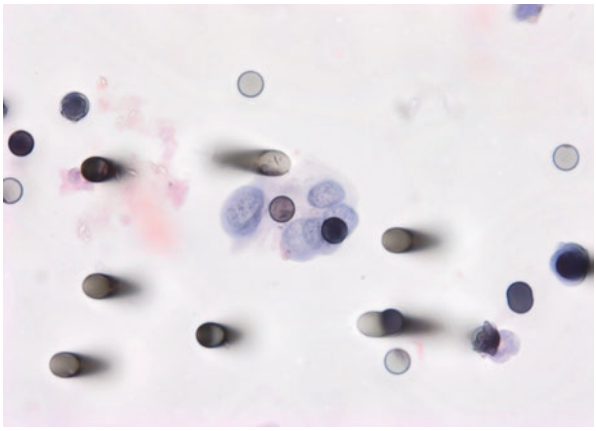

Fig. 4.16 Patient with 57 years old, man, with stage IIIB. The CTC count was 2.5 CTCs/mL at baseline (at diagnosis)

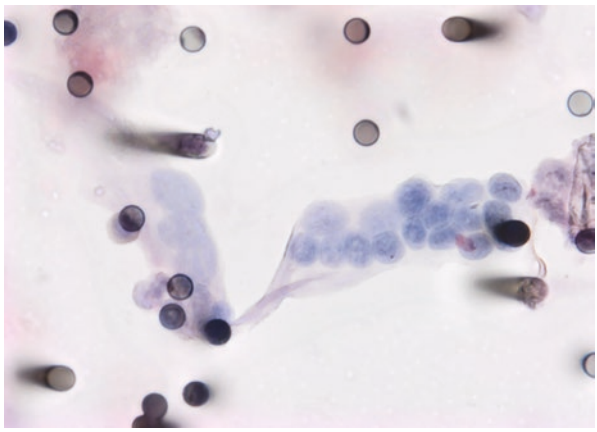

Fig. 4.17 Same patient of Fig. 4.16

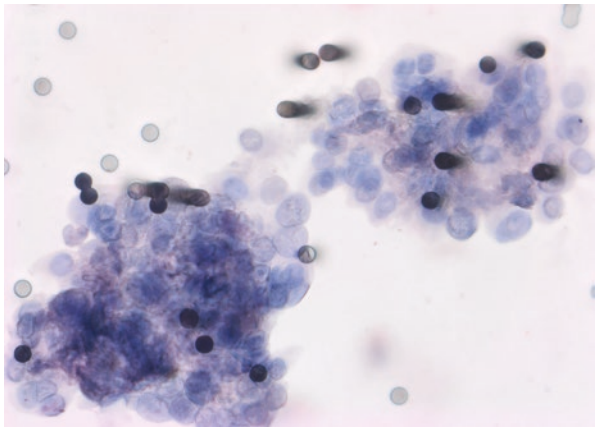

Fig. 4.18 Same patient of Fig. 4.16. Three-dimensional cluster of neoplastic epithelial cells

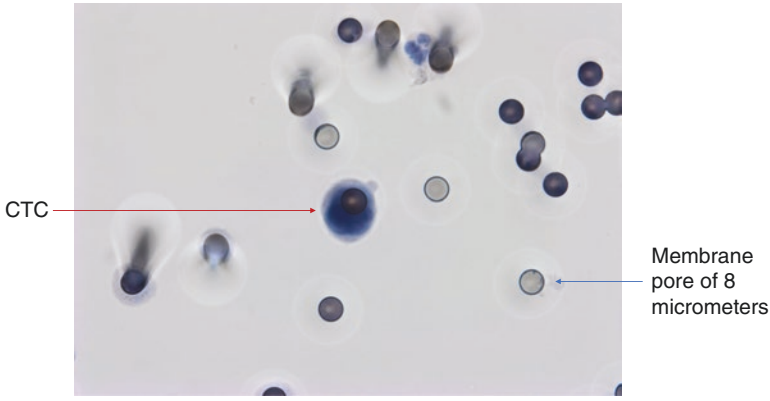

Fig. 4.19 Patient with 69 years old, male, with stage IIA. The CTC count was 7 CTCs/mL at baseline. This figure is of 3rd collection, made after adjuvancy (3.33 CTCs/mL)

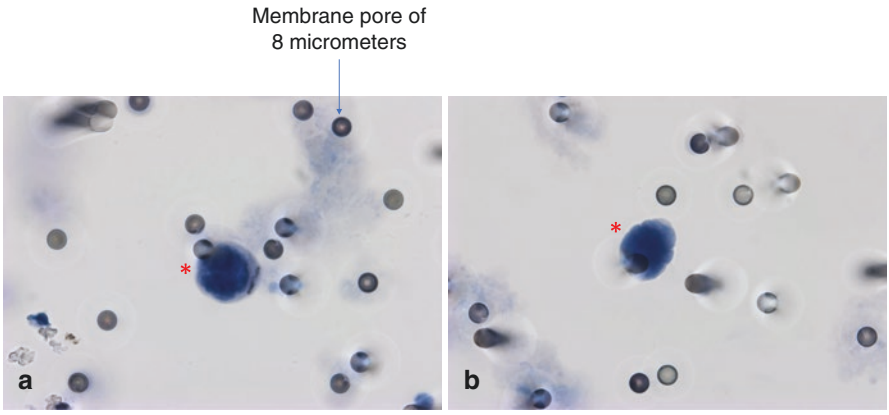

Fig. 4.20 Patient with 59 years old, woman, with stage IIIC. The CTC count was 2.80 CTCs/mL at diagnosis. This figure is of 3rd collection (after adjuvancy) and the count was 5.33 CTCs/mL. The asterisk represents CTCs stained with hematoxylin

feasible tool for evaluating CTC kinetics in patients with mCRC, which can be promising in their clinical evaluation.

These data are reinforced by the meta-analysis with 13 studies that showed that the rate of disease control was significantly higher in patients with CRC with low CTC compared to high CTC (RR = 1354, 95% CI [1002–1830], $p = 0.048$). CRC patients in the CTC-high group were significantly associated with poor progression-free survival (PFS; HR = 2500, 95% CI [1746–3580], $p < 0.001$) and poor overall survival (OS; HR = 2856, 95% CI [1959-4164], $p < 0.001$). Patients who converted from low CTC to high CTC or who were persistently high CTC had a worse disease progression (OR = 27.088, 95% CI [4960–147,919], $p < 0,001$), PFS (HR = 2095, 95% CI [1105–3969], $p = 0.023$) and OS (HR = 3604, 95% CI [2096–6197], $p < 0,001$) than patients who converted from high CTC to low CTC. Thus, it concludes that CTCs can be used as a new marker capable of predicting the response to chemotherapy in patients with metastatic CRC [15].

Another more recent meta-analysis with 15 published studies containing 3129 patients reinforces that the presence of CTCs was significantly associated with poor mortality (overall survival: HR = 2.36, 95% CI: 1.87–2.97; $P = 0.006$) along with aggressive disease progression (progression-free survival: HR = 1.83, 95% CI: 1.42–2.36; $P < 0.00001$) (Yi Tan et al. 2017).

Another study by our group in the metastatic setting evaluated the expression of TYMS in CTCs, in 34 samples and was TYMS considered positive in 9 (26.5%). Six of these patients had tumor progression after treatment with 5-FU. An association was found between CTC TYMS staining and disease progression (PD), although without statistical significance ($p = 0.07$). Patients who had a CTC count above the median (2 CTCs / mL) had higher TYMS expression ($p = 0.02$) correlating with a worse prognosis. These results suggest that TYMS analysis may be a useful tool as a biomarker predictor of 5-FU resistance if analyzed in CTCs of

Table 4.1 Studies showing that the presence of CTCs can predict future metastasis (disease progression) and unfavorable outcome

Author, year	Number of patients	Population	CTC's evaluation	Treatment	Main results
Sastre et al. (2012) [16]	1202	mCCR	CellSearch System	Chemotherapy + Mab	bCTC presented in 41% of patients; association with worse ECOG, stage IV, >3 metastatic sites and CEA levels
Bidard et al. (2019) [7]	131	mCCR	CellSearch System	Chemotherapy + surgery (metastasectomy)	bCTC was associated with OS; no association of CTC and metastatic hepatic resection
Tan et al. (2018) [18]	9	mCCR	Size-exclusion method	Chemotherapy +/− Mab	CTC kinetics during chemotherapy was associated with disease progression and trends in CEA levels
Yang et al. (2017) [37]	2363 (metanalysis)	Non-metastatic CCR	RT-PCR	Adjuvant chemotherapy for III and part of II	CTC positive was associated with shorter OS (HR = 3.07, $P < 0.001$) and disease-free survival (HR = 2.58, $P < 0.001$)
Chen et al. (2017) [10]	90 (and 151 healthy donors)	CCR and healthy donors	RT-PCR in marker genes in RNA extracted of CTCs	–	The expression of ECT2 in the CTC could serve as an alternative measurement in the diagnosis and monitoring of colorectal cancer patients

Table 4.1 (continued)

Author, year	Number of patients	Population	CTC's evaluation	Treatment	Main results
Souza e Silva et al. (2016) [26]	54	mCCR	Isolation by size of epithelial tumor (ISET) cells	Chemotherapy +/− Mab	ISET was proved a feasible tool for evaluating CTC kinetics, that, together with CTC levels were associated with prognosis
Abdallah et al. (2015) [1]	54	mCCR	Isolation by size of epithelial tumor (ISET) cells	Chemotherapy +/− Mab surgery +/− metastasectomy	Thymidylate synthase (TYMS) expression in CTC was a predictor biomarker of 5-FU resistance
Barbazan et al. (2014) [5]	50	mCCR	Multimarker CTC detection panel	Chemotherapy +/− Mab	A multimarker model based on expression levels of a six-gene panel of tissue-specific and EMT-related markers in CTC was associated with of OS and PFS
Sastre et al. (2012) [25]	108	mCCR	CellSearch System	Chemotherapy + bevacizumab	CTC count is a strong prognostic factor for PFS and OS
De Albuquerque et al. (2012) [11]	60	mCCR	Immunomagnetic enrichment with BM7 and VU1D9 Ab	Chemotherapy +/− Mab	CTC positivity was prognostic factor and associated with radiographic disease progression

(continued)

Table 4.1 (continued)

Author, year	Number of patients	Population	CTC's evaluation	Treatment	Main results
Matsusaka et al. (2011) [21]	64	mCCR	CellSearch System	Chemotherapy +/− bevacizumab	CTC number before and during treatment was associated with PFS and OS in oriental population
Tol et al. (2010) [31]	477	mCCR	CellSearch System	Chemotherapy +/− Mab	CTC count before and during treatment was associated with PFS and OS and provides additional information to CT imaging
Cohen et al. (2008) [28]	430	mCCR	CellSearch System	Chemotherapy +/− Mab	CTC number before and during treatment was associated with PFS and OS in occidental population

Abbreviations: *bCTC* baseline CTCs, *Mab* monoclonal antibody, *ECT2* epithelial cell transforming sequence 2, *BM7* antibody which target mucin 1, *EMT* epithelial-mesenchymal transition, *mCCR* metastatic colorectal cancer, *OS* overall survival, *PFS* progression-free survival, *VU1D9* antibody which target EpCAM

patients with mCRC [1]. In addition, in another study developed by our group, we analyzed the immunocytochemical expression of MRP1 and ERCC1 in patients with metastatic CRC who had previously detectable CTCs. Among patients treated with irinotecan-based chemotherapy, 4 out of 19 cases with MRP1-positive CTCs showed a worse progression-free survival (PFS) compared to those with negative MRP1 CTCs (2.1 months vs. 9.1 months; $p = 0.003$). These results show MRP1 as a potential biomarker of resistance to treatment with irinotecan when found in CTCs of patients with mCRC [2].

4.5 CTCs as a Predictive Factor in the Treatment of Locally Advanced Rectal Cancer

Neoadjuvant chemoradiation (NCRT) followed by total mesorectal excision (TME) is the standard treatment for locally advanced rectal cancer (LARC). Our group developed a study aiming to explore the role of CTCs in patients undergoing NCRT followed by surgery for treatment of LARC. In addition, we evaluated the predictive values of TYMS and RAD23B expression in CTC before and after NCRT. The initial analysis of 30 patients was published and demonstrated that the complete pathological response (*p*CR; $p = 0.02$) or the partial response ($p = 0.01$) could correlate with CTC counts. Regarding protein expression, TYMS was absent in 100% of CTCs from patients with pCR ($p = 0.001$) yet was expressed in 83% of non-responders at S2 ($p < 0.001$). Meanwhile, RAD23B was expressed in CTCs from 75% of non-responders at S1 ($p = 0.01$) and in 100% of non-responders at S2 ($p = 0.001$); 100% of non-responders expressed TYMS mRNA at both timepoints ($p = 0.001$). In addition, TYMS/RAD23B was not detected in the CTCs of patients exhibiting pCR ($p = 0.001$). Thus, TYMS mRNA and/or TYMS/RAD23B expression in CTCs, as well as CTC kinetics, have the potential to predict non-response to NCRT and avoid unnecessary radical surgery for LARC patients with pCR [32].

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Chapter 5
Circulating Tumor Cells in the context Non-small Cell Lung Cancer

Jacqueline Aparecida Torres

5.1 Introduction

Lung cancer is the neoplasm with the highest incidence rate and mortality, affecting men and women. In 2018, the global annual incidence of lung cancer was 2.1 million cases (11.6%), in addition to being responsible for 1.8 million deaths. Based on these data, we can observe that lung cancer is a serious public health problem [25].

Non-small cell lung cancer (NSCLC) is the most incident lung cancer, accounting for about 80–85% of cases being subdivided into three main types: adenocarcinoma, squamous carcinoma, and large cell carcinoma. The overall survival rate of NSCLC is approximately 50% in 5-year but the progression from stage I to stage IV decreases this rate to 1% [50].

The main obstacles to the treatment of NSCLC are late diagnosis, metastatic behavior, and disease recurrence. A small percentage of patients with NSCLC, approximately 20%, are diagnosed in the early stages of the disease (I or II), where they could be treated by surgical resection; however, about 80% are diagnosed late and present with locally advanced disease (22%) or metastatic disease (57%), requiring chemotherapy and/or radiotherapy. Even patients eligible for surgical resection may have recurrences due to distant metastases within the first 24 months [41, 50, 65, 67].

A characteristic of NSCLC is histological heterogeneity. There are variations within the main groups, such as adenocarcinomas, with distinct subtypes, diagnostic, prognostic, therapy, and demography, being necessary for the notification of the NSCLC, the realization of an immunohistochemical profile for differentiation [52].

Histological heterogeneity can be explained by intratumoral heterogeneity (ITH), present in the NSCLC. ITH is understood to be the molecular and genetic changes that occur in this neoplasm. The origin of molecular heterogeneity can be

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explained by several mechanisms such as genomic or chromosomal instability, epigenetic modifications, adaptations to the microenvironment, clonal evolution due to selective pressure from the tumor microenvironment, or by chemotherapy action. In addition to these molecular changes, the NSCLC expresses biomarkers such as the PD-L1 protein whose ligand, programmed death receptor 1 (PD-1), is expressed by T cells that may be present in the composition of the tumor microenvironment. This discovery enabled the targeting of the immune response to the target tumor cells [2, 3, 52].

Currently, the tumor material used to characterize NSCLC histologically, to identify molecular alterations and protein expression, is obtained by conventional biopsy. However, this examination is invasive and locally restrictive, making it impossible to perform with the frequency necessary to understand the molecular changes that occur in tumor dynamics [33].

In search of new methods to reduce obstacles in the treatment of NSCLC, liquid biopsy, which is the ex vivo analysis of a body fluid sample for the purpose of detecting and quantifying targets of interest, has shown a diagnostic approach with the potential to reveal health changes that include the onset and development of diseases [13].

Liquid biopsy performed by blood is feasible in patients with NSCL, because, unlike tissue biopsy, is performed in minimally invasive and safely procedure. In addition, the blood presents circulating biomarkers that, if analysed, allow a whole understanding of the tumor biology, since they come from the primary tumor and metastatic site [32, 53].

Among these biomarkers, circulating tumor cells (CTCs) are present, which are fragments of the primary tumor that circulate spontaneously individually or in groups of three or more CTCs (clusters), exclusively by lymphatic vessels and blood and precedes the metastatic behavior of neoplasms. CTCs have several components that can be analyzed, such as intact tumor DNA for mutation analysis, tumor RNA for gene expression and profile identification, and several biomarkers for proteomic analysis [33, 46].

Although not yet approved by the Food and Drug Administration (FDA) for use in clinics, CTCs have the potential to complement testing in patients with NSCLC and, in this review, we will focus on the contribution of CTCs to the comprehension of this neoplasm.

5.2 Expression of biomarkers in CTCs of patients with NSCLC

Immunotherapy revolutionized the treatment of patients with NSCLC, as it enabled the targeting of the immune response to tumor cells, allowing patients affected by different types of NSCLC to have a longer survival due to its ability to increase or restore antitumor immune function [2].

PD-L1 protein is expressed in several cell types, among them cancer cells and antigen-presenting cells (B lymphocytes, dendritic cells, macrophages) after being exposed to cytokines. Binding PD-1 to PD-L1 results in a signal that inhibits the full activity of T cells. However, in cancer patients, this inhibition mechanism causes tumor cells to pass unharmed to the immune system [3].

The most clinically advanced ICIs are directed to PD-1/PD-L1, performing immunosuppressive function in patients, obtaining authorization from the Food and Drug Administration (FDA) as a treatment option for NSCLC. Among the ICIs, there are nivolumab and pembrolizumab whose target is the PD-1 receptor and atezolizumab and durvalumab that target the PD-L1 protein [2, 11, 47].

To analyze the efficacy of treatment with ICIs, it is necessary to evaluate in real time the status of PD-1/PD-L1 expression; however, in clinical practice, it is difficult to perform this evaluation due to the invasive nature of conventional biopsy. However, through CTCs, there is the potential to monitor, via liquid biopsy, the dynamics of PD-1/PD-L1 expression of patients treated with ICIs over time.

Some groups have studied PD-1/PD-L1 expression in CTCs. The study by Kallergi et al. [26] demonstrated that CTCs PD-1+ and PD-L1+ can be detected before and after first-line chemotherapy in patients with metastatic NSCLC. For this, CTCs were isolated from 30 patients with NSCLC before chemotherapy and from 11 patients after the third treatment cycle, using the ISET Technology® (Rarecells Diagnostics, France) methodology. To identify the CTCs, Giemsa staining and immunofluorescence staining (IF) were used.

Using Giemsa staining, CTCs were identified in 28 out of 30 patients (93.3%) at baseline and in 9 out of 11 patients (81.8%) after the third chemotherapy cycle. On the other hand, with immunofluorescence staining (FI), CTCs were detected in 17 out of 30 patients (56.7%) at baseline and in 8 out of 11 patients (72.7%) after the third chemotherapy cycle. At the beginning of the study, the expression of PD-1 and PD-L1 was observed in 53% and 47% of patients, respectively. After the third treatment cycle, the corresponding numbers were 13% and 63%, respectively. Median progression-free survival (PFS) was significantly lower in patients with >3 PD-1 CTCs (+) at baseline compared to those with 3 < PD-1 CTCs (+) ($p = 0.022$) [26].

The pilot study conducted by Dhar et al. [7] also aimed to evaluate the expression of PD-L1 in CTCs. Twenty-two patients with metastatic NSCLC treated with pembrolizumab, nivolumab and avelumab were recruited, of whom 31 samples were collected before and after chemotherapy. Using the Vortex Chip HT device, CTCs were isolated in 30 of the 31 samples (96.8%), and samples with CTCs had 1 or more PD-L1+ CTCs. The PD-L1+ CTCs fraction ranged from 2.2 to 100%. It was possible to verify the agreement of PD-L1 expression of CTCs with tissue biopsy in only 4 patients of 22. This group demonstrated that quantification of PD-L1 CTCs levels when combined with tissue biopsy results can help identify patients with a higher probability of responding to therapy or, by monitoring throughout treatment, the patients most likely to become resistant to treatment.

Ilie et al. [20] isolated CTCs, using the ISET Technology® (Rarecells Diagnostics, France) platform, in samples of 106 patients, as a non-invasive method to evaluate the status of PD-L1 in patients with advanced NSCLC and compared them with the

status of PD-L1 in tumor tissue. CTCs were detected in 80 (75%) patients. In 71 samples, it was possible to compare the tissue and CTCs; 6 patients (8%) presented 1 PD-L1(+) CTCs and 11 patients (15%) presented 1% of PD-L1(+) tumor cell in the tumor tissue, with 93% agreement between tissue and CTCs, demonstrating that the status of both tissues correlate, revealing the potential of CTCs to assess real-time PD-L1 expression in patients with NCSLC.

In view of the results presented here, it is observed that CTCs can contribute to the analysis of expression levels PD-1/PD-L1 before the start of treatment and progressively over this course.

5.3 Circulating Tumor Cells: Source of Early Detection and Recurrence of NSCLC

On average, 80% of the patients are diagnosed late, that is, with the disease in advanced stages, where surgical treatment is not an option. Even with the advancement of therapies, a large portion of the patients do not survive the 5 years after diagnosis. Reducing tobacco consumption is a very important factor in controlling the number of NSCLC cases, but in addition, there is an imminent need to diagnose patients in the early stages of the disease.

The American College of Radiology Imaging Network conducted The National Lung Screening Trial (NLST) which aimed to compare two forms of early detection of lung cancer: computed helical low-dose Tomography (CT) – often referred to as spiral CT – and standard Chest X-ray [40]. The study was conducted with 53.454 smokers and ex-smokers aged between 55 and 74 years, who smoked at least 30 packs-a-year, who had no previous symptoms or history of lung cancer. The results of this study showed that low-dose CT screening was 24.2% while X-ray was 6.9%. However, among the positive results, 96.4% in the low-dose CT group and 94.5% in the X-ray group were false-positive results.

The amount of false-positive results raised the question about expanding this type of screening, which could increase the rate of consultations based on indeterminate cause nodules, generating concerns and high costs. On the basis of this study and given the imminent need for new methods for the early detection of lung cancer (LC), Ilie et al. [21] analyzed patients with chronic obstructive pulmonary disease (COPD), which, regardless of stage of development, is a risk factor for NSCLC. In addition, based on the invasive behavior of the NSCLC and data from experimental models where tumors measuring less than 1 mm can release CTCs in the bloodstream, the group proposed to investigate whether patients with COPD had CTCs, which could be an early marker of NSCLC.

For this, they analyzed the peripheral blood of 168 patients with COPD, who did not present any lung cancer detectable by imaging tests. Using ISET Technology (Rarecells Diagnostics, France), researchers detected CTCs in 3% (5 patients). The patients were followed-up and after an average of 3.2 years, all presented nodules in

the lung detected by computed tomography. The 5 patients underwent surgery and analysis showed that the cancer was stage I, which means that they had not spread to lymph nodes or developed metastases. This study demonstrated, for the first time, the potential of CTCs as an early marker of invasive CL in patients at high risk [21].

CTCs are considered the primary metastatic source of cancer due to their ability to colonize organs and tissues. To this end, CTCs undergo several molecular and cellular changes, through the epithelium-mesenchymal transition process (EMT), granting a mesenchymal phenotype to epithelial cells making them more effective in their mobility due to the weakening of cell-cell adhesion and fusiform shape gain fundamental for metastatic behavior to be effective [31, 36].

The study by Xie et al. [62] investigated the possible correlations between CTCs and pathological types and staging of NSCLC during the early postoperative period. Sixty-nine patients with NSCLC were recruited. CTCs were analyzed by multiple mRNA in situ after enrichment by nanotechnology for lysis of red blood cells.

The presence of epithelial or mixed CTCs had no significant correlation with tumor size, lymph node metastasis, and distant metastasis TMN in patients with NSCLC ($P > 0.05$), but higher TNM levels were related to the presence of mesenchymal CTCs ($P < 0.05$). After surgery, the patients were divided into pathological types: 48 patients had adenocarcinoma of which 40 were positive for CTCs. Of the 16 cases of squamous cell carcinoma, only 2 were negative for CTCs and among the 5 patients with large cell carcinoma only 1 had CTCs ($P < 0.5$) [62].

Frick et al. [12] analyzed CTCs as a prognostic marker to measure the risk of NSCLC recurrence after stereotactic body radiotherapy (SBRT) treatment. The treatment is effective in early stage of NSCLC; however, failures occur at the primary tumor site in about 10–15% and 20–25% in distant locations. For the study, 92 patients with stage I NSCLC treated with SBRT were recruited. The samples for analysis of CTCs were obtained before, during, and in series up to 24 months after treatment with SBRT. CTCs were quantified by a trial using adenoviral-based probe that expresses green fluorescent protein (GFP) that detects high telomerase activity in cancer cells.

The CTC test was positive before SBRT treatment in 38 of 92 (48%) patients. During treatment, CTCs were observed in 35 patients with a count of 0.5 CTC/mL. In the 3-month period after SBRT treatment, CTCs continued to be detected in 10 out of 35 patients (29%). The persistence of CTCs was associated with increased risk of treatment failures in distant locations and ($P = 0{,}04$) tended to increase the regional failure ($P = 0{,}08$) and local failure ($P = 0{,}16$). This study suggests that CTCs before treatment and its post-treatment maintenance are associated with the risk of recurrence outside the target treatment site, suggesting that CTCs have the potential to identify patients at higher risk of recurrence [12].

In order to identify the prognostic value of the presence and characterization of CTCs in the peripheral blood of NSCLC patients undergoing radical resection, Bayarri-Lara et al. [1] analyzed samples of 56 patients with pathological stage between IA and IIIA, obtained before and 1 month after surgery, the mean follow-up of these patients was from 3 to 16 months (variation 3–23).

In the samples prior to surgery, CTCS were detected in 29 of 56 patients (51.8%) and after 1 month of surgery, 18 patients (32.1%) presented CTCs. During follow-up, 16 patients (28.6%) presented signs of cancer recurrence in an average of 8 months; 50% of the patients who had CTCs after surgery developed recurrence, compared to 18.4% of the patients who did not have post-surgery CTCs, thus correlating the presence of CTCs after surgery to a higher risk of early recurrence.

The results of these studies demonstrated the potential of CTCs as an early marker of diagnosis and recurrence in the NSCLC, which would enable more rigorous and early decision-making, in addition to the individualization of treatment.

5.4 Identification of the NSCLC Molecular Profile in CTCs

Knowing the molecular heterogeneity of NSCLC was an important factor for the development of new precision therapies, because some of these tumors are dependent on oncogenes, that is, depend on key point mutations of signaling pathways to grow and survive.

Among NSCLC subtypes, adenocarcinoma is the most incident and may present at least one driver mutation. The main changes identified were in the epidermal growth factor receptor (EGFR) and in the anaplastic lymphoma kinase (ALK), both protein tyrosine kinases (PTKs) receptors, proteins responsible for gene expression, acting in cell growth, survival, migration, and apoptosis, these being, until now, the main targets for the treatment of NSCLC.

The discovery of these molecular changes changed the course of the treatment of patients with NSCLC, as it enabled the development of tyrosine kinase inhibitors (TKIs), whose function is to prevent the enzymatic activity of these oncogenes. EGFR TKIs are gefitinib, erlotinib, afatinib, and osimertinib, and ALK inhibitors are crizotinib, ceritinibe, and alectinib. The response to the use of TKIs has been promising, with very significant clinical benefits. Objective response rates of 60–70% are reported with the use of these different TKIs and a disease control rate of up to 80–90%. However, patients tend to develop drug resistance within 1 to 2 years due to somatic mutations [24, 27, 38, 55].

Mutations in EGFR occur mainly at sites where EGFR binds to TKIs and are detected in exons 18 to 21 of the tyrosine kinase coding gene. More than 85% of adenocarcinomas present exon 19 deletions or L858R point mutation in exon 21, targets that are clinically actionable. At exon 18, point nucleotide substitutions occur at codon 719. In the exon 20, there are point mutations and insertions including T790M, and this mutation is responsible for about 50% of all acquired resistance mutations. In ALK rearrangements, EML4-ALK is the dominant rearrangement. This mutation is found in 3–7% of NSCLC [5, 10, 51].

It is necessary to develop new ways of detecting somatic mutations in NSCLC. Studies have shown that CTCs have predictive, diagnostic, and prognostic value to identify mutations in NSCLC, in addition to identifying and monitoring mutations related to resistance to TKI treatments.

The study by Yang et al. [64] aimed to isolate and quantify CTCs after treatment with osimertinib, TKI) with activity against the T790M mutation in EGFR. Patients ($n = 68$) had samples collected at baseline and on day 28. CTCs were evaluated by the CellSearch system. CTCs were divided into favorable (<5 CTCs) and unfavorable (≥5 CTCs) groups. Patients in the favorable group at the beginning of the study showed significantly longer median progression-free survival (PFS) compared to patients in the unfavorable group (9.3 vs.6.5 months; $p = 0.0002$). The PFS interval for patients in the favorable group on day 28 was 9.7 months, significantly higher than the mean time of PFS of 6.2 months achieved by patients in the unfavorable group ($p = 0.011$). This is the first report on the presence of CTCs and its prognostic role in T790M-positive NSCLC EGFR patients after disease progression with treatment with EGFR-TKI.

The objective of the study by Pailler et al. [45] was to verify whether the sequencing of CTCs could provide information on acquired resistance to ALK inhibitors in addition to tumor heterogeneity in NSCLC mutated in ALK. Patients treated with TKI-ALK ($n = 17$), crizotinib ($n = 14$) or lorlatinib ($n = 3$) were recruited after progression of the disease.

The samples were filtered with ISET Technology® (Rarecells Diagnostics, France), CellSearch, and Rosettesep system. Pools of CTCs ($n = 126$) and 56 unique CTCs were isolated and sequenced. Hotspot regions over 48 cancer-related genes and 14 ALK mutations were examined to identify ALK-independent and ALK-dependent resistance mechanisms. Various mutations were observed in crizotinib-resistant patients in several genes on independent pathways of ALK. RTK-KRAS (EGFR, KRAS, BRAF) and TP53 pathways have been mutated recurrently. In a patient resistant to lorlatinib, two single CTCs in 12 showed mutations in the compound ALK. Mutation of the compound ALK G1202R/F1174C was observed practically similar to ALK G1202R/F1174L and ALK G1202R/T1151 mutation of the compound not detected in tumor biopsy. These results highlight the genetic heterogeneity and clinical utility of CTCs to identify TKIs-ALK resistance mutations. Therefore, CTC sequencing can be a unique tool to evaluate resistance mechanisms and assist in the personalization of treatments [45].

By means of hypermetabolic CTCs, detected by the increased uptake of glucose, Turetta et al. [58] demonstrated that it is possible to evaluate the mutational status of the NSCLC. Thirty patients with stage IV NSCLC were included in the study, of which the blood samples were incubated with 2-NBDG, a fluorescent glucose analog, and analyzed by flow cytometry. Using ddPCR, they detected mutations in EGRF and KRAS in 85% of patients, corresponding to the primary tumor in 70% of cases. Multiple mutations in KRAS were found in two patients, other two had mutations different from those detected in the primary tumor and two patients with wild primary tumor new mutations were detected: EGFR p.746_750del and KRAS p.G12V. This study demonstrated the potential of CTCs to detect distinct mutations of the primary tumor, allowing us to know the heterogeneity of the NSCLC.

Analyzing samples of 125 patients with stage IIIB-IV NSCLC, using CellSearch technology and anti-vimentin antibody to detect mesenchymal CTCs, Lindsay, et al. [34], observed that 51/125 patients (40.8%) had CTCs and 26/125 (20.8%)

were CTC + vim at the beginning of the study. A multivariate analysis showed that patients with 5 CTCs (total) significantly reduced to OS but not PFS compared to patients with <5 total CTCs.

The researchers divided the patients according to the mutation of the NSCLC driver, where they observed an increase of vim + CTCs in the mutated subgroup EGFR (*N* = 21/94 patients), a reduction of total CTCs in the rearranged subgroup ALK (*N* = 13/90 patients), and a total absence of vim + CTCs in adenocarcinomas mutated with KRAS (*N* = 19/78 patients. This study demonstrated that EGFR mutant CTCs express epithelium-mesenchymal transition characteristics not observed in CTCs of KRAS-mutant adenocarcinoma patients [34].

Chromosomal rearrangements of ROS1 in CTCs of patients with NSCLC mutated in ROS1 and treated with crizotinib were evaluated by Pailler et al. [43]. A sample of four patients was analyzed using ISET Technology® (Rarecells Diagnostics, France), and the ROS1 rearrangement was detected by filter-adapted-fluorescence in situ hybridization (FA-FISH). In CTCs of all patients, ROS1 rearrangement was detected, initially confirmed by conventional biopsy. The mean number of CTCs at the beginning of the study was 34.5/3 ml of blood. Tumor heterogeneity, assessed by the number of copies of ROS1, was significantly higher in baseline CTCs compared to tumor biopsies. The number of CTCs increased significantly in two patients who progressed during crizonitinibe treatment. This study showed for the first time the ability of CTCs to detect mutated NSCLC in ROS1.

The combination of the studies exposed in this chapter (Table 5.1) demonstrates the potential of CTCs as an auxiliary and/or independent source for mutation analysis, a tool for prognosis in treatments with TKIs and ICIs, as also for early diagnosis of NSCLC. It is essential to develop more research in order to contribute to the validation of CTCs in clinical practice, composing the biomarkers used in liquid biopsies.

Table 5.1 Main studies with CTCs in NSCLC

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC adenocarcinoma		44	CellCollector	PD-L1/EpCAM/ CK7/CK/ CK19/ panCK/CD45	Duan et al. [9]	2020	Of the patients (n = 44) suspected of LC, (n = 34) were diagnosed with the disease, (n = 10) were diagnosed with benign pulmonary diseases.CTCs were detected in 18/34 patients with LC (52.94%) and in 1/10 (10%) patients with benign pulmonary disease	LC: lung cancer
NSCLC adenocarcinoma; squamous cell carcinoma; small cell carcinoma	I-IV	38	Metallic MCA filter	CK/CD45	Ichimura et al. [19]	2020	The patients were divided according to clinical stage disease. CTCs were detected in 63.2% stage I (n = 19); 33.3% stage II (n = 6); 66.7% stage III (n = 6) and, 71.4% stage IV (n = 7)	

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC	I/II	24	CellCollector	EpCAM/CKs/ CD45/TP53/ ERBB2/ PDGFRA/ CFS1R/FGFR1/ PD-L1	He et al. [16]	2020	CTCs (+) were detected in 15/24 patients and CTCs clusters in 40% of patients. In 13/24 patients, the genetic mutations of TP53 (ERBB2, PDGFRA, CFS1R, and FGFR1) in the CTCs were 71.6%, similar to the mutations in the tumor tissue. The PD-L1 expression detected in 40% of CTCs compared to 26.7% tumor tissue	
NSCLC metastatic		35	ISET	PD-L1	Castello et al. [4]	2020	At baseline, CTCs detected in 16/35 patients (45,7%) and 10/24 8 weeks after ICI initiation. Numbers of CTCs before and after 8 weeks were 15 ± 28 and 11 ± 19, respectively. The combination of mean CTC and median MTV after 8 weeks was associated with PFS ($p < 0.001$) and OS ($p = 0.024$)	PFS: progression-free survival; OS: overall survival; ICIs: immune checkpoint inhibitors

NSCLC	I-IV	50	Flow cytometry	CD45/EpCAM	Wu et al. [61]	2020	MMRM analysis indicated that surgery could contribute to decrease the amount of CTCs in all patients with statistical significance ($p = 0.0005$). The daily decrease of CTCs was statistically different between patients with and without recurrence ($p = 0.0068$). An early recovery of CTC counts on postoperative days 1 and 3 was associated with recurrence months later	MMRM: mixed model repeated measures
NSCLC/SCLC	II/III/IV	67	Automated MCA system	PD-L1	Koh et al. [28]	2019	CTCs were detected in 66/67 patients and more than 5 CTCs were detected in 78% of patients. PD-L1expression in CTCs was detected in 73% of patients, ranging from 3% to 100%	

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC adenocarcinoma	II-III	103	CanPatrol ™/ RNA-ISH	PD-L1/EpCAM/ CK8 CK18/ CK19/Vimentin/ Twist	Dong et al. [8]	2019	CTCs in the PV were detected in 101/103 (98.1%) patients, in the PPA in 92/103 (98.1%) patients. The PFS in the group with PV CTCs≥ 16/5 ml was shorter than that in the group with PV CTCs < 16/5 ml (11.1 months vs. 21.2 months, respectively; $P < 0.001$). The PFS in the group with PPA CTCs ≥ 3/5 ml was shorter than that in the group with CTCs < 3/5 ml (14.8 months vs. 20.7 months, respectively; $P < 0.001$). More CTCs were found in the stages II-III compared stage I ($P = 0.025$). 50.5% of patients had CTCs PD-L1 (+)	PV: pulmonary vein; PPA: preoperative peripheral artery PFS: progression-free survival

NSCLC adenocarcinoma/ squamous cell carcinomas	IIIB-VI	104	CellSearch	PD-L1/PD-1	Tamminga et al. [54]	2019	Patients (n = 104) treated with PD-L1-ICI's were included. CTC were present in 33/104 (32%) patients at T0 and 17/63 (27%) at T1, 9/63 (14%) patients had CTC at both time points. The presence of CTC, both at T0 (OR = 0.28, p = 0.02,) and T1 (OR = 0.07, p < 0.01), was an independent predictive factor for a lack of durable response and was associated with worse progression free and overall survival	ICI's: Immune Checkpoint Inhibitors; OR: Odds ratio
NSCLC adenocarcinoma/ squamous cell carcinomas		17	ISET	PD-L1	Monterisi et al. [39]	2019	10/17 (59%) patients had CTCs. A significantly lower number of CTCs was found in patients previously treated with chemotherapy (P = 0.04). Patients with an extensive tumor burden, MTV, and TLG were associated with a higher number of CTCs (P = 0.004 and P = 0.028, respectively). Likewise, patients with a higher metabolism result had higher CTCs count (P = 0.048)	MTV: metabolic tumor volume; TGL: total lesion glycolysis

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC	I-IV	73	Nano-enrichment	Cy5/ anti-CD45-FITC	Wei, et al. [60]	2019	Among patients with CTCs > 5/7.5 mL, 17.4% were in stage I and 60% in stage IV. During chemotherapy cycles, the average CTC number decreased from 5.8/7.5 ml in cycle 1 to 2.4/7.5 ml in cycle 4. Patients (n = 14) were EGFR (+) of whom 7 had mutated EGFR (5 patients with E19del and 2 with L858R). The average progression-free survival (PFS) in the favorable group (CTC ≤ 5/7.5 ml) was 11.3 months, which was longer than that in the unfavorable group (CTC > 5/7.5 ml, 7.2 months)	PFS: progression-free survival
NSCLC advanced		96	ISET	PD-L1	Guibert et al. [14]	2018	CTCs were detected in 89/96 samples at baseline (93%). PD-L1 was detected in 83% of patients and IN 17.2% of CTCs	

NSCLC advanced		30	On-chip Sort/ CellSearch	EGFR	Watanabe et al. [59]	2018	The On-chip Sort methodology detected CTCs in 22/30 patients, while CellSearch detected 9/30. EGFR mutations in CTCs captured by On-chip Sort were observed in 40.0% (8/20) patients	
NSCLC adenocarcinoma	IIIB-VI	43	Cyttel method	EGFR /ALK	Tong, et al. [56]	2018	33/43 patients had CTCS(+) mutations in EGFR were detected in 36/43 patients (L858R [n = 11] and deletions in exon 19 [n = 25]) and 7 patients had Alk rearrangement. Patients (n = 29) were evaluated in the progression of the disease, of which n = 14 had increase of CTCs; in n = 13 the CTCs decreased and n = 2 remained stable. The median PFS and OS of the favorable compared to the unfavorable group were longer (11.6 vs. 8.5 months, P = 0.004 for PFS; 21.00 vs. 17.7 months, P = 0.013 for OS)	

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC		47	CanPatrol	GALC/CEA/ CYFRA21-1/ NSE/CK8/18/19/ EpCAM/ Vimentin/Twist/ CD45	Liu et al. [35]	2018	CTCs were detected in 91.5% patients. Increased CTCs were associated with advanced tumor stages (6/5 mL) compared with early stages (3.5/5 mL). Epithelial, hybrid, and mesenchymal CTCs were detected in 55.4%, 78.7%, and 61.7% of the patients, respectively. The GALC expression was positive in 80.6% of the CTCs	GALC: galactocerebrosidase
NSCLC adenocarcinoma/ squamous cell carcinomas	IIIB-VI	127	Cyttel method	EGFR /ALK	Tong et al. [57]	2017	Patients who had baseline of CTCs < 8 CTCs/3.2 mL, had longer OS and PFS (20.0 vs. 10.4 months [$P = 0.009$] and 7.2 vs. 5.5 months [P 0.001], respectively). Patients with increased post-treatment CTCs had lower OS and PFS compared to patients with stable CTC count (12.0 vs. 13.3 months [$P = 0.028$] and 5.2 vs. 6.4 months [$P = 0.022$], respectively)	PFS: progression-free survival; OS: overall survival

NSCLC	IIIB-VI	107	CellSearch	EGFR	Yang et al. [63]	2017	Before of the TKIs, 47/107 (44%) had CTCs ≥2 and 17/107 had CTCs ≥5. Patients were divided into favorable (CTC-d0 of 0–4, $n = 90$) and unfavorable (CTC-d0 of $^{3}5$, $n = 17$) prognostic groups. The median PFS time in the favorable versus unfavorable groups (11.1 vs. 6.8 months). In the day 28, patients favorable had significantly longer PFS compared with patients unfavorable (11.6 vs. 6.3 months; p 0.0001)	TKIs: tyrosine kinase inhibitors
NSCLC adenocarcinoma		39	ISET/CellSearch	ALK	Pailler et al. [44]	2017	At baseline, the median numbers of ALK-rearranged CTCs and ALK-CNG CTCs were 14/3 mL and 12/3 mL. We observed a significant association between the decrease in CTC number with ALK-CNG on crizotinib arm and a longer PFS (likelihood ratio test, $P = 0.025$) and the dynamic change of CTC with ALK-CNG was the strongest factor associated with PFS (HR, 4.485; 95% confidence interval, 1.543–13.030, $P = 0.006$)	HR: hazard ratio

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC adenocarcinoma	III-IV	362	ISET/CellSearch	MET	Ilie et al. [23]	2017	CellSearch: CTCs were detected in 83/256 (32%) patients evaluated, 30 patients (12%) with ≥5 CTCs/7.5 ml blood. ISET: 80/106 patients (75%) had CTCs and 79 patients (75%) exhibited ≥5 CTCs/4 ml blood. MET expression on ISET CTCs was positive in 72% of cases. MET expression on tissue was positive in 65% patients. Patients (n = 9) were positive for MET with CellSearch	
NSCLC	I-IV	23	ScreenCell		Chudasama et al. [6]	2017	The two pathologists identify CTCs in 78.3% (18/23) and 73.9% (17/23) with overall 80.6% (n = 29) in early stages compared to 60.0% (n = 6) in late stages. The median survival times of positive vs. negative for CTC patients were 1011 and 711 days, respectively, with a survival percentage rate of 77.8% and 60% in positive and negative CTC cohorts, respectively	

NSCLC metastatic		19	ESP technology (VERSA and SLIDE)	PD-L1/MUC1/ EpCAM/CD11b/ CD45	Schehr et al. [49]	2016	Large populations of CD11b+CD45lo cells were identified. The amount of CD11b+ cells as CTCs varied among patients accounting for 33–100% of traditionally identified CTCs. Cells captured with vimentin had a higher frequency of CD11b+ cells at 41%, compared to 20% and 18% with MUC1 or EpCAM, respectively. Wrong identification of CTCs distorted the PD-L1 expression to varying degrees	
NSCLC	I-IV	32	Microfluidic chip technology	EpCAM/CK/ CD45/EGFR/ CD133	Reddy et al. [48]	2016	20/32 (62.5%) patients had CTCs detected. The average number of CTCs in the PV was 340.0 (range, 0.0–5422.50; $P > .01$). When PV CTCs were present, the number of CTCs was correlated with pathological tumor size ($P = .0236$). The number of PV CTCs was significantly higher when preoperative bronchoscopic biopsy was performed, compared with computed tomography–guided biopsy ($P = .0311$). Patients ($N = 7$) had evidence of CTC clusters	PV: peripheral vein

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC	I-IV	111	RT-PCR	CK7/ELF3/ EGFR/EphB4/	Yu et al. [66]	2016	Patients with enhanced expression of CK7, ELF3, EGFR, and EphB4 mRNA in PBMCs had poorer DFS (OS) than those without (all $p < 0.0001$). The expression of at least one of these four markers (a combination of the four markers) was considered as CTC positive	
NSCLC		48	Adna-EMT-2 test/ CellSearch	PIK3CA/ AKT2/ TWIST/ALDH1/ EGFR/ HER2/ EpCAM	Hanssen et al. [15]	2016	CellSearch identified 15% of patients as CTC positive, whereas a multiplex RT-PCR for PIK3CA, AKT2, TWIST, and ALDH1 following EGFR, HER2 and EpCAM based enrichment detected CTCs in 29% of the patients. Only 11% of the patients were CTC-positive by both techniques	

NSCLC locally advanced/ metastatic	IIIB-VI	37	CellSearch/ NGS	EGFR	Marchetti et al. [37]	2014	41% of the patients had CTCs. EGFR mutations were identified by NGS in CTCs of 31 (84%) patients, corresponding to those present in tumor tissue. 25/26 (96%) of deletions at exon 19 and 6/11 (55%) of mutations at exon 21 were detectable ($P = 0.005$). In 4 (13%) cases, multiple EGFR mutations, suggesting CTC heterogeneity, were documented	NGS: next-gen sequencing
NSCLC metastatic		32	ISET/CellSearch	ALK/vimentin/ cytokeratins/ CD45/ N-cadherin/E--cadherin/CD45	Pailler et al. [42]	2013	All ALK-positive patients had ALK-rearranged ≥4 CTCs/ml of blood (median, 9 CTCs/mL). ALK-rearranged CTCs harbored a unique (3′5′) split pattern, and heterogeneous patterns (3′5′, only 3′) of splits were present in tumors. ALK-rearranged CTCs expressed a mesenchymal phenotype contrasting with heterogeneous epithelial and mesenchymal marker expressions in tumors. Variations in ALK-rearranged CTC levels were detected in patients being treated with crizotinib	

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
NSCLC	IIIA-IV	40	ISET/CellSearch	EpCAM/CK (4, 5, 6, 8, 10, 13, 18)/EGFR/ VE-cadherin/ Ki67/igG control	Krebs et al. [29]	2012	CTCs were detected using ISET in 32 of 40 (80%) patients compared with 9 of 40 (23%) patients using CellSearch. CTMs were observed in 43% patients using ISET but were undetectable by CellSearch. About 62% of single CTCs were positive for the proliferation marker Ki67; however, cells within CTM were nonproliferative	CTM: circulating tumor microemboli
Lung cancer	I, II, III, and IV	87	ISET/FISH/ Immunoreactivity	ALK-rearrangement	Ilie et al. [22]	2012	5 patients showed ALK-gene rearrangement and ALK protein expression in CTCs and in the corresponding tumor samples. Both ALK-FISH and ALK immunoreactivity analyses show negative results in CTCs and corresponding tumor samples	

NSCLC	I-VI	210	ISET/CellSearch	Anti-cytokeratin/ anti-vimentin/ EpCAM/CD45/ CK-PE/ pan-cytokeratin	Hofman et al. [18]	2011	CTCs were detected in 144/210 (69%) patients through CellSearch and/or ISET. By ISET, CTCs were detected in 104/210 (50%) and in 82/210 (39%) patients by CellSearch. With ISET, 23/210 (11%) patients had vimentin-positive cells. DFS was worse for patients with CTCs compared to patients without CTCs detected by CellSearch alone ($p < 0.0001$; log rank = 30.59) or by ISET alone ($p < 0.0001$; log rank = 33.07)	DFS: disease-free survival

(continued)

Table 5.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
	III-IV	101	CellSearch	EpCAM	Krebs et al. [30]	2011	Patients (n = 60) with stage IV NSCLC had in 7.5 mL higher CTCs (range, 0 to 146) compared with patients with stage IIIB (n = 27; range, 0 to 3) or IIIA disease (n = 14; no CTCs detected). In univariate analysis, PFS was 6.8 vs. 2.4 months with $P < .001$, and OS was 8.1 v 4.3 months with $P < .001$ for patients with >5 CTCs compared with ≥5 CTCs before chemotherapy, respectively. In addition, CTC number was the strongest predictor of OS (HR, 7.92; 95% CI, 2.85 to 22.01; $P < .001$)	OS: overall survival; PFS: progression-free survival
NSCLC	I/II	208	ISET	TTF1	Hofman et al. [17]	2011	102/208 (49%) patients showed CNHCs. A level of ≥50 CNHCs corresponding to the third quartile was associated with shorter overall and disease-free survival, independently of disease staging, and with a high risk of recurrence and death in early-stage I + II-resectable NSCLC	CNHCs: circulating nonhematologic cells

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Chapter 6
Circulating Tumor Cells in Prostate Cancer

Milena Shizue Tariki

6.1 Introduction

Prostate cancer is the second most common cancer diagnosed in men worldwide with 1,276,106 new cases annually and 358,989 deaths estimated in 2018 [8]. It is a disease of the elderly, with a peak of incidence in 65–74 years old [51].

Recently, an increase in cancer mortality has seen, in part, due to modifications in screening recommendations since 2012 [37], which led to a decrease in prostate cancer incidence and increase in diagnosis of metastatic disease at presentation [6, 30]. It is estimated that at the time of diagnosis, 76% of patients have localized cancer, 13% have regional lymph node involvement, and 6% have distant metastases [51].

Although 5-year overall survival (*OS*) is 97.8% in general, it can vary from 100% of those patients with localized or locally advanced disease to only 30.2% in distant metastases. In this scenario, prostate cancer has a predicted timeline natural evolution, from biochemical recurrence (*PSA only increase*) to evidence of metastasis at first only in lymph nodes and bone to visceral disease. Also, biologically prostate cancer cells change from castration sensitive status to castration resistance along time.

Since docetaxel pivot studies in 2004 [42, 54], which was the first drug to improve OS in metastatic castration resistant prostate cancer (*mCRPC*), a lot has changed. New hormonal agents such as abiraterone and enzalutamide [7, 16, 46, 49], immunotherapy with Sipuleucel T [28], radiopharmaceutical Radium 223 [41], and chemotherapy with cabazitaxel [15] have also shown OS improvement.

The next step was to test earlier some of those life-prolonging treatments: in the context of hormone-sensitive disease. The results were that in seven of eight major trials, an impressive gain such as 17.8 months in OS was achieved and changed

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clinical practice forever [3, 9, 13, 20, 24, 26, 27, 53]. In addition, in non-metastatic-resistant disease, three trials demonstrated an important prolongation in metastasis-free survival around 2 years [21, 25, 52].

Despite recent developments in prostate cancer drugs, a little has changed in terms of personalizing choice of therapy. There is no clear evidence of better efficacy from one drug to another besides side-effect profile and patients' comorbidities. Available therapies have not been directly compared in randomized clinical trials. Moreover, issues such as the best sequencing after progression to one drug and the best evaluation of response need to be answered.

There is a clear need in finding biomarkers to better guide treatment, so patients can benefit the most from impressive advances that took decades to come up. Therefore, liquid biopsies and specially circulating tumor cells have surged as an important tool not only in predicting treatment outcomes but also as a prognostic marker.

6.2 CTCs in Localized Prostate Cancer

It is assumed that cancer cells disseminate from primary tumor by CTCs (*circulating tumor cells*) and that these cells can be found and isolated from peripheral blood of cancer patients [40]. The presence of CTCs in blood infers less favorable outcome than the absence in almost all cancers, independent of the technique used [22, 43, 56].

Since the 1990s, some authors have published identification of CTCs in localized prostate cancer and its correlation with prognosis. In 1992, Moreno et al. [35] detected PSA RT-PCR positive only in patients with locally advanced or metastatic prostate cancer but not in control group. The positivity was 33%. This study generated the hypothesis that circulating tumor cells could be an early event in prostate cancer. Two years later, researchers from Columbia University found a significant correlation with PSA RT-PCR positivity and higher pathologic stage in 65 patients submitted to radical prostatectomy [29]. Unfortunately, studies that came after and more recently showed that monitoring CTC level after localized treatment is not yet ready for practical use since some of them failed to demonstrate relationship with clinical outcome [12, 32]. In the largest of them, only 11% of 152 patients had detectable CTCs before surgery and did not translate into prediction of biochemical recurrence in the 48 months that followed [34]. This finding could be attributed to the technique involved to isolate CTCs (*immunomagnetic* × *isolation based on physical properties*) as they have different sensitivities [19]. Also, maybe the molecular characterization of CTCs matters more than levels.

Screening for prostate cancer has been challenging and controversial since the main biomarker (*PSA*) is far from being ideal. PSA can be increased not only in

prostate cancer but also in benign conditions such as prostatitis and benign prostatic hyperplasia. Actually, PSA test confers only 25% of true-positive and 14.5% of true-negative patients [37]. This has generated a special need in finding a better tool to help detect early prostate cancer.

In this case, CTC detection can possibly add accuracy to PSA test. In 2020, Ried et al. [45] studied 45 CTCs detected in a group of 2000 patients screened and compared those findings to PSA. CTCs were also tested for PSA expression. For 20 patients diagnosed with prostate cancer and CTCs detected, blood PSA was elevated in only 35%; 100% of patients with prostate cancer had expression of PSA in CTC. Combination of CTC detection with CTCs with PSA expression conferred a very high positive predictive value (*99%*) and also negative predictive value.

6.3 CTCs in Advanced Prostate Cancer

The magnitude of CTC levels also correlated with outcomes. In 2001, Moreno et al. [36] found that in ten patients submitted to chemotherapy, high levels of CTCs correlated with shorter disease-free survival and low levels, with slow progression. In 2007, Danila et al. isolated CTCs from 120 prostate cancer patients and found higher levels in patients with bone metastases and in those previously submitted to chemotherapy [10].

In clinical trials, CTC detection has been incorporated as a parameter of clinical outcomes after a paper in 2008 showed CTC measurement after treatment correlated with prognosis as a continuing variable, especially when combined to DHL levels. This correlation was even stronger than PSA decrease in 50% or more [14].

Since then, CTCs have been included in other clinical trials as a measurement of outcomes. Baseline levels of CTCs have shown correlation with survival. The cut-off level of < or > 5 cells/7.5 mL blood at baseline identified patients with more or less favorable outcome [4, 11, 44, 48]. It is important to take into account that the level of CTCs detected varies from line of treatment, being more unfavorable (*>5 CTCs/7.5 mL*) in more late lines [33].

Not only baseline counts but also changing in this value over treatment as a favorable or unfavorable rate could also predict survival and in some studies even better than PSA response rate [14, 39, 47]. These findings were seen in treatment with different agents. In Docetaxel trials, decrease in CTC counts to less than 5 CTCs/7.5 mL was associated with decrease in 50% of OS [1, 23, 38, 57]. During treatment with abiraterone also, CTC detection was checked in parallel with other end-points and revealed the same worse outcome with levels >5 CTCs/7.5 mL after treatment.

Sometimes, CTC change was a better predictor of treatment response than classical imaging evaluation (*RECIST*) [55].

6.4 CTCs as a Biomarker of Treatment Resistance

Another focus of CTCs in prostate cancer is their molecular study. Treatment response to docetaxel, for example, was linked to decrease in expression of KLK3, PCA3, and TMPRSS2-ERG in the CTCs [18].

Interestingly, in the publication of Reid et al. [44], CTCs were included as a part of response evaluation to abiraterone in phase II clinical trial. Changes in CTC count during treatment were also significantly correlated with PSA response rate but only in patients whose tumor had ERG rearrangement.

Evidence of cross resistance between novel hormonal agents (*abiraterone and enzalutamide*) have emerged. Some trials looked at sequential use of those after progression on the other ended up with low PSA response rate (*such as 5%*) and low progression-free survival (*<6 months*) [5, 31]. In another trial, even chemotherapy sequentially would be better option than the other hormonal agent [17].

Some explanation came from the study of androgen receptor and its variants, specifically AR-V7 that is found more frequently during the use of abiraterone or enzalutamide and that is not expressed in primary tumor. Splice variants can activate AR constitutively and avoid new hormonal agents connection and their antitumor inhibition. It is known that these mutated receptors may exist on a primary or as an acquired resistance. In this case, CTCs became a very useful tool to demonstrate this important mechanism of resistance since mRNA from CTCs can be isolated in patients exposed to these agents. By studying mRNA from CTCs, researchers detected splice variants from androgen receptor that was linked to worse outcomes when patients were treated with abiraterone or enzalutamide [2].

More recently, researchers from Memorial Sloan Kettering Cancer Center found that not only identification of mRNA AR-V7 (*CTCs or whole blood*) is sufficient, but the protein localization in the CTCs is a stronger predictor of response to novel ARSi or taxane therapy [50]. In this observational study, 142 patients with mCRPC who progressed to first-line therapy were tested for AR-V7 positivity before and after starting second-line therapy (*novel ARSi or taxane*) by EPIC science test. Positive patients were considered nuclear localized AR-V7 and negative, AR-V7 cytoplasmic or absent. In AR-V7 negative patients, treatment with ARSi resulted in a superior overall survival than chemotherapy (*16.9* vs. *9.7 months, hazard ratio, 2.38; 95% CI, 1.12–5.06; p = 0.02*) and in AR-V7 positive patients, performance with novel ARSi was inferior than those treated with taxanes (*overall survival 5.6 × 14.3 months, respectively, hazard ratio, 0.35; 95% CI, 0.14–0.88; p = 0.03*). This finding lost significance when positive patients were also considered to have cytoplasmic AR-V7 localization (*HR 0.73, 95% CI 0.26–2.04; p = 0.55*).

6.5 Conclusion

The role of detecting CTCs in prostate cancer has evolved a lot during recent years, from diagnostic to prognostic and to prediction of response. In the localized disease, CTC levels can be a promising tool to identify patients at higher risk of recurrence and so, select patients to a more intensive follow-up (Table 6.1). Unfortunately, studies in this scenario are few with limited number of recruitment and do not validate CTC collection for this purpose yet. Also, CTCs can be detected in patients submitted to screening program and in association with PSA contribute to better find early disease.

In advanced disease, CTC kinetics over time have demonstrated more accuracy for response evaluation, sometimes more than PSA levels and earlier than images. As the treatment improved, liquid biopsy with the study of CTC biology has been a promising tool to select which patient can benefit from one strategy rather than the other, considering that both are proved to be effective.

Table 6.1 Examples of studies that evaluate CTCs in prostate cancer

Tumor	Stage	*N*	Method	Markers	Authors	Year	Main results
Prostate	I–IV	29	RT-PCR	PSA	Moreno JG et al.	1992	33% detection of RNA + PSA in locally advanced or metastatic disease 0% detection in control group
Prostate	I–III	148	RT-PCR	PSA	Katz AE et al.	1994	67% of T3 patients had PSA RT-PCR + and in 86% with positive margin
Prostate	I–III	152	CellSearch	EpCAM, CK	Meyer CP et al.	2016	Biochemical recurrence did not increase with CTC positivity before surgery ($p = 0.7$)
Prostate	IV	120	CellSearch	EpCAM, CK	Danila DC et al.	2007	Higher CTC numbers were seen in bone metastases disease and in prior cytotoxic chemotherapy Baseline CTC count were strongly associated with survival

(continued)

Table 6.1 (continued)

Tumor	Stage	*N*	Method	Markers	Authors	Year	Main results
Prostate	IV	231	CellSearch	EpCAM, CK	De Bono JS, et al.	2008	CTC detection after treatment occurred in patients with shorter OS in a better way than blood PSA. Prognosis improved in conversion to CTC unfavorable to favorable after treatment (*6.8 to 21.3 months*) and worsened in conversion of favorable to unfavorable (*>26 to 9.3 months*)
Prostate	IV	62	AdnaTest	AdnaTest Prostate Cancer Detect kit with additional primers targeting ARV7 and AR-FL	Antonarakis ES et al.	2014	AR-V7-positivity correlated with lower PSA response, shorter PSA, clinical or radiographic PFS and OS in patients treated with abiraterone or enzalutamide
Prostate	IV	33	CellSearch	EpCAM, CK	Thalgott M et al.	2015	CTC counts predicted better overall survival and treatment response than RECIST by conventional images
Prostate	IV	142	EPIC Test	AR-V7 (*nuclear and cytoplasmic*)	Scher HI et al.	2018	AR-V7 + associated with OS benefit of chemo × novel ARSi and AR-VT – benefited more from chemo
Prostate		45	ISET	Filtration + PSA	Ried K et al.	2020	CTC detection with PSA expression conferred 99% of PPV and 97% NPV

Abbreviations: *PFS* progression-free survival, *PPV* predictive positive value, *NPV* negative predictive value

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Chapter 7
Circulating Tumor Cells in Gastric Cancer

Jacqueline Aparecida Torres and Victor Hugo Fonseca de Jesus

7.1 Introduction: Epidemiology

Gastric cancer is currently the sixth most frequent malignant neoplasm worldwide, with 1,003,701 cases estimated in 2018 [1]. Also, its occurrence varies greatly, with East Asia and Western South America representing the areas with the highest incidence rates of the disease [2]. However, the frequency of proximal gastric tumors in most Western countries has constantly risen in the past decades, and the cardia represents the most common primary tumor site in these locations. Additionally, gastric cancer represents the second most common cause of cancer-related mortality, with 782,685 deaths estimated in 2018.

Exposure to many agents is associated with increased risk of developing gastric cancer [3]. The infection by the Gram-negative bacteria *Helicobacter pylori* has been recognized as the most important factor leading to the development of gastric cancer. Other factors associated with increased risk are cigarette smoking, consumption of salty or smoked food, and low consumption of fruits and vegetables. Obesity is also considered to be a significant risk factor, especially for tumors arising in the cardia. Among the non-modifiable risk factors, advanced age and male gender are associated with higher chances of developing gastric cancer.

Figures separated by Ludmilla T. D. Chinen and revised by Mauro Saieg (Cytopatologist from AC Camargo Cancer Center, São Paulo, SP – Brasil)

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7.2 Treatment of Early Disease

The cornerstone of localized gastric cancer treatment is gastrectomy with adequate (D2) lymphadenectomy [4]. For many years, surgery alone was considered the standard of care in the management of this disease. Nonetheless, with the development of active chemotherapy regimens, a multidisciplinary approach has become standard. The Intergroup INT0016 trial was the first to establish the role of adjuvant (postoperative) 5-Fluorouracil/Leucovorin and radiotherapy in the management of this disease. Patients submitted to surgery alone experienced inferior overall and relapse-free survivals [5]. Subsequently, the role of adjuvant chemotherapy was further confirmed in the ACTS-GC and CLASSIC trials. In the first study, the use of adjuvant S-1 for 1 year was associated with improvements in overall and relapse-free survivals [6]. In the latter trial, adjuvant XELOX (Capecitabine plus Oxaliplatin) for 6 months was associated with decreased risks of death and relapse [7]. Thus, postoperative chemo(radio)therapy became one the preferred treatment strategies for patients with pathological stage II or III gastric cancer, especially in the East.

Concurrently, groups in the West tested the activity of perioperative chemotherapy. This approach has potential advantages over adjuvant chemotherapy, such as early treatment of metastatic disease, potentially higher rates of complete resection, tumor downstaging, and an in vivo evaluation of chemotherapy activity [8]. Two studies, the MAGIC and the FFCD 9703 trials, evaluated the role of perioperative chemotherapy in localized gastric cancer. In the MAGIC trial, patients treated with ECF (Epirubicin, Cisplatin, and Fluorouracil) for three cycles before surgery (neoadjuvant) and three cycles thereafter experienced improved overall and progression-free survival compared to those treated with surgery alone [9]. Likewise, patients in the FFCD 9703 trial who were treated with 2–3 cycles of neoadjuvant CF (Cisplatin and 5-Fluorouracli) and 3–4 postoperative cycles had lower risks of death and relapse [10]. More recently, results of the FLOT4 trials have established FLOT (Fluorouracil, Oxaliplatin, and Docetaxel) as the preferred chemotherapy regimen in the setting of perioperative chemotherapy for gastric cancer [11]. In this trial, patients treated with four cycles of FLOT before surgery and four cycles thereafter experienced improved overall and progression-free survival when compared to those treated with ECF or ECX (Epirubicin, Cisplatin, and Capecitabine). Thus, FLOT is currently considered the standard perioperative chemotherapy regimen in clinical stage II or III gastric cancer.

One of the main controversies in the management of localized gastric cancer is whether patients should undergo upfront surgery followed by adjuvant chemotherapy or perioperative chemotherapy plus surgery. Early studies showed no differences in survival outcomes according to the treatment strategy [12, 13]. However, more recent studies have shown decreased risk of disease relapse for those treated with perioperative chemotherapy [14–16]. While this benefit might stem from differences in the chemotherapy regimens used in the perioperative and adjuvant settings, no trial so far has shown inferior outcomes for patients treated with neoadjuvant chemotherapy, and therefore perioperative chemotherapy is currently perceived by many as the most adequate treatment strategy.

7.3 Treatment of Advanced Disease

7.3.1 First-Line Treatment

Chemotherapy is associated with significant improvements in overall survival for patients with recurrent or metastatic gastric cancer compared to best supportive care [17]. For many years, the combination of Epirubicin, Cisplatin, and 5-Fluorouracil (ECF) was considered to be the standard treatment in this setting [18]. However, data from recent meta-analysis do not support the use of anthracyclines in gastric cancer [19]. Therefore, in many parts of the world, Cisplatin plus 5-Fluorouracil (or another fluoropyrimidine, such as Capecitabine or S-1) was considered the treatment of choice of advanced gastric cancer for many years. Following that, Cisplatin plus 5-Fluorouracil (plus Leucovorin; FLP) was compared to FLO (infusional 5-Fluorouracil plus Oxaliplatin). That trial showed non-inferiority of FLO in terms of overall and progression-free survival [20]. The results of this study and of the REAL2 [21] trial established the role of Oxaliplatin in the management of advanced gastric cancer, and regimens based on infusional 5-Fluorouracil plus Oxaliplatin (such as FOLFOX and FLO) are currently among the most frequently used chemotherapy regimens in the West. Conversely, in the East, where the use of oral fluoropyrimidines is very common, the combination of Cisplatin plus S-1 (CS) was shown to be more effective than S-1 alone [22]. Recently, SOX (S-1 plus Oxaliplatin) was shown to be at least as effective (REF) [23] or more active than CS [24]. Thus, both SOX and CS are currently considered standard regimens in the East.

One alternative to the use of platinum plus fluoropyrimidine is to employ regimens that combine 5-Fluorouracil and Irinotecan (e.g., IF or FOLFIRI). The results of two randomized [25, 26] trials support this concept, and these regimens are generally used when platinum-based regimens are contraindicated (e.g., when patients have grade 2 or higher peripheral neuropathy at baseline or when patients develop disease progression within 3 months of the end of adjuvant platinum-based chemotherapy) [27]. One other important issue is the use of taxane-based triplet regimens (e.g., DCF [Docetaxel, Cisplatin, and 5-Fluorouracil]) in first-line treatment. Clinical trials have shown that these regimens are associated with improved survival [28, 29], despite an increase in toxicity. In this sense, the use of modified regimens, such as modified DCF [30] or FLOT [31], can sustain the anti-cancer activity of the treatment while keeping side effects in an acceptable range. Thus, triplet-based regimens are often used in patients with adequate performance status and organic function, especially when symptom or disease burden is high.

Further understanding of the molecular biology of gastric cancer brought insights to the development of relevant treatment strategies. The discovery that up to 38% [32] of all gastric cancers present hyperexpression of the HER2 protein led to clinical trials that added anti-HER2 treatments to the backbone chemotherapy regimens. In the ToGA trial [33], patients with HER2 hyperexpression where randomized to treatment with Cisplatin plus a fluoropyrimidine (Capecitabine or 5-Fluorouracil) with or without Trastuzumab, an anti-HER2 monoclonal antibody previously shown to be active in breast cancer. In this trial, patients whose tumors had presented high

HER2 expression (3+ on immunohistochemistry or 2+ on immunohistochemistry with positive in situ fluorescence hybridization [FISH]) experienced significantly longer overall survival. Therefore, the use anti-HER2 antibodies is considered to be standard of care for those patients with tumors with high expression of HER2. In the first-line setting, many other potential therapeutic targets were tested, including immunotherapy, with disappointing results. Apart from patients with tumors with high frequency microsatellite instability (MSI-H) [34], for whom immunotherapy with Pembrolizumab is considered to be the standard of care in first line, no other targeted therapy has demonstrated significant benefits in the this setting.

7.3.2 Second and Further Lines Treatment

Randomized trials have also established the role of chemotherapy after progression on first-line treatment. Irinotecan, Docetaxel, and Ramucirumab were shown to improve overall survival compared to best supportive care [35–37]. Additionally, Paclitaxel was shown to be at least as active as Irinotecan in two randomized trials [38, 39]. Recently, the addition of the anti-VEGFR2 monoclonal antibody Ramucirumab to Paclitaxel was associated with increased overall response rate, progression-free survival, and overall survival in the RAINBOW trial. According to the results of this study, Paclitaxel plus Ramucirumab is likely the most active second-line chemotherapy regimen currently available for patients with gastric cancer.

While the addition of Trastuzumab to the backbone of chemotherapy improved survival in the first-line setting, no other anti-HER2 treatment was associated with improved outcomes in the second-line setting, including Lapatinib, TDM-1, and maintenance Trastuzumab beyond progression [40–42]. Only recently, the antibody-drug conjugate (ADC) Trastuzumab deruxtecan was shown to be superior to single-agent chemotherapy in third- or further lines of treatment [43]. Regarding immunotherapy, while Pembrolizumab failed to demonstrate improved outcomes in the second-line setting in the KEYNOTE-061, data from the cohort 1 of the KEYNOTE 059 and from the ATTRACTION-2 trials support the use of Pembrolizumab and Nivolumab in the third-line setting, respectively [44, 45]. Importantly, Pembrolizumab is approved only for patients with tumor CPS (a marker of programmed cell death ligand 1 [PD-L1] expression) higher or equal to 1, while the evaluation of the expression of PD-L1 is not a prerequisite for the treatment with Nivolumab.

7.4 CTC in Gastric Cancer

Circulating tumor cells (CTCs) have been the target of several studies and have been identified in patients with several cancers including gastric cancer (GC). The interest in knowing more about this compartment of the liquid biopsy is due to the fact that, when they detach from the primary tumor, CTCs can circulate individually or

in the form of circulating tumor microemboli (CTMs), making possible to visualize in a more comprehensive way and in real time the reality of the tumor. CTCs are an important biomarker that can be used as a source of early detection, to follow-up of the efficacy of treatments, to discover new therapeutic targets, and to bring new understanding about the biology of metastases [46–49].

Kuroda et al. [50] analyzed the overexpression FGFR2 (fibroblast growth factor receptor) in CTCs of GC patients. Patients (n = 100) with CG who underwent gastrectomy were recruited, from which 8 ml of total blood was collected before surgery. CTCs positive for FGFR2 were enumerated by flow cytometry, and through immunohistochemistry (IHC), the expression of FGFR2 of the primary tumor was evaluated. The IHC was divided into 4 groups (0, 1+, 2+ and 3+) according to the FGFR2 expression and the number of cases in each group was 39, 35, 17, and 9, respectively. The number of CTCs FGFR2$^+$ in 2 ml of blood was 0.6 ± 1.2; 2.4 ± 4.2; 2.6 ± 2.9 e 8.3 ± 11.2 (mean ± SD) in IHC groups 0, 1+, 2+, and 3+, respectively. It can be observed that the level CTCs FGFR2+ increased proportionally to the level of FGFR2+ IHC. Recurrence-free survival was analyzed and 50/89 patients with CTCs FGFR2+ ≥ 1 CTC/2 ml had significantly worse survival (P = 0.018, log-rank test) than patients without CTCs FGFR2+. In conclusion, overexpression of FGFR2 in CTCs of GC patients can be used to identify overexpression of FGFR2 in the primary tumor and act as a prognostic factor.

Abdallah et al. [51] analyzed 88 peripheral blood samples from patients (n = 55) with non-metastatic gastric adenocarcinoma (CAG) to evaluate the presence of CTCs and CTMs, in addition to the expression of HER2 and plakoglobin. Samples were obtained before treatment and after surgery, before the administration of adjuvant chemotherapy. The isolation of CTCs was done using the ISET methodology (Rarecells Diagnostics, Paris, France). Immunocytochemistry (ICC) was used to analyze the expression of markers in CTCs and compared to GAC IHC. Baseline CTCs were observed in 90.9% of patients (50/55) with median of 2.8 CTCs/mL. The analysis of follow-up CTCs was also high (93.9%) but with reduction of the median when compared to baseline (1.0 vs. 2.8 CTCs/mL; p = 0.005). CTMs were identified in 22/55 patients (41.8%) in baseline and 2/55 patients (6.1%) in follow-up. Patients with CTMs had a worse PFS than those who did not have CTMs (18.7 months vs. 21.6 months, respectively; p = 0.258). The HER2 expression was analyzed in 45/55 CAG samples, of which 5/45 (11%) were HER2$^+$ and in 42/55 CTCs samples with positivity of 18/42 (42.9%). Negative HER2 patients in CTCs tended to have better PFS (p = 0.092). There was overlap between 36 patients in the analysis of HER2 expression (CTCs and primary tumor), with an agreement of 69.4% (κ = 0.272; p = 0.04). In 10/36 cases (27.8%), HER2 expression was positive only in CTCs. Plakoglobin was evaluated in 47/55 patients and positive in 59.6% of CTC baseline cases. Patients 9/47 had the protein identified in CTM being related to a worse trend of median PFS (15.9 months vs. 21.3 months; p = 0.114). These results suggest that CTC count and HER-2 and plakoglobin analysis contribute to evaluate the response and determine prognosis in patients with GAC. (Please see some pictures of CTCs isolated from metastatic and localized gastric cancer by ISET in Figs. 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 7.10, 7.11, 7.12, 7.13, 7.14, 7.15, 7.16, 7.17, 7.18, 7.19, 7.20, and 7.21).

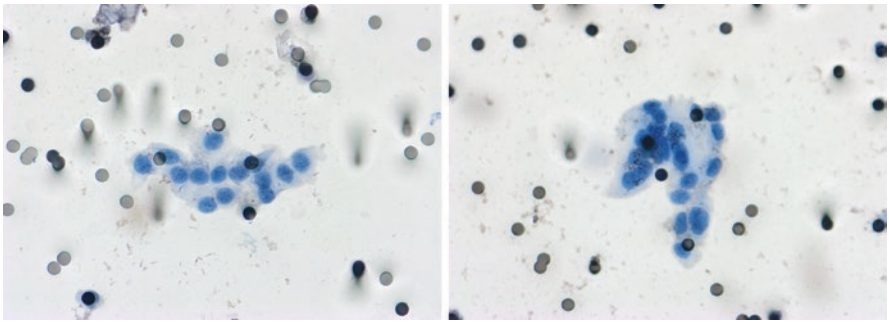

Fig. 7.1 CTM from patient (woman) with non-metastatic gastric cancer, 43 years old. Blood was collected at diagnosis, 13.25 CTCs/mL. Here, we can observe clusters of neoplastic cells with a three-dimensional arrangement and moderate atypia. Objective magnification: 20×

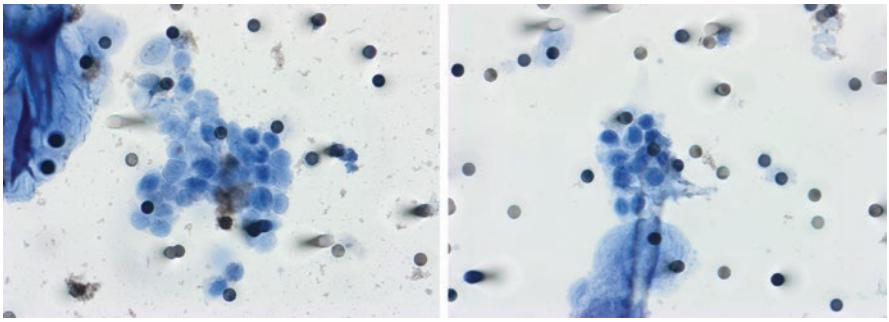

Fig. 7.2 CTM from patient (man) with non-metastatic gastric cancer, 40 years old. Blood was collected at diagnosis, 4.22 CTCs/mL. The patient was selected from neoadjuvant treatment with 5-Fluorouracil. Here, we can observe three-dimensional clusters of neoplastic cells with nuclear irregularity. Objective magnification: 20×

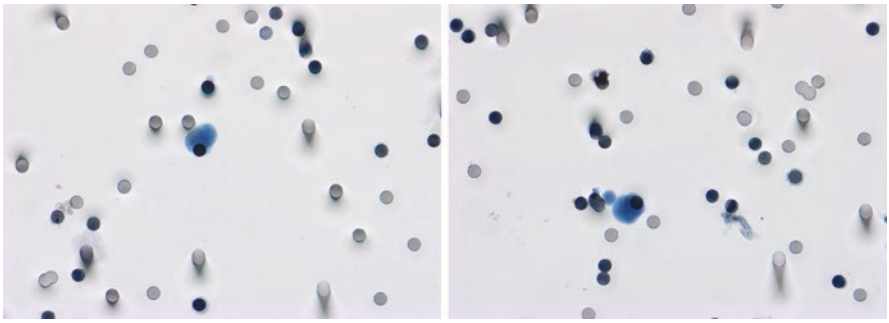

Fig. 7.3 CTCs from patient (man) with metastatic gastric cancer, 67 years old, with peritoneum metastasis. Blood was collected at diagnosis, 9.0 CTCs/mL. Objective magnification: 20×

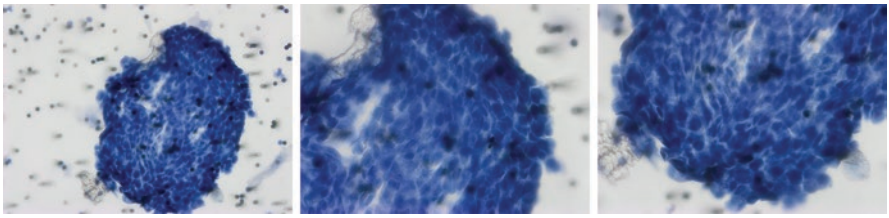

Fig. 7.4 CTM from patient (man) with metastatic gastric cancer, 39 years old. Blood was collected at diagnosis (1.16 CTCs/mL) and after 6 months of follow-up. The patient had peritoneum metastasis at diagnosis. Here, CTM from the second blood collection (44.5 CTCs/mL). We can observe a group of neoplastic cells with scaly phenotype, showing three-dimensional blocks with hyperchromic and irregular nuclei in 20× and 40×

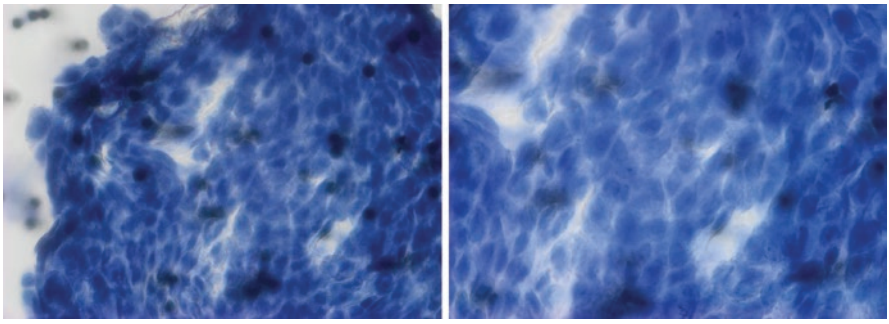

Fig. 7.5 CTM from patient of Fig. 7.4, in 60×

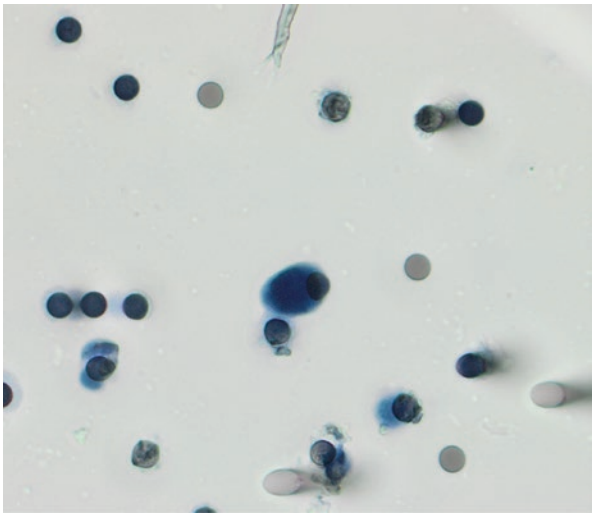

Fig. 7.6 CTC from patient (woman) with non-metastatic gastric cancer, 34 years old. Blood was collected at diagnosis, 2.83 CTCs/mL. Here, we can observe irregular nuclei

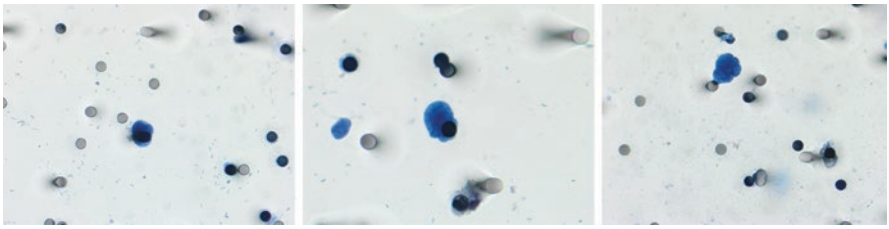

Fig. 7.7 CTCs from patient (man) with non-metastatic gastric cancer, 39 years old. Blood was collected at diagnosis, 1.0 CTCs/mL. The patient was submitted to neoadjuvant treatment with 5-FU

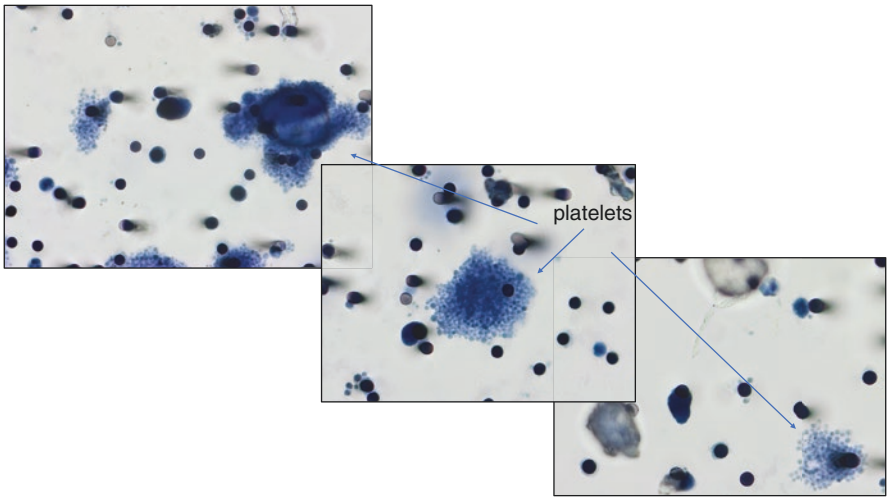

Fig. 7.8 CTCs from patient (woman) with non-metastatic gastric cancer, 58 years old. Blood was collected at diagnosis, 4.66 CTCs/mL and after neoadjuvant treatment with 5-FU (4.33 CTCs/mL)

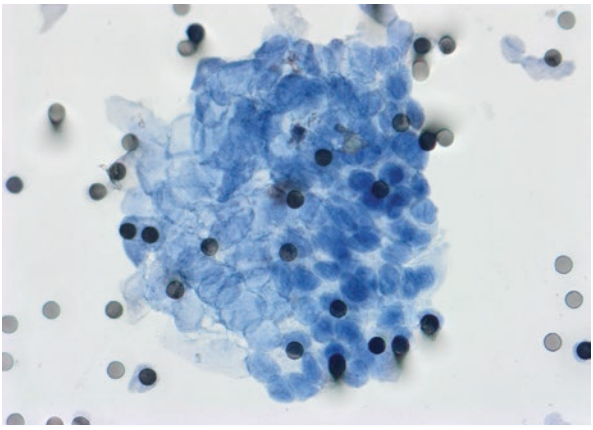

Fig. 7.9 CTM from patient (woman) with non-metastatic gastric cancer, 72 years old. Blood was collected at diagnosis, 10.5 CTCs/mL

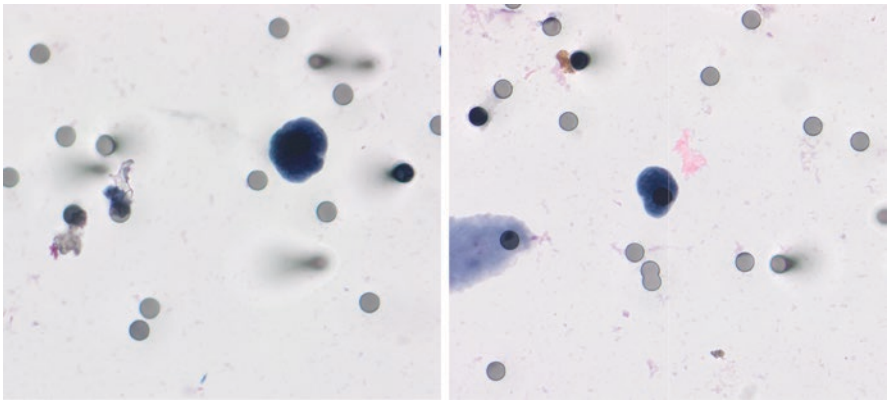

Fig. 7.10 CTCs from the same patient Fig. 7.9. Here, we can observe irregular and hyperchromatic nuclei

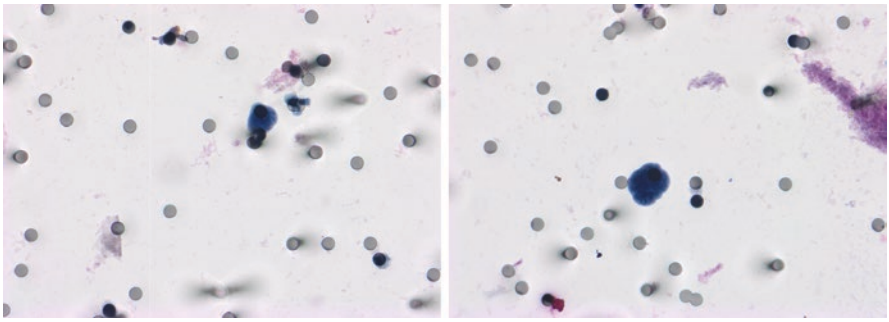

Fig. 7.11 CTCs from the same patient of Fig. 7.9

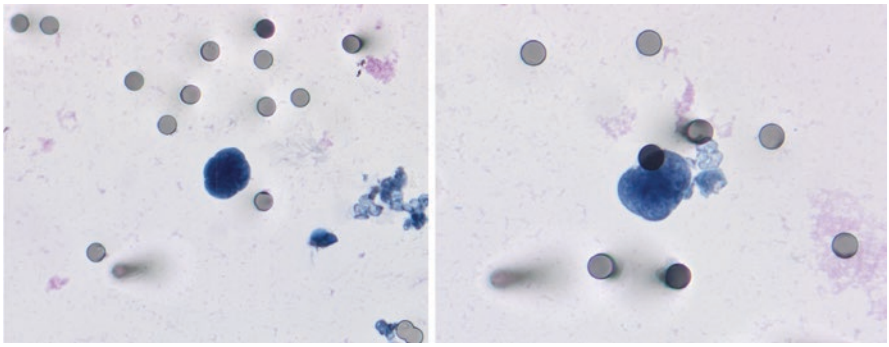

Fig. 7.12 CTCs from patient (man) with non-metastatic gastric cancer. Blood was collected at diagnosis, 1.33 CTCs/mL and after neoadjuvant treatment with 5-FU (2.0 CTCs/mL). Here, CTCs from the second collection. At the right side, nuclear details are visible, with hyperchromic and irregular nuclei and scarce cytoplasm

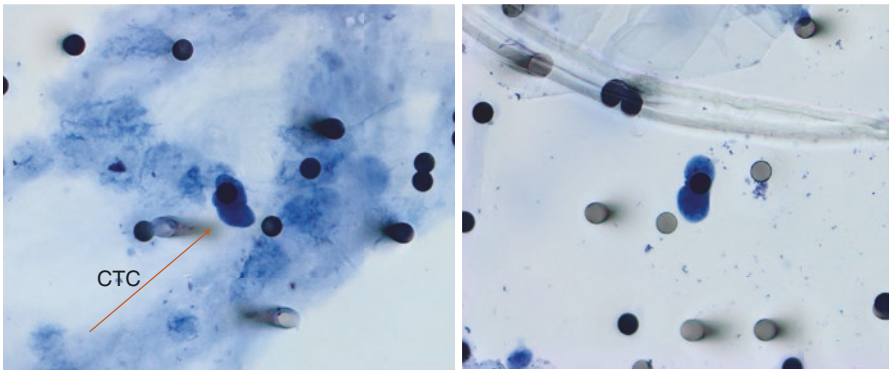

Fig. 7.13 CTM from patient (man) with non-metastatic gastric cancer, 70 years old. Blood was collected at diagnosis, 0.5 CTCs/mL and after a year (1.5 CTCs/mL). Here, we can observe cells of the second collection, with irregular shaped and hyperchromic nuclei

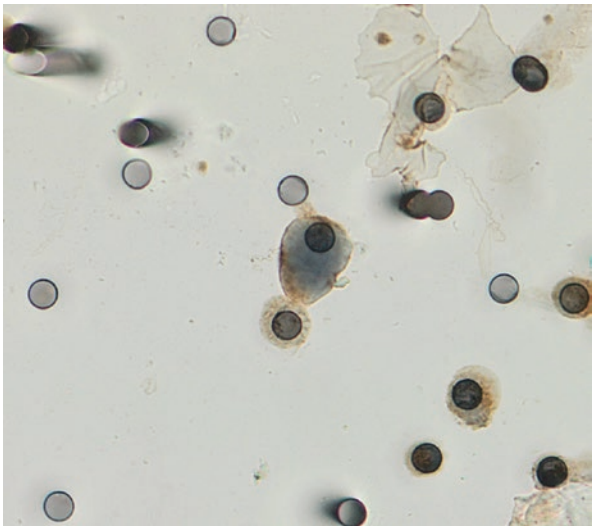

Fig. 7.14 CTC from the same patient of Fig. 7.13. In brown, cytoplasm staining with DAB for HER-2

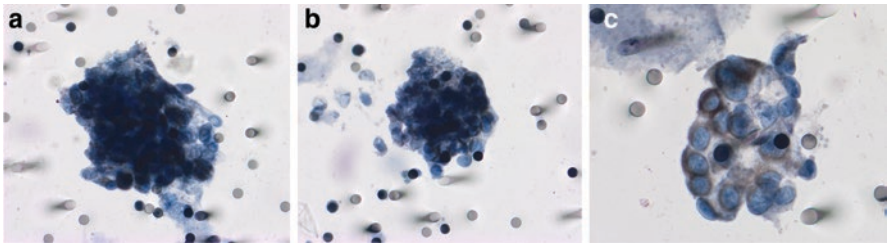

Fig. 7.15 CTM from patient (man) with non-metastatic gastric cancer, 50 years old. Blood was collected at diagnosis, 8.33 CTCs/mL. Here, we can observe CTM (**a**, **b**) and in (**c**), HER-2 staining in microemboli cells in three-dimensional arrangement and nuclear irregularity

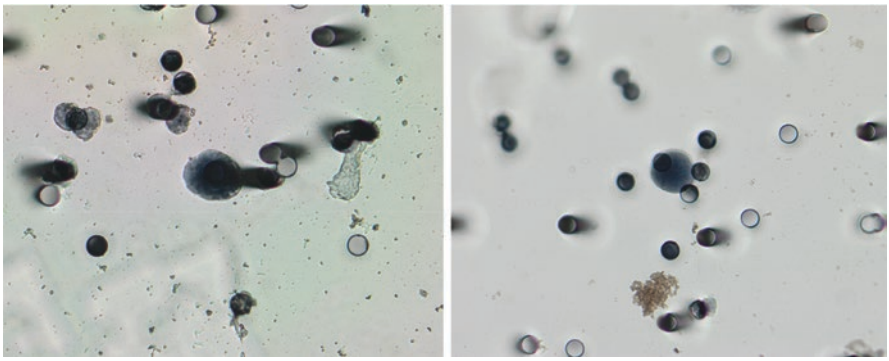

Fig. 7.16 CTCs from patient (woman) with non-metastatic gastric cancer, 53 years old. Blood was collected at diagnosis, 0.5 CTCs/mL and after neoadjuvancy with 5-FU (5 CTCs/mL). Here, we can observe cell staining for HER-2 in the second blood collection

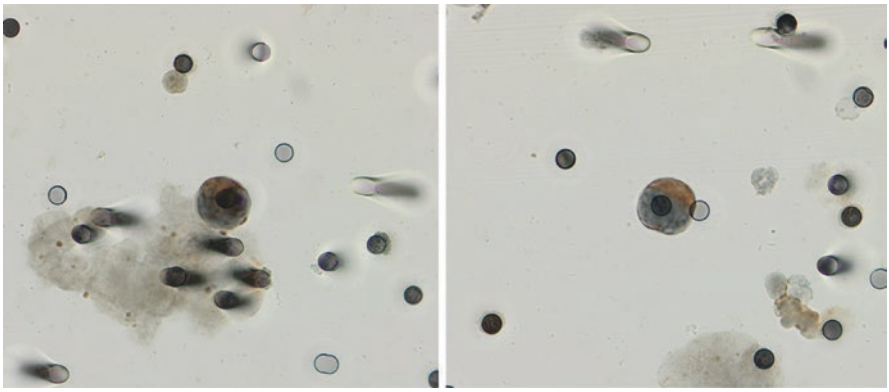

Fig. 7.17 CTCs from patient (man) with metastatic gastric cancer, 36 years old. Blood was collected at diagnosis, 3.0 CTCs/mL and after 3 months (3.0 CTCs/ml). Here, we can observe cytoplasm staining for HER-2 at the second blood collection

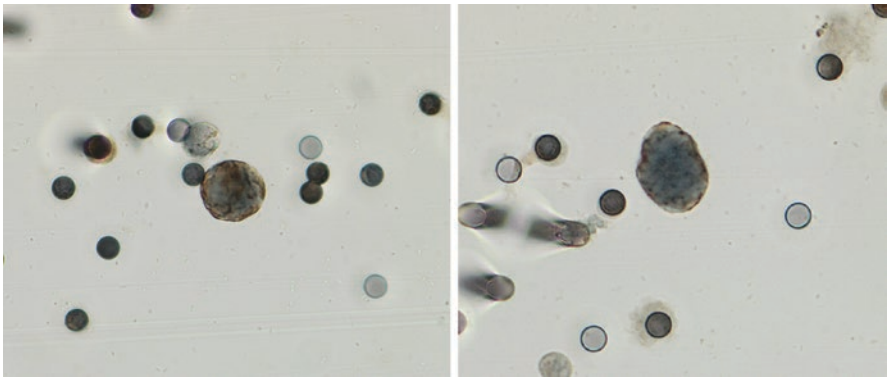

Fig. 7.18 CTCs from the same patient of Fig. 7.17. Cytoplasm staining for HER-2. We can observe nuclear irregularity and irregular chromatin, with high nucleus/cytoplasmic ratio

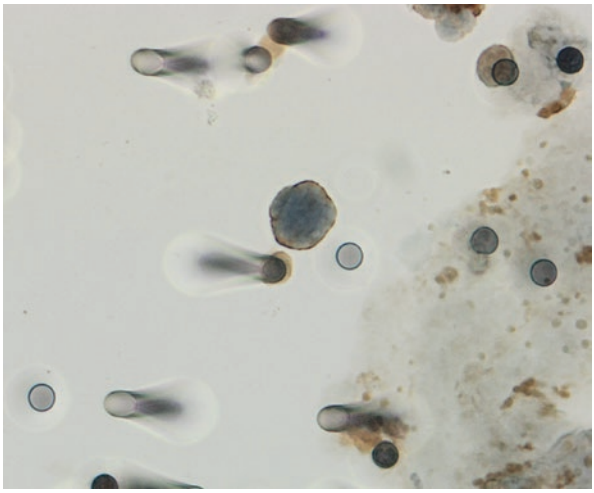

Fig. 7.19 CTC from patient (woman) with non-metastatic gastric cancer, 55 years old. Blood was collected at diagnosis, 2.33 CTCs/mL and after neoadjuvant treatment with 5-FU (1.0 CTCs/ml). Here, we can observe cytoplasm staining for plakoglobin at the second blood collection

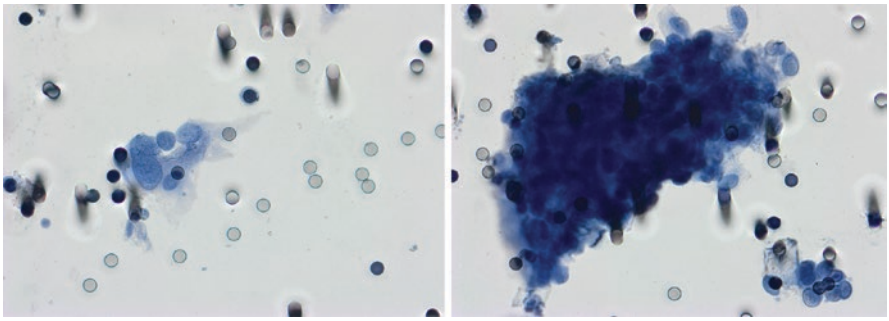

Fig. 7.20 CTM from patient (woman) with metastatic gastric cancer to peritoneum, 80 years old. Blood was collected at diagnosis, 40.0 CTCs/mL. Here, we can observe on the right, neoplastic cell blocks in three-dimensional arrangement and hyperchromic nuclei

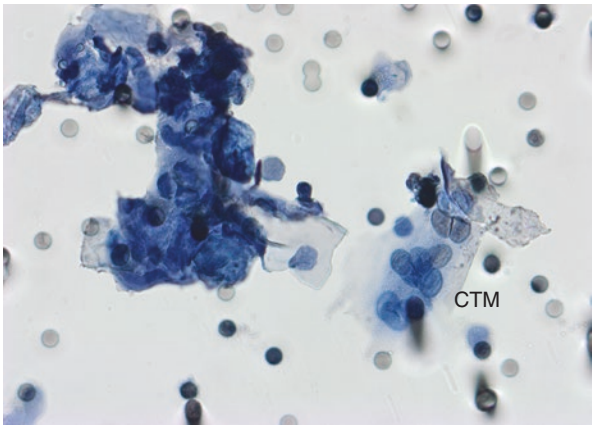

Fig. 7.21 CTM from the same patient of Fig. 7.20

Epithelial CTCs undergo epithelium-mesenchymal transition (EMT) acquiring mesenchymal characteristics that result in an increased ability to reach distant sites and colonize them, forming metastases. During this process, there is a cell surface vimentin (CSV) overexpression that can be used as a marker to identify EMT CTCs. Liu et al. [52] studied the expression of PD-L1 (programmed death ligand 1), a protein that when expressed in tumor cells allows immune system evasion, in CSV+ CTCs of patients with GC. Total blood (5 ml) was collected from patients ($n = 70$) with ressectable ($n = 38$) and non-ressectable ($n = 32$) CG. The samples were analyzed using CVS microsphere and EpCAM. CTCs were detected in 60/70 patients (86%) (0–512 CTCs/mL). VCS+ PD-L1+ CTCs were identified in 50/70 patients (71%) (0–261 CTCs/mL). When compared, the total counts of CSV + PD L1+ CTCs showed a significant difference in the distinction between ressectable and non-ressectable populations (2 vs. 8 mL; $P = 0.001$), respectively. The total CTC count in an average follow-up of 12.9 months resulted in HR of 2.364 for PFS (IC 95%: 1.038–5.381; $P = 0.040$) and 1.817 for OS (IC 95%: 0.8025–4.114; $P = 0.152$). However, patients with a higher amount of CTCs CSV+ PD L1+ had worse PFS (HR: 2.437; IC 95%: 1.074–5.529; $P = 0.033$) and worse OS (HR: 3.762; IC 95%: 1.629–8.691; $P = 0.002$) when compared to patients with fewer CTCs CSV+ PD L1+. This study demonstrated that it is possible to predict therapeutic and prognostic response in CG patients using CTCs PD-L1+ detected by a CVS-based methodology.

All these studies together demonstrate the potential of CTCs for the treatment and follow-up of patients with CG. Other studies that relate CTCs in CG are described in Table 7.1.

Table 7.1 Overview of studies evaluating CTCs in gastric cancer

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
Gastric cancer	I–IV	41	Cyttel/FISH	Vimentin/EpCAM/ ULBP1/ CD45/CEP8/ DAPI/CEP17/TGF-β1	Hu et al. [53]	2020	CTCs were detected in 29/44 patients (70.7%). Count = 0 to 13 CTCs/3.2 ml PB. CTCs of the GC patients were divided into three CTCs EMT subgroups: E+CTC (93.1%); M+CTC (75.86%) and E+/M+ CTC (86.21%). ULBP1 expression was observed in the CTCs EMT subgroups. The subgroup M+CTC expressed lower level compared to the E+/M+ CTC and E+ CTC subgroups	GC: gastric cancer; PB: peripheral blood; EMT: epithelium-mesenchymal transition; E+CTC: epithelial CTCs; M+ CTC: mesenchymal CTCs; E+/M+ CTC: biphenotypic
Gastric adenocarcinoma		55	ISET	HER2/plakoglobin/ anti CD45	Abdallah et al. [51]	2019	90.9% of patients (20/55) baseline had CTCs (median = 2.8 CTCs/ml blood. CTCs follow-up were present in 93.9% patients (median = 1.0 CTC/ml blood. CTMs were observed in 22/55 (41.8%) baseline and 2/55 (6.1%) in the follow-up. CTMs were associated with poor PSF vs. patients without CTMs (18.7 vs. 21.6 months, respectively; $p = 0.258$). CTCs HER2+ were present in 42/55 (42.9%) patients vs. 5/45 samples of GAC. In 36 cases overlapping in the analysis of HER2, in 10 cases the expression was positive only in CTCs. The presence of plakoglobin was observed in 47 CTCs baseline with 59.6% positive and 9/47 patients had CTM plakoglobin +, associated with poor median PFS 15.9 vs. 21.3 months; $p = 0.114$)	CTMs: circulating tumor microemboli; PFS: progression-free survival; GAC: gastric adenocarcinoma

Adenocarcinoma (stomach)	I–IV	228	Flow cytometry	CD45/CK 8, 18 e 19/ CD44	Szczepanik et al. [54]	2019	CTCs were present in 31 patients and DTC in 106. Patients ($n = 93$) 41% presented tumor cells+ for CK in the PB (CTCs) or on bone marrow (DTC) and the expression of the CD44 was observed in 22 cases (10%). Patients with distant metastasis present more cells CK+ and CD44+ (50 vs. 19%, $P = 0.001$). Cells CD44+ were associated with worse median survival (6.7 months) compared to patients with CD44- (22.3 months)	CK: cytokeratin; DTC: disseminated tumor cells
Advanced gastric cancer		115	Cytelligen/iFISH	HER2/CEP8/CD45	Li et al. [55]	2018	91% of the patients hHER2+ and 76.2% hHER2- acquired the cHER+ phenotype, being related to the development of resistance to therapy with Trastuzumab (hHER2+) e chemotherapy (hHER2-). The Chr8 aneuploidy is related to obtaining the HER2+ phenotype in CTCs, facilitating growth and acquisition of resistance to treatment	HER2: human epidermal growth factor receptor-2; hHRE2: histopathologic HER2

(continued)

Table 7.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
Gastric cancer	I–III	93	CellSearch	Anti-EpCAM/ anti-CD45/ anti-cytokeratins (CK-8, −18, and −19)/DAPI	Zhang et al. [56]	2018	Preoperative CTCs ($n = 93$) (≥ 1, ≥ 2, ≥ 3, ≥ 4, and ≥5 CTCs/7.5 ml) were found in 31 (33.3%), 13 (14.0%), 9 (9.7%), 6 (6.5%), and 5 (5.4%) patients, respectively. Postoperative CTCs ($n = 63$) (≥1, ≥2, ≥3, ≥4 e ≥5 CTCs/7.5 ml) were found in 21 (33.3%), 11 (17.5%), 10 (15.9%), 8 (12.7%), and 4 (6.3%). DFS in 3 year for patients CTCs ≥5/7.5 5/7.5 ml was lower than for patients with <5/ml CTCs (40.0% vs. 66.4%, p 0.001 for pre-surgery; 25.0% vs. 62.2%, $p = 0.001$ for post-surgery). The OS of the patients with ≥5CTCs/ml was of 10.0 vs. 34.9 months; $p = 0.001$ than other patients	DFS: disease-free survival; OS: overall survival
Gastric cancer		147	Fast disc	IgG/anti-CD45 / anti-CK/anti-pan-CK /EpCAM/DAPI	Kang et al. [57]	2017	This study included 116 GC patients and 31 healthy volunteers. CTCs were detected in 105/116 (91%) patients and the median CTC count was 19.5 CTC/7.5 ml. Among the healthy volunteer 3/31 presented CTCs (CTC count 2.5, 5, and 5/7.5 mL). Sensitivity and specificity were calculated based on the threshold of 2 CTC/7.5 ml of blood. Patients 99/102 (97.1%) with CTC level ≥ 2/7.5 blood had GC and patients 28/45 (62.2%) with CTC level <2/7.5 mL had no disease. The sensitivity and specificity to differentiate patients with GC or healthy patients were 85.3% and 90.3%, respectively	GC: gastric cancer

Gastric cancer	II–IV	59	CELLection™ Epithelial Enrich	EpCAM/DAPI/ anti-CK7/8/18/19	Liu et al. [58]	2017	Median CTC count in all patients = 5.95 ± 8.4/5 ml blood. CTC+ rate in advanced GC was 83.05%. Patients (n = 29) tested before and after the first cycle chemotherapy did not present differences in the median CTC count between the two moments (8.10 ± 12.64/5 ml vs. (7.24 ± 10.942/5 ml). After 3 months, patients with decreasing CTC counts after first cycle of chemotherapy had complete, partial response or stable disease, besides longer (PFS) ($P \leq 0.001$) and (OS) ($P = 0.002$), while the increasing CTCs had progressive disease. High CTC counts were associated with poor tumor differentiation and high serum CEA levels ($P = 0.021$ and 0.005, respectively)	GC: gastric cancer; OS: overall survival; PFS: progression-free survival
Gastric adenocarcinoma	I–IV	65	OBP-401	Anti-CD45/CD326 (EpCAM)	Ito et al. [59]	2016	Patients were observed for 60 months. The progression disease was correlated with increased in CTCs count. Among the GA stages I and III, there was statistical difference in CTC number ($P = 0.0460$). Patients with CTC count >5 CTC/ml had worse OS and relapse-free survival compared to patients with ≤5 CTC/ml ($P = 0.183$; $P = 0.034$ respectively)	OS: overall survival GA: gastric adenocarcinoma

(continued)

Table 7.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
Advanced gastric cancer		31	SE-iFISH	Anti-CD45/(PanCK) (CK4, 5, 6, 8, 10, 13, and 18) CEP8/DAPI	Li et al. [60]	2016	CTCs were present in 93.5% of the patients. CTCs with aneuploidy of chromosome 8 (subtype multiploid and multiploid plus triploid) correlated with poor PSF and OS: patients with ≥2 CTCs multiploid post-therapy had poor PSF and OS compared with <2 CTCs multiploid post-therapy (4.5 months vs. 8.4 months, $P = 0.011$; 11.4 month vs. 22.6 month, $P = 0.001$)	OS: overall survival; PFS: progression-free survival
Gastric cancer with diffuse bone metastases		39	CellSearch/ CellTracks AutoPrep system	CEA/CA19–9	Shimazu et al. [61]	2016	Among all patients with GC, 12.8% ($n = 5$) had subtype gastric cancer with diffuse bone metastases. CTC count had 235–6440 CTCs/7.5 ml PB (median = 1724). The high number of CTCs is characteristic of this GC subtype. In patients responding to chemotherapy, CTC number decreased in 14 days of 275. 235 and 1724 for 2. 7 and 66, respectively, however, the CTCs level increased when treatment did not work (2. 7 and 6440 to 787. 513 and 7885). This change may early indicate the outcome of the treatment	PB: peripheral blood; GC: gastric cancer

Gastric cancer		24	Fluorescence-activated	HER2	Meulendijks et al. [62]	2016	Patients ($n = 22$) were treated with B-DOC or ($n = 2$) B-DOC + trastuzumab. 16/24 patients (67%) were CTC+ at baseline (median = 36 CTC/8 ml; 2–428 CTCs). The presence of CTCs correlated with poor PFS and OS (HR, 6.7; IC 95%, 1.43–31.03 [$P = 0.016$]) and a tendency to lower OS (HR, 4.3; IC 95%, 0.82–22.90 [$P = 0.084$])	B-DOC: bevacizumab, docetaxel, oxaliplatin, capecitabine OS: overall survival; PFS: progression-free survival
Advanced gastric cancer		136	CellSearch	HER2	Li et al. [63]	2016	CTCs baseline ⩾1($n = 76$–55.9%); ⩾2 ($n = 65$–47.8%); ⩾3 ($n = 57$–41.9%); ⩾4 ($n = 52$–38.2%); ⩾5($n = 48$–35.3%) were found in 7.5 ml of blood. CTC number ⩾3, ⩾4, and ⩾5 were present more frequently in tumor in the stomach. ORR and DCR in patients with ≥3 CTCs/7.5 ml vs. < 3 CTCs/7.5 ml baseline were 24.4% and 86.7% months vs. 24.6% and 93.4% months, respectively. After treatment (6 weeks) ORR and DCR was 7.7% and 76.9% months, respectively. PFS and OS baseline (≥3 CTCs/7.5 ml vs. < 3 CTCs/7.5 ml) were 5.7 and 11.9 months, respectively vs. 7.1 and 17.7 months, respectively). After (6 weeks) PFS and OS were 3.6 and 8.0 months respectively vs. 7.7 and 17.7 months	ORR: overall response rate; DCR: disease control rate; OS: overall survival; PFS: progression-free survival

(continued)

Table 7.1 (continued)

Tumor	Stage	Number of patients	Method	Markers	Authors	Year	Main results	Abbreviations
Gastric cancer		44	CanPatrol	Keratins (KRT) 8, 18 and 19/EpCAM/ Vimentin and Twist/ CD45/DAPI/HER2	Li et al. [64]	2015	CTCs were present in 79% of patients (35/44) median 7.5 CTCs. Patients (n = 4) had CTC M+ and (n = 10) CTCs M+ or M+> E+. The patients with gastric SRCC and adenocarcinoma had mesenchymal CTCs	E+: epithelial; M+: mesenchymal; E+/M+: biphenotypic SRCC: signet-ring cell carcinoma
Metastatic gastric cancer		100	CellSearch	CK8, CK18 e CK19/ EpCAM/anti CD45/ DAPI	Lee et al. [65]	2015	Patients 43/95 had 1 CTC/7.5 ml (45.3%) (CTC-). Patients (n = 27) had 5 or more CTCs (28.45) (CTCs+). In patients with CTC+ progressive disease is more frequent (60.0% vs. 23.4%, p = 0.004) due to poorer response to cytotoxic chemotherapy; patients with CTCs+ had poor median PFS (59 days vs. 141 days p 0.004 and median OS was 120 days vs. 220 days (p = 0.030) in patients with CTCs-.	OS: overall survival; PFS: progression-free survival
Gastric cancer		136	CellSearch	EpCAM/CD45/CK 8/18/19	Okabe et al. [66]	2015	CTC+ was present in 18.4% (25/136) patients (1–1123 CTCs/7.5 ml). 33% of metastatic patients had CTC+ vs. 12% not metastatic. PSF of patients <1 CTC was poor compared with negative CTC (HR 2.03; P = 0.016), the OS too was poor HR 2.20; IC 95% 1.120–4.03; P = 0.009)	HR: hazard ratio; OS: overall survival; PFS: progression-free survival
Gastric cancer		45	Cytospin	CK19/CD44/CD45	Li et al. [67]	2014	27/44 patients had CTCs associated with lymph node and distant metastasis (P = 0.007, P = 0.035, and P = 0.035, respectively). 19/27 patients were CTCs CD44+, more likely to develop metastasis and recurrence	

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Chapter 8
Circulating Tumor Cells in Mesenchymal Tumors

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8.1 Mesenchymal Stem Cells

Many authors have previously isolated CTCs from carcinomas and demonstrated their prognostic value in different tumors. However, the majority of methods used for isolating these CTCs are based on epithelial antigen-targeted antibodies, and thus they neither allow the isolation of the CTCs undergoing epithelial-mesenchymal transition (EMT) nor the detection of CTCs from sarcomas [35, 60].

The ability to differentiate along different lineages and the ability to self-renew are characteristic of stem cells [49]. Embryonic stem cells (ESCs) and adult stem cells compose two large groups and the first are associated with tumorigenesis [2, 4]. Based on this observation at the turn of the 1960s and 1970s, Friedenstein was one of the pioneers of the theory that bone marrow is a reservoir of stem cells of mesenchymal tissues in adult organisms. In his study, Friedenstein noted in vitro cultivation that ectopic transplantation of bone marrow (BM) into the kidney capsule resulted in the formation of bone, not only in the proliferation of bone marrow cells [24]. According to McCulloch, cells from the BM can give rise to multilineage descendants while retaining the ability to self-renew [45, 59, 61].

Proposed by Caplan in 1991, the term "mesenchymal stem cells" (MSCs) was used due their ability to differentiate into more than one type of cells capable to form connective tissue in many organs [10]. The MSCs are multipotent cells that are present in several adult tissues, such as the umbilical cord, adipose, peripheral blood, liver, and bone marrow [21, 29].

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The mesenchymal stem cell phenotype is characterized by the presence of CD73, CD90, CD105 surface antigens and the absence of protein expression CD45, CD34, CD14, CD11b, CD79a, or CD19 or class II histocompatibility complex antigens (HLA II, human leukocyte antigens class II). Furthermore, these cells must have the ability to differentiate osteoblasts, adipocytes, and chondroblasts [8, 18, 31] (Fig. 8.1).

8.2 Detection of Circulating Tumor Cells of Sarcomas

Sarcomas are a heterogeneous group of soft tissue and bone neoplasms that arise out of mesenchymal tissues and consequently may arise from mesenchymal stem cells [42, 62]. In patients with localized disease, distant metastases develop in 50% of cases, with lungs being the most common metastatic site [42]. Detection of circulating tumor cells (CTCs), as a measure of metastatic potential, could provide a way to diagnose and monitor patients. However, the clinical significance of CTCs, as a prognostic or predictive marker in sarcoma, is poorly explored (Table 8.1).

The detection of CTCs in sarcomas are relatively recent due to the limited number of patients, the absence of specific markers expressed by sarcoma tumor cells, and their high diversity/heterogeneity.

Considering that most CTCs are frequently larger than that of normal circulating cells in blood, cell size represents a potential criterion for isolating sarcoma CTCs. Chinen et al. [16] and Braun et al. [9], were the first to describe the isolation by size method to isolate sarcoma CTCs, but other studies, with other techniques, have been

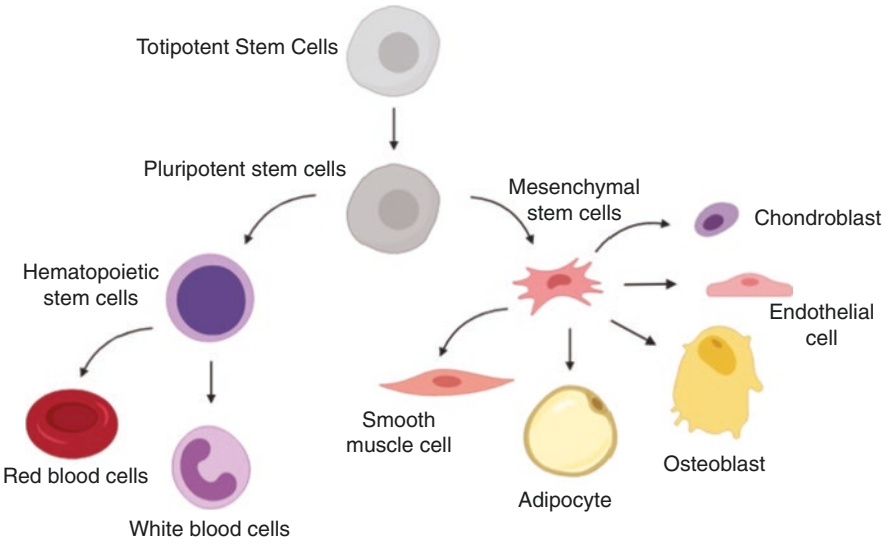

Fig. 8.1 The ability of mesenchymal stem cells (MSCs) to differentiate in other cells

Table 8.1 Studies with CTCs and their main findings in diverse types of sarcomas

Author	Year of publication	Patients (*n*)	Histology	Method of detection	Source	Finding
Peter et al.	1995	36	Ewing's sarcoma	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Pfleiderer et al.	1995	16	Ewing's sarcoma	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Kelly et al.	1996	11	Rhabdomyosarcoma	RT-PCR	BM	Detection of PAX3-FKHR fusion transcript
West et al.	1997	16	Ewing's sarcoma/PNET	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Fagnou et al.	1998	62	Ewing's sarcoma	RT-PCR	BM	CTC detection in BM but not PB associated with reduced survival
Zoubek et al.	1998	35	Ewing's sarcoma	RT-PCR	BM	EWS/ets-oncogene fusion transcripts
de Alava et al.	1998	28	Ewing's sarcoma	RT-PCR	PB	EWS-FLI-1, EWS-ERG
Thomson et al.	1999	12	Alveolar rhabdomyosarcoma, Ewing's sarcoma	RT-PCR	PB and BM	PAX3-FKHR, EWS-FLI1
Wong et al.	2000	11	Osteosarcoma	RT-PCR	PB	Quantification of COLL mRNA
Burchill et al.	2001	49	Neuroblastoma	RT-PCR	PB	Tyrosine hydroxylase mRNA
Schleiermacher et al.	2003	172	Ewing's sarcoma	RT-PCR	PB and BM	CTC detection in PB or BM associated with poor outcome among patients with localized disease
Avigad et al.	2004	26	Ewing's sarcoma	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Gallego et al.	2006	16	Rhabdomyosarcoma	RT-PCR	PB	Isolation of PAX3-FKHR correlated with prognosis
Hoshino et al.	2009	1	Alveolar sarcoma	RT-PCR	PB	Isolation of ASPSCR1-TFE3-positive cells
Dubois et al.	2010	1	Ewing's sarcoma	Flow cytometry	PB and BM	Isolation of CD99+CD45− cells

(continued)

Table 8.1 (continued)

Author	Year of publication	Patients (*n*)	Histology	Method of detection	Source	Finding
Chinen et al.	2013	11	Soft-tissue sarcomas	ISET	PB	Isolation by size of tumor cells (antivimentin and CD45)
Przybyl et al.	2014	38	Ewing's sarcoma	RT-PCR	PB and BM	EWS-FLI-1, EWS-ERG
Satelli et al.	2014	28	Ewing's sarcoma/osteosarcoma/ leiomyosarcoma/Angiosarcoma	Flow cytometry	PB	Detection by a novel monoclonal antibody (cell-surface vimentin)
Braun et al.	2018	18	Soft-tissue Sarcomas	ISET	PB	Isolation by size of tumor cells and EGFR expression detected
Mihály et al.	2018	15	Synovial sarcoma	ddPCR	PB	Detection of SS18-SSX2 fusion transcript
Przybyl et al.	2019	38	Synovial sarcoma	RT-PCR	PB	Detection of SS18-SSX fusion transcript

performed involving these tumors. Filtration methods are relatively rapid, sensitive, and easy technique. After isolation, CTCs are characterized by immunocytochemistry. Chinen et al. [16] investigated the feasibility of using isolation by size of tumor cells (ISETs) for isolation, identification, and characterization of CTCs derived from patients with high-grade and metastatic sarcomas. The researchers studied 11 patients and blood samples (8 ml) were collected from patients with advanced soft tissue sarcomas (STSs). In these studies, all patients showed CTCs, with numbers ranging from 2 to 48 per 8 mL of blood.

Braun et al. [9] quantified CTCs and identified CTM as well as the EGF receptor (EGFR) protein expression in these cells and correlated with clinical outcome in metastatic STS. Blood was prospectively collected from patients with different types of high-grade STS, before the beginning of chemotherapy. The samples were processed and filtered by ISET (Rarecells, France) for the isolation and quantification of CTCs and CTMs. EGFR expression was analyzed by immunocytochemistry (ICC) on CTCs/ CTMs (Fig. 8.2).

The authors analyzed 18 patients with median age of 49 years (18–77 y). The positivity for EGFR protein expression in CTCs was observed in 93.75% of the patients. The authors were the first to demonstrate the expression of EGFR protein in CTCs from sarcoma patients. These results may open an area for future investigations.

Another strategy for CTC detection in sarcomas is the use of common mesenchymal cell markers such as vimentin. Satelli et al. [57] used a new marker on

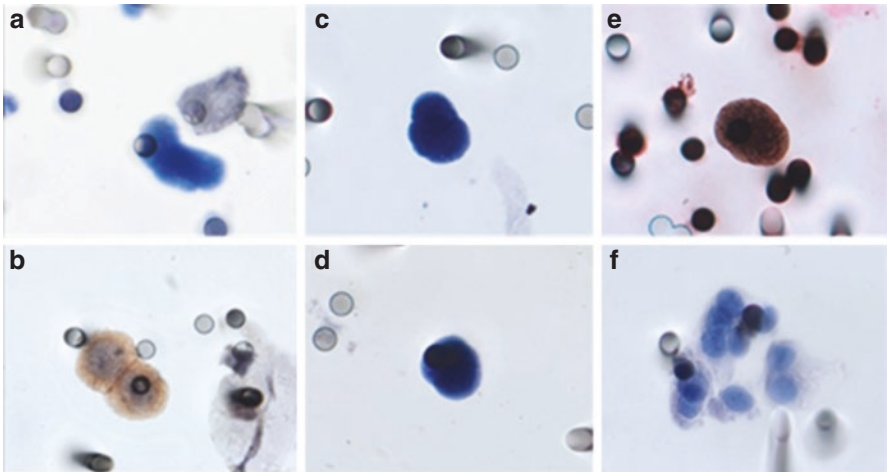

Fig. 8.2 (**a**) Negative control, A-549 cell line "spiked" in healthy blood and negative for EGFR. (**b**) Positive control, FaDu cell line "spiked" in healthy blood and stained for EGFR. (**c**, **d**) Examples of an isolated CTC of sarcoma patient with cytomorphological features (negative staining for CD45, nucleus size ≥12 μm, hyperchromatic and irregular nucleus, visible presence of cytoplasm, and a high nucleus–cytoplasm ratio (Krebs et al. [43]) 15. (**e**) Immunocytochemistry of CTC with anti-EGFR antibody and counterstaining with DAB. (**f**) One CTM from STS patient observed in the blood filtered using the ISET. (Ref. Braun et al. [9])

sarcoma CTC regardless of the tissue origin of the sarcoma as detected by a novel monoclonal antibody. In this study, the authors reported cell-surface vimentin (CSV) as an exclusive marker on sarcoma CTC. Using flow cytometry and FISH, they suggested that this new marker established the first universal and specific CTC marker described for enumerating CTCs from different types of sarcoma, thereby providing a key prognosis tool to monitor cancer metastasis and relapse.

Gallego et al. [25] used detection of muscle markers for CTC detection in rhabdomyosarcoma patients. They performed the analysis combining the detection of a fusion gene product and muscle-specific markers, including MyoD1 and myogenin. In this study, patients with positivity in peripheral samples at the end of treatment showed a poorer prognosis than patients with negative samples.

Circulating tumor cells of sarcoma subtypes associated with specific chromosomal translocations leading to the expression of a unique fusion product are more easily identified, and most studies were performed on Ewing's sarcoma by RT-PCR analysis for the research of the fusion gene product associated with the disease: EWS-FLI-1 and EWS-ERG markers [46]. Results from clinical studies of patients with Ewing's sarcoma suggest that the detection of CTCs at diagnosis may be associated with worse clinical outcomes and that CTCs may be an early marker of recurrent disease.

West et al. [63] studied 16 patients with nonmetastatic disease, three of 16 were RT-PCR positive for EWS/HumFLI1 RNA in BM and three of 10 were positive in PB. In this study, they showed that it is possible to amplify the EWS/HumFLI1 RNA by RT-PCR from the BM and PB of a subset of patients with both nonmetastatic and metastatic ES or PNET, which implies that occult tumor cells are present at these sites.

In the study of Schleiermacher et al. [58], the researchers studied 172 patients with Ewing tumor. RT-PCR targeting EWS-FLI-1 or EWS-ERG transcripts was used to search for occult tumor cells in peripheral blood and bone marrow at diagnosis. The presence of circulating tumor cells (CTCs) was more frequently observed in patients with large tumors ($P = .006$), and CTCs were associated with a poor outcome among patients with clinically localized disease ($P = .045$). The study's conclusion was that patients with localized Ewing tumor and BM micrometastasis or CTC are comparable to patients with metastases in terms of the localization of the primary tumor and relapse pattern.

Avigad et al. [1] reported the prognostic potential of the positive chimeric transcript (EWS/FLI1) in bone marrow (BM) and/or peripheral blood (PBL) in 26 patients with EFTs (Ewing family tumors), during a long follow-up period (median, 61 months), and the results suggested that occult tumor cells in BM and/or PBL samples during long follow-up are strong predictors of recurrent disease in patients with nonmetastatic EFTs.

Semi-quantitative RT-PCR was described by Wong et al. [64]. The researchers correlated mRNA levels of "osteoblast-related genes like" in CTCs from peripheral blood of osteosarcoma patients and found that type I collagen levels were significantly higher in osteosarcoma patients than in healthy subjects.

Hatano et al. [30] developed a similar methodology. They used a system with a polymerase chain reaction assay based on an enzyme-linked immunosorbent assay (PCR-ELISA) to detect circulating osteosarcoma cells in a mouse metastatic model. Osf2/Cbfa1, hereafter called Osf2, a member of the runt family of transcription factors, was used as a target gene, and the amount of the splicing variant of Osf2 mRNA was significantly higher in the blood of mice with metastasis than in the blood of the control group. The researchers demonstrated that PCR-ELISA using Osf2 mRNA was a potential method to detect circulating osteosarcoma cells in peripheral blood.

Multiple studies use flow cytometry to detect CTCs. To isolate these cells, pre-enrichment steps are required in combination with specific antigen recognition for discriminating CTCs from circulating hematopoietic cells (anti-CD45 marker) and epithelial cells (pan-cytokeratin-related marker) [17]. Dubois et al. [19] studied Ewing sarcoma cell line A673, peripheral blood mononuclear cells (PBMCs), and bone marrow mononuclear cells (BMMCs). In this study, the cells were stained for CD99 and CD45 in order to detect CD99+CD45− cells by flow cytometry. Known quantities of A673 Ewing sarcoma cells were spiked into control PBMCs to test the accuracy of this method, and control PBMCs were evaluated to access the level of background staining. The authors suggested that multicolor flow cytometry for CD99+CD45− cells provides a new strategy for detecting circulating Ewing sarcoma cells.

8.3 Epithelial-Mesenchymal Transition (EMT)

To initiate metastasis, tumor cells (CTs) need to leave the primary site to colonize distant tissues. Within the cascade of events that would allow migration, the so-called epithelial-mesenchymal transition (EMT) is presented, a process present during embryogenesis, when epithelial tissue healing is performed. Carcinoma cells can also pass through this process, by loss of epithelial properties and acquisition of partially or totally mesenchymal ones [33, 34].

Carcinoma cells are of epithelial origin and so, undergo to cell-to-cell interaction through adhesion molecules such as cadherins, claudins, or plakoglobin [27, 41].

EMT is a transformation that, apart from being highly dynamic, can be reversible, and in the case of tumor cells, it is characterized by stimulating the invasiveness toward other tissues, by a series of events such as the detriment of cell-cell adhesion proteins within the tumor, in addition to the loss of cellular-atomic-basal polarity [33, 44].

It is documented that EMT is probably triggered by paracrine signaling of the transforming growth factor beta (TGF-β), the Wnt signaling pathway, platelet-derived growth factors, interleukin-6 (IL-6), and some different agents such as nicotine, alcohol, and ultraviolet light. These activators would stimulate transcription factors, such as the basic helix-loop-helix factor (TWIST) and zinc-finger

E-box-binding homeobox (ZEB), which help to maintain the mesenchymal phenotype by autocrine signaling. Due to the breakage of tight and adherent junctions, together with the cytoskeleton variations, epithelial markers such as EpCAM and E-cadherin are negatively regulated, and at the same time the expression of keratins is altered, together with a positive regulation of mesenchymal markers, such as vimentin [32].

To invade the extracellular matrix, tumor cells enter and exit the bloodstream using different cell forms and alternating between the rounded (or amoebic) and the elongated (or mesenchymal) shape, directed by Rho GTPases (RHO) – RHO-associated protein kinase (ROCK) RHO-ROCK [39, 40, 52]. The mesenchymal mode demands the Rac small GTPase (Rac). Cells with amoebic motility exhibit rounded or ellipsoid morphology. These cells also present weak interaction with surrounding matrix, induced by elevated RHO levels, that stimulate membrane blebbing by ROCK-dependent myosin II phosphorylation and consequent actin-myosin contractility [53, 56]. The balance of activated RAC and RHO may determine the mesenchymal or amoebic mode, and the mutual antagonism contributes to maintain different modalities of cell motility [28, 65]. However, the activation mechanism is still confusing [36, 53, 55].

According Li et al. [38] and Caramel et al. [11], mesenchymal tumors are characterized by early metastasis, frequent relapse, and unfavorable clinical outcomes; thus, sarcomas exhibit an aggressive clinical phenotype [26]. EMT has been observed mainly in carcinomas; however, EMT-like processes have also been reported in non-epithelial cancers. Based on that, some studies indicate that sarcomas can undergo phenotypic changes reminiscent to the EMT/MET (mesenchymal-epithelial transition) [15, 20, 22] (Fig. 8.3).

Studies with melanoma have shown that cells spread in a mesenchymal state throughout the body during embryogenesis and settle in the skin. These studies suggested that a subpopulation of melanoma cells transiently acquires a mesenchymal-like state [13, 38].

In Ewing sarcoma, several research groups have shown that individual tumor cells can switch back and forth between more epithelial and more mesenchymal phenotypes.

Chaturvedi et al. [14], using an orthotopic xenograft model, showed that EWS/FLI-induced repression of α5-integrin and zyxin expression promotes tumor progression by supporting anchorage-independent cell growth. This selective advantage was paired with a trade-off in which metastatic lung colonization is compromised, demonstrating that phenotypes can change.

Franzetti et al. [23] demonstrated in their study that cell-to-cell heterogeneity of EWSR1-FLI1 activity determines proliferation/migration choices in Ewing sarcoma cells, using proteomic analysis.

These data together suggest that certain sarcomas can undergo to an EMT- and MET-related process through pathways classically involved in the EMT/MET in carcinomas. The activation of one or another pathway appears to be crucial for the

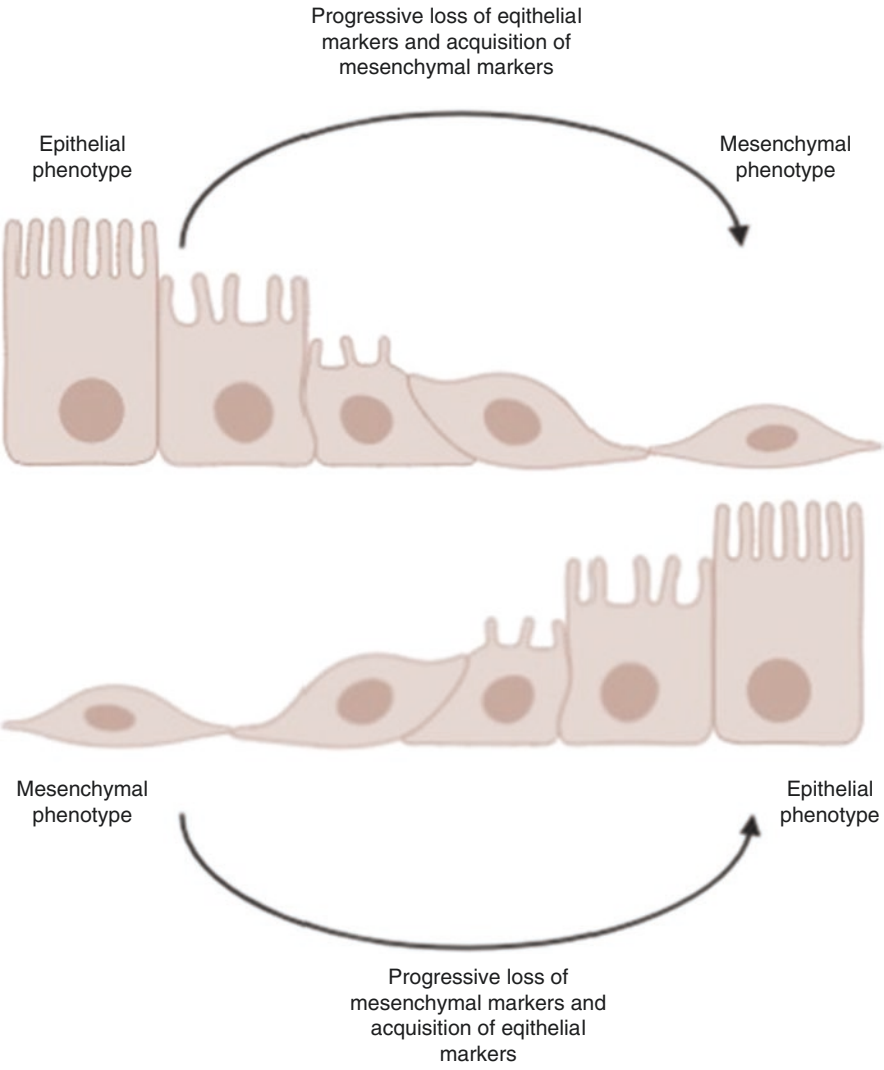

Fig. 8.3 Illustration of the EMT/MET

phenotypic switching of sarcomas toward either a more epithelial or mesenchymal phenotype.

Sannino et al. [54] proposed in a review that certain sarcoma subtypes reside in a peculiar metastable state that enables individual tumor cells to undergo EMT/MET-related processes due to specific cues, combining both epithelial and mesenchymal biological features in a single tumor, which makes metastable sarcomas highly aggressive.

8.4 Plasticity of Circulating Tumor Cells

As previously described, EMT is a complex process that occurs in a broad range of tissue types and developmental stages. EMT involved various mechanisms of the dissemination of cancer including the release of CTCs [6, 7, 47].

Most of the assays for detecting CTCs use cell surface proteins, which pose a challenge to any detection system. In addition, not all steps of EMT are required for carcinoma cells to become invasive and enter the circulation [3, 5, 51].

In 2009, Aktas et al. analyzed blood samples of 39 patients suffering from metastatic breast cancer using the AdnaTest Breast Cancer and observed that 97% of 30 healthy donor samples investigated were negative for EMT and 95% for ALDH1 transcripts. CTCs were detected in 69/226 (31%) cancer samples. In the positive CTC group, 62% were positive for at least one of the EMT markers and 69% for ALDH1. In the negative CTC group, the percentages were 7% and 14%, respectively. In non-responders, EMT and ALDH1 expression were found in 62% and 44% of patients, in responders the rates were 10% and 5%, respectively.

CTCs were detected in 69/226 (31%) cancer samples. Those results indicate that a major proportion of CTC of metastatic breast cancer patients shows EMT and tumor stem cell characteristics.

Lecharpentier et al. [37] found the presence of hybrid epithelial-mesenchymal CTCs in six NSCLC patients that was reported in a pilot study. They observed the presence of clusters of dual CTCs strongly co-expressed vimentin and keratin in all patients (range 5–88/5 ml) and showed for the first time the existence of hybrid CTCs with an epithelial/mesenchymal phenotype in patients with NSCLC.

Alix-Panabières et al. [12] in a review exposed that CTCs with mesenchymal features in patients with various tumor entities can be attributed to higher disease stages, presence of metastases, and in some studies even to therapy response and worse outcome.

Future studies should focus more on the detection and characterization of CTCs with mixed epithelial mesenchymal features.

8.5 Perspectives

The use of CTCs detection in sarcoma patients might be an important diagnostic tool for the earlier detection of metastatic disease for monitoring therapeutic response and for identifying the time point during treatment at which an adjustment in therapy is indicated. CTCs, CTM, and EMT/MET in these cells can be used as tools to measure the effectiveness of treatment and better select patients for clinical intervention. Studies with a larger cohort of patients, with well-defined treatment and follow-up are necessary to confirm data.

Advances may help clarify the extent to which EMT is involved in the various disease states and point to avenues through which our current understanding of the

EMT pathway and transitional events can be exploited to target tumors and/or make them more susceptible to treatment regimes.

8.6 Pictures from Patients

Here, we show some pictures (Figs. 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 8.10, 8.11, 8.12, 8.13, 8.14, 8.15, 8.16, 8.17, 8.18, 8.19, 8.20, 8.21, and 8.22) of CTCs and CTM from patients with diverse types of sarcomas, treated and followed-up at ACCamargo Cancer Center, São Paulo, Brazil. All pictures were selected by Dr. Ludmilla T.D. Chinen and reviewed by Dr. Mauro Saieg (cytopathologist).

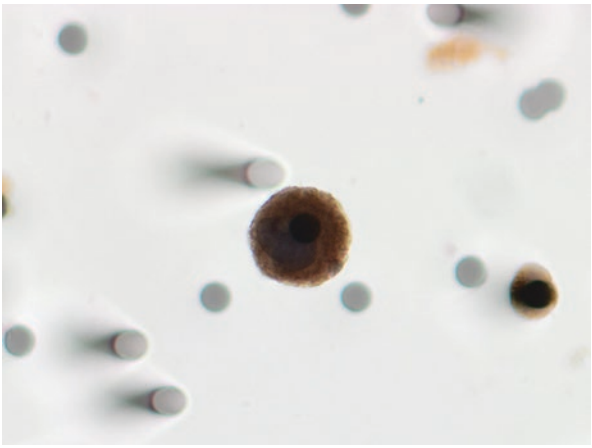

Fig. 8.4 Macrophage isolated from blood, by ISET. Woman, 57 years old, with pleomorphic sarcoma. At the time of blood collection, before first-line treatment with epirrubicin and ifosfamide, she showed 4.5 CTCs/ml. In brown: DAB (anti-β-galactosidase). Microscope magnification: 60×

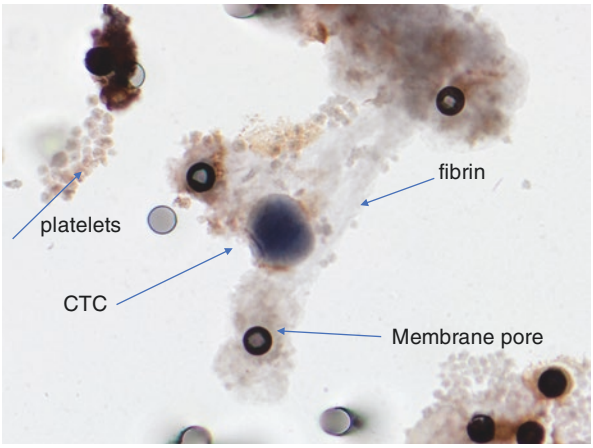

Fig. 8.5 CTC isolated from blood, by ISET. Woman, 59 years old, with leiomyosarcoma. At the time of blood collection, before first-line treatment with gemcitabine, she showed 9.6 CTCs/ml. In brown: DAB (anti-β-galactosidase). Microscope magnification: 40×. Membrane pore diameter of 8μm and CTCs nucleus size ≥ 12 μm

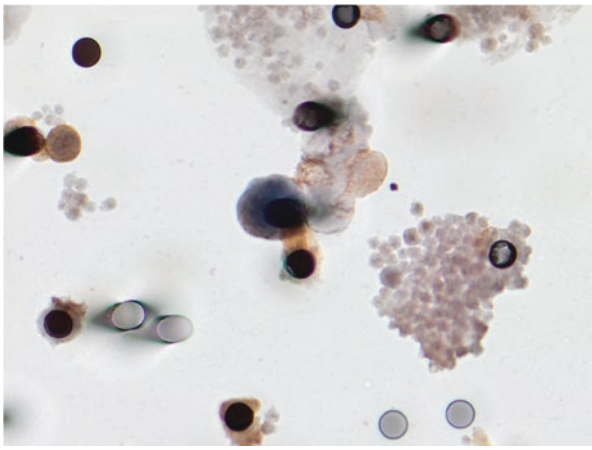

Fig. 8.6 CTC from the same patient Fig. 8.5. In brown: DAB (anti-βgalactosidase). Blue: hematoxylin

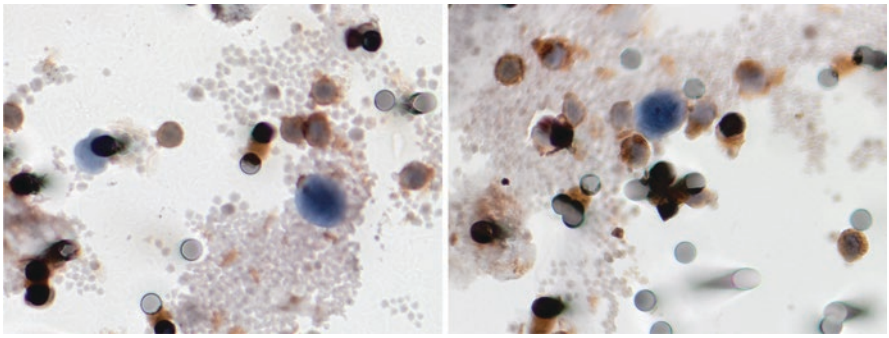

Fig. 8.7 CTC from the same patient Fig. 8.5. CTCs with evident nuclei

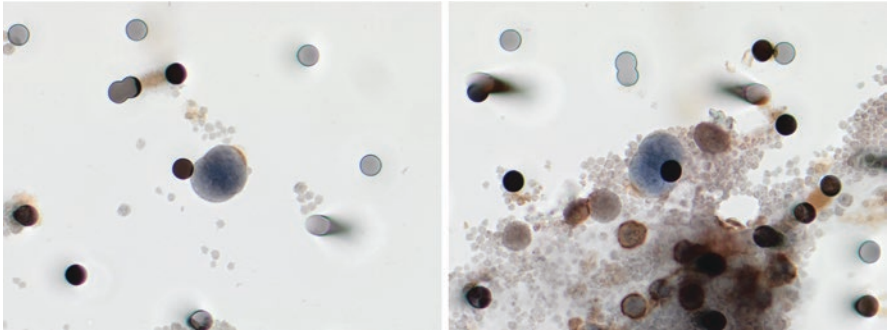

Fig. 8.8 CTC from the same patient Fig. 8.5. In brown, cell on left side, with evident nuclei and anti-EGFR staining with DAB. Cell on right side: CTC in the middle of the field showing changes in the N/C ratio and binucleation, with irregular chromatin and high nuclear/cytoplasmatic ratio

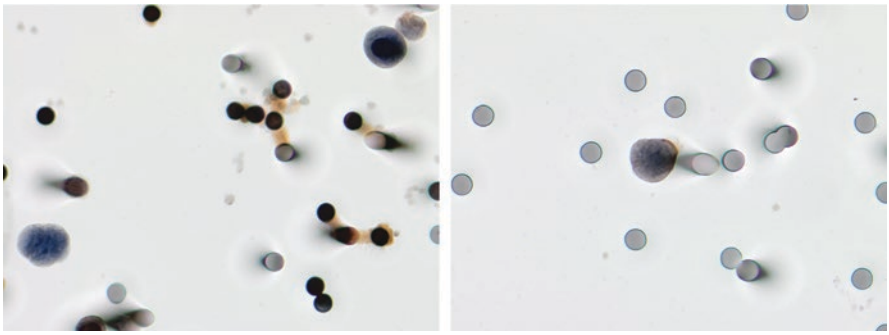

Fig. 8.9 CTC from the same patient Fig. 8.5. In brown: DAB (anti-EGFR). In blue: hematoxylin

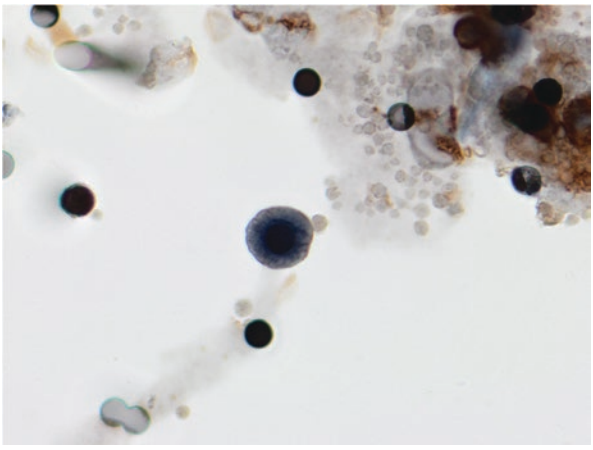

Fig. 8.10 CTC from the same patient Fig. 8.5. In brown: DAB (anti-βgalactosidase); in blue: hematoxylin. CTC in the middle of the field showing changes in the N/C ratio and binucleation, with irregular chromatin and high N/C ratio

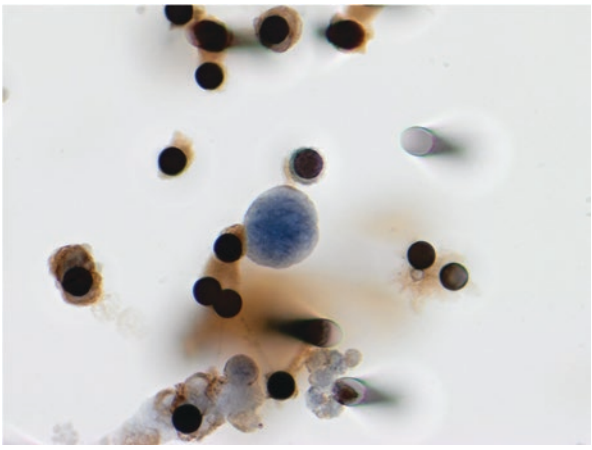

Fig. 8.11 CTC from the same patient Fig. 8.5. In brown: DAB (anti-EGFR), in blue: hematoxylin. CTC in the middle of the field showing chromatin irregularity and alteration of the nuclear/cytoplasmatic ratio

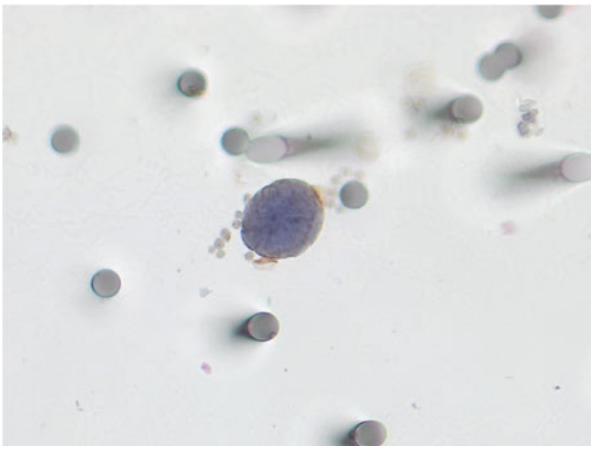

Fig. 8.12 CTC from the same patient Fig. 8.5. In brown: DAB (anti-TGF-β-RI). In blue: hematoxylin. CTC showing high N/C ratio, nuclear and chromatin irregularity

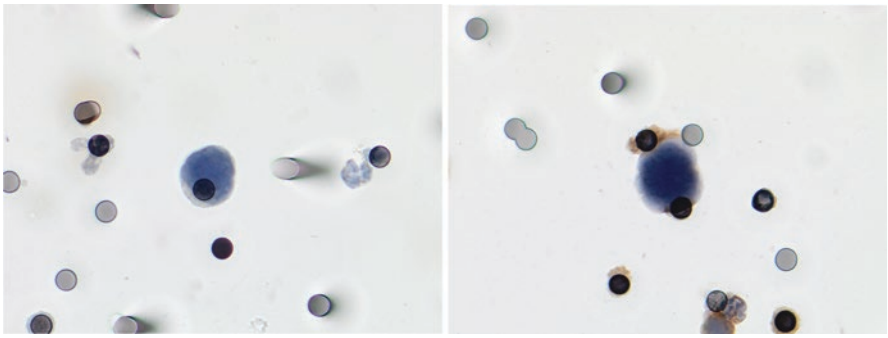

Fig. 8.13 CTC isolated from blood, by ISET. Woman, 46 years old, with leiomyosarcoma. At the time of blood collection, before surgical rescue, she showed 0.75 CTCs/ml. Microscope magnification: 40×

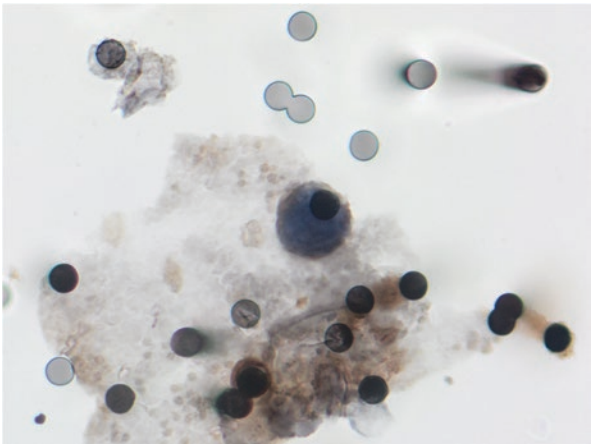

Fig. 8.14 CTC isolated from the same patient Fig. 8.13. In brown: DAB (anti-EGFR). Microscope magnification: 40x

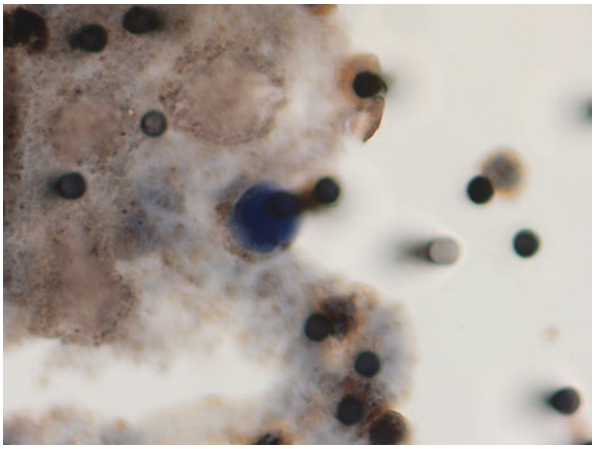

Fig. 8.15 CTC isolated from blood, by ISET. Man, 69 years old, with liposarcoma. At the time of blood collection, before treatment with doxorubicin, he showed 11.25 CTCs/ml. Microscope magnification: 40×

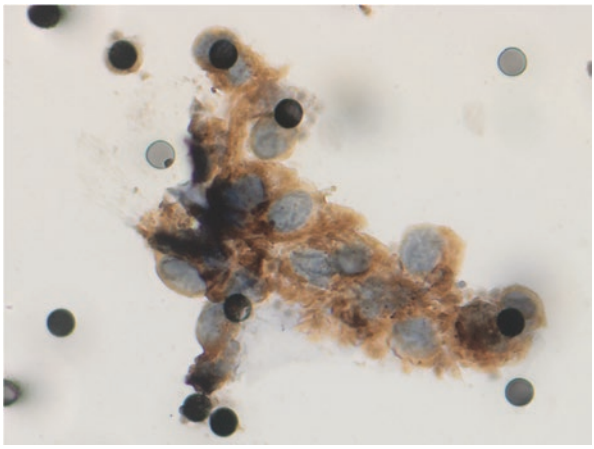

Fig. 8.16 CTC isolated from the same patient of Fig. 8.15. Neoplastic cell block, sometimes spindle shaped, with cytoplasmic marking for vimentin (DAB). Microscope magnification: 40×

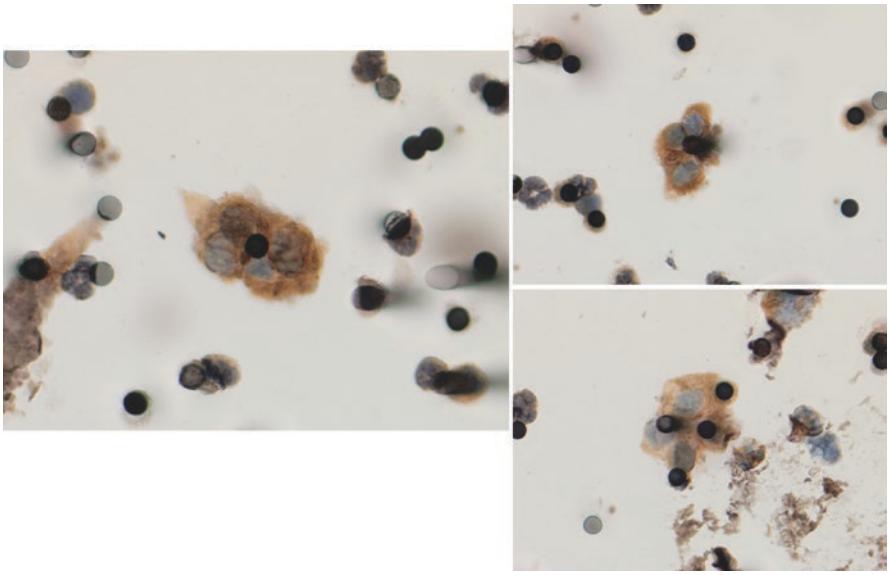

Fig. 8.17 CTM isolated from the same patient of Fig. 8.15. Neoplastic cell blocks, with cytoplasmic marking for vimentin (DAB). Microscope magnification: 40×

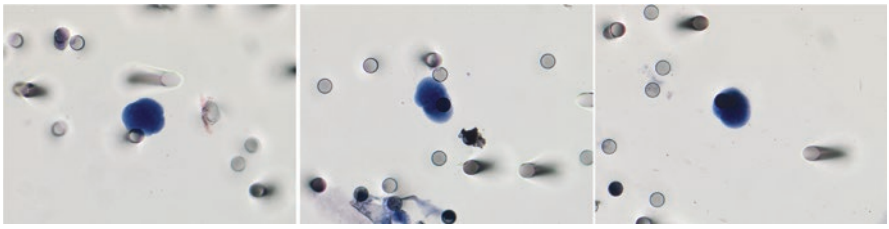

Fig. 8.18 CTC isolated from blood, by ISET. Man, 76 years old, with pleomorphic sarcoma. At the time of blood collection, before treatment with gemcitabine and docetaxel, he showed 5.62 CTCs/ml. Microscope magnification: 40×

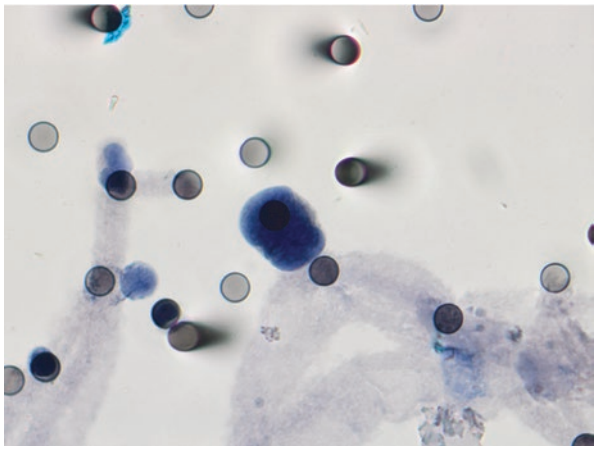

Fig. 8.19 CTC from the same patient of Fig. 8.18. We can observe intense atypia, with multilobulated nucleus, irregular chromatin, and hyperchromic nucleus

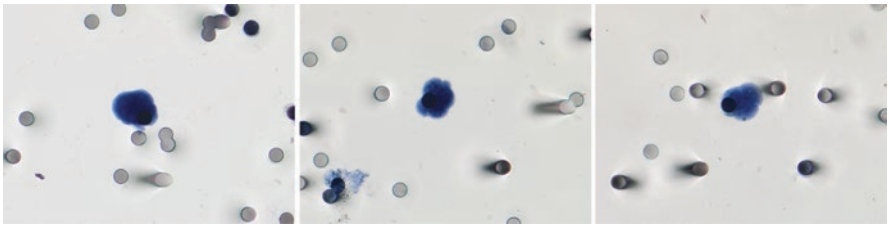

Fig. 8.20 CTCs from the same patient of Fig. 8.18

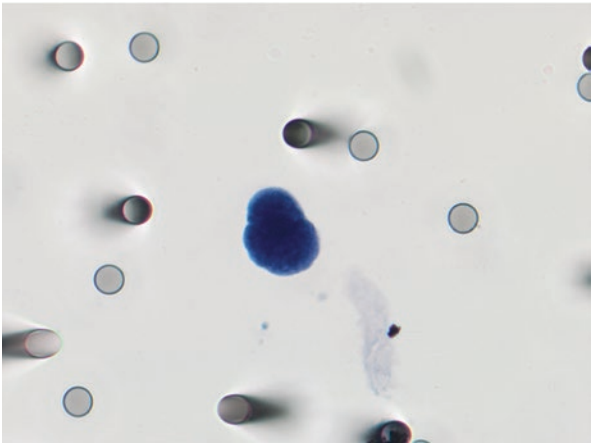

Fig. 8.21 CTCs from the same patient of Fig. 8.18. We can observe an irregular, multilobulated nucleus, with hyperchromasia and nuclear irregularity

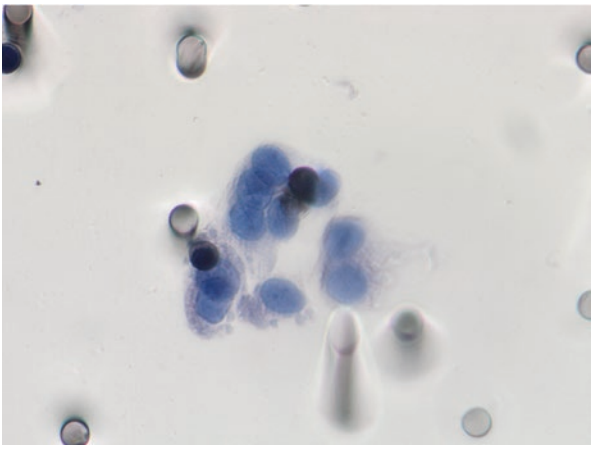

Fig. 8.22 CTC isolated from blood, by ISET. Woman, 27 years old, with synovial sarcoma. At the time of blood collection, before treatment with epirrubicin and ifosfamide, she showed 1.0 CTC/ml. We can observe a group of neoplastic cells showing nuclear irregularity and three dimensionality. Microscope magnification: 40×

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Chapter 9
Circulating Tumor Microemboli: Characteristics and Clinical Relevance

Emne Ali Abdallah

9.1 Brief History

The first observation of clusters of non-hematological cells in bloodstream was postulated by Rudolf Virchow in 1858. He postulated that the entrapment of these structures in the vasculature could contribute with tumor dissemination. However, due to misunderstanding of the clinical importance as well as lack of skilled technology to enrich these clustered-cells, only from 1950s, researches started to show an increased metastatic potential in clustered cells when compared to single cells in animal models' studies [1, 2].

Coman and collaborators (1951) sought to study the reason why specific tumor types progress to secondary tumors with a preferential distribution. The first explanation was the hypothesis that the local chemical composition and the attraction of tumor cells is the factor that influence on the preference (the "soil" theory). The second hypothesis was about the tumor emboli in blood, mechanically lodging in secondary sites, targeting that organ in the route of blood. Therefore, the investigators performed an in vivo assay with tumor emboli from Brown-Pearce rabbit tumor cells. They fixed and stained cells before heart injection and sacrificed the animal after 1–3 min in order to compare the site of lodging with tumor formation. After that, they performed heart injection using living cells, and the animals were sacrificed within 1–3 weeks. They concluded that the distribution of stained/fixed cells observed in capillaries was similar to the secondary tumors formed by living cells, reinforcing the influence of the shear forces and the route of capillaries on cell lodging [1]. Later, researchers injecting clusters of B16 melanoma cells in C57 BL/6

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mice observed those cells were more prone to cause lung metastasis when compared to the group with the same amount of single B16 cells [2].

The lack of methodologies able to enrich, identify, and preserve in an intact manner these cell structures is the most important factor of these years with this scarcity of research about clustered-cell migration.

Discoveries of some of the mechanisms – from detachment, circulation/migration, aggregation, half-life, to the clinical importance – of CTM have been addressed in recent years. It is well known that there are unlimited technical challenges that restrict advances in this area. Recently, many microfluidic and size-based methodologies were developed and showed promising results. These technologies are able to isolate and to characterize CTM, in order to improve the understanding of the role of CTM on tumor development and progression. Here, we provide some examples of CTM captured from blood of patients with cancer by ISET (Isolation by SizE of Epithelial Tumors, Rarecells, France) (Figs. 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, and 9.8). Some of them were related to poor clinical outcome.

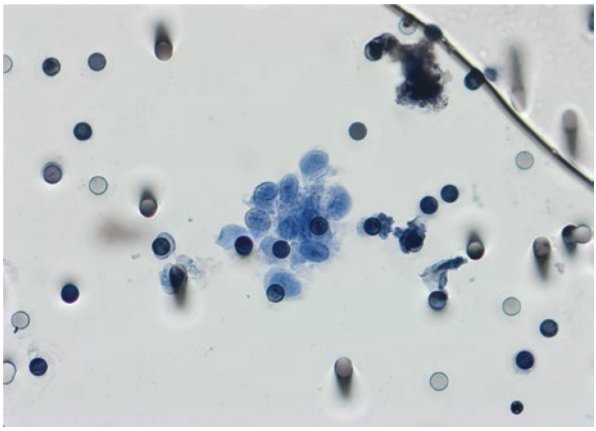

Fig. 9.1 Photomicrography of a circulating tumor microemboli (CTM) isolated from a patient with gastric cancer. The cells were stained with haematoxylin-eosin

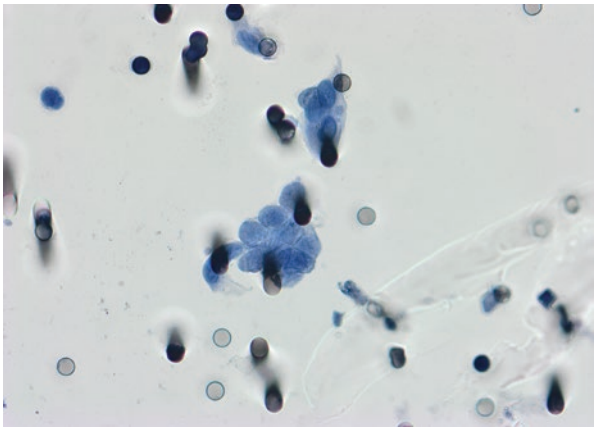

Fig. 9.2 Photomicrography of two circulating tumor microemboli (CTM) isolated from a patient with gastric cancer. The CTM above containing 4 CTCs within; and the CTM below containing approximately 10 CTCs

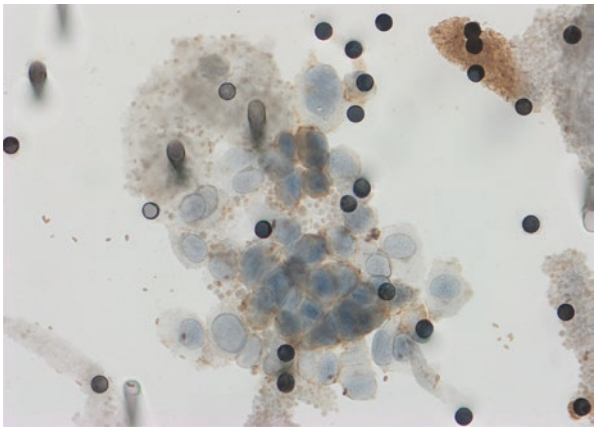

Fig. 9.3
Photomicrography of a circulating tumor microemboli (CTM) from a patient with gastric cancer. Cell staining with anti-plakoglobin and counterstaining with haematoxylin-eosin. At the center of CTM, observation of a strong cytoplasmic expression of plakoglobin; this expression is weaker at the periphery. Aggregated platelets can be seen within the CTM

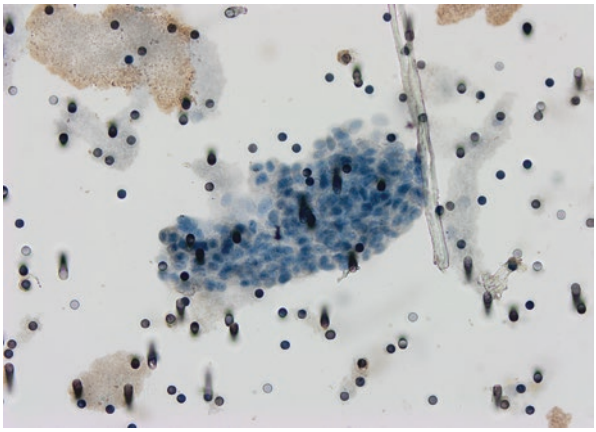

Fig. 9.4
Photomicrography of a circulating tumor microemboli (CTM) from a patient with gastric cancer. Cell staining with anti-plakoglobin and counterstaining with haematoxylin-eosin. In this figure, is possible to observe more than 70 CTCs within the CTM

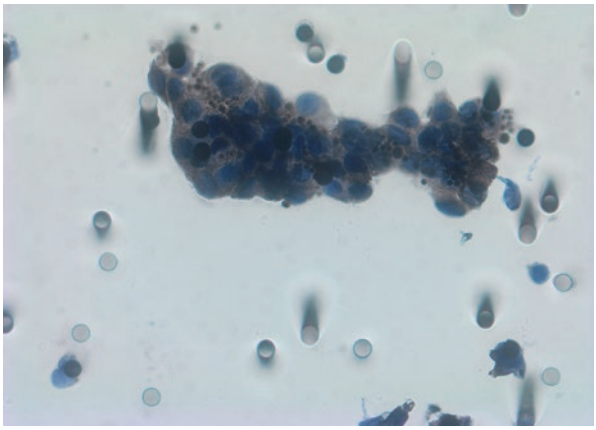

Fig. 9.5
Photomicrography of a circulating tumor microemboli (CTM) from a patient with gastric cancer. Cell staining with anti-plakoglobin and counterstaining with haematoxylin-eosin. In this figure, is possible to see CTCs highly connected with each other and with hiperchromatic nuclei within the CTM

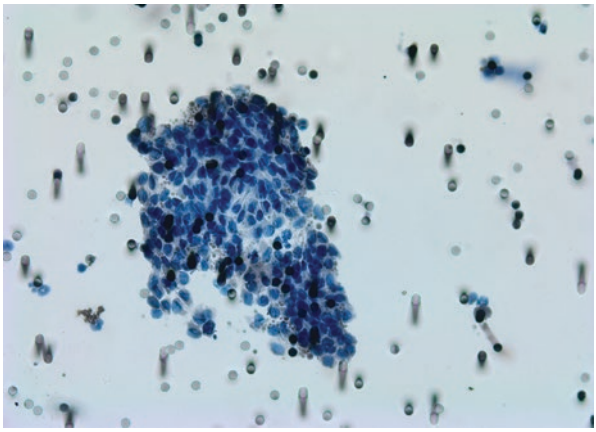

Fig. 9.6
Photomicrography of a circulating tumor microemboli (CTM) from a patient with gastric cancer. Cell staining with anti-plakoglobin and counterstaining with haematoxylin-eosin. In this figure, a large CTM (this CTM may represent "collective migration" of tumor) with weak expression of plakoglobin

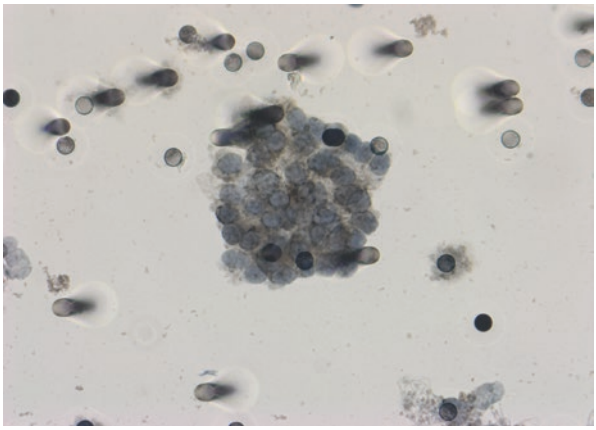

Fig. 9.7
Photomicrography of a circulating tumor microemboli (CTM) from a patient with gastric cancer. Cell staining with anti-plakoglobin and counterstaining with haematoxylin-eosin. In this figure, the CTM presents moderate expression of plakoglobin

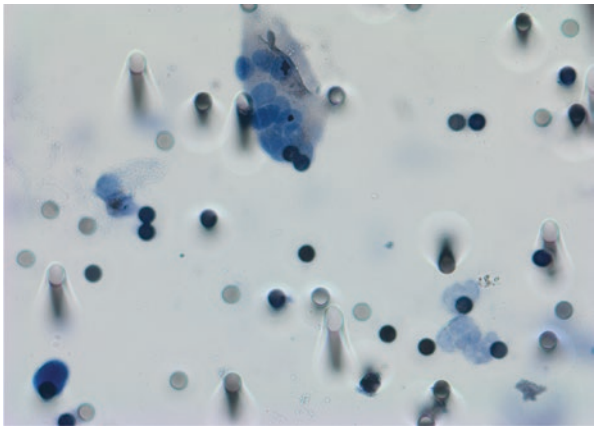

Fig. 9.8
Photomicrography of a circulating tumor microemboli (CTM) isolated from a patient with gastric cancer (upper center). Also, a circulating tumor cell (CTC) in the lower left of figure. The cells were stained with haematoxylin-eosin

9.2 Physiobiology and Cell Composition of CTM

The cell-to-cell communication within CTM microenvironment certainly provides advantages in a successful – tumor – cell survival in bloodstream. Studies have been demonstrating that circulating clusters can be composed not only by tumor cells, but also by other cell types, such as, neutrophils, lymphocytes, platelets, pericytes, and stromal cells [3, 4].

However, limited information was obtained in order to understand the biological characteristics and the interaction within these aggregated cells. Moreover, it is important to understand which are the fundamental mechanisms for these different cell population – when aggregated – to drive the increased survival success forward. There is much to be done in order to characterize these circulating entities in other types of cancer, since the majority of studies focused on breast cancer.

There is an interest in knowing the physiological condition of CTM. Cells within CTM are known to be negative for Ki67, a proliferation marker [5]. This lack of proliferation may implicate that CTMs are resistant to anoikis and hence, resistant to the majority of the currently available therapies. This is mainly because the target of most chemotherapeutic agents focus on cell proliferation [6], showing the relevance of studying its composition for developing treatments targeting CTM.

It was demonstrated that CTMs are highly capable to form metastases up to 50 times more than single CTCs [7]. In addition, investigators recently showed a marked upregulation in plakoglobin expression in CTM (219 times) versus single CTCs. Plakoglobin is a cell-junction protein that seems to be important in keeping the CTM structures in bloodstream, and it is also a potential biomarker in CTM. This data was later confirmed in an independent study with breast cancer patients [8].

CTC-white blood cell (WBC) clusters are prone to induce an increased tumor growth and metastasis formation and decreased progression-free survival (PFS) and overall survival (OS) in mice when compared with single CTCs and CTM. By single-cell RNA sequencing and cytokine network analysis in mouse models and breast cancer patients with these CTC-WBC clusters in the bloodstream, it was shown that CTCs are more commonly found in association with neutrophils and monocytes. Further characterization of CTC-WBC cluster showed that these groups of cells are more prone to promote cell cycle progression in comparison with single cells [9] (Fig. 9.9).

9.3 Mechanisms of CTM Migration

There is a concern in the literature over the way these aggregated cells work as well as their origin. Aceto et al. (2014) have proved that these CTMs are not random spontaneous events formed into vascular environment [7]. Instead, these structures are released actively from primary tumor and supplied by an orchestrated

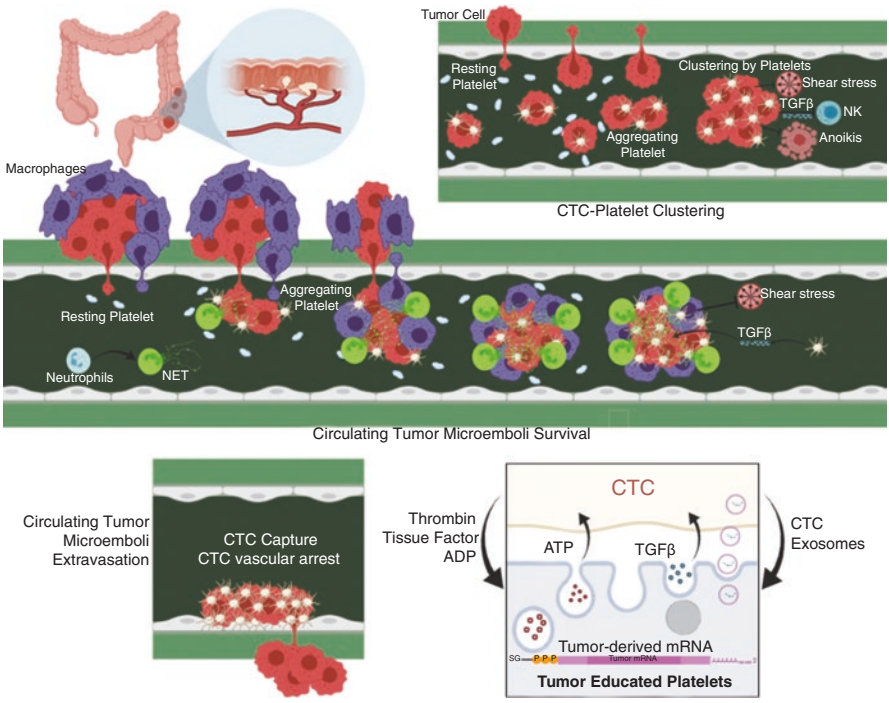

Fig. 9.9 The interaction among circulating tumor microemboli (CTM) and blood microenvironment. CTM can be constituted both by pure tumor cells and with blood cell types (neutrophils, macrophages, and platelets). This last composition can promote the communication between tumor cells and immune cells and increase the chances of tumor cell survival in the blood. Moreover, neutrophil can release genetic material, forming "neutrophil extracellular traps," that can be able to capture CTCs and CTM, stimulating adherence and survival of these structures. Circulating tumor cells (CTCs) and CTM can induce platelet activation and "educate" them, by genetic material (mRNA) transference. On the other hand, platelets can stimulate interactions between CTCs and endothelial cells as well as CTC spread. Platelets release high levels of TGF-β (transforming growth factor-β) and ATP, which can be activators of the epithelial-to-mesenchymal transition (EMT), and help in CTCs' immune evasion. Also, platelets can assist CTC clustering and CTM formation. CTMs are biologically designed to protect single CTCs from shear stress and anoikis, but platelets can help in these processes as well

machinery of cell communication. In general, only a little percentage of cells (even when aggregated) will achieve success in surviving and moving to distant sites.

One unexpected feature of CTMs is that their half-life is shorter than single CTCs, which is explained by the size of the structure and rapid entrapment in small capillaries placed around the tumor [7]. So, when found in circulation, it probably means something in terms of invasiveness and metastatic potential of tumor.

9.4 Clinical Significance of CTM

A high success rate of metastasis achieved by clustered cells in comparison with single cells is well described by animal models, as mentioned in the beginning of this chapter. By injecting DHD/K12/TRb colon cancer cells in portal vein from syngeneic BD 1X rats, Topal and collaborators (2003) showed a significant higher liver metastases formation rate when compared to the injection of 0.5×10^6 of aggregated cells versus single cells, leading to an efficiency in liver metastasis formation of 81% and 16%, respectively [10]. This result allows the investigators to transpose this hypothesis on clinical aspects and behaviors of the tumors.

The significance of CTM on clinics has been demonstrated in some small prospective studies, with the inclusion of patients ranging from 1 to 128 [11–14]. The smaller one was the first and the only detecting CTM in glioblastoma and high-grade glioma, demonstrating that CTM from glioblastoma patients can overcome the blood–brain barrier and reach the peripheral circulation. The data obtained was confirmed by exome sequencing [11].

Breast cancer is the type of cancer most commonly studied to explain the importance of CTM. Three studies with stage III and IV of breast cancer patients, using the CellSearch® system, showed that the prevalence of CTM among them was similar at baseline (16.4%, 17.3%, and 17.4%). Moreover, the presence of CTM at different time points was correlated with poor PFS in all three studies [13, 15, 16], and with poor OS in two of them [13, 16]. Similar results, about prevalence as well as poor PFS and OS, were observed in advanced and metastatic colorectal cancer, using a size-based platform followed by immunofluorescence [17]. Furthermore, a greater number of CTM in metastatic colorectal cancer was a significant indicator of non-response to treatment [18].

There are few studies attempting to explain the importance of CTM in non-small cell lung cancer, but the clinical significance is not yet clear [5, 19–21], although these studies provided a high contribution focusing on CTM molecular characterization. On the other hand, the presence of CTM was already associated with shorter PFS and OS [6] in small-cell lung cancer, besides the development of pulmonary metastases from renal cell carcinoma patients [22].

In relation to diagnosis, a study was made searching for CTC/CTM in patients with suspicious lesion in the lung at the moment of percutaneous CT-guided fine needle aspiration (FNA) or core biopsy. They found at least 1 CTC/CTM per 3 mL of blood in 75% patients with extrapulmonary metastasis, 69% of patients with primary lung cancer, and in none of patients with nonmalignant nodules. These results indicate that CTC/CTM can be found in very early stages of the disease. However, this tool cannot replace the current gold-standard methods [23]. A more detailed information about clinical studies on CTM can be seen in Table 9.1 [6, 11–18, 22–32].

Table 9.1 Studies with circulating tumor microemboli (CTM) and the relation with clinical outcome in cancer patients

Study, year	Cancer topography	PMID	AJCC Stage	Method	Number of patients	Outcome
Vona et al., 2004	Liver cancer	14999698	I–III	ISET	44	CTMs were found in 4.5%. CTM/CTC presence was more observed in patients with diffuse tumors and was associated with portal vein tumor thrombus and shorter survival
Molnar et al., 2008	Colorectal cancer	18334735	IV	Immunomagnetic	30	Non-responder patients had a significantly higher number of CTM
Kats-Ugurlu et al., 2009	Renal cancer	19731255	I–IV	Filtration (75 μm mesh nylon sieve), centrifugation and processing to paraffin-embedded AgarCyto-blocks	42	Tumor fragments in renal venous outflow were observed in 33% of patients and was correlated with pulmonary metastases
Giesing et al., 2010	Prostate cancer	19818074	Diagnosed: with and without tumor, after RP: low- and high risk	Mononuclear cell fractionating followed by filtration, and RT-PCR for antioxidant genes	129 diagnosed, 111 after RP	Overexpression of antioxidant genes in CTM was considered a risk factors for distant recurrence
Hou et al., 2012	Small cell lung cancer	22253462	Limited and extensive disease	CellSearch	97	CTMs were found in 25.7% of patients. Patients with >1 CTM had poor PFS and poor OS
Chinen et al., 2013	Non-small cell lung cancer	24255771	IV (case report)	ISET/MACS System	One (case report)	CTMs were identified by ISET method after two cycles of chemotherapy and indicated non-response to treatment and further clinical progression
Mu et al., 2015	Breast cancer	26573830	III–IV	CellSearch	115	CTMs were found in 17.4% of patients. CTM presence was correlated with poor PFS (HR: 2.83)

Chang et al., 2016	Pancreatic ductal adenocarcinoma	26861552	I–IV	Anti-EpCAM-conjugated supported lipid bilayer-coated microfluidic chips	63	CTMs were found in 81% of patients. CTM presence was independent prognostic factor of poor PFS and OS
Jansson et al., 2016	Breast cancer	27390845	IV	CellSearch	52	CTMs were found in 17.3% of patients at BL, 10.4% at first FU, and 9.7% at second FU. CTM presence in FU samples indicated poor PFS and OS
Long et al., 2016	Melanoma	26945789	III–IV	ISET + May Grünwald Giemsa (MGG) and immunocytochemistry	128	CTMs were found in 49.2% of patients. Presence of CTM was an independent prognostic factor of poor OS
Fanelli et al., 2017	Head and neck	28815787	III–IV	ISET + immunocytochemistry	53	CTMs were found in 28.3% of patients at BL and in 23.3% at FU. Persistence of CTM at FU correlated with poor PFS
Lee et al., 2017	Ovarian cancer	28274569	I–IV	Biotin-doped Ppy-deposited microfluidic device	54	CTMs were found in 59.2% of patients. CTM presence indicated platinum resistance
Mascalchi et al., 2017	Lung cancer diagnosis	28819424	I–IV	ScreenCell	75 screened, 55 diagnosed	70% of patients with lung cancer diagnosis had at least 1 CTC/CTM. None of non-tumoral patients had CTC/CTM
Wang et al., 2017	Breast cancer	27771841	IV	CellSearch	128	CTMs were found in 16.4% of patients at BL and 24.7% at FU. In both instances, CTM presence was determinant of poor PFS and OS
Zhang et al., 2017	Colorectal cancer	28070168	III–IV	Size-based platform + immunofluorescence	98	CTMs were found in 18.3% of patients. CTM presence indicated poor PFS and OS

(continued)

Table 9.1 (continued)

Study, year	Cancer topography	PMID	AJCC Stage	Method	Number of patients	Outcome
Zheng et al., 2017	Gastric cancer	28448959	I–IV	ISET + Diff-Quick staining or immunofluorescence	86	CTMs were found in 18.6% of patients. CTM presence was determinant of poor PFS and OS (all patients) and independent prognostic factor for stage IV patients
Krol et al., 2018	Glioblastoma or high-grade glioma	30065256	Recurrent or progressive disease	Parsortix	13	First evidence of CTM from a glioblastoma patient, confirmed by exome sequencing
Kulasinghe et al., 2018	Head and neck	29335441	I–IV	Spiral microfluidics + Cytospin + CellSearch antibody cocktail	60	CTMs were found in 25% of patients (all stage IV) and were associated with metastasis development
Wu et al., 2018	Pancreatic ductal adenocarcinoma	29725451	I–IV	Cytelligen + iFISH	19	CTMs were found in 21% of patients. CTM presence was determinant of poor DFS and OS
Abdallah et al., 2019	Gastric cancer	30846515	I–III	ISET + immunocytochemistry	55	CTMs were found in 41.8% of patients at BL and in 6.1% at FU. HER2 expression in CTC plus plakoglobin expression in CTM was determinant of poor PFS in patients with diffuse histological subtype Study, year: Krol et al., 2021 Cancer topography: Breast cancer PMID: 33762721 AJCC Stage: I-III Method: SBS-CTC technology Number of patients: 28 Outcome: A proof-of-concept study showing presence of CTM in early-stage breast cancer (5/28 patients CTM+)

Abbreviations: *AJCC*: American Joint Committee on Cancer, *CTM* circulating tumor microemboli, *CTC* circulating tumor cells, *EpCAM* epithelial cell adhesion molecule, *RP* radical prostatectomy, *BL* baseline, *FU* follow-up, *PFS* progression-free survival, *OS* overall survival, *DFS* disease-free survival

9.5 Hypotheses/Perspectives on CTM

The aspects related to origin of CTM, survival, and destruction remain unclear. An evaluation of methylation profile in CTM versus single CTCs from breast cancer patients and in blood from breast cancer xenografts showed high hypomethylation in transcription factor binding sites related to stemness and proliferation in CTM, while these regions were hypermethylated in single CTCs. Moreover, the treatment with the FDA-approved Na^+K^+/-ATPase inhibitor showed promising results in dissociation of CTC clusters and to reverse the profile of methylation.

The persistent observations of CTM and their importance on metastatic development, consequently leading to an inferior survival, raised hypotheses on using these clustered cells as targets for treatment. A study used in vitro methods to mimic CTC cluster formation from breast cancer; such clusters increased in vivo metastatic potential. These cells presented a high heparanase (HPSE) expression, a molecule suitable for inhibition and knockdown, showing a suppression on tumor cell aggregation, thus suggesting HPSE as a target for inhibition of CTM formation [33].

The phenomenon in which tumor cells are replacing endothelial cells and/or being found intermediating with endothelial cells around the tumor is known as "vasculogenic mimicry." This phenomenon was described in a xenograft model of colon carcinoma, in vitro and in vivo assays from melanoma cases, and in tissues from individuals with glioblastoma [34–36]. The hypothesis generated from these studies is that this condition of vasculogenic mimicry can contribute with tumor cell migration (in both single and clustered (CTM) forms), as well as with treatment failure, mainly with angiogenic inhibitors. These observations reinforced the undifferentiated phenotype of tumor cells and a high level of plasticity, since these cells present potential stem/embryonic-like features.

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Chapter 10
Circulating Endothelial Cells: Characteristics and Clinical Relevance

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In the long way of cancer research, many studies were carried out by the scientific-medical community. Day by day, new results of studies clarify many questions, but in turn new questions arise that need to be clarified to improve and direct new therapies [16]. The tumor microenvironment (and all cellular elements that compose it) is considered as determinant for cancer development and progression and has been exhaustively evaluated by many authors [1, 2, 8, 9, 13, 20, 26, 32, 35, 37, 39, 41, 44, 45]. Endothelial cells are involved with tumor development/progression, due to its close proximity to the primary constituent element of the tumor and serving as a pavement for the oxygen and biochemical transport. These cells also act as a barrier and stimulus for cellular migration, together with one or several circulating tumor cells, giving them the advantage to start a neovasculature directly inside the blood vessel.

In recent years, circulating endothelial cells (CECs) have materialized as markers of vascular damage. Although they are present in healthy individuals, they increase in cardiovascular diseases, vascular infections, vasculitis, and type 2 diabetes. Furthermore, these cells are predictive factors of a possible cardiovascular disease in patients with coronary cancer and in patients with chronic hemodialysis treatment. Other studies related endothelial damage in women with a history of pre-eclampsia (Tuzcu et al. 2015). These cells are also seen to be increased in patients with cancer, inflammatory, infectious, ischemic, and autoimmune processes such as systemic lupus erythematosus [7, 12, 22].

The development of new blood vessels, or neovascularization, is necessary for embryonic development and stimulation of injured tissues, but also promotes the growth of tumors and inflammatory diseases [11, 40]. Vascular and lymphatic endothelial cells are activated by pro-angiogenic growth factors such as vascular

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endothelial growth factor (VEGF), which stimulates the proliferation and migration of endothelial cells, promoting the formation of new vessels [17, 21, 31, 36].

In 1997, a research team described for the first time the bone marrow-derived circulating endothelial progenitor cells (EPCs) [4]. Subsequently, studies showed that endothelial cells would eventually ascend from cells derived from the bone marrow. They also demonstrated that endothelial cells derived from human bone marrow could infiltrate tumors and contribute to the angiogenesis [6, 33].

It has recognized that postnatal neovascularization is stimulated by proliferation and in situ migration of pre-existing endothelial cells (ECs). It is also evident that EPC would be housed in neovascularization sites and differentiate in EC in situ (vasculogenesis), well described for embryonic and postnatal neovascularization [5].

Studies have proposed that neovascular ECs are produced from bone marrow stem cells or tumors that express VEGF receptor 2 (VEGFR-2+) [3, 19, 23, 29, 34, 42, 43].

The amounts of CEC and EPC, kinetics, and viability can be measured by positive enrichment by immune beads and flow cytometry. However, since there is no one specific antigen for endothelial cells, a multiparametric analysis is necessary [7, 15, 46].

Nakajima and colleagues isolated endothelium from surgical specimens of pancreatic cancer and normal pancreas by magnetic selection. The primary culture of tumor CEs was confirmed by positive expression of endothelial markers, CD31 and ERG1. The cells showed short vessel formations and capillary network initiation, revealing little angiogenic vigor, in addition, peripheral blood lymphocytes exhibiting fewer adherences to the tumor CE [30]. Preclinical and clinical studies revealed that circulating endothelial progenitor cells (EPCs) are incorporated in centers of physiological or pathological neovascularization as in tumor vessels [5, 7], usually at low frequencies. They also suggested that EPCs are crucial in the vasculogenesis as well in the later stages of cancer. For this reason, anti-angiogenic drugs could, in principle, prevent the growth of cancer [7, 10, 28, 47].

Among the obstacles to the success of immunotherapy for the cure, there is the fact that cancer patients develop resistance to the immune response. Possibly this is due to phenomena such as the deployment of tumor-associated antigens or tumor secretions and/or the use of endothelium associated with tumors that could act as a guardian of the infiltration of immune cells in the tumor [30].

Circulating endothelial cell clusters would originate from the tumor vasculature, and it has hypothesized that the count of the clusters will decrease after tumor resection. To test this, a study collected samples and data from 17 patients with colorectal cancer before and after surgical resection of the tumor ($n = 34$ samples in total). The results indicated that tumor resection significantly decreased the number of these circulating endothelial cell clusters, supporting that these structures are derived from the tumor. Furthermore, it would indicate that the clusters of these cells are not produced from the peripheral circulation by the growth of single circulating endothelial cells, but that they would be released as groups of the tumor vasculature [14].

A study conducted with 42 patients with gastric cancer showed that the number of endothelial progenitor cells (EPCs) and endothelial cells (ECs) in patients with stage III was higher than in stages I and II. The number of EPC in patients in stage IV was

reduced, while the number of EC increased significantly compared to those in patients in stages I, II, or III. In addition, the number of EPC decreased in patients with tumors that had not invaded the serosa or with distant metastases. In addition, the number of EPC and EC in patients with lymph node metastasis increased significantly compared to patients without metastases. This would indicate that EPC could be involved in lymph node metastasis in gastric cancer. This study hypothesizes that EPCs are involved in angiogenesis in stages I and II, CE and EPCs are linked in angiogenesis in stage III, and EC would be the main cell involved in angiogenesis in stage IV. Factors such as hypoxia, neovascularization, and cell adhesion molecules stimulate the recruitment of EPC [25]. EPCs have demonstrated their promising value as markers of tumor diagnosis in renal cell and lung adenocarcinoma [25, 27], breast cancer [18, 38], and colorectal cancer [24]. It has been found that adrenomedullin receptor antagonists achieve targeted therapy of pancreatic and renal tumors in mice by inhibiting the mobilization of tumor endothelial cells and EPC [25].

As demonstrated here, knowledge about EPC, CEC, associated angiogenic factors, inhibitory factors of endogenous angiogenesis, and synthetic inhibitors of exogenous angiogenesis may encompass angiogenic inhibition therapy and may be a promising anticancer treatment (Fig. 10.1). Studies are needed to investigate the factors that affect the mobilization, migration, and differentiation of EPC and CEC in different clinical stages.

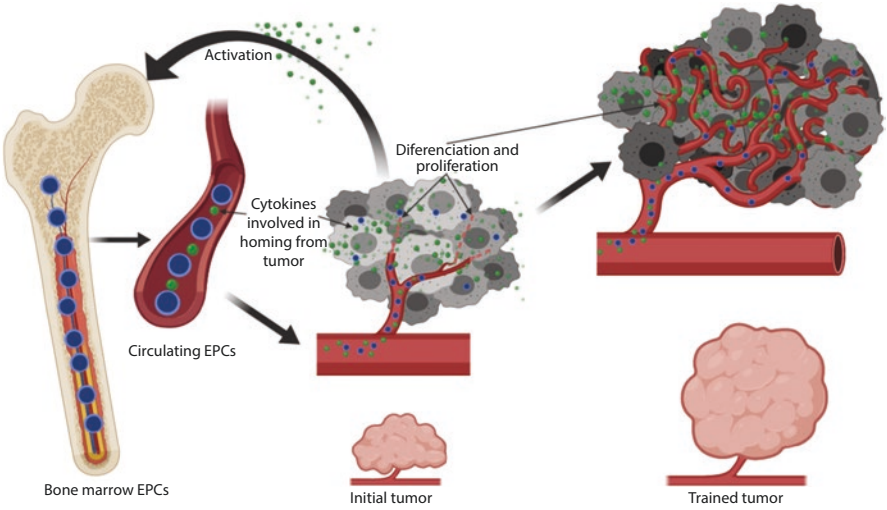

Fig. 10.1 Cytokines secreted by the tumor activate the bone marrow cells, resulting in the mobilization of subsets of EPCs from the bone marrow en route to the tumor bed in response to chemotaxis. Subsequently, EPCs enter the blood and interact with the wall of the blood vessels; this interaction activates integrins which mediate intercellular adhesion and facilitate transendothelial migration of the EPCs to the tumor. Both integrins and proteases are essential for tissue invasion. EPCs differentiate into mature endothelial cells in three steps: (i) integrin-mediated adhesion to the extracellular matrix, (ii) production of paracrine/juxtacrine factor, and (iii) expression of genes that regulate endothelial maturation. EPCs regulate the angiogenic process through the paracrine secretion of pro-angiogenic factors and provide a structural function to the new vessels

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Chapter 11
Giant Macrophages: Characteristics and Clinical Relevance

Julie Earl and Bruno Sainz Jr.

11.1 Introduction

The role of macrophages in tumor development and dissemination has been known for several years [1–3] and was more recently reviewed by Yang and Zhang in 2017 [4]. Herein, we discuss in depth the role of macrophages and specifically that of tumor-associated macrophages (TAMs) in these various processes, as well as their potential role as clinical biomarkers and therapeutic targets. The following terms describe the different macrophage phenotypes mainly associated with these processes [5].

Inflammatory monocytes are recruited to inflammatory sites and are characterized as follows: CD14+(high), CD16−, CCR2+(high), CSF1R+(high), and LY6G-.

Tumor-associated macrophages (TAMs) are present in the tumor microenvironment and promote tumor development and progression. Human TAMs have the following marker profile: CD11b+, CD14+, CD23+, CD34−, CD45+, CD68+, CD117−, CD133−, CD146−, CD163+ (h), CD204+, CD206+, CCR2+, CSF1R+,

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CXCR4+, MHC class II+, VEGFR1+, and VEGFR2−. However, different markers are expressed by TAMs with specific tumor-associated functions.

Metastasis-associated macrophages (MAMs) are a subset of inflammatory monocytes that promote tumor dissemination and invasion and the formation of the metastatic niche. Studies in mice have shown that MAMs originate from inflammatory monocytes and have the following marker profile: CD11b+, CD31−, CD45+, CCR2+, CXCR4−, F4/80+, LY6C−, LY6G−, TIE2−, VEGFR1+, and VEGFR2-.

11.2 The Interplay Between Circulating Tumor Cells (CTCs) and Macrophages

The tumor microenvironment (TME) is composed of fibroblasts, immune cells, and vascular endothelial cells. Monocyte-derived macrophages are immune cells that originate from bone marrow-derived blood monocytes. Tumor-associated macrophages (TAMs) are involved in cancer-related inflammation, form part of the tumor microenvironment, and facilitate the dissemination of circulating tumor cell (CTC) and their subsequent seeding in metastatic niches [6, 7]. TAMs are either tissue resident or derived from peripheral sources such as monocytes of bone marrow and spleen [8], although their exact origin and the mechanisms underlying their pro-metastatic function in human tumors is unknown.

Macrophages are extremely plastic and can fluctuate between two states of polarization: “M1” or “M2” state. Classically activated macrophages are known as M1-polarized macrophages, whereas TAMs more closely resemble M2-polarized macrophages, which express higher levels of anti-inflammatory cytokines and angiogenic factors compared to their M1-type counterparts [4]. It is important to note that while TAMs do resemble M2-polarized macrophages, there exist several subpopulations of TAMs that share features of both M1 and M2 macrophages. Thus, the traditional M1 or M2 classification of TAMs may not accurately reflect the differentiated or biological state of these cells, and therefore, researchers have proposed functionally classifying TAMs (e.g., metastasis-promoting macrophage or immunosuppressive macrophage) in lieu of using the traditional M1 and M2 nomenclature [9–12].

Until a consensus is established, however, the use of binary M1/M2 classification continues to be widely used [13]. Based on this system, it is believed that macrophage polarization toward a pro-inflammatory, classically activated or “M1” phenotype is mediated by activation of Toll-like receptors upon engagement with bacterial components (e.g., lipopolysaccharides) or via type I helper T (Th1)-secreted cytokines [e.g., interferon (IFN)-γ]. In general, it is assumed that M1 macrophages are involved in Th1 responses to microorganisms, are involved in clearance of dead/apoptotic cells, have enhanced cell killing activity, and are characterized by secretion of a battery of pro-inflammatory cytokines that include IL-12, IL-1β, IL-6, and tumor necrosis factor α (TNFα) and generation of reactive oxygen and nitrogen

intermediates [14]. Alternatively and in response to different stimuli or signals, macrophages can polarize toward an alternatively activated "M2" phenotype participating in Th2-type immunity, wound healing, and tissue remodeling [10]. These alternate stimuli can include, but are not limited to IL-4, IL-10, and IL-13 [10]. While M1 macrophages are characterized by secretion of pro-inflammatory cytokines, M2 macrophages are characterized by high expression of scavenging molecules, mannose and galactose receptors, activation of the arginase pathway, production of IL-10, vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and efficient phagocytic activity [10, 14].

Cells of the myeloid lineage constitute one of the dominant immune cell populations present within tumors. While their initial infiltration into a tumor is dependent on the release of macrophage chemoattractants from tumor cells, such as colony stimulating factor 1 (CSF-1), the chemokines, CCL 2, 3, 4, and 8, vascular endothelial growth factor (VEGF), macrophage inflammatory protein-1 alpha (MIP-1α), and macrophage migration inhibition factor (MIF) [6, 7], once within the tumor, tumor cells promote the differentiation of monocytes (or M1 macrophages) into tumor-conditioned macrophages, also known as TAMs [11]. As mentioned above, while TAMs resemble M2 macrophages and express many of the same cell surface markers as M2 macrophages, to date no single panel of cell surface markers can accurately discriminate TAMs from non-TAMs. In the murine setting, the absence of Gr1 (Ly6G) and the expression of the canonical markers CD11b, F4/80, and CSF-1R in combination with mRNA analysis of additional markers are routinely used to classify macrophage subtypes [9]. In the human setting, antibodies to the glycoprotein CD68, the LPS-co receptor CD14, CD312, CD115, HLA-DR, or FcγRIII (CD16) have been used to identify macrophages, but with mixed and often times contradictory results [15]. Thus, combinations of these markers provide higher specificity and should be used when possible. To more specifically identify M2-like TAMs and subsets, the hemoglobin-scavenger receptor CD163 [16, 17], the macrophage scavenger receptor 1 CD204 [11, 18, 19], the mannose receptor CD206 [20], and more recently the T-cell immunoglobulin and mucin-domain containing protein-3 (Tim-3) [21] have been used with great success.

TAMs directly participate in tumor initiation, progression, and metastasis via numerous mechanisms including the following: (1) the secretion of proteolytic molecules such as MMPs to facilitate ECM remodeling [22–25]; (2) the expression of non-proteolytic proteins like chemokines [26, 27], TGF-β1 [28, 29], ISG15 [30], and hCAP/LL-37 [31, 32] to facilitate tumor cell proliferation, migration, and invasiveness; (3) the expression of angiogenic mediators such as TGF-β, VEGF-A, VEGF-C, platelet-derived growth factor (PDGF), and MMP-9 to sustain the growth of the tumor stroma and promote de novo tumor blood vessel formation [9, 26, 33, 34]; or (4) the expression of immunosuppressive factors including TGF-β, inducible nitric oxide synthase (iNOS), arginase-1, IDO (indoleamine 2,3-dioxygenase), and IL-10 to facilitate T-cell proliferation and activity [35, 36]. While the mechanisms underlying the pro-tumor effects of TAM-secreted factors on bulk tumors has been extensively studied, there is now growing evidence to support that TAMs also enhance tumor cell migration via physical interactions with tumor cells.

11.2.1 Pro-Tumorigenic Function of TAMs

It is well accepted that high TAM content in the tumor microenvironment is associated with a poor prognosis due to their pro-tumor and pro-angiogenesis role [37], and macrophages are found in large numbers at the leading invasion edge of many primary tumors where they degrade the basement membrane to promote tumor progression [1]. Thus, they play an important role in the primary tumor, and at the same time are essential for CTC intravasation. Regarding the latter, previous studies have hypothesized that CTCs intravasate into the circulatory system with TAMs via transendothelial migration [1]. Disseminated tumor cells need to survive in the hostile environment of the blood stream in order to develop metastatic foci at distant sites. Immune cells including macrophages, platelets, and T cells are thought to protect CTCs from the immune system and the environment within the blood vessels [5]. CTCs migrate through the blood stream as single cells or microemboli cell clusters, which consist of cells from the TME such as leukocytes, endothelial cells, or fibroblasts. This hinders the detection and destruction of CTCs by the immune system and also provides a physical protective barrier against damage and destruction in the harsh environment of the blood stream. Thus, the role of TAMs in CTC intravasation may be more complex and dynamic than previously recognized.

11.2.2 TAMs Enhance the Invasive Nature of Tumor Cells

Clinical and experimental evidence both in vivo and in vitro show that macrophages play an important role in tumor progression and dissemination and are therefore, potential targets for therapy. The relationship between poor disease prognosis and the presence of TAMs has been shown in tumor types such as breast, lung, and pancreas [38–40].

Macrophages are associated with chronic inflammation, and Balkwill et al. showed in 2005 that treatment with anti-inflammatory agents reduced cancer risk [41]. NF-κB appears to be important in the inflammatory response. In fact, it has been shown in vivo that NF-κB activation leads to the upregulation of anti-apoptotic genes, such as BCL-XL, BFL1, and GADD45β and therefore prevents apoptosis of tumorigenic cells [42]. Inhibition of NF-κB could be a potential therapeutic strategy to target macrophages, as this would not only restore apoptosis of malignant cells but also inhibit the expression of growth and survival factors in macrophages.

Furthermore, Lin et al. in 2001 showed that a homozygous null breast cancer mouse model for the colony-stimulating factor-1 (CSF-1) gene had reduced tumor progression with almost no metastasis. Whereas, overexpression of CSF-1 accelerated tumor progression and metastasis [43]. Furthermore, blocking the expression of CSF-1 in a human xenograft mouse model reduced tumor growth and metastatic capacity [44], thus supporting the notion of macrophages as enhancers of tumor progression. Tumors cells appear to “educate” macrophages in order to promote

tumor invasion and intravasation into the blood vessels and the circulation to form secondary metastatic lesions [5]. In 2010, Wu et al. showed using co-cultures of macrophages or macrophage-conditioned medium with tumor cells an enhanced invasive phenotype, which appears to be dependent on NF-κB and SNAIL [45]. Thus, the tumor-macrophage interaction is fundamental for tumor invasion and dissemination. Grivennikov et al. showed in 2012 that TAMs secrete inflammatory cytokines such as IL-23 and IL-17 that promote cancer progression [46]. IL-23 was mainly produced by tumor-associated myeloid cells in response to tumor-elicited inflammation by microbial products in colon tumors. A recent study by Krug et al. in pancreatic neuroendocrine tumors (PNET) showed that TAMs play a critical role in the malignant phenotype of PNET. The number of infiltrating TAMs correlated with tumor invasiveness and metastatic potential. Specifically, in vivo and in vitro experiments of myeloid cell inhibition with liposomal clodronate showed a reduced malignant transformation of insulinomas with an associated reduction in angiogenesis and the number of infiltrating TAMs [47]. Similarly, Michl and colleagues showed in a genetic model of pancreatic cancer that clodronate-mediated depletion of macrophages markedly reduced metastasis formation and was associated with reduced CD4+CD25+ T cell levels and impaired angiogenesis. Interestingly, tumor incidence was only slightly reduced, suggesting that TAMs likely are more important in dissemination rather than tumorigenesis, at least for pancreatic cancer [48].

TAMs also produce proteases, such as Cathepsin B, matrix metallopeptidase (MMP) 2, MMP7, and MMP9 that destroy the components of the extracellular matrix (ECM), and therefore facilitate the invasion and migration of tumor cells [4]. In fact, Finkernagel et al. recently demonstrated in ovarian tumors that the transcriptional profile of TAMs was similar to that of resident macrophages. This included functions such as bacteria phagocytosis and antigen presentation. However, there was a subset of genes that were specifically upregulated in TAMs that were associated with the re-organization of the extracellular matrix [49].

TAMs are also involved in angiogenesis and promote the formation of intratumoral blood vessels that provide essential nutrition to the growing tumor. They also secrete pro-angiogenesis factors including colony stimulating factor-1 (CSF-1), various chemokines such as IL-8 and IL-1β, CCL2, CCL3, CCL4, CCL5, and CCL8, as well as macrophage migration inhibition factor (MIF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), tumor-necrosis factor-α (TNF-α), and thymidine phosphorylase [6]. Specifically, macrophage infiltration of the tumor site was significantly reduced in a CSF-1-null mouse model of breast cancer, with a consequent impaired development of the vasculature of the tumor and reduced vessel density due to VEGF depletion in the surrounding stroma [33]. Furthermore, human breast cancer spheroids had an increased angiogenic response and more pronounced vascularization when implanted into nude mice if they were infiltrated with macrophages. This was likely due to the release of VEGF by the spheroid cultures [6].

11.2.3 TAMs, Inflammation, and the Immune Response

The role of inflammation in cancer development is clear, and TAMs connect inflammation and cancer. The recruitment of macrophages to the primary tumor is crucial to establish and maintain an inflammatory TME. Epithelial-mesenchymal transition (EMT) increases the motility and invasiveness of tumor cells and is a key mechanism in the metastatic process. The transcription factor, Snail, induces EMT via the repression of the cell adhesion protein E-cadherin and is a crucial factor for inflammation-initiated invasion and metastasis. The inflammatory cytokine, TNFα, stabilizes Snail via the activation of the NF-κB pathway [45].

Metastatic tumor cells are immunogenic and should be recognized and neutralized by immune cells such CD8+ T cells natural killer (NK) cells. However, these metastatic tumor cells develop several strategies to overcome detection and destruction by the immune system, such as the recruitment of immunosuppressive cells [5]. TAMs are involved in immune suppression in the TME via the inhibition of the cytotoxic T lymphocyte (CTL) response via IL-10 and the induction of the expression PD-L1 in monocytes [4]. The anti-inflammatory cytokines produced by M2 TAMs reprogram the immunosuppressive microenvironment to promote tumor progression.

11.2.4 TAMs Play an Important Role in Metastasis

TAMs are important players in the development of a premalignant niche for the establishment of metastatic lesions [4] and also play a crucial role in the regulation of EMT, which enhances the metastatic capabilities of tumor cells [4]. TAM-derived TNF-α, VEGF, and TGF-β induce macrophages to produce S100A8 (aka SMA1) and serum amyloid A3, which recruit macrophages and tumor cells to the metastatic site [4]. Metastasis-associated macrophages (MAMs) are characterized by the expression of the markers CD11b, VEGF receptor 1 (VEGFR1), CXC-chemokine receptor 3 (CXCR3), and CC-chemokine receptor 2 (CCR2) and do not express GR1, angiopoietin 1 receptor (TIE2), and CD11c [5].

In pancreatic cancer, IFNβ produced by primary human PDAC cells can induce TAMs to secrete IFN-stimulated gene 15 (ISG15), a protein with both anti-viral and pro-tumorigenic properties. TAM-derived ISG15 can then stimulate pancreatic cancer stem cell (CSC) self-renewal and tumor-initiating properties, for example increased EMT [30]. Sainz et al. demonstrated that PDAC CSCs secrete the TGF-β superfamily members Nodal/Activin A and TGF-β1, which promote macrophage polarization to an M2 phenotype. As a consequence, polarized TAMs secrete a number of pro-tumoral proteins, including the antimicrobial peptide hCAP-18/LL-37. This antibacterial peptide binds to its receptors, including formyl peptide receptor 2 (FPR2) and P2X purinoceptor 7 receptor (P2X7R) and enhances the metastatic potential of pancreatic tumor cells. Specifically, the authors show that tumor cells

pre-treated with LL-37 have significantly higher metastatic capacity than those treated with a scrambled peptide control [32].

11.3 Macrophages as a Therapeutic Target

The targeting of TAMs represents a novel strategy in cancer treatment and may be achieved in various ways such as blocking the recruitment of macrophages to tumors and re-educating the TME to a more anti-tumor phenotype and macrophage cytoreductive strategies. In mouse models, the CCL2-blocking agent (carlumab, CNTO88) was shown to inhibit tumor growth in a phase 2 study in metastatic castration-resistant prostate cancer in 2013 [50].

Furthermore, Sanford et al. showed in 2013 in a pancreatic cancer mouse model that the CCR2 antagonist (PF-04136309) blocks the mobilization of CCR2+ monocytes, which leads to a depletion of TAMs, reducing the metastatic potential [51]. Inflammatory macrophages were increased in the blood compared to the bone marrow in pancreatic cancer patients, which was a predictor of poorer survival in patients that had undergone a surgical resection. Pancreatic tumors with high CCL2 expression and low CD8 T-cell infiltrate have significantly decreased survival rates as tumor cells secrete CCL2, which recruits immunosuppressive CCR2+ macrophages to the primary tumor [51]. In a recent dose-finding study by Nywening et al., researchers were able to translate these findings directly to patients, by showing that the addition of an inhibitor of monocyte recruitment, specifically a small molecule CCR2 inhibitor PF-04136309 to FOLFIRINOX resulted in tumor shrinkage in 48% of patients with pancreatic cancer [13], double the historical response rate of FOLFIRINOX alone.

Re-education of TAMs to an M1-like phenotype using bioconjugated manganese dioxide nanoparticles or *Pseudomonas aeruginosa* mannose-sensitive hemagglutinin have been shown to enhance chemotherapy [39, 52]. In this way, antitumor macrophages scavenge and destroy phagocytosed tumor cells [53]. The CSF1/CSF1 receptor (CSF1R) is critical for monocyte progenitor generation and TAM polarization and is therefore a potential cytoreductive treatment target [4]. Furthermore, macrophage-specific surface markers are potential therapeutic targets, such as the mannose receptor CD206, the scavenger receptor A, and CD52 [4]. Several phase I clinical trials have been performed with antibodies against CSF1, which leads to a reduction in the number of TAMs (ClinicalTrials.gov identifier: NCT01316822, NCT01444404 and NCT01004861). However, there are currently no phase II or III clinical trials that specifically target macrophages or TAMs [5]. TAMs appear to modulate the cytotoxic effects of chemotherapy in animal models via various mechanisms. The M2 subtype of TAMs has been shown to be involved in revascularization of the tumor after chemotherapy, resulting in tumor relapse that is partly regulated by VEGF-A. In fact, the number of M2 TAMs around the blood vessels reduced after pharmacological inhibition of CXCR4, which subsequently diminished tumor revascularization and regrowth [54].

More recently, in vitro experiments with sorafenib, an oral multikinase, was shown to inhibit polarized macrophage-induced epithelial mesenchymal transition (EMT) in hepatocellular carcinoma cell lines [55]. Specifically, secretion of the EMT stimulation factor, hepatocyte growth factor (HGF), was decreased in macrophages after sorafenib treatment. Consequently, HGF-Met signaling activation by polarized macrophage-conditioned medium was reduced. These effects were not observed in normal hepatocytes. Furthermore, pre-treatment of polarized macrophages with sorafenib reduced the migration of hepatocellular carcinoma cells.

In humans, histological examination of hepatocellular carcinoma tumors treated with sorafenib showed a reduced number of tumor-infiltrating CD68+ macrophages and a reduced expression of the EMT markers, fibronectin and vimentin. Furthermore, the plasma levels of the EMT stimulation factor, hepatocyte growth factor (HGF), were significantly reduced after 24 weeks of therapy with sorafenib in patients with hepatocellular carcinoma, thus, suggesting that sorafenib inhibits HGF secretion.

11.4 TAMs as a Biomarker in Oncology

A high number of infiltrating TAMs in the primary tumor are associated with an aggressive behavior and poor prognosis [4]. The cell surface markers CD163, CD14, CD204, and CD206 may be used to identify TAMs, although they are not tumor-site specific [4, 56]. Serum CD163 levels may also be used as a prognostic marker in some tumor types [4]. Cell-surface vimentin–positive macrophage-like circulating tumor cells were identified in blood from patients with gastrointestinal stromal tumors (GISTs). These cells express the macrophage markers CD68 and CD14, tumor cell markers DOG-1, C-kit and are negative for CD45 [57].

11.4.1 Circulating Tumor Microemboli (CTM)

Circulating tumor clusters or microemboli (CTMs) have been reported in various tumor types including lung, breast, colon, prostate, and pancreas. CTMs have been identified via a variety of approaches including cell microscopy, immunocapture, and microfluidic chips [58–61]. As with the detection of CTCs, higher numbers of CTMs per ml of blood correlates with a poorer progression-free and overall survival. CTMs are thought to provide a survival advantage for CTCs in the harsh environment of the bloodstream and protect them from anoikis [62]. CTMs are thought to be cell clusters that have collectively shed from the primary tumor and consist of cells with both epithelial and mesenchymal phenotypes [63].

11.4.2 CTC-Macrophage Fusions

Cell fusion occurs when two or more cells become merged via the plasma membranes, and the progeny are known as hybrids. The tumor-leucocyte cell fusion theory of metastatic potential was proposed many years ago, whereby a tumor cell fuses with a migratory blood cell in order to promote tumor cell dissemination around the body [64–66]. Many tumor cell types have fusogenic properties and this was proposed as a mechanism to promote their malignant potential, resistance to drugs, and apoptosis [67]. In fact, malignant plasma cells in multiple myeloma (MM) are highly fusogenic and form multinucleated osteoclasts that express CSC markers with a high metastatic potential [68]. This concept of leukocyte-tumor cell fusion as a driver of cancer progression has been recently reviewed by Sutton et al. [69].

Giant circulating cancer-associated macrophage-like cells (CAMLs) were identified in 2013 [70] and are thought to be exclusively found in cancer patients. These cells range from 25 to 300 μm in size, with enlarged nuclei and express the pro-angiogenic markers CD14 and CD11c as well as CD45, cytokeratin, and epithelial markers CK 8, 18, and 19, and EpCAM [70] (Fig. 11.1). CAMLs are disseminated TAMs with the ability to seed, proliferate, and neovascularize the metastatic niche and are also involved in the phagocytosis of neoplastic cells within the primary tumor. In fact, higher CAML counts were found after chemotherapy treatment compared to untreated or hormone-based therapy. CAMLs or tumor cell-macrophage hybrids have been found in various tumor types such as breast, prostate, esophageal, colorectal, and pancreas, and the majority (over 83%) of patients with early and advanced stage disease are positive for CAMLs [69]. However, healthy controls and patients with benign disease were negative [70]. Here below we summarize the most relevant studies of tumor cell-macrophage fusions in various tumor types (Figs. 11.2 and 11.3).

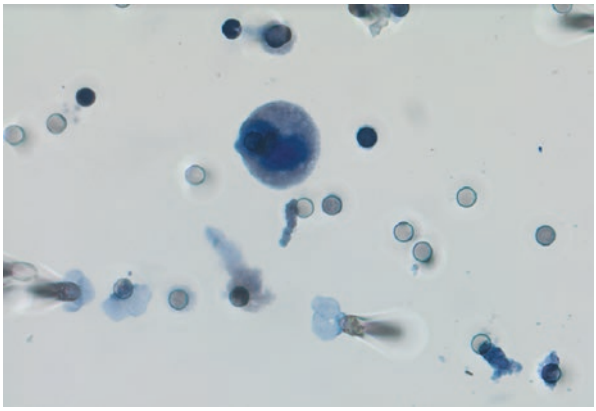

Fig. 11.1 Example of a giant macrophage. Although there is a high nuclei/cytoplasm ratio, the cytoplasm is vacuolized

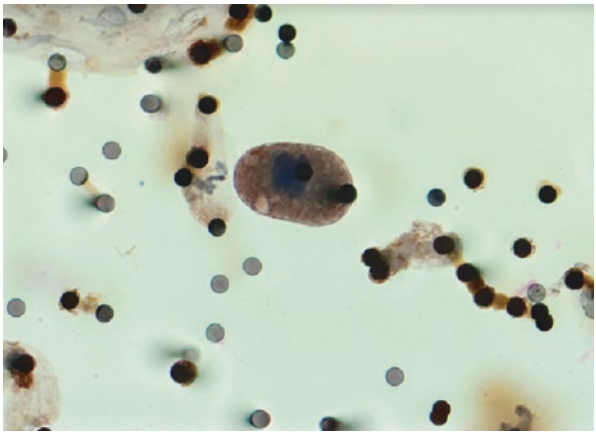

Fig. 11.2 Giant macrophage stained with anti-CD45 (visualization with DAB)

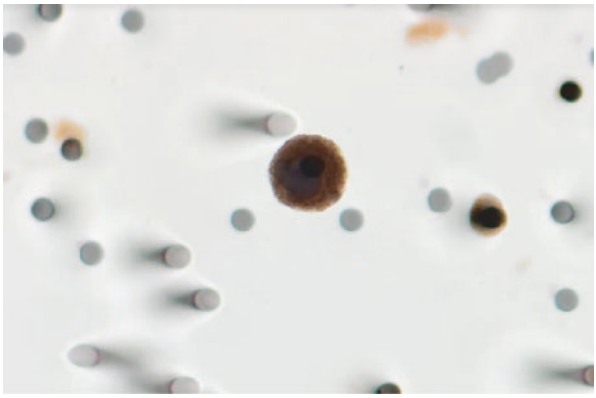

Fig. 11.3 Example of a giant macrophage, with a riniform nuclei and vacuolized cytoplasm. Cell stained with anti-CD45 (visualization with DAB)

A recent study in breast cancer cells by Zhang et al. showed that tumor-macrophage hybrid cells had enhanced tumorigenic and metastatic capacities such as increased proliferation, colony formation, migration, and invasion capacity with resistance to apoptosis. These effects appeared to be induced by EMT and Wnt/β--catenin signaling, with an associated downregulation of E-cadherin and an increased expression of N-cadherin, vimentin, Snail, as well as MMP-2, MMP-9, and S100A4 [71]. Another study showed that MCF-7 breast tumor cells and macrophage hybrids occurred by spontaneous fusions at a rate of around 2%. These fusions showed phenotypic and genetic traits from both maternal cells such as CD163 and CD45 expression and short tandem repeat (STR) genetic markers [72]. Another recent study described the isolation, cultivation, and characterization of macrophage-tumor cell fusions (MTFs) from the blood of pancreatic ductal adenocarcinoma (PDAC) patients. The MTFs consisted of M2-polarized macrophages, and the cells were generally aneuploid with characteristics associated with epithelial, macrophage, and stem cells and also expressed markers associated with tumor progression and metastasis. Furthermore, when transplanted orthotopically into a murine

pancreas, MTFs grew as well-differentiated cell colonies in many different organs, without forming an established tumor. Thus, suggesting that these structures disseminate from the primary tumor and form a metastatic niche [73]. Furthermore, a study in melanoma showed that 2 circulating tumor cell (CTC) populations were detectable, one cytokeratin positive only and a second that was also positive for CD45 and the monocyte differentiation marker CD14, thus, suggesting the presence of leukocyte/macrophage-tumor cell fusion hybrids in these patients [74]. In fact, these macrophage-CTC fusions enter into the blood stream and generate metastatic lesions due to their ability to secrete cytokines to prepare the metastatic niche and colonize the secondary organ [75]. A recent study by Lindström et al. in breast cancer showed that cell fusions of MCF-7 cells with macrophages resulted in an increased radio resistance and enhanced DNA-repair capacity after exposure to Gy γ-radiation [76]. Another study in breast cancer, regarding the tumor-initiating and metastatic capacities of M2 macrophages and MCF-7 or MDA-MB-231 cell line hybrids in NOD/SCID mice showed that the hybrids had a more aggressive phenotype, including increased migration, invasion, and tumorigenicity. However, their proliferative ability was reduced and the hybrid phenotype was CD44+CD242/low with overexpression of EMT associated genes, indicative of stem-like properties [77]. Although, a study in a murine model of spontaneous neu+ breast cancer demonstrated that even though macrophages are most commonly fused with tumor cells, they were present at low levels in the primary tumor and undetectable in metastasis [78]. These studies suggest that TAMs may promote the metastatic potential of breast cancer cells via cell fusion, and the hybrids may gain a BCSC phenotype, compared with the parental lines (Fig. 11.4).

Several studies have addressed the specific mechanism by which macrophages promote the metastatic potential of tumor cells via cell-cell fusion. In fact, acute myeloid leukemia (AML) cells spontaneously fused with macrophages, dendritic and endothelial cells in a murine in vivo model. The hybrid cells gave rise to leukemia with 100% penetrance when implanted into mice, and data suggest that tumor cell-macrophage fusion may be a mechanism of gene transfer to promote tumor

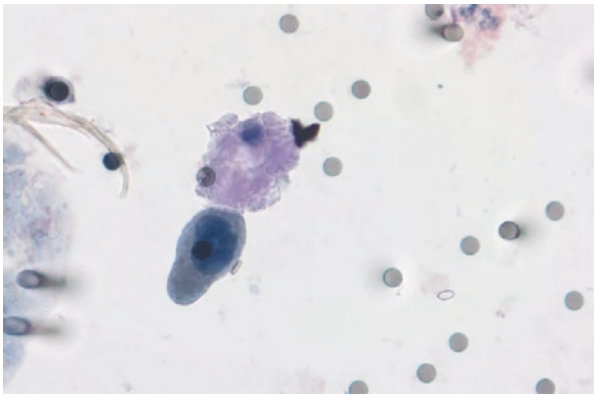

Fig. 11.4 Giant macrophage stained with haematoxilin

dissemination [79]. Furthermore, a study by Powell et al. in 2011 suggested that the cellular properties of macrophages, such as migration and immune evasion, are transferred to tumor cells via cell fusion as a mechanism of the metastatic conversion of cancer cells [80].

These cell fusions could provide new diagnostic, prognostic, and treatment response biomarkers in oncology as their presence seems to correlate with many clinical criteria. However, this presents a challenge due to their low prevalence in blood and the difficulty to isolate these cells in sufficient quantities in order to perform profiling studies. Likewise, the concept and existence of giant macrophages or CTC-macrophage fusions is still under scrutiny, and the true biological relevance of these cells has yet to be definitely determined. Without a doubt, the challenges associated with identifying and isolating these cells are many and include the scarcity of these cells, and the methodologies available to isolate them. Regarding the latter, many systems that have been developed to isolate CTCs include an immune cell elimination step. Thus, giant macrophages or CTC-macrophage fusions would be eliminated based on the expression of immune cell markers. Such systems include the CellSearch® platform (Menarini Silicon Biosystems, Inc. 2019). Thus, for the detection of these cell hybrids, other methodologies based on cell size would likely prove more beneficial. For example, the OncoQuick® system represents a simple-to-use, rapid, and efficient system for the enrichment of CTCs. OncoQuick® tubes consists of 50 ml polypropylene tube with a porous barrier which is inserted above a specially developed separation medium, which allows for density gradient centrifugation of cells from up to 30 ml of anticoagulated whole blood. Disseminated CTCs are enriched in the interphase, and following centrifugation, cells can be validated via various techniques, including immunofluorescence, RTqPCR, WB, or in vitro cell culture. This technique was used by Clawson GA et al. to identify and culture macrophage-tumor cell fusions from blood of patients with pancreatic ductal adenocarcinoma [81]. Indeed, other systems exists, but more research is needed to determine the best method for isolating these cells. Until then, we can only speculate that these cells play an important role in tumor cell dissemination.

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Chapter 12
In Vitro and In Vivo Models of Circulating Tumor Cells

Anna Paula Carreta Ruano and Fernanda Cristina Sulla Lupinacci

12.1 Introduction

The presence of CTCs in the blood of cancer patients is being investigated for their potential as real-time noninvasive liquid biopsies. Such a method can provide complete information on the genetic profile of cancers and track genomic changes [1–3].

In addition, the molecular characterization of individual CTCs revealed important information about the genotype and phenotype of these tumor cells and demonstrated a remarkable heterogeneity of CTCs. Although the presence of CTCs is known in several cancers [4], little is known about the proportion of viable CTCs [5–7]. Currently, works have been done seeking the molecular characterization of CTCs, as a manner to increase their diagnostic specificity. Some groups have been able to grow these cells in vitro and analyze the proteins secreted by them, as well as induce tumor in animals with CTCs from cancer patients [8, 9].

CTC-derived xenografts (CDXs) or CTC-derived cell lines at relevant times during disease progression are decisive in achieving a complete characterization of CTCs, along with in vivo and in vitro pharmacological tests. Despite this continuing task being challenging due to the scarcity of CTCs in peripheral blood and limitations related to enrichment methodologies, significant studies have been done to establish clinically relevant systems for the study of CTC biology in different types of cancers. In this chapter, we discuss the basic knowledge of CTCs and evaluate existing CTC-derived models, including in vivo CDXs and in vitro functional culture assays in different types of cancer.

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12.1.1 Model Organism Databases (MODs)

Model organism databases (MODs) are designed to facilitate researchers to find specific needs of each model organism and integrate biomedical research data. To study the development and progression of diseases and to test new treatments, researchers can use animal models, allowing the development of a better comprehension of animal and human anatomy, physiology, pathology, and pharmacology. The possibility of experimenting under controlled situations and mimicking biological conditions of diseases or situations, guaranteed the development of scientific methods and the creation of the concept of animal models [10].

Regarding biomedical research, many species can be used such as *Drosophila, Caenorhabditis elegans, Zebrafish, Xenopus*, and also mammals, such as *Mus musculus* (mouse), dogs, pigs, and monkeys [10, 11]. Due to characteristics such as its short life cycle, gestation period, and lifespan, as well as its high fecundity and breeding efficiency, the laboratory mouse (*Mus musculus*) is widely recognized as an important vertebrate animal model and is the most frequently used animal in biomedical research [12]. Mice are considered the model organism of choice for studying the diseases of humans, with whom they share 99% of their genes [13], and this high degree of conservation with humans is reflected in its anatomy, physiology, and genetics [14]. They can be used to investigate genetic and cellular systems relevant to human biology and disease, in a variety of ways to comprehend the mechanisms, genetics, genomics, and environmental contributions [15].

Modern molecular biology approaches and cost reductions in next generation sequencing have opened avenues for direct application of model organism research to elucidating mechanisms of human diseases. The scientific understanding of how genes, environment, and behavior could interact to generate chronical diseases such as cancer and obesity remains insufficient, as does current treatment in most cases, that cannot reach a cure, but only momentary stabilization of the disease.

12.1.2 CTC-Derived Xenografts (PDXs)

Patient-derived xenograft (PDX) technology has emerged as a research platform to better elucidate the understanding of cancer biology and the evaluation of new therapeutic strategies [16]. PDXs are generated by implanting surgically removed tumor tissue (primary or metastatic) in immunodeficient mice. Despite these models present utility as preclinical tool in many types of cancer, their practicality is a challenge due to the limited availability of tumor tissue [17]. This limitation can be overcome by the generation of CDX models after the enrichment of CTCs collected from one of an easily accessible blood sample, followed by injection in immunodeficient mice [18–20]. However, it is important to note that the development of CDX is still a huge challenge due to the amount of viable CTC in various types of cancer. So far, CDXs been established in breast cancer, melanoma, lung, and prostate (Table 12.1).

Table 12.1 Studies about CTC-derived xenografts

CTC-derived xenografts					
Type of cancer	Stage	Live CTC isolation technique	Transplantation of CTCs	Main findings	Ref
Breast cancer	Metastatic luminal	FACS isolation ($PI^-CD45^-EpCAM^+$) or RosetteSep	1109 CTCs	Expression of $EpCAM^+CD44^+MET^+CD47^+$	[21]
			$EpCAM^+$ (CellSearch)	Demonstration of the metastatic phenotype of patients in CDX metastases	
				No drug sensitivity study	
	Metastatic triple-negative	Histopaque®	969 CTCs/7.5 mL	RT-qPCR for genomic profiling of CTC/CDX samples before and after injection	[22]
			$EpCAM^+$ (CellSearch)	No drug sensitivity study	
	Metastatic triple-negative	FACS ($CD45^-/CD34^-/CD105^-/CD90^-$ $CD73^-$)	N/A	Identification of a TNBC liver metastasis CTC-specific signature (whole-transcriptome) – survival analyses for signature transcripts	[23]
Melanoma	Stage IV	RosetteSep	N/A	Demonstration of patient response to *trametinib* in the CDX	[24]
				Concordance in SNV profiles (WES/RNAseq)	
SCLC	Metastatic	RosetteSep	400 CTCs per 7.5 mL blood	Demonstration of CTC genomic profile by CDX tumors	[9]
			$EpCAM^+$ (CellSearch)		
				CDX simulated donor's response to chemotherapy	
	Limited or extensive stage	CTC-iChip + RosetteSep Ficoll	N/A	Stable demonstration of tumor genome	[25]
				Importance of the evolution of the treatment of the patient's tumor	

(continued)

Table 12.1 (continued)

CTC-derived xenografts					
Type of cancer	Stage	Live CTC isolation technique	Transplantation of CTCs	Main findings	Ref
NSCLC	Metastatic	RosetteSep	>150 CTCs by FACS (CD45/CD144/vimentin/CK)	Importance of mesenchymal CTCs with tumorigenic capacity	[26]
CRPC	Metastatic	DLA/RosetteSep	19.998 CTCs	Demonstration of genome characteristics in CTC, patient tumor, and CDX (WES)	[27]
			$EpCAM^+$ (CellSearch)	Tumorigenic CTCs with characteristics acquired from CRPC-NE	

N/A not available, *FACS* fluorescent-activated cell sorting, *CDX* CTC-derived xenograft, *TNBC* triple-negative breast cancer, *SCLC* small-cell lung cancer, *SNV* single nucleotide variant, *NSCLC* non-small cell lung cancer, *CRPC* castration-resistant prostate cancer, *NE* neuroendocrine, *WES* whole-exome sequencing. (Reference table [28])

The first CDX CTC-derived xenograft was reported in 2013 by Baccelli et al. from an immunodeficient mice that developed bone, lung, and liver metastases after tibial bone injection of CTCs from patients with metastatic breast cancer. The injection of CTCs of 110 patients was performed. Six receiver mice developed bone, liver, and lung metastases within 6–12 months after transplant CTC (approximately 1000 CTCs) from three patients with advanced metastatic breast cancer. These metastases were analyzed and found to express EpCAM, CD44, CD47, and MET. The authors also showed that the number of CTCs positive for these markers was strongly correlated with decreased progression-free survival of patients with metastatic breast cancer. Therefore, this study revealed that CTCs can be an attractive tool for tracking and directing metastatic development in breast cancer [21]. A second group developed a CDX model from a patient with metastatic triple negative breast cancer (TNBC) for the first time. The patient selected for the CDX establishment had advanced TNBC with a high CTC count, analyzed by CellSearch (969 CTCs and 74 CTC clusters/7.5 mL). The enriched cells were injected subcutaneously into mice and a noticeable tumor after 5 months. The samples were collected at two different times (metastasis and progression), which allowed the real-time assessment of the molecular changes between tumor samples from patients, CTCs and CDXs. The CDX showed a phenotype equal to that of the patient's tumor. Furthermore, the analysis of circulating tumor cells (CTCs) also deciphered a panel of potential tumor biomarkers [22]. In 2019, Vishnoi et al. developed a TNBC CDX model with the addition of liver metastasis. The authors identified a first CTC signature of 597 genes related to liver metastasis in TNBC that can provide information about the mechanics of TNBC disease progression in the liver [23].

A study with CDX and melanoma developed by Girotti et al. (2016) reported a success rate of 13% of CDX established. The CDX tumor was palpable from 1 month after CTC implantation and was sustainable in secondary hosts. In addition, CDXs represented patients' tumors [24].

In the study with lung cancer, Hodgkinson et al. (2014) showed that CTCs in chemosensitive SCLC are tumorigenic. CTCs were isolated from 6 patients with advanced SCLC who never received chemotherapy. CTCs were injected into NSG mice (NOD scid gamma mice). Each patient had more than 400 CTCs and 4 CTC samples gave rise to CDX tumors detected after 2.4 months. The CDXs of CTCs enriched with CellSearch reproduced the chemotherapeutic response of donor patients (platinum and etoposide), proving the clinical importance of these models [9].

Drapkin et al. (2018) also developed CDX models of a patient with SCLC undergoing combined treatment based on olaparib and temozolomide after relapse. By the way, the models also portrayed the evolution profiles of the patient's malignancy, which highlights the potential usefulness of CDXs in the treatment of SCLC [25]. Regarding the NSCLC, Morrow et al. (2016) used CTC samples recovered and analyzed by CellSearch, in two different moments: baseline and post-brain radiotherapy. No CDX was developed at the baseline. But the injection of CTCs after radiotherapy resulted in a remarkable tumor 95 days after injection. Despite not having CTCs with epithelial characteristics, it was possible to notice a considerable

population with mesenchymal expression (vimentin). This study suggests that the absence of EpCAM + CTCs in the NSCLC does not exclude the existence of CTCs and highlights the importance of investigating CTCs undergoing EMT [26].

And for last, a patient with specific castration-resistant prostate cancer (CRPC), with an extremely high CTC (126 CTCs per 7.5 mL blood, obtained by CellSearch and 19,998 CTCs obtained by DLA) gave rise to a palpable tumor in 165 days. Overall, the genomic characterization of CDX revealed some genomic changes found in CRPC-NE, such as TP53 mutations, loss of RB1, and PTEN [27].

12.1.3 Ex Vivo Models Derived from CTCs

The expansion of viable CTCs ex vivo can offer an attractive alternative, allowing molecular analysis and screening of high-yield drugs in a shorter time. The CTC culture was demonstrated by only a few groups [25, 29–35].

The first CTC cell line for colon cancer was derived by Cayrefourcq et al. (2015). Blood samples enriched negatively of 71 patients with metastatic colon cancer and successfully established a permanent cell line from a patient with a CTC count ≥300 detected by CellSearch platform. It is important to note that the cell line CTC-MCC-41 characterized, provided the main genomic characteristics of the primary tumor of the donor patient and lymph node metastases [8]. In a second study, the authors established and characterized eight additional cell lines from the same patient with CTCs collected at different times. Functional experiments showed that these cells favor angiogenesis in vitro, which was consistent with the secretion of VEGF and FGF2 (angiogenesis inducers) [8, 29].

Drapkin et al. (2018) generated 16 additional models of SCLC CDX by CTCs collected at initial diagnosis or progression, with 38% efficiency. Somatic mutations were maintained between patients' tumors and CDX, and the genomic outlook remained stable throughout the initial passages of CDX, showing clonal compliance [25]. Still in lung cancer, Zhang et al. (2014) focused on lung cancer and developed a new in situ capture and culture methodology for ex vivo expansion of CTC using a 3D co-culture model. CTC was successful in 14 out of 19 early-stage lung cancer patients, using a three-dimensional co-culture model, including fibroblasts, to support tumor development. This group developed a new model derived from CTC ex vivo using a 3D co-culture system, developing the tumor through microenvironment stimulation. In the cultured CTCs, several mutations, such as TP53, were found by sequencing, corresponding to primary tumors of patients [30].

Andree et al. (2018) generated the first CDX model of castration-resistant prostate cancer (CRPC) that resulted in a permanent ex vivo culture of CDX tumor cells. Samples ($n = 22$) were collected from patients with metastatic CRP, 7 of which were obtained by diagnostic leukapheresis (DLA) [31]. Notably, the cell line obtained derived from CDX in vitro responded to the genetic characteristics and

tumorigenicity of CDX and corresponded to treatments for patients with CRPC based on enzalutamide and docetaxel [27, 32]. Another group generated 7 first organoid strains, faithfully characterized with a patient's CRPC, in addition to a 3D organoid system derived from the CTC of a patient who had more than 100 CTCs per 8 mL of blood. There was a high agreement between 3D models and the molecular diversity of prostate cancer, yet the organoid graft derived from CTC in vivo showed tumors corresponding to primary cancer [33].

In 2014, Yu et al. reported the establishment of CTC lines from 6 patients with breast cancer, metastatic luminal subtype. Three out of five cell lines tested were tumorigenic in vivo, giving rise to tumors similar to the patient's primary tumor, elucidating the importance of monitoring the tumor's mutational evolution throughout the disease [34]. Jakabova et al. (2017) cultured CTCs of breast cancer patients at different stages. There was no significant difference between the tested subgroups, but the highest occurrence of CTC was observed in the group undergoing surgery (86.6%) and similarly in the group before the start of neoadjuvant and adjuvant treatment (82.3%) [35].

Recently, Koch et al. [36] introduced a new line (called CTC-ITB-01) derived cell CTC from a patient with metastatic breast cancer positive for estrogen receptor (ER). The *CellSearch*® system was used to identify CTCs in parallel. The blood of the same patient was processed for cell culture by *Rosette Sep*™ (*StemCell Technologies*). For characterization of this cell line, the authors analyzed protein expression of *ER* and *ERBB2* (relevant receptors for major breast cancer subtypes) by Western blot and confirmed by immunocytochemistry, resulting in cells positive for ER and negative for *ERBB2*, in correspondence with the primary tumor. It was also analyzed the whole exome (WES) of CTC cells-ITB-01, both primary tumors (left and right breast) and the distant vaginal metastasis. Data were analyzed for mutations of common genes related to cancer. There was an agreement in genes of the *PIK3CA* protein, in addition to others associated with hereditary predisposition to breast cancer, such as *BRCA1/2, P53, PTEN, STK11*, *or CHEK2*. Then the metastatic potential of the CTC-ITB-01 cell line was verified by injection into the mammary glands of female immunodeficient mice. It was possible to observe the constant increase in tumor burden in the 8.5 months until the sacrifice. Immunohistochemical staining also revealed that the ER + status of the cell line was maintained in CTC-derived xenografts (CDXs), confirming that histopathological characteristics are preserved [36].

These examples show that the establishment of line of functional models of CTC cells is feasible. The isolation and in vitro culture of CTC may provide an opportunity to noninvasively monitor the varying patterns of drug resistance in individual patients, while their tumors acquire new mutations and can improve treatment. The methodologies to cultivate CTCs are still in development, there is no ideal protocol for the culture of CTC derived from the patient, and, in fact, the cell of each patient may demonstrate different growth conditions. Therefore, development and optimization of isolation technologies require specific attention.

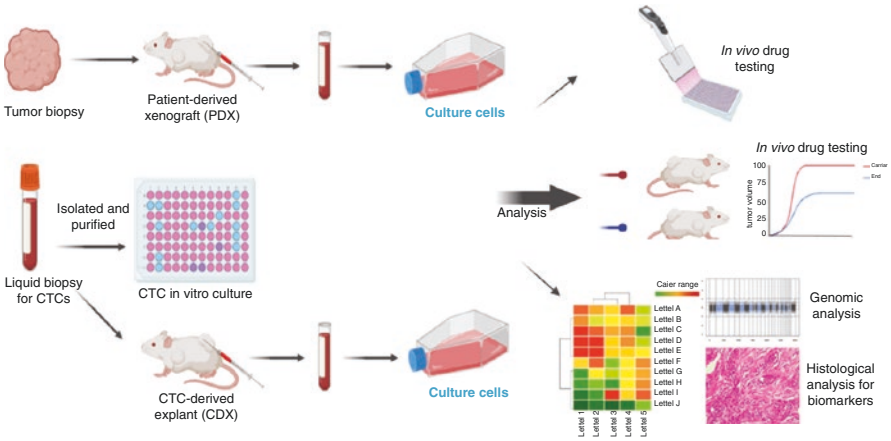

Fig. 12.1 The study of circulating tumor cells can benefit from animal models to establish new methodologies of CTC quantification and isolation, thus enabling expansion and analysis of these cells

12.2 Concluding Remarks

There has been a great expansion in techniques to safely detect, quantify, and characterize CTCs at the phenotypic, genetical, and functional levels. The characterization of CTC-derived models provides a better understanding of the tumor mechanism of these cells (Fig. 12.1). As shown in Table 12.1, the procedures for developing CDXs may vary from one study to another.

Future improvements in the detection of CTCs in vivo, such as individual cells or clusters, will be invaluable in elucidating their modes of generation and developing strategies to direct them to their source.

Some emerging technologies can complement CTC analyses and demonstrate important steps in cancer detection, monitoring, and management. Identification of the role and importance of CTCs in cancer metastasis and progression, whether by identifying potential biomarkers, gene signatures, survival mechanisms, or new mechanisms, could provide new tools for preclinical studies [37].

Still, the increasing in the numbers of studies with ex vivo CTCs is promising, but very far from being applied in clinical practice, as the culture conditions are still under elucidation. Therefore, cultures derived from CDXs present a better way to characterize this population, showing possibilities of providing information about the biological mechanism of the metastatic process.

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Chapter 13
Brief Summary and Perspectives for CTCs

José Gabriel Rodríguez Tarazona and Ludmilla Thomé Domingos Chinen

As discussed in the previous chapters, the analysis of circulating tumor cells (CTCs) is an important tool to provide information on the biology of solid cancers and disease monitoring [5]. Tumors are heterogeneous entities, so in classical biopsy, there is the possibility that some characteristics, even aggressive subclones, remain undetectable, causing loss of important information [11]. Furthermore, because of its low invasiveness and low risk, blood biopsies can be used repeatedly and can monitor the dynamics and molecular landscape of the disease [12].

Finding CTCs in patients, time after resection of the primary tumor, is common, probably due their capacity to re-circulate from secondary metastatic sites into the bloodstream, or to come back to the site of the primary tumor; but how these CTCs contribute to metastasis is unclear [26]. Studies in animal models indicate that tumor cells may return to the primary site, a process called self-seeding [2]. New approaches must unravel if CTCs re-infiltrate the tumor to give it some resistance gain and clarify these mechanisms. Another question is whether the microenvironment could cause these released CTCs to be guided through the exosomes as biological magnets that corroborate both new metastatic sites and the primary tumor from which the CTCs originate. In fact, we must know whether self-seeding selects populations of cancer cells, how it does, and if cells have been subjected to selection through circulation and survival in the blood.

It should be emphasized that the detection of disseminated tumor cells (DTCs) in the bone marrow of patients with breast cancer is related to locoregional and metastatic recurrence with more hostile metastatic variants. Next, analyses should emphasize comparative genomic studies of CTCs and DTCs, along with primary and metastatic lesions of the same patients [2].

In fact, tumor cells travel through the bloodstream or lymphatic vessels to establish metastases. Theoretically, the CTCs are generated within the primary or

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metastatic tumor with ability to separate from the basement membrane, colonize and cross the tissue stroma to enter the blood vessels [14]. It is believed that this phenotype happens by the increase of the hypoxia in the tumor by development and competition by resources [1]. A relevant concept for CTCs is the epithelial-mesenchymal transition (EMT), a process that allows epithelial cells to lose apical-basal polarity, with consequent removal of neighboring cells, as well as to acquire rounded or elongated morphology, invading the surrounding stroma and becoming less susceptible to apoptosis. In the process, cells decrease the expression of epithelial markers and increase expression of mesenchymal proteins and growth factors [7].

One current idea is that if a tumor cell is to be a CTC, it needs to make the EMT happen and thereby escape the primary/metastatic tumor and cross the blood vessel. However, if the epithelial-like CTCs are diffused from a tumor into a blood vessel and during this process passes through the EMT is still a debate. CTCs expelled from epithelial tumors can be identified with multiple epithelial markers. Many of clinical trials of CTCs have used platforms that detect CTCs that express epithelial markers. However, more promising are clinical trials that incorporate CTC subpopulations, joining EMT, stem-like, and epithelial markers [7]. Future studies could identify and classify cytopatologically the CTCs as cells recognized by the two types of morphology (epithelial and mesenchymal) and marking with epithelial, mesenchymal, sarcomatoid, and/or stem antibodies. An assay that could unify all these markers would undoubtedly leave no CTC unobserved (Fig. 13.1).

Tumor cells undergo EMT to enter and survive in the bloodstream, and perhaps CTCs that left the blood vessels, by disseminating into the tissues, could revert to the former form through a process called mesenchymal-epithelial transition (MET), and this reversion to the epithelial state implies the presence of a state between

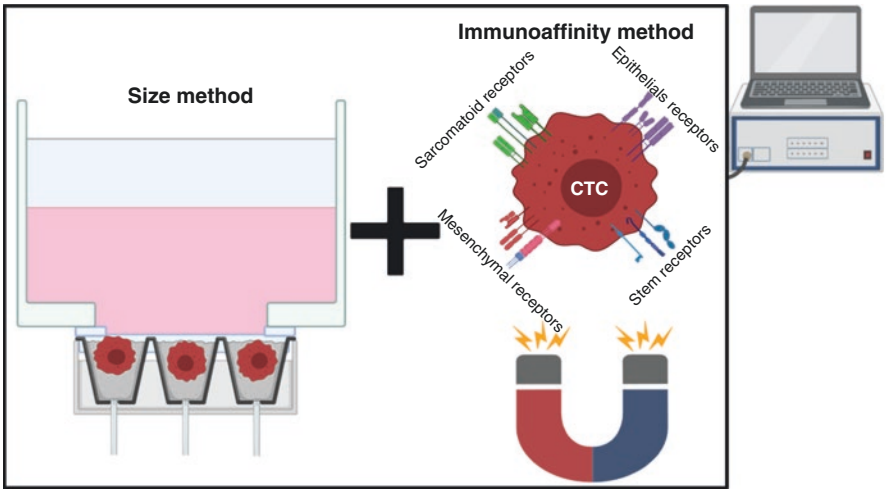

Fig. 13.1 A method that combines immunological affinity with a method based on size, within a systematized electronic platform, could evolve current methodologies, because it would not be dependent on cell labeling, nor would it depend exclusively on cell size

epithelial and mesenchymal [8]. The concept of dynamic flow between epithelial and mesenchymal states would better explain the metastasis process [24], assuming that the mesenchymal subtype colonizes distant sites and then returns to the epithelial state, once the niche found is appropriate [8]. It should be emphasized that, to date, no single model of metastasis encompasses all observational findings. In addition, a number of other factors, including the site of tumor cell origin and the degree of cellular heterogeneity within a tumor, may influence the process. However, the EMT/MET model of dissemination has advantages in explaining the wide variety and plasticity of observed CTCs and has important implications for the direction of future research [7]. Another consequence of CTC heterogeneity is that not all tumor cells will continue to develop or are related to metastasis. The future in this field should focus on identifying subsets or subpopulations of cells exhibiting these aggressive properties [13].

Another major benefit of using CTCs in clinical practice would be their ability to diagnose undetectable micrometastases. Minimal residual disease (MRD) is the presence of malignant cells in organs distant from the primary tumor that are undetectable by conventional imaging tests and laboratory tests used for tumor staging, all after the surgery to remove the primary tumor. CTCs and DTCs are considered substitutes for MRD because they are cells with the potential to initiate metastasis [10]. Studies show that the finding of CTCs can serve as a biomarker for MRD in ovarian cancer [19], pancreatic cancer [21], breast cancer [6, 15, 22], colon cancer [16, 18], colorectal cancer [25], prostate cancer [4, 17], and esophageal cancer [9]. Ultrasensitive assays that allow the reliable detection of minute amounts of tumor cells should be implemented in clinical trials of neoadjuvant and/or adjuvant therapies and can complement current classic post-surgical surveillance procedures for tumor relapse. Early detection of micrometastatic relapse would lead to intervention and updating of post-adjuvant therapies before overt metastasis. Therefore, liquid biopsy analyses would change the current landscape of oncology diagnosis, evolving cancer therapies targeting MRD [20].

In preliminary studies of detection of CTCs, the major problem for the development of a detection assay was the unfamiliarity about the presence of tumor cells in a blood sample and about their number. Advances were made using the expression of epithelial markers such as EpCAM, Cytokeratin 8, 18, or 19, but it is important to emphasize that the detection made by these assays might be marginal as they limit the detection to epithelial expression. Progress is imperative to detect significant heterogeneity of CTCs [3].

New and relevant studies with the aim to identify varieties of CTCs are necessary and urgent. Maybe, these new studies will use physical characteristics, such as size, rigidity, dielectric attributes together with diverse protein expression detection methods. Further research will be needed to identify the biological differences between these tumor cells and to determine whether specific subsets are responsible for the metastatic phenotype [3]. The window of the new technologies for CTC characterization and its gene expression, as well the expansion of CTCs in culture and animal models, is open [23]. It is also expected that the unification of technologies helps to develop a personalized medicine.

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