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

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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

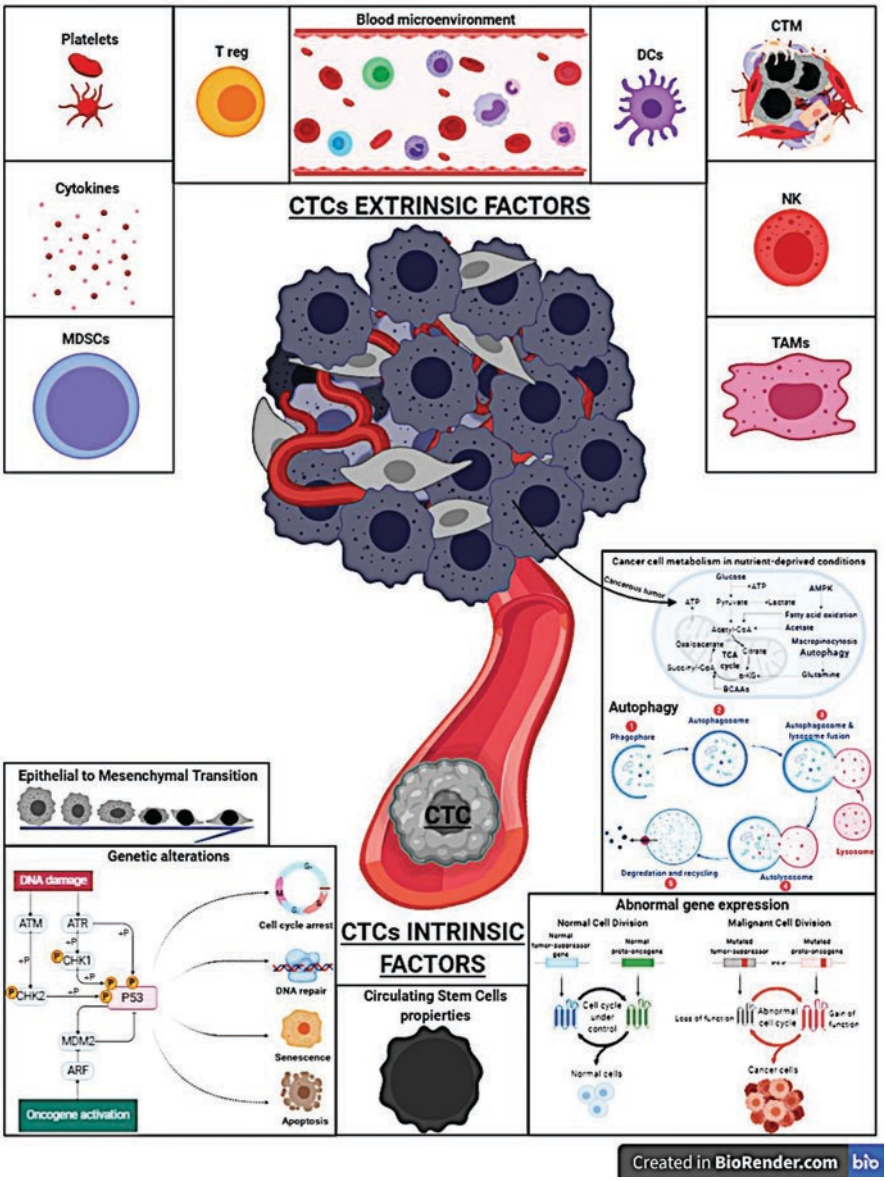

Fig. 1.1 Extrinsic and intrinsic factors related to CTCs/CTM survival

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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