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TITOLO TESI

CIRCULATING TUMOR CELLS INVESTIGATION IN ESOPHAGEAL CANCER

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Introduction

Since the Greek physician Galen had associated of an imbalance in "black bile" with cancer, to the post-mortem observation of tumor-like cells in the blood by Thomas Ashworth in 1869, and to the validation of circulating tumor cells (CTCs) as a prognostic tool in the early 2000's, the scientific community has always demonstrated an ever-deepening interest in these rare cells and their role in the metastatic process [1,2]. CTCs are exceedingly rare cells found into whole blood of cancer patients. They have the potential to serve as a 'blood biopsy', enabling population-wide screening for early diagnosis, highly sensitive prognostic monitoring for cancer patients, and serial non-invasive molecular profiling to bring the science of personalized medicine to practical fruition in the clinic. Furthermore, while much has been hypothesized about their potential role in the hematogenous dissemination of cancer, the biological characterization of these cells could lead to an entirely new class of therapies targeting metastasis. Metastasis is the spread and growth of tumor cells from the primary site to distant organs, is conceivably the most devastating and deadly attribute of cancer, and is ultimately responsible for more than 90% of cancer-related deaths, resulting in over 500,000 deaths each year in the United States alone. Circulating tumor cells have the potential to be highly informative concerning our understanding of metastasis. Present technologies, however, enable only a subset of potential analyses to be conducted, principally due to sub-optimal cell isolation sensitivity, purity, throughput, or handling method [details in Appendix 1].

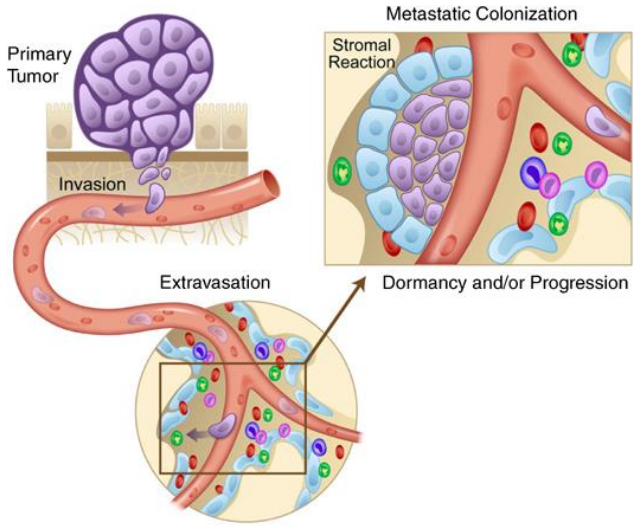


FIGURE 1 MODEL OF TUMOR PROGRESSION [3]

Origin of CTCs

Ten years ago, I.J. Fidler postulated that a primary tumor composed by 10^9 tumor cells can sheds millions of tumor cells into circulation every days, but the development of lung metastases was very rare in an experiment where $2x10^6$ cancer cells were injected into rabbits[4].

These findings lead to consider the cancer cells spreading as an inefficient process where only a little percentage of circulating tumor cells could generate metastases. What happens to the vast majority of the tumor cells that fail to form metastases is still matter of debate since the metastatic process consists of a long series of sequential, interrelated steps. . Each of these can be relate limiting, as a failure or an insufficiency at any of the steps can stop the entire process. The outcome of the process is dependent on both the intrinsic properties of the tumour cells and the responses of the host.

The very first step of metastatic dissemination is thought to include the detachment of epithelial cells from the extracellular matrix (ECM) and the disruption of the actin skeleton, leading to cells rounding. Both of these events normally trigger apoptotic process, which termed anoikis and amorphosis respectively.

The ability to survive in the absence of normal matrix components represents a crucial property of metastatic cells, as tumor cells that intravasate into the circulation and extravasate into secondary tissue sites are either deprived of matrix or exposed to foreign matrix components [5,6].

To enter the bloodstream a tumor cell has to negotiate and survive the process of intravasation. A fluorescence-based in vivo study using orthotopically injected metastatic versus non-metastatic tumor cells showed that a large number of the non-metastatic cells fragment when interacting with blood vessels, whereas the metastatic cells display increased survival during this process [7], indicating that the entry of cancer cells into the vasculature constitutes an important barrier to metastasis.

The efficiency of metastasis inhibition by apoptosis induction in the early phases of entrance into the circulation and extravasation is probably another important regulatory mechanism that counteracts metastasis development. Cell death might occur as a result of two main effects: cell destruction by mechanical stress and cell death mediated by the immune system.

Furthermore, to form metastases circulating cancer cells have to pass through several stressful and highly selective steps, including arrest in the capillary bed and resumption of proliferation in distant organs.

TUMOR PROGRESSION MODELS: LINEAR VS PARALLEL

The debate about metastases development and consequently the role of CTCs is still open: on one hand there is the linear progression model and on the other the parallel progression model.

The linear progression model is based on Leslie Fould's description of a stepwise progression of morphological abnormalities [8] accompanying cancer [Figure 2].

Accumulation of genetic and epigenetic alterations was subsequently associated with this process [9]. At its simplest, the model states that cancer cells pass through multiple successive rounds of mutation and selection for competitive fitness [10] in the context of the primary tumor. After a significant number of such rounds the cells may be able to proliferate relatively autonomously at a competitive rate. These tumor cell clones expand and individual cancer cells leave the primary site to seed secondary growths.

The final clonal expansion of fully malignant clones is linked to tumor size. For example, mutations of the tumor suppressor gene TP53 are rare in T1 stage (<2 cm; sizes refer to diameter throughout) breast cancers, and significantly more frequent in T3 stage (>5 cm) tumors [11]

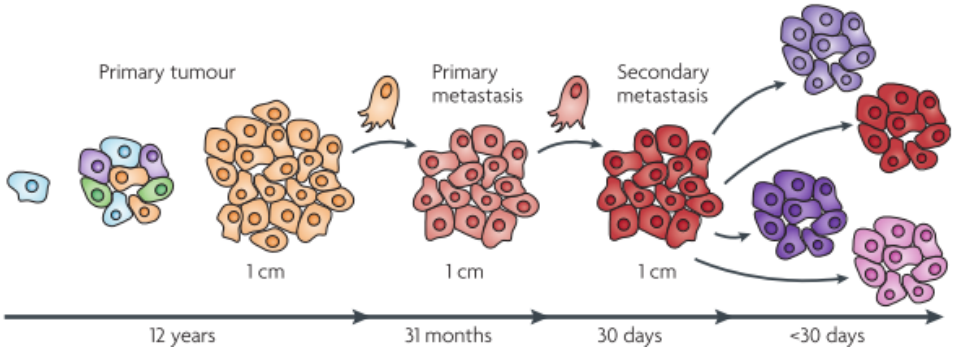


FIGURE 2 TUMOR LINEAR PROGRESSION [12]

Such observations and the well-known association of tumor size with higher frequency of metastasis, which is the basis of the routinely used TNM classification system, have promoted the concept that only tumor cells that are shed late in primary tumor progression have the possibility of eventually spawning macroscopic metastases [13].

However, the possibility that dissemination of tumor cells occurs early (when tumors are small), after acquisition of fully malignant traits, cannot be excluded. Such cells, having departed early, are also thought to share most of their characteristics with the primary tumor.

The parallel progression model dates back at least to the 1950s [14], when considerable effort was made to quantify human cancer growth rates. These studies concluded that metastasis must be initiated long before the first symptoms appeared or the primary tumor was diagnosed [14,15]; given their growth rates, metastases were simply too large to be accounted for by initiation at a late stage of primary tumor development [Figure 3].

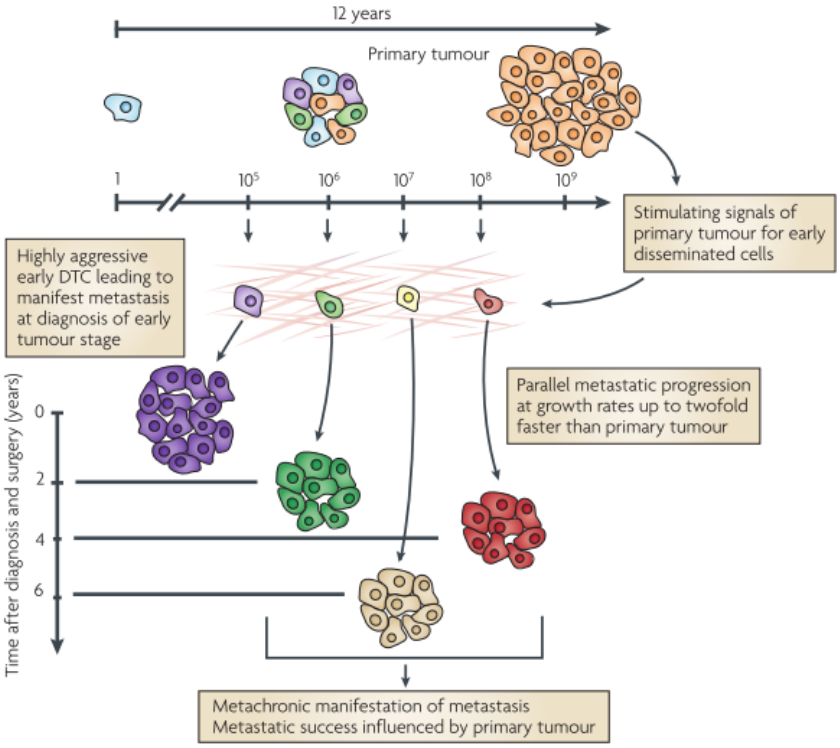


FIGURE 3 TUMOR PARALLEL PROGRESSION [12]

Parallel progression does not necessarily place metastatic founder cell dissemination near the end of primary tumor development. On the contrary, dissemination of tumor cells that are still evolving may lead to allopatric selection and expansion of variant cells adapted to specific microenvironments [14] .

Owing to selection pressure and the inherent genetic instability of tumor cells, parallel progression predicts greater disparity between metastatic founder and primary tumor cells than does linear progression and emphasizes the mechanistic importance of site-specific selection of genetic and epigenetic alterations. Finally, the parallel progression model makes three testable predictions. The first is parallel and independent accumulation of genetic and epigenetic alterations in the primary tumor compared with the metastasis. The second is seeding of tumor cells to different distant sites in parallel, independent, niche-related adaptation during outgrowth towards metastasis. The third is parallel ontogenesis of metastatic cells that disseminated from the primary site at different times. Because, in parallel progression, metastatic founder cells in most cases disseminate long before clinically detectable disease, there is ample time for multiple waves of dissemination or continuous spread.

Both models of tumor progression consider the emergence of multiple metastases. In parallel progression, metastatic founder cells are selected in parallel at various sites and grow independently. Linear progression predicts metastasis from metastasis, in part predicated on the assumption that a metastasis is at least as able to spawn metastases as is the primary tumor, as it has been selected to do so. This view emerged from autopsy studies and selection of highly metastatic cell lines in vivo [16]. The autopsy studies were taken to support linear progression from primary tumors to metastatic cascades. However, growth rates and therapy

trials argue against a linear progression from first to secondary metastasis. For example, the median time taken for 50% of breast cancer patients to develop a single metastasis after diagnosis of M0 disease and surgery is shortest for the liver, taking 29 months. However, the median time taken for M0 stage patients to be diagnosed with multiple metastases, including liver (such as liver and lung or liver and brain), after surgery is 28 months [17].

Parallel progression diminishes the emphasis on metastatic cascades in favor of direct (and early) seeding from the primary lesion. Favored metastatic sites may thus reflect the probability and rate of specific tumor cells growing in a given niche. In counter- point to this, the prognostic value of circulating tumor cells (CTCs) in the blood of patients with metastasis may be considered as support for metastatic cascades.

Epithelial to Mesenchymal Transition

Changes in cell phenotype between epithelial and mesenchymal states, defined as epithelial–mesenchymal (EMT) [Figure 4] and mesenchymal–epithelial (MET) transitions, have key roles in embryonic development an wound healing processes, and their importance in the pathogenesis of cancer and other human diseases is increasingly recognized [18–21].

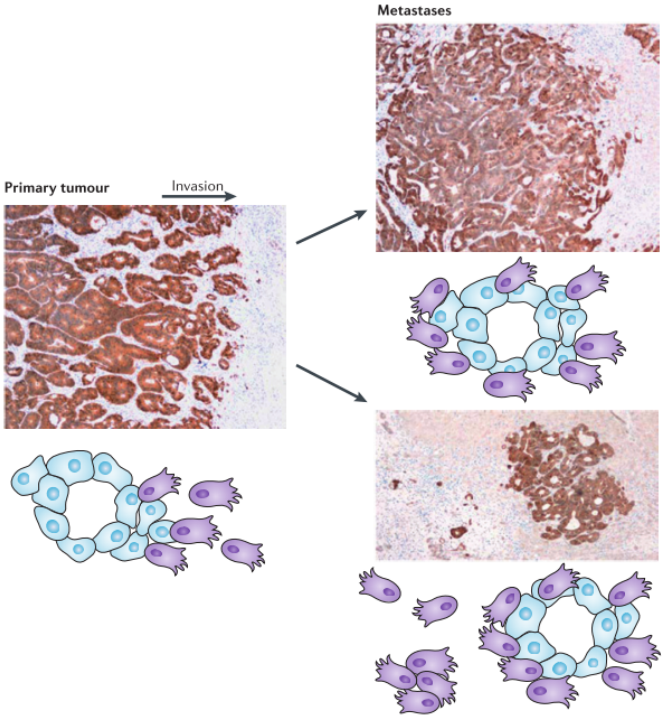


FIGURE 4 EMT HALLMARKS IN DEDIFFERENTIATED TUMOR THE PRIMARY [22]. TUMOUR AND METASTASES SHOW THE SAME HETEROGENEITY, WITH A TUBULAR DIFFERENTIATED PHENOTYPE IN THE CENTER AND AN UNDIFFERENTIATED PHENOTYPE AT THE PERIPHERY. THIS IS ALSO INDICATED SCHEMATICALLY (A DIFFERENTIATED PHENOTYPE IS INDICATED WITH BLUE AND AN UNDIFFERENTIATED PHENOTYPE IS INDICATED WITH PURPLE). A SMALL DIFFERENTIATED METASTASIS WITH ADJACENT SINGLE, UNDIFFERENTIATED TUMOUR CELLS IS SHOWN (BOTTOM PANEL).

However, even though developmental and pathological EMT forms have many common features, they also have obvious differences, leading to the classification of three different EMT types [23] [Figure 5]. Type 1 refers to developmental EMTs; type 2 denotes those related to wound healing, tissue regeneration, and organ fibrosis; and type 3 indicates the EMT associated with cancer. Developmental EMTs imply the conversion of epithelial into mesenchymal cells, but embryos lack inflammatory responses, typical of type 2 and 3 EMTs that occur in the adult [23,24]. Type 2 EMTs are also characterized by the appearance of myofibroblasts with the ability to secrete an excess of extracellular matrix molecules in response to inflammation [23]. In contrast to the situation in embryos, in cancer cells and during regeneration, the acquisition of a mesenchymal state in fibrosis can be considered an end stage, which leads to organ degeneration and failure. During cancer progression, tumor cells undergo type 3 EMT, which, in addition to invasion and motility, involves intravasation of delaminated cells into lymphatic and blood vessels and their subsequent extravasation.

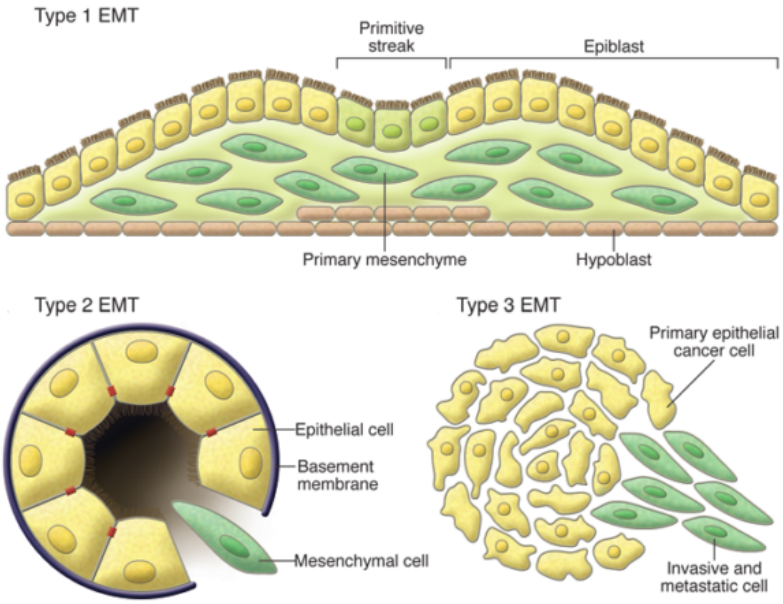


FIGURE 5 DIFFERENT EMT TYPES[23] TYPE 1 EMT IS ASSOCIATED WITH IMPLANTATION AND EMBRYONIC GASTRULATION AND GIVES RISE TO THE MESODERM AND ENDODERM AND TO MOBILE NEURAL CREST CELLS. THE PRIMITIVE EPITHELIUM, SPECIFICALLY THE EPIBLAST, GIVES RISE TO PRIMARY MESENCHYME VIA AN EMT. EMTS ARE RE-ENGAGED IN THE CONTEXT OF INFLAMMATION AND FIBROSIS AND REPRESENT THE TYPE 2 EMTS. UNLIKE THE TYPE 1 EMT, THE TYPE 2 EMT IS EXPRESSED OVER EXTENDED PERIODS OF TIME AND CAN EVENTUALLY DESTROY AN AFFECTED ORGAN IF THE PRIMARY INFLAMMATORY INSULT IS NOT REMOVED OR ATTENUATED. FINALLY, THE SECONDARY EPITHELIA ASSOCIATED WITH MANY ORGANS CAN TRANSFORM INTO CANCER CELLS THAT LATER UNDERGO THE EMTS THAT ENABLE INVASION AND METASTASIS, THEREBY REPRESENTING TYPE 3 EMTS

Another essential difference between the embryonic and tumorigenic processes is that the tumorigenic processes involve genetically abnormal cells that progressively lose their responsiveness to normal growth-regulatory signals and possess the ability to evolve. Such evolution derives from the genetic and epigenetic instability that is inherent in most neoplastic cell types.

The hallmarks of the EMT can be summarized as follows: loss of cell-cell junctions, loss of apico-basal polarity, and acquisition of migratory and invasive properties [25].

Epithelial cells form layers kept together by specialized membrane structures (tight junctions, adherens junctions, desmosomes and gap junctions). The organization of these junctions as a lateral belt, the localized distribution of adhesion molecules such as cadherins and some integrins, the polarized organization of the actin cytoskeleton, and the presence of a basal lamina used as substratum define the characteristic ‘‘top–bottom’’ apico-basal polarity of epithelial cells.

In contrast, mesenchymal cells interact with their neighbours only at focal contacts without forming organized cell layers, and do not have an apico-basal polarized actin cytoskeleton. Instead, mesenchymal cells are elongated and motile in a three-dimensional space defining a front–back polarity with a leading edge enriched in integrins and matrix metalloproteinases (MMP). EMT describes the process in which epithelial cells lose their epithelial characteristics, gain mesenchymal features, and become motile

Recently it has become clear that EMT is driven by at least four fundamental regulatory networks, which are closely interrelated, and modulation of any of these networks affects the others. The most extensively studied network is undoubtedly the network of the nuclear factors (described below). There is growing evidence that three additional layers of regulation support the EMT programme: the expression of small non- coding RNAs [26], differential splicing [27,28], and translational and post-translational control (which affect protein stabilization and localization).

EMT is controlled by at least five major signalling pathways [Figure 6] that are used in early embryonic development (the WNT, TGFb, Hedgehog, Notch and receptor tyrosine kinase pathways) [29,30]. These pathways are often disregulated in cancer [31–35] , and they often cooperate to drive EMT [36,37].

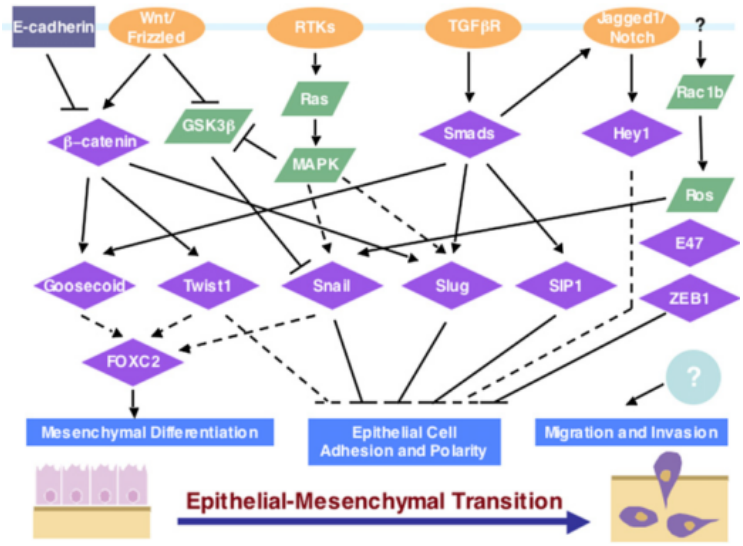


FIGURE 6 THE MOLECULAR NETWORK OF KNOWN SIGNALING PATHWAYS AND TRANSCRIPTION FACTORS THAT REGULATE THE EPITHELIAL-MESENCHYMAL TRANSITION PROGRAM IN CARCINOMA CELLS [21]

There are many examples where induction of EMT in cultured tumor cells results in increased metastatic potential in animal models [29]. The transcriptional programs activated by TGF-β [38,39], PDGF [40] and NFKB, in vitro [36] have also been detected in vivo in animal models and in human tumor samples [40–43].

TGF-β, for example, induces EMT in H-Ras-transformed mammary epithelial cells, a process that has recently shown to be dependent on NF-kB [44]. Importantly, inhibition of NF-kB activity caused a reversal of EMT and also abrogated the metastatic potential of the tumour cells in vivo, providing further evidence for a link between EMT and metastasis. Snail family genes have also been implicated in tumour-associated EMT and metastasis, and constitute tumour progression markers that can be expressed at the invasive front of tumours [45]

Alongside to pathways implicated in EMT, specific markers have been identified, trying to distinguish between epithelial and mesenchymal cells, which often play key functional roles [29]

An example is E-cadherin, a transmembrane protein localized to the adherens junctions and basolateral membrane that plays a central role in organizing the cell–cell adhesion complexes typical of epithelial cells [46]. It is expressed in epithelial cells but not in mesenchymal cells, and is generally down-regulated during EMT.

Due to the pivotal role of E-cadherin loss, EMT-TFs are sometimes referred to as E-cadherin repressors; also, a decrease in functional E-cadherin is often associated with the activation of EMT. However, it is important to bear in mind that the EMT program and EMT-TFs are much more than E-cadherin transcriptional repressors, that E-cadherin down-regulation (or endocytosis) is not necessarily associated with the EMT program, and that E-cadherin re-expression is not sufficient to revert the fibroblastic phenotype [47]

On the other hand, mesenchymal cells express intermediate filaments, such as vimentin, specific cadherins such as N-cadherin and OB-cadherin, cytosketletal proteins, including smooth muscle actin, c-actin, β- filamin and talin, and extracellular matrix components, such as fibronectin and collagen precursors, as well as specific integrins and MMPs

The ability of the transcription factor SNAI1 to directly repress E-cadherin has been considered dogma since its initial observation [48,49], and this conviction has been reinforced over the years. The list of potent EMT-inducing transcription factors (EMT-TFs) has been growing ever since [50], revealing regulatory networks that are often described as signalling pathways converting stimuli to transcriptional reprogramming [20,51].

EMT-TFs have been involved not only in migration and invasion but also in the suppression of senescence and apoptosis, attenuation of cell-cycle progression and resistance to radiotherapy and chemotherapy [25,52,53].

For an epithelial cell to undergo a full EMT, the cell should be permissive: the mesenchymal end-stage can be considered the ultimate goal and will only be achieved when necessary component pathways of the network are activated. There is good evidence to support the idea that dedifferentiation is triggered if the balance between different regulatory networks is fundamentally disturbed; this probably requires the disruption of more than one layer.

A recent study by Rhim and colleagues further supports the concept of permissiveness, using a model of pancreatic cancer in which yellow fluorescent protein (YFP)-labelled pancreatic epithelial cells have a mutant K-RAS combined with loss of Trp53 [54] [Figure 7].

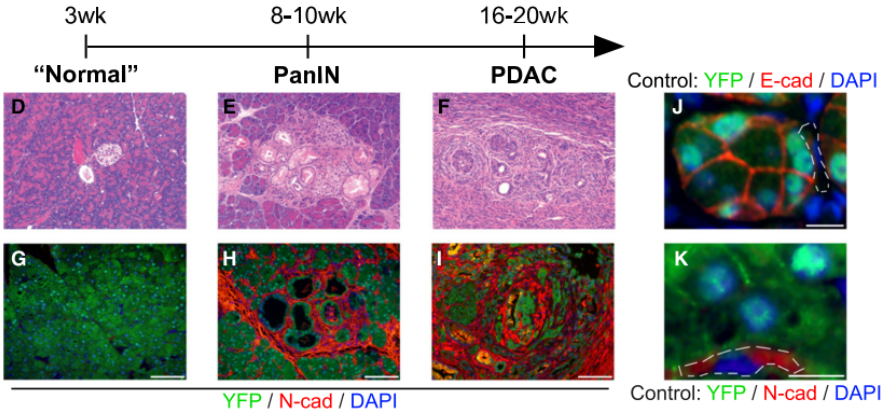


FIGURA 7 LINEAGE-LABELED MOUSE MODELS OF PANCREATIC CANCER AND DETECTION OF EMT [54] TIME COURSE OF MALIGNANT PROGRESSION IN PKCY MICE. (D–F) REPRESENTATIVE IMAGES OF MALIGNANT PROGRESSION. PRIOR TO WEANING, PKCY MICE HAVE HISTOLOGICALLY NORMAL PANCREATA (D) BUT DEVELOP PANIN LESIONS (E) AND EVENTUALLY PDAC (F). (G–I) IMAGES OF PANCREATA FROM (D)–(F) STAINED WITH AN ANTIBODY AGAINST YFP (GREEN) AND N-CADHERIN (N-CAD, RED); PRIOR TO WEANING, SCANT N-CAD STAINING IS SEEN (G). (J AND K) FLUORESCENT IMAGES OF LINEAGE-LABELED CELLS DERIVED FROM THE PANCREATIC EPITHELIUM.

On the one hand, induction of EMT at an early stage is sufficient for the cells (that is, permissive cells) to escape the primary lesions, supporting the possibility of parallel tumour progression. On the other hand, 42% of the YFP-positive cells in these tumours had undergone EMT, supporting the existence and potency of EMT during primary tumour progression. In fact, all of these in vivo studies provided a better understanding of why EMT downstream of an EMT-TF is much easier to detect in vitro: cell lines are genetically aberrant, resulting in different degrees of plasticity and allowing a range from partial to full EMT to occur.

While it is clear that EMT-like processes are not an obligate for invasion and metastasis, there is nevertheless compelling evidence that these processes can play an important role in determining the dissemination of tumours.

This is consistent with the concept of "migratory cancer stem cells" [55] also and provides a link between the EMT program and the characteristics associated with cancer stem cells (CSCs), that is self-renewal, proliferation rate decrease, drug resistance and the capacity to seed secondary tumors and produce differentiated non-stem cells [56]. Recent evidence suggests that cells that undergo EMT acquire stem cell-like properties (Figure 6A). These undifferentiated mesenchymal cells are characterized by a shift from E- to N-cadherin expression, the expression of Snail factors, vimentin, and metalloproteases.

However, the link between EMT and stemness is another controversial issue in tumorigenesis, as it was later shown that fibroblasts must undergo a MET to complete the initial reprogramming of fibroblasts to iPSCs [57,58]. Again, help may come from studies on development biology [59–61], although CSCs differ from canonical embryonic stem cells (ESCs) in that they revert to the phenotypes of only the primary tumor and, therefore, have a more restricted potency than ESCs and iPSCs [62].

Circulating cancer stem cells

The concept that tumor cells within a primary tumor are heterogeneous in terms of their metastatic and renewal potential is not a recent one. Studies done more than 50 years ago demonstrated that only some tumors could be grown from a single tumor cell, and that the daughter cells could be morphologically different from the parent [63]. This work laid the foundation for the modern cancer stem cell hypothesis, which posits that within tumors there exists a subset of cells that are capable of continuous self-renewal and that can give rise to differentiated progeny [62].

The discovery of cancer stem cells (CSCs) has stimulated great excitement, as well as heated debates, for both stem cell and cancer biologists. How the CSC theory fits into the general scheme of cancer progression, particularly with respect to metastasis, has not been completely defined. In order to sustain a lifelong supply of blood cells, hematopoietic stem cells (HSCs) must have the ability to self-renew and generate differentiated blood cells. With the landmark work accomplished by John Dick and his co-workers in human leukemia studies, the existence of CSCs has now been established in many solid tumors including those arising in the brain [64,65], colon [66–70], breast [71–73], skin [74,75], prostate [76], and pancreas [77,78].

Similar to normal stem cells, CSCs can reproduce themselves through the process of self-renewal, which can be studied in serial transplantation assays. Additionally, cancers derived from purified CSCs recapitulate the heterogeneous phenotypes of the parental cancer from which they were derived, reflecting the differentiation capacity of CSCs [79,80] .

The origin of these cells remains unclear; are they derived from normal stem cells with a cancerous phenotype? Or do previously differentiated progenitor cells with oncogenic mutations regain the ability to self-renew? A third theory hypothesizes that CSCs may come from a rare fusion event between stem cells and other cells. Normal stem cells may be ideal target cells for accumulating mutations that are necessary for stepwise malignant transformation due to their inherent self-renewal capacity. Since multiple pathways are involved in self-renewal of stem cells, it seems conceptually more difficult for a differentiated cell to regain this ability through mutations. But the rareness of stem cells in tissues may counter this theory because of the low probability that they could be targeted by mutations. The relative abundance of transient amplifying immediate progenitor cells, derived from stem cells retaining partial self-renewal capacity, makes them likely candidates for initial transforming events.

Kang et al proposed a CSC-based model for both tumorigenesis and metastasis, in which either adult stem cells, their more differentiated progenies or fused cells, could be subject to initial transforming events. Gain of self-renewal capacity at early phases of cancer is essential for further accumulation of oncogenic transformations and eventual development of cancer. The genetic and/or epigenetic changes could determine the cancer malignancy, metastatic potential and the tissue tropism upon the CSC pool establishment. The initial origin of CSCs may influence the phenotypes of developing cancer, including the metastatic property. Molecular crosstalk between the primary tumor and the pre-metastasis niche through secreted stimulatory signals helps govern the homing of metastatic CSCs.

Like in embryogenesis, metastatic stem cells tropism towards preferred tissues and organs is guided by cues such as oxygen gradients or other chemo-attractants derived from niche sites [81–84].

It is increasingly clear that the cell heterogeneity characteristic of many cancer types results from a hierarchical organization that resembles that of the tissue of origin (Figure 8). CSCs comprise the apex of this hierarchy and appear to be the phenotypic and functional equivalents of normal stem cells harboring oncogenic mutations. CSCs in primary tumors self-renew their own population and can generate short-lived progeny.

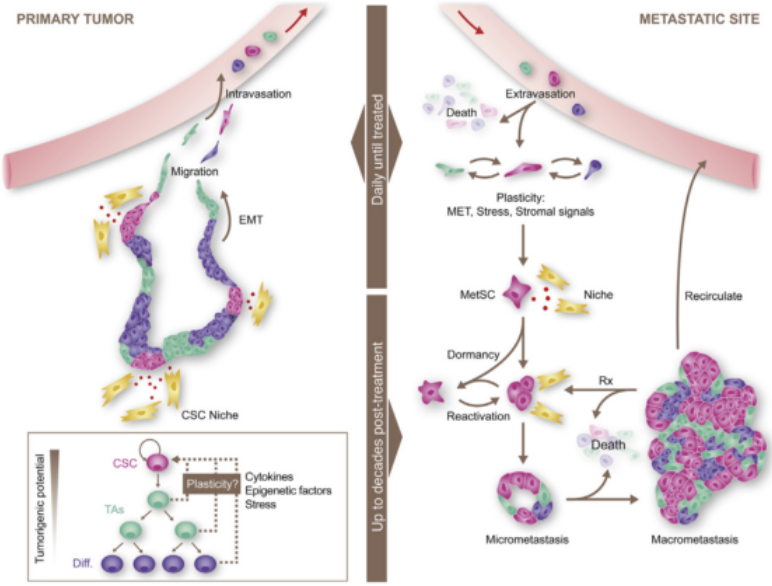


FIGURE 8 SOURCES, DISSEMINATION, DORMANCY, AND OUTGROWTH OF METASTATIC STEM CELLS [85] SEVERAL TYPES OF CANCER DISPLAY A HIERARCHICAL ORGANIZATION WITH CSCS BEING THE ONLY CELL TYPE WITH LONG-TERM SELF-RENEWAL POTENTIAL.. CSCS INTERACT WITH MICRO- ENVIRONMENTAL NICHES THAT SUSTAIN THE TUMOR- PERPETUATING POTENTIAL OF THE CELLS. BOTH CSCS AND NON-CSCS CAN DISPLAY MIGRATORY BEHAVIOR AT THE INVASIVE FRONT OF PRIMARY TUMORS, FREQUENTLY ASSOCIATED WITH AN EMTDISSEMINATED NON-CSCS MAY CONVERT INTO METASTATIC STEM CELLS THROUGH STILL POORLY UNDERSTOOD PROCESSES OF PHENOTYPIC PLASTICITY. METASTATIC STEM CELLS MAY GENERATE PROGENY AND GIVE RISE TO OVERT METASTASIS RIGHT AFTER INFILTRATING THE HOST TISSUE. MORE FREQUENTLY, HOWEVER, DISSEMINATED CANCER CELLS ENTER A DORMANT STATE THAT CAN LAST FOR DECADES AND IS LARGELY RESISTANT TO CURRENT THERAPIES. UPON EXIT FROM QUIESCENCE, METASTATIC STEM CELLS MAY REGENERATE THEIR LINEAGE IN THE HOST ORGAN AND RELEASE METASTATIC PROGENY INTO THE CIRCULATION TO START SECONDARY LESIONS IN THE SAME OR OTHER ORGANS.

Recent lineage tracing experiments in mouse models provide genetic evidence that primary tumors of the brain, colon, and skin comply with this organization [64,70,74,86]. It remains to be determined whether metastases arising from these tumors retain a similar hierarchy. Nevertheless, because the long-term growth capabilities of these and other cancers relies on CSCs, the idea that a so-called metastatic stem cells in these cases may be primary CSCs that resume their regenerative potential at metastatic sites is of interest .

An alternative route to metastatic stem cells status is based on regaining tumor-initiating capacity through phenotypic plasticity. This possibility is suggested by several observations.

In cell line xenograft models, breast cancer stem-like cells can arise from non-stem populations as a result of stochastic transitions between both states [87].

EMT has been shown to result in cancer cells with stem cell-like characteristics that have a propensity to invade surrounding tissue and display resistance to certain therapeutic interventions.

Recent studies have demonstrated that the EMT can induce non-CSCs to enter into a CSC-like state [72,88]. As such, the EMT confers on epithelial cells precisely the set of traits that would empower them to disseminate from primary tumors and seed metastases [20]. Hence, it is an attractive solution to understanding the mechanics of dissemination. Moreover, the

heightened resistance to apoptosis that is integral to cells generated by an EMT is surely critical to the ability of carcinoma cells to survive the rigors of the voyage from primary tumors to sites of dissemination [89]. In addition, the CSC-like state approached by carcinoma cells that have passed through an EMT may be critical in their sites of dissemination for launching new colonies of cancer cells. Merely because the EMT is an attractive solution does not make it unique, however, and it remains possible that other still-undiscovered biological programs operate in carcinoma cells as drivers of malignancy.

Recent data, for example, also implicate a role for TGFβ in regulating breast cancer stem cell phenotypes [72,88] and have demonstrated its essential role in maintaining the pluripotency of human embryonic stem cells [90]; the latter may indicate a role of this pathway in inducing stem cell states during all phases of ontogeny.

As mentioned, Notch signaling also has a role in the regulation of EMTs occurring during both embryogenesis and tumorigenesis [91] . The complexity of Notch signalling derives from the involvement of multiple receptors, ligands and downstream mediators. Moreover, the outcome of Notch activation is cell type-specific and can be either oncogenic or tumor suppressive [91] . Notch can also induce an EMT by activating the nuclear factor-κB (NF-κB) pathway [92] or by modulating the activity of TGFβ signaling. Hedgehog signaling has also been implicated in EMT and cancer metastasis [91].

Thus, it appears that signaling pathways involved in the regulation of stem cell function and niche–stem cell interactions can play some part in triggering EMT programs, potentially connected with the role of these programs in establishing and maintaining stem cell-like characteristics.

This is consistent with the concept of “migratory cancer stem cells” [55] and provides a link between the EMT program and the characteristics associated with CSCs [Figure 9].

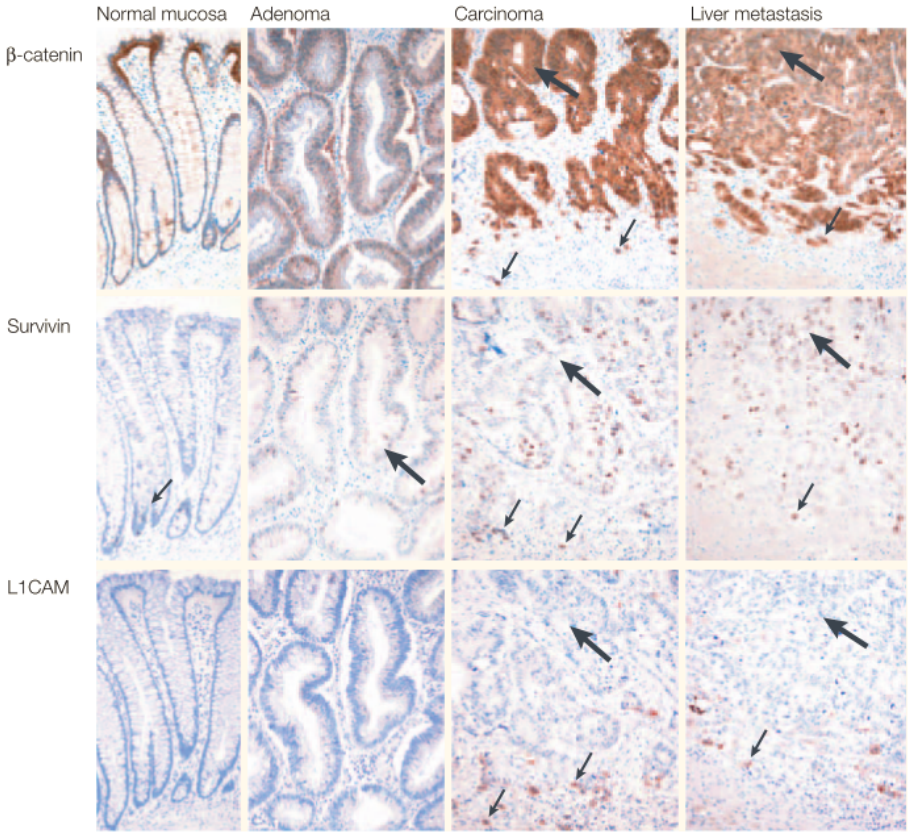


Figura 9 Expression of stem cell- and EMT-markers in colorectal cancer progression [55]. The images show serial histological sections for each progression step with specific protein staining shown in brown. Stem-cell-associated Wnt target genes, such as survivin, are expressed only in the basic region of normal colonic crypts (thin arrow in left panel of images), but are distributed throughout all areas of benign adenomas, carcinomas and metastasis, including differentiated (thick arrows) and dedifferentiated (thin arrows) tumour cells. In contrast, epithelial to mesenchymal transition (EMT)-associated Wnt targets (such as **L1CAM** and **LAMC2**) are not expressed in normal mucosa, adenomas and differentiated tumour areas (thick arrows). Expression is mainly seen in disseminated tumour cells that have highest nuclear β-catenin at the tumour host interface (thin arrows), most of which retain expression of stem cell markers. **L1CAM**, L1 cell adhesion molecule; **LAMC2**, γ2 chain of laminin.

However, the relationship between EMT and stemness is another controversial issue in tumorigenesis, as it was later shown that fibroblasts must undergo a MET to complete the initial reprogramming of fibroblasts to iPSCs [57,58].

Several characteristics of CTCs make them likely candidates to occupy and thrive in these foreign sites. It is theoretically possible that only CSCs within tumors have the ability to initiate and sustain cancer growth. It has been known for years that just one cell can initiate a metastatic lesion [93].

The inherent plasticity of stem cells makes them more adept to survive in a foreign environment (albeit primed by the pre-metastasis niche) where growth factors and other signaling molecules are different than in the primary tumor site. Increased genetic instability in CSCs is also likely to provide a selective advantage in adapting to foreign sites. However, it remains to be seen how CTCs are capable of forming metastasis at different sites.

Cell populations capable of generating metastasis when transplanted into mice can be isolated from primary tumor samples via stem cell marker genes [75,77]. Circulating tumor cells expressing both mesenchymal and stem cell markers have been detected in the blood of breast cancer patients , and when inoculated into immunodeficient mice these cells generated bone, liver, and lung metastases [94,95].

Esophageal cancer

The worldwide overall incidence of esophageal carcinoma (EC) is on the rise. Esophageal cancer is a global problem for which no formalized screening programs currently exist.

This cancer is the eighth most common cancer worldwide, with an estimated 456,000 new cases in 2012 (3.2% of the total), and the sixth most common cause of death from cancer with an estimated 400,000 deaths (4.9% of the total) [source http://globocan.iarc.fr/].

Esophageal cancer is extremely aggressive nature and poor survival rate [96,97], it exhibits epidemiologic pattern distinct from all other cancers [98,99]. Worldwide simple maps of Age-standardized incidence rates per 100,000 of 2008 VS 2012 is showed in Figure 10; Worldwide simple maps of esophageal cancer mortality of 2008 VS 2012 is showed in Figure 11.

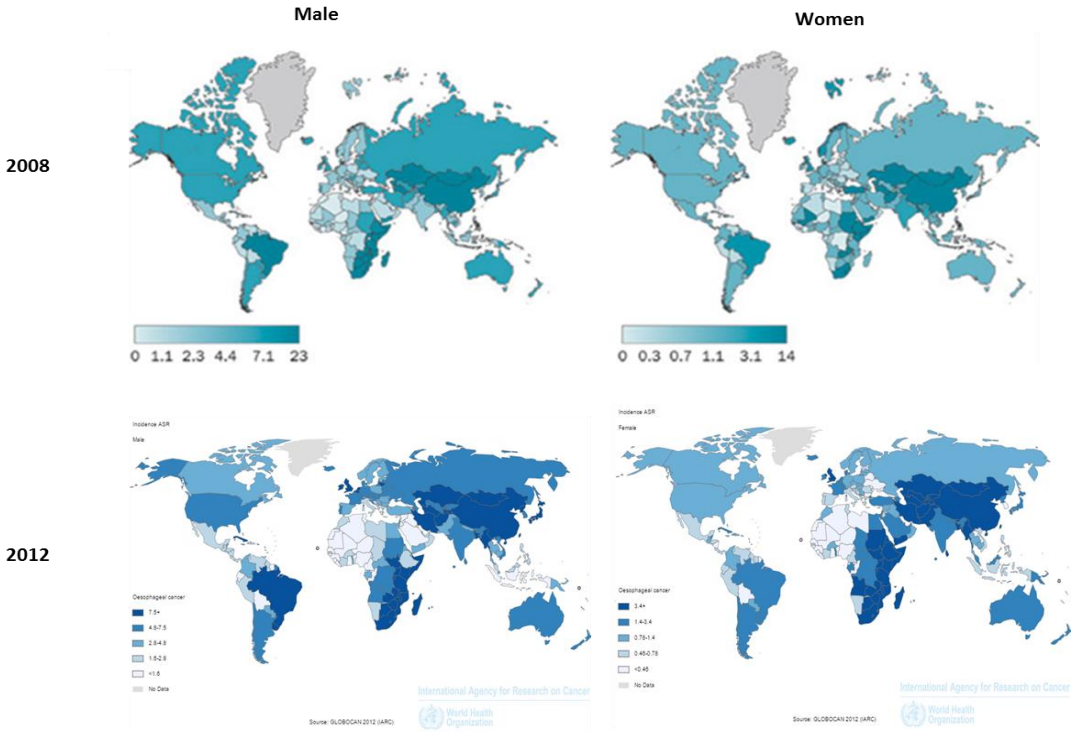


FIGURE 10 ESOPHAGEAL CANCER- AGE-STANDARDIZED INCIDENCE RATES PER 100,000 WORLDWIDE—2008 [100] VS 2012 ESTIMATES [FROM GLOBOCAN . AVAILABLE FROM: HTTP://GLOBOCAN.IARC.FR ACCESSED GEN. 2016]

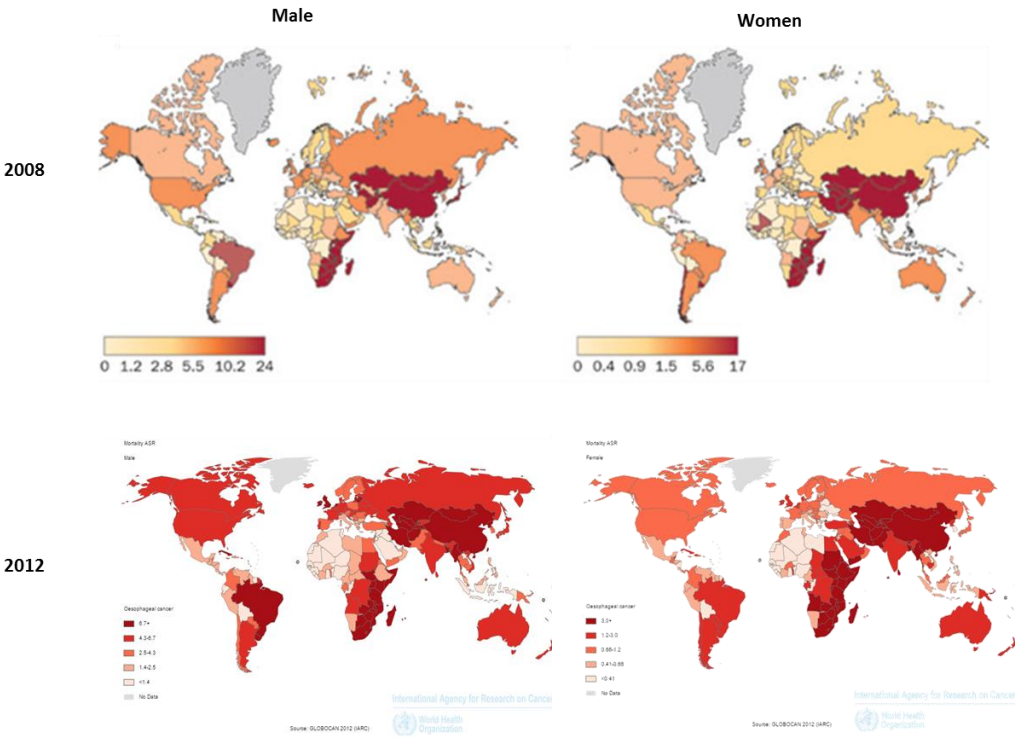


FIGURE 11 ESOPHAGEAL CANCER MORTALITY WORLDWIDE—2008 [100] VS 2012 ESTIMATES [FROM GLOBOCAN . AVAILABLE FROM: HTTP://GLOBOCAN.IARC.FR ACCESSED GEN. 2016]

The pathogenesis of esophageal cancer remains unclear. Data from studies in animals suggest that oxidative damage from factors such as smoking or gastro-esophageal reflux, which cause inflammation, esophagitis, and increased cell turnover, may initiate the carcinogenic process [101].

Pathology and anatomy

Cancer of the esophagus typically occur in one of two forms, squamous cell carcinomas (ESCC), arising from the stratified squamous epithelial lining of the organ, and adenocarcinomas (EAC), affecting columnar glandular cells that replace the squamous epithelium [102]. Despite the epidemiological differences, both types of esophageal cancer have a dismal prognosis.

The natural histories of squamous cell carcinomas and adenocarcinomas of esophagus appear to differ substantially. For squamous cell cancers, transition models have described squamous epithelium undergoing inflammatory changes that progress to dysplasia and in situ malignant change [103].

Most adenocarcinomas, however, tend to arise in the distal esophagus from columnar-lined metaplastic epithelium, commonly known as Barrett's esophagus [104], which replaces the squamous epithelium during the healing reflux esophagitis and may progress to dysplasia.

For both esophageal cancer subtypes there is a well-described pre-invasive stage illustrated in Figure 12.

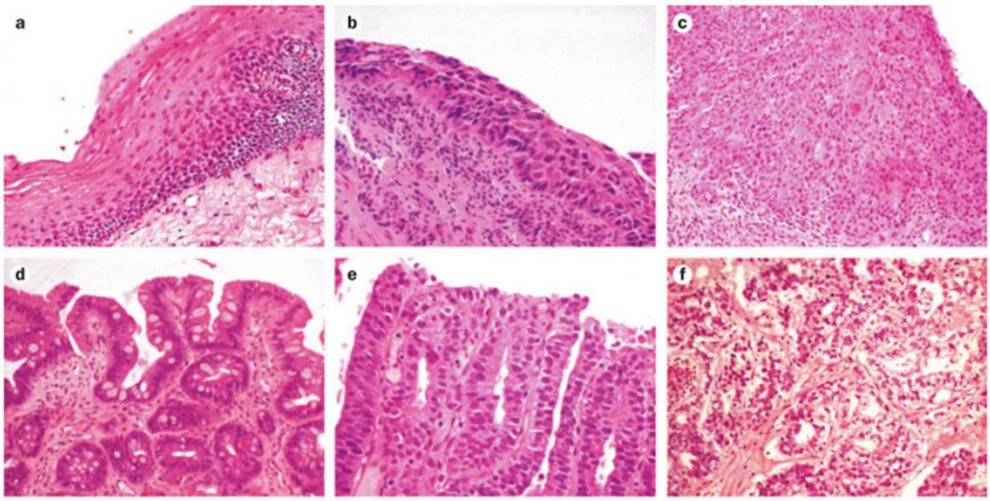


FIGURE 12 | PATHOLOGICAL SEQUENCE OF OESOPHAGEAL CANCER PROGRESSION [100]. PROGRESSION OF "A" NORMAL SQUAMOUS EPITHELIA TO "B" SQUAMOUS HIGH-GRADE DYSPLASIA AND "C" SQUAMOUS-CELL CARCINOMA. PROGRESSION OF "D" BARRETT'S OESOPHAGUS TO "E" GLANDULAR HIGH-GRADE DYSPLASIA AND "F"ADENOCARCINOMA. THE SECTIONS WERE STAINED WITH HAEMATOXYLIN AND EOSIN AND ARE DISPLAYED AT ×100 MAGNIFICATION

Cancers that start at the area where the esophagus joins the stomach (the GE junction), which includes the cardia, tend to behave like esophagus cancers (and are treated like them, as well), so they are grouped with esophagus cancers. Approximately three quarters of all adenocarcinomas are found in the distal esophagus, whereas squamous-cell carcinomas are more evenly distributed between the middle and lower third.

Prognosis

Esophageal cancer is the sixth most common cause of cancer deaths and its five- year survival is about 16% and 10% in USA and in Europe, respectively [105].

After esophageal resection the five-year survival rates were 20.6% in a Western meta-analysis for unselected patients [106] and 50% for EAC patients operated with two-field lymphadenectomy [107]. At the time of diagnosis, more than half of the patients were suffering from inoperable disease [108]. In a large analysis of 1059 operated EC patients from a single German centre, Siewert et al. found better long-term prognosis for EAC than for ESCC. During the years 1982 to 2000, the overall resection rate was 68.2% and 75.5% for patient with EAC and 64% for patients with ESCC.

More than two thirds of all patients with EC develop local recurrence or distant metastases and die despite complete resection of the primary tumor and multimodal treatments. Recurrence or metastasis results from clinically occult minimal residual disease caused by circulating tumor cells (CTCs) or disseminated tumor cells [109,110].

Staging system

Esophageal cancer staging is defined by the American Joint Committee on Cancer (AJCC) Staging System that establishes tumor-node-metastasis (TNM).

Sub-classifications based on the depth of invasion of the primary tumor (T), lymph node involvement (N), and extent of metastatic disease (M). The most recent, 7th edition of the AJCC Cancer Staging Manual for esophagus and esophagogastric junction cancers was

developed based on a database of 4,627 esophagectomy patients who were not treated with induction or adjuvant therapy [111] see Figure 13. This data from 13 institutions in five countries and three continents was collected by the Worldwide Esophageal Cancer Collaboration (WECC) [112].

Stage	Adenocarcinoma				Squamous cell carcinoma				
	T	N	M	Grade	T	N	M	G	Location
0	is	0	0	1	is	0	0	1	Any
IA	1	0	0	1-2	1	0	0	1	Any
IB	1	0	0	3	1	0	0	2-3	Any
	2	0	0	1-2	2-3	0	0	1	Lower
IIA	2	0	0	3	2-3	0	0	1	Upper, middle
					2-3	0	0	2-3	Lower
IIB	3	0	0	Any	2-3	0	0	2-3	Upper, middle
	1-2	1	0	Any	1-2	1	0	Any	Any
IIIA	1-2	2	0	Any	1-2	2	0	Any	Any
	3	1	0	Any	3	1	0	Any	Any
	4a	0	0	Any	4a	0	0	Any	Any
IIIB	3	2	0	Any	3	2	0	Any	Any
IIIC	4a	1-2	0	Any	4a	1-2	0	Any	Any
	4b	Any	0	Any	4b	Any	0	Any	Any
	Any	3	0	Any	Any	3	0	Any	Any
IV	Any	Any	1	Any	Any	Any	1	Any	Any

Cancer location definitions: upper thoracic, 20-25 cm from incisors; middle thoracic, 25-30 cm from incisors; lower thoracic, 30-40 cm from incisors.

FIGURE 13 AJCC7TH EDITION- ESOPHAGEAL CANCER STAGING GROUPING [MODIFIED FROM [113]]

The Society of Thoracic Surgeons has published guidelines on the diagnosis and staging of patients with esophageal cancer [114]. The work-up for esophageal cancer often starts when patients present with symptoms such as dysphagia and weight loss in the setting of an unremarkable physical exam [115]. Therefore, the most common tests used to initially identify and diagnosis esophageal cancer are upper gastrointestinal (GI) tract contrast studies and upper endoscopy with biopsy. After a histologic cancer diagnosis has been obtained, subsequent studies are performed to determine clinical stage as accurately as possible before treatment is initiated. Obtaining a computed tomographic (CT) scan of the chest and abdomen with both oral and intravenous contrast should be the first staging study when esophageal cancer is diagnosed histologically. The CT scan is somewhat limited in defining the local extent and nodal involvement of esophageal cancer but is most useful in identifying the presence of distant disease such as liver or lung metastases.

Positron-emission tomography (PET) scans improve staging by detecting previously unsuspected metastatic disease in up to 15-20% of patients and should be considered in place of CT scans or as an additional study when the CT scan does not show metastatic disease [116,117]. Performance of the above staging modalities establishes the pre-treatment clinical stage which can be used to guide subsequent treatment. Distant metastases are unfortunately missed even with completion of the staging evaluation described above. Small liver or lung metastases can be missed by both PET and CT scans, and patients can also have undetected pleural or peritoneal disease [118]. Staging via minimally invasive surgical techniques of thoracoscopy and laparoscopy improves the accuracy of the above non-invasive testing [118–120]. Use of these invasive techniques is relatively uncommon but should be considered in select patients, such as those who may be considered to have a high risk of treatment-related complications.

An appropriate staging system is essential for determining treatment strategies, especially those involving neo-adjuvant treatments, in patients with EC. Despite the availability of

several preoperative diagnostic techniques, accurate pretreatment staging remains inconsistent. Therefore, a novel tool for early tumor detection, adequate prognostic staging, and accurate therapy monitoring in EC is urgently needed.

CTC & esophageal cancer

Despite the aggressiveness of esophageal cancer, a little number of research was conducted to understand the role of CTCs on this disease.

Pioneering studies from 2004 using reverse transcriptase-polymerase chain reaction provided a CTC detection rates ranging from 2% to 32.9% in esophageal cancer patients [121,122]

Lack of methodological uniformity, nucleic acid-extraction protocols and molecular marker selection as well as the inconsistent definition of CTC positivity in polymerase chain reaction-based methods may account for this large variation. Despite methodological shortcomings, early study in patients before and after surgery, brought attention about CTCs presence as an independent predictor of disease recurrence in esophageal squamous cancer [121].

With the introduction of CellSearch system, designed to detect CTC in peripheral blood samples by using immunomagnetic enrichment, there was an outbreak of studies enumerating circulating tumor cells in different cancers starting from those metastatic.

It took about ten years from the establishment of CTCs (EpCAM+/CKs+/CD45-) as an independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast cancer (MBC) [1] to investigate these cells in esophageal cancer.

In 2013 a small pilot study in a cohort of 18 patients with advance esophagogastric cancer illustrated a 44% proportion of patients with more than 2 CTC/7.5ml of blood before first line chemotherapy [123]. Only 11 patients of the 18 displayed exhibited cancers ascribable to esophagus: 9 esophagogastric junction, 2 esophagus. Only 4 patients had more than 2 CTCs of the 9 with esophagogastric cancers whereas none of the patients with esophageal cancer showed more than 2 CTCs. Unfortunately this study didn't explain anything about the relationship between CTCs and esophageal cancer. Moreover the established cutoff of 2 CTCs per 7.5ml of blood is supported only by a large study including a wide variety of cancers at different stages [124] bringing the cutoff-choice perhaps too rough.

A larger and well-defined study to assess CTCs as a staging tool for non-metastatic esophageal cancer into defined prognostic subgroups was carried out by the group of Reeh [125].

Using a strict cutoff of 1 CTC or more they found that CTC-positive patients with non-metastatic disease had a significantly shorter overall and relapse-free survival than patients without CTCs (Figure 14-15).

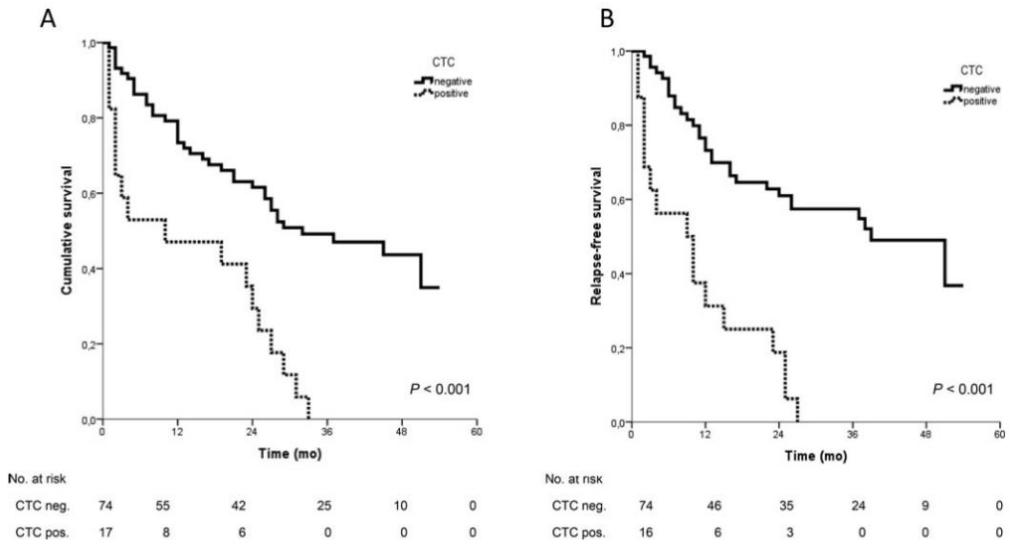


FIGURE 14 OUTCOMES OF PATIENTS WITH EC WITH CTCS COMPARED WITH PATIENTS WITHOUT CTCS. CORRELATION OF OVERALL (A) AND RELAPSE-FREE SURVIVAL (B) WITH CTCS IN PATIENTS WITH EC (N = 91)

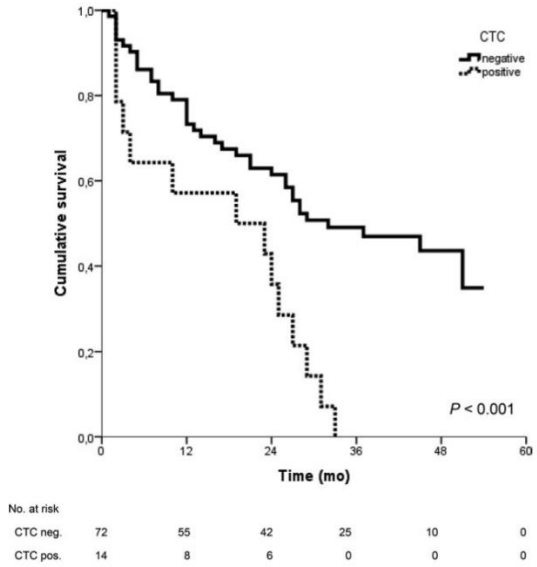


FIGURE 15 OUTCOMES OF PATIENTS WITH EC WITH CTCS COMPARED WITH PATIENTS WITHOUT CTCS. CORRELATION OF OVERALL SURVIVAL WITH CTCS IN PATIENTS WITHOUT DISTANT METASTASES (M0) (N = 86).

In particular, 3 of the 29 patients (10.3%) with SSC showed 1 CTCs or more, 14 of the 68 patients (20.6%) with adenocarcinoma showed 1 CTCs or more this could be due to different characteristics between esophageal cancer subtypes (SSC vs EAC) [125].

Consistent with this hypothesis, the elegant study of Stoecklein et al reported that EpCAM expression in early esophageal cancer may vary [126]. Analysis of EpCAM status on disseminated tumor cells (DTC) derived from lymph nodes and bone marrow showed that CK18 positive DTCs often lack of EpCAM expression [Figure 16].

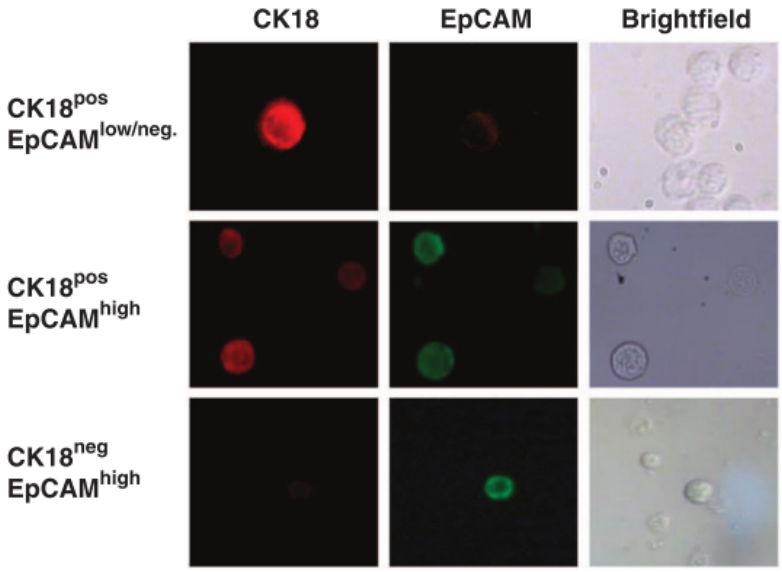


Figure 16 Variable expression of EpCAM in disseminated tumor cells [126]. Bone marrow aspirates were stained with antibodies specific for EpCAM and CK18 with fluorescence-labeled secondary antibodies. Representative fluorescence microscopy pictures of $EpCAM^{HIGH}/CK18^{NEGATIVE}$, $EpCAM^{HIGH}/CD18^{POSITIVE}$ and $EpCAM^{NEGATIVE}/CK18^{POSITIVE}$ samples are displayed.

CK18 DTCs were detected in 38.9% of esophageal cancer patients but co-expression of EpCAM was seen in 37.1% of cases, 62.9% revealed a DTC CK18+/$EpCAM^{low/negative}$; comparison of EpCAM expression on 14 antilogous pairs of primary tumors and their associated DTCs revealed an absence of EpCAM on DTCs in 64% of patients with EpCAM ^{high} expression primary tumors.

This discrepancy was not due to an intrinsic characteristic of primary tumor but EpCAM knockdown and EMT induction experiments using esophageal squamous cancer cell lines indicate that the reduction of EpCAM expression is associated with cell migration.

Both EpCAM positive and EpCAM negative/low cancer cells sustain metastatic progression during esophageal cancer in a context-dependent manner.

Taking together these findings lead to the hypothesis that CTC studies supported by CellSearch may be most likely partial due to the EpCAM down-regulation observed in DTCs. EpCAM must not to be used as the only CTCs identification marker as it could underestimate the amount of circulating and/or disseminated tumor cells.

Focus on markers

Epithelial CTCs (epithelial-tag)

At single cell level, besides lack of expression of CD45 (leucocytes marker), we can define a CTC on at least two criteria of expression of specific proteins, e.g., epithelial cellular adhesion molecule EpCAM and Cytokeratin 8,18,19 [127]. These identification criteria provide the FDA approved definition of CTC [128]. Such a definite statement during the last five-years encountered many revisions, most notably because of major advances in the field of EMT [129,130]. Indeed, in the last three years some pioneering laboratories identified non-canonical CTCs able to grow in vitro [94,131] bringing solid confirmations of CTC heterogeneity.

The three most well-known families of antigen characteristics that may be present alone or in different combination on CTCs are the epithelial, the mesenchymal and the stemness markers. Six markers (plus CD45 as a negative marker) were choosen to identify circulating esophageal cancer cells and to comprehend a "wide" range of CTC characteristics.

EpCAM (epithelial tag)

EpCAM is a cell surface glycoprotein highly expressed in epithelial cancer cells and at lower level in normal epithelial cells [132]. EpCAM was the starting point of detection and enumeration of CTCs and is particularly exploited by CellSearch system (Veridex, Warren, New Jersey, USA) [133]. Yet, expression of this protein is dependent on the stage of EMT.During EMT, it can be down-regulated in parallel to the increase of expression of N-cadherin and vimentin, and could finally disappears, as E-cadherin does. Numerous antibodies against EpCAM were used to target epithelial cancer cells. It is well known from the paper of Rao et al. that EpCAM expression is down-regulated in many CTCs [134] [Figure 17]. If patients with undetectable CTCs by EpCAM-based technology have a more favorable prognosis compared to those with detectable CTCs, there may be other CTC-negative patients with a bad prognosis. The latter could have undetectable CTCs due to EMT which down regulates epithelial marker expression. This is particularly true for EpCAM and cytokeratins (Cks).

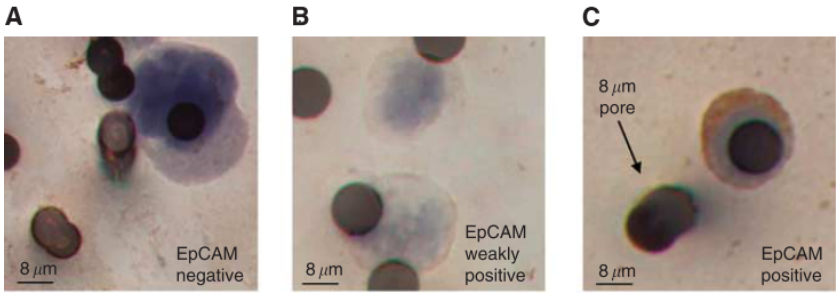


Figure 17 CTCs Positive for EpCAM [135]. Different levels of expression of EpCAM from negative A, weekly B and positive C

E-cadherin (epithelial tag)

E-cadherin is a component of adherens junctions and one of the hallmarks of epithelial cells. This molecule functions in close cooperation with the actin cytoskeleton and enables resistance to forces causing cell detachment. It is implicated to support epithelial tissue architecture. Expression regulation of E-cadherin has a major role in the EMT progression.

When a cell is going towards the mesenchymal state, expression of this protein is decreased [136]. Once cancer cells escape from primary tumor, E-cadherin can be inactivated or down-regulated by different mechanisms. Its down-regulation can be done through promoter hypermethylation, histone deacetylation, and more importantly, transcriptional repression [137]. Major E-cadherin repressors are Snail and Slug. Snail appears to be the most important E-cadherin repressor related to tumor progression. TGFβ represses both Id proteins (Inhibitor of DNA-binding proteins) and activates Snail family members, explain the links between TGFβ signaling, E-cadherin repression and EMT initiation [138]. Examples of CTCs positive for E-cadherin in Figure 19

Cytokeratins (Cks)

Cks are the largest and most diverse class of intermediate filaments which constitutes cytoskeleton components. At least twenty different Cks can be expressed. They are markers of normal epithelial differentiation, but they can be used as diagnostic tool to detect different circulating cells of carcinoma [Figure 18]. To this aim, antibodies must be able to recognize most of the more conserved epitopes. Epithelial cancer cells often express Cks 8, 18 and 19, and are used by Cell Search™ in addition to EpCAM to count epithelial CTCs. They have also been used to identify disseminated tumor cells (DTCs). However, when EMT is ongoing their expression is often down-regulated [29]. Cells leaving the primary tumor intravasate into the blood and often lose their Ck expression, escaping "standard" CK-based detection. Joosse et al. demonstrated that the use of an anti-Ck antibody cocktail is able to track cells loosing partial epithelial phenotype. These results suggest that CTCs showing mesenchymal state could have, among numerous Cks, a residual expression of some of them [139].

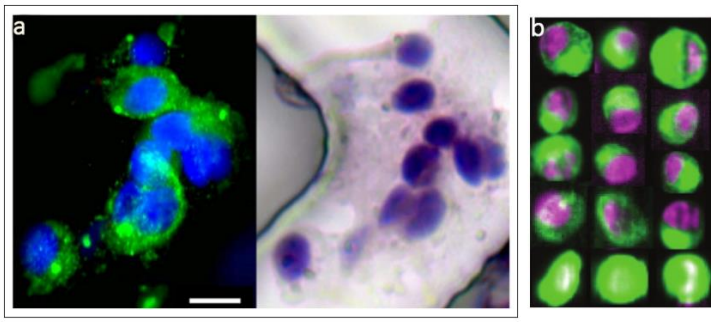


Figure 18 Examples of CTCs cytokeratins positive. A CTC counterstained with DAPI (blue) panCK (green) by iChip technology [140]; B examples of CTCs identified with CellSearch DAPI (purple) panCK (green)[141]

Mesenchymal/Stem CTCs (mesenchymal/stem-tag)

Differentiated epithelial carcinoma cells can be transformed into a mesenchymal state and during this process, different degrees of EMT occur. Then it can be difficult to define a pure mesenchymal CTC subpopulation. However the mesenchymal character of CTCs can be assessed by the expression of protein/s reflecting EMT process.

In addition, during EMT, cancer cells are endowed with stemness characteristics acquiring migratory properties. This is one of the hypothetical origin of circulating-cancer stem cells (CSC) [130].

Thus the diversity of CTCs having EMT features, which can be relevant in either CSCs or differentiated cancer cells, underlines the difficulty to choose significant identification markers.

N-CADHERIN

Adherens junctions have a major role in the shape organization of epithelial tissue. Phenotypic changes leading to epithelial sheet movements are based on their modifications. Among proteins of adherens junctions, classic cadherins are the E-cadherin and N-cadherin subfamilies. N-cadherin is expressed in many cells and particularly in mesenchymal cells. Therefore it is normally used as a marker of EMT. During this process E-cadherin is switched off while N-cadherin is switched on. Abnormal expression of N-cadherin associated to a dramatic decrease of E-cadherin is a hallmark of mesenchymal character of CTCs resulting from E- to N-cadherin switch [142] [Figure 19]. Loss of E-cadherin in tumor cells is due to methylation of its promoter or to overexpression of transcriptional repressors which target its promoter [143,144].

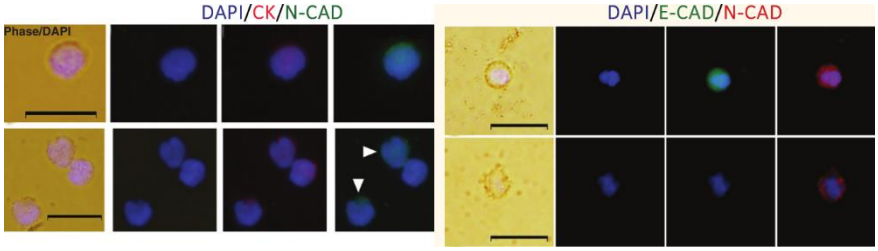


FIGURE 19 EXAMPLES OF CTCS DIFFERENTLY POSITIVE FOR N-CADHERIN, E-CADHERIN OR CKS.[145]

CD44v6

CD44 is a widely expressed polymorphic integral membrane adhesion molecule that binds hyaluronic acid and contributes to cell-cell and cell-matrix adhesion, cell growth and trafficking, EMT, and tumor progression [146]. CD44 transcripts undergo complex alternative splicing, resulting in functionally different isoforms expressed primarily on epithelial cells [147]. Among CD44 isoforms (CD44v*n*), CD44v6 seems to play a major role in cancer progression for its ability to bind hepatocyte growth factor (HGF), osteopontin (OPN), and other major cytokines produced by tumor micro- environment [146]. The major role of CD44v6 involves cell migration and invasion. CD44v6 interacts with hepatocyte growth factor receptor (MET) in the presence of HGF and potentiates its signaling [148]. Studies indicated that increased CD44v6 expression would induce a high metastatic potential in some tumors, such as non-small cell lung cancer[149], gastric cancer [150], and pharyngolaryngeal cancer [151]. In a recent meta-analysis where single studies concerning esophageal cancer and presence of CD44v6 were pooled together, the results suggested that the expression of CD44v6 was higher in esophageal cancer tissue than in normal tissue [152]. Results also found that the high expression of CD44v6 was associated with poor survival in esophageal cancer patients with lymphoid nodal metastasis, which suggests that the abnormal expression of CD44v6 in tumor cells may enhance their potential form metastasis in the regional lymph nodes [Figure 20]. However, no association was observed between the expression of CD44v6 and the tumor stage and histological types, indicating that CD44v6 may not be involved in the biological function of tumor cells but may promote the metastasis of tumor cells.

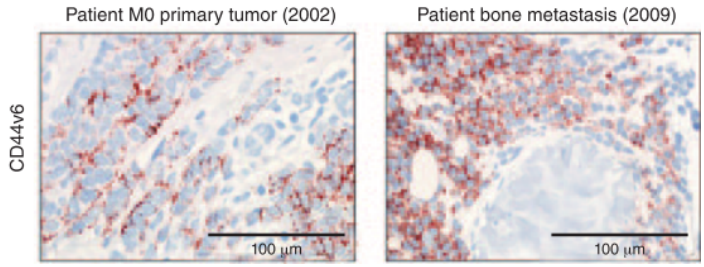


FIGURA 20 CD44V6 EXPRESSION DURING TUMOR PROGRESSION [94]. IMMUNIHISTOCHEMISTRY OF TWO SECTIONS OF PRIMARY TUMOR IN PATIENTS M0 AND AFTER PROGRESSION TO BONE METASTASES, BOTH EXPRESSED CD44V6 BUT AT DIFFERENT LEVELS

ABCG2

ATP binding cassette (ABC) transporters form one of the largest trans-membrane protein families. These proteins use cellular ATP to drive the transport of various substrates across cell membranes including drugs, metabolites and other compounds. Human ABCG2 is the second member of the G-subfamily of ABC transporters. ABCG2 was first cloned from doxorubicin-resistant human MCF-7 breast cancer cells and named as breast cancer resistance protein (BCRP) [153] ABCG2 is widely distributed in normal tissues and is highly expressed in a subpopulation of stem cells. Its conserved expression in stem cell populations suggests an important role in stem cell biology. In addition, ABCG2 is one of the most important multidrug-resistance transporters and its substrates include many commonly used drugs in cancer chemotherapy [154]. There is increasing evidence that ABCG2 correlates with unfavorable prognosis in a variety of tumors [155]. In a study conducted in a large cohort of esophageal cancer patients (100), ABCG2 expression was found in 61% of tumor tissues [Figure 21]. The expression of ABCG2 was correlated with poor survival being an independent prognostic factor [156].

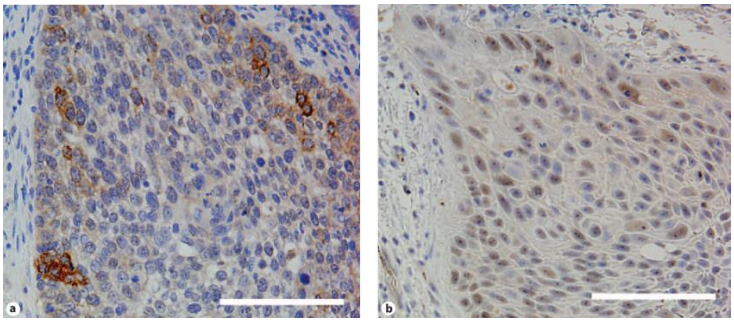


FIGURE 21 IMMUNOHISTOCHEMICAL OF ABCG2 IN TUMOR TISSUES OF ESCC. A SLIDE REPRESENTATIVE OF POSITIVE ABCG2 EXPRESSION, IN POORLY DIFFERENTIATED ESCC. B SLIDE REPRESENTATIVE OF NEGATIVE ABCG2 EXPRESSION, SHOWN IN MODERATELY DIFFERENTIATED ESCC.

Aims and Study design

Primary aims

- To assess the feasibility of circulating esophageal cancer cells enrichment and their characterization
- To evaluate the prognostic value of Circulating Esophageal Cancer Cells in esophageal cancer

Secondary aim

- to characterize the identified cells, by molecular assays through cancer-related genetic alterations that will be identified by next generation sequencing (NGS) approaches.

Design

Blood samples (15-20 ml) from untreated/naive patients affected by metastatic and non-metastatic esophageal cancer will be assessed, including cervical esophagus, upper-middle-lower esophagus, esophagogastric junction.

Samples will be collected before primary therapy of choice (radio-therapy, radio-chemiotherapy, radio-chemiotherapy), one month after therapy and, when possible, after surgery. For experimental Workflow see Figure 1.

Expected Outcomes

- To confirm the feasibility of the detection of CTCs in a pathology where they are still poorly studied
- To characterize CTCs in this disease
- To investigate the correlation of CTCs and survival, progression-free survival and anatomical district
- To investigate a potential biological explanation for esophageal cancer aggressiveness

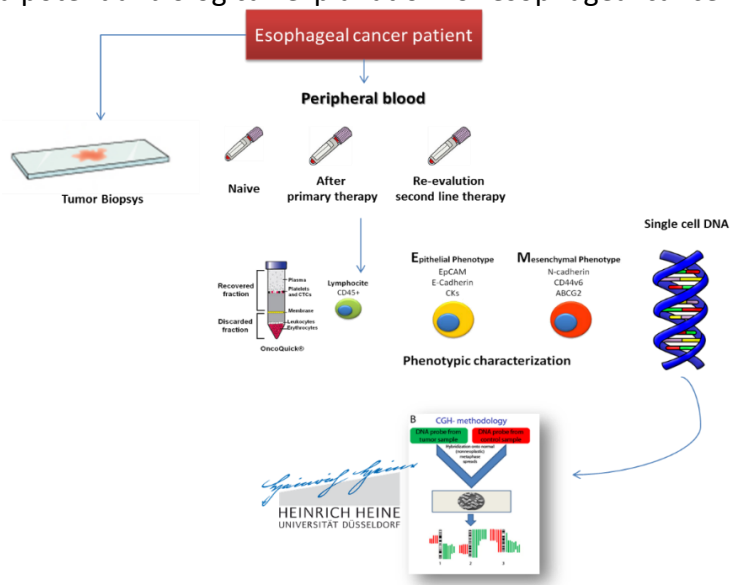


FIGURE 1 EXPERIMENTAL WORKFLOW

Materials and Methods

Cell culture

Breast cancer cell lines MDA-MB 436, MDA-MB 231, MCF7 were originally purchased from American Type Tissue Culture. The non-tumorigenic breast epithelial cell line MCF10A, immortalized human mammary epithelial cells hTERT-HMECs (named HME), and the modified HME-TWIST1, HME-ZEB1, HME-ZEB2 over-expressing the EMT master transcription factors TWIST, ZEB1, ZEB2 respectively, were gifted by Professor Alain Puisieux laboratory.

MDA-MB 436, MDA-MB 231 cell lines were cultured in Leibovitz's L-15 medium (GIBCO) with 10 mcg/ml insulin(Sigma Aldrich), 100U/ml penicillin-streptomycin (GIBO), 2mM glutamine (GIBCO) complemented with 10% FBS (GIBCO), in a non free-gas exchange condition.

MCF7 cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (GIBCO) 100U/ml penicillin-streptomycin (GIBCO), 2nM glutamine (GIBCO) complemented with 10% FBS (GIBCO). HMEC-derivatives were cultured in 1:1 DMEM/HAMF12 medium (GIBCO) complemented with 10% FBS (GIBCO), 100 U/ml penicillin-streptomycin (GIBCO), 2 mM L glutamine (GIBCO), 10 ng/ml human epidermal growth factor (EGF) (Millipore), 0.5 mg/ml hydrocortisone (Sigma Aldrich) and 10 mg/ml insulin (Sigma Aldrich) as previously reported [88]

MCF10A cell line were cultured in DMEM/F-12, GlutaMAX™ medium (GIBCO) complemented with 5% Horse Serum (New Zealand origin) (GIBCO), 100 U/ml penicillin-streptomycin (GIBCO), 2 mM L glutamine (Invitrogen), 100 mg/ml human epidermal growth factor (EGF) (Millipore), 1 mg/ml hydrocortisone (Sigma), 1mg/ml cholera toxin (Sigma Aldrich) and 10 mg/ml insulin (Sigma Aldrich), 100U/ml penicillin-streptomycin (GIBCO) as mentioned [157] .

All cell lines were maintained as a monolayer in a 37°C incubator with 5% CO2 and sub-cultured at 70-80% confluence (approximately twice weekly).

EMT induction

MCF10 cell line were cultured on 19,5cm^2 petri dish (BD Falcon), using previously described culture medium. Epithelial to mesenchymal transition were induced by treating cells with 10ng/ml TGF beta1 (Peprotech) every 48 hours or during sub-culturing.

Peripheral Blood Monuclear cells (PBMC) isolation

PBMC were isolated from healthy donor peripheral blood (PB). About 3ml of PB were drawn in EDTA tubes (BD Vacutainer, Becton Dickinson).A density gradient separation by Lymphocyte Separation Media (BioWest) was subsequently performed (400g centrifugation for 30 minutes). From the resulting blood stratification, the mononuclear cells (PBMC) interphase was carefully collected, washed and re-suspended in PBS1X. If samples had an erythrocyte contamination, a red blood lysis was performed by ACK lysis buffer (Lonza).

Immunofluorescence

Since original HME and their modified descendant cells are putative models of different levels of EMT, they were tested with a immunofluorescence assay based on a two antibodies combination to verify their levels of Epithelial-to-mesenchymal transition.

All the four types of HME cells were cultured on cover-glass and stained with anti-Vimentin FITC (BD Biosciences) and anti-E-cadherin (Miltenyi) both with a diluition of 1:100. Differences between the positivity of these two markers reflect different levels of EMT: E-cadherin + / Vimentin – identifies epithelial cells, E-cadherin – / Vimentin + identifies mesenchymal cells, whereas E-cad+/Vim+ reveals intermediate levels of EMT.

DEPArray calibration settings

Since DEPArray system is a fluorescence microscope-based lab-on-a-chip (see Appendix 2 for details), a preliminary setup was needed in order to detect only specific signals.

The brightest fluorochromes respectively PE and APC were selected for Epithelial and Stem/Mesenchymal tags, respectively. FITC channel was designed to identify lymphocytes marker CD45. DAPI was used to counterstain cell nuclei.

A three steps setup was carried out with MDA MB436 cell line and PBMC stained with different clusters of antibodies (one tag per step). The first the two cell types mix was stained for the Epithelial tag and CD45, the second for the Stem/Mesenchymal tag and CD45 and the third with all tags and CD45.

Briefly, cells were firstly stained with primary antibodies for membrane targets. After membrane labeling, samples were rinsed and fixed by Inside Fix Buffer (Miltenyi), permeabilized using Inside Perm buffer (Miltenyi) and labeled for intracytoplasmic targets. All the antibodies used were monoclonal and conjugated with a specific fluorochrome.

For a detailed specification of antibodies refers to Table1

Antibody	Fluorochrome	Clone	Manufacturing	Dilution
EpCAM	PE	HEA-125	Miltenyi	1:10
Pan CK	PE	C11	Aczon	1:10
E-cadherin	PE	67A4	Miltenyi	1:10
N-cadherin	AlexaFluor 647	8C11	BD Pharmingen™	1:20
CD44v6	APC	2F10	R&D Systems	1:20
ABCG2	APC	5D3	R&D Systems	1:10
CD45	AlexaFluor 488	GA90	Aczon	1:14

TABLE1 ANTIBODIES SPECIFICATIONS

For a detailed scheme of samples and antibodies, see Tables 2-4.

Cell types	Experiment 3	Antibody	Fluorochrome
		EpCAM	PE
		Pan CK	PE
	EPITHELIAL TAG	E-cadherin	PE
Lymphocytes	STEM/MESENCYMAL TAG	N-cadherin	AlexaFluor 647
	LYMPHOCYTE	CD44v6	APC
		ABCG2	APC
		CD45	AlexaFluor 488

TABLE 2 EXPERIMENT 1 SPECIFICATIONS

Cell types	Experiment 3	Antibody	Fluorochrome
		EpCAM	PE
MDA MB-436	EPITHELIAL TAG	Pan CK	PE
Lymphocytes	LYMPHOCYTE	E-cadherin	PE
		CD45	AlexaFluor 488

TABLE 3 EXPERIMENTS 2 SPECIFICATIONS

Cell types	Experiment 3	Antibody	Fluorochrome
		N-cadherin	AlexaFluor 647
MDA MB-436	STEM/MESENCHYMAL TAG	CD44v6	APC
Lymphocytes	LYMPHOCYTE	ABCG2	APC
		CD45	AlexaFluor 488

TABLE 4 EXPERIMENT 3 SPECIFICATIONS

During DEPArray "Scan" step every channel exposure times was adjusted to minimize unspecific signals (e.g. autofluorescence traceable by FITC channel) and to display only distinct signals.

Grab all assay on EMT inducted cell lines

The muti-phenotypic tag Grab all-assay was tested on the two different EMT models: MCF10A treated with TGF beta and HME transfected with EMT master regulators TFs.

In order to arrange a wide EMT spectrum modification, samples of MCF10A treated cells were collected at specific time points reflecting morphological modifications. Four different stages of EMT on MCF10A cells were selected: at baseline, after four days, after six days and after ten days of treatment.

HME, HME-TWIST1, HME-ZEB1, HME-ZEB2 cell lines were cultured at selective conditions i.e. for transfected cells, culture media were addicted with selective antibiotics.

All collected samples were stained as previously described for DEAParray calibration experiments.

All the analysis were performed by DEPArray system using settings previously determined.

Grab all-assay on esophageal cancer patients and healthy donors

Patients aged ≥18 years with a diagnosis of M0 and M1 esophageal cancer were enrolled. M1 patients were selected as potential CTC-positive controls. Patients were treated at Irccs Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (I.R.S.T.) (Italy) between January 2013 and December 2015. All M0 patients received adjuvant therapy, referred as primary therapy (chemotherapy and radiotherapy), before surgery (referred as secondary therapy according to the local institutional practice).

The blood draw from M0 patients was performed before primary treatment (1° CTC analysis), after primary treatment/before-secondary treatment (2°CTC analysis) and after secondary treatment/follow-up (3°CTC analysis). Blood draw from M1 patients was performed at baseline before I line chemotherapy (1° CTC analysis) after I line chemotherapy (2°CTC analysis) and eventually after II line chemotherapy (3° CTC analysis).

Patients and healthy donors signed a written informed consent. This study was approved by the Ethics Committee of the IRST IRCCS AVR (CEIIAV) (reference L3P957) and an amendment dated March 2015 was approved by the CEIIAV.

Blood sampling was carried out in EDTA Vacutainer tubes (BD). Three tubes were collected: a first 3ml, which was discarded due to possible skin epithelial cell contamination, and two 9 ml tubes, that were utilized for the CTC test.

For CTCs enrichment, Oncoquick™ system was used according to manufacturer's protocol and previous studies [158].Enriched samples was stained with Grab all-assay as DEPArray calibration protocols; the complete experiment workflow is listed in Figure 1

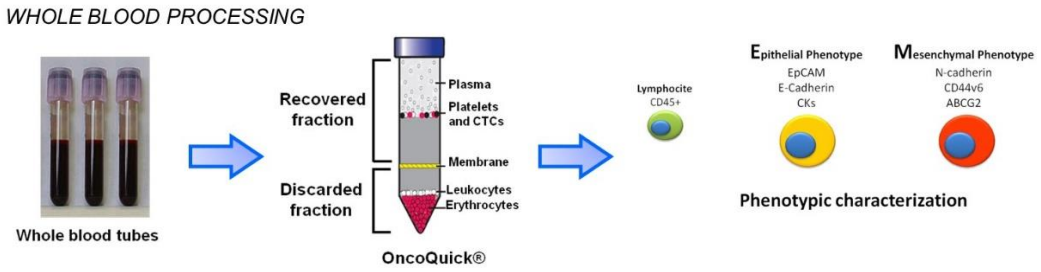


FIGURE 1 CTC ENRICHMENT-GRAB ALL ASSAY WORKFLOW

Analyses were conducted using DEPArray system adopting the setting previously determined. After identification, CTCs were sorted as a single cell for subsequent molecular analyses by the "recovery manager" software of DEPArray system. For a complete scheme of the DEPArray system referred to Figure 2

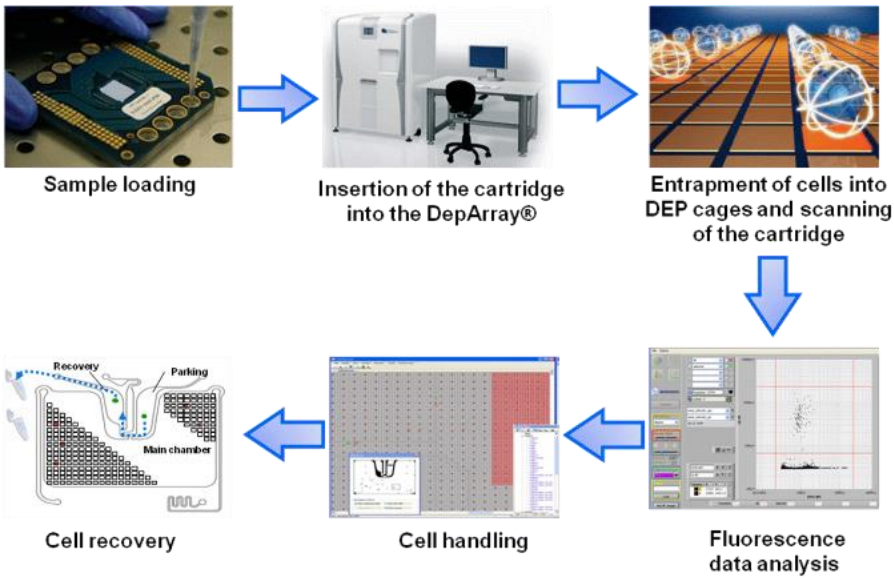


FIGURE 22 DEPARRAY ANALYSIS WORKFLOW

Whole Genome Amplification

Identified CTCs were recovered singly (with a 100% of purity guaranteed by DEPArray technology). Internal controls were 1 leucocyte, 5 leucocytes and buffer (SB115), collected at every DEPArray analysis.

On single CTCs, and their respective controls we performed whole genome amplification (WGA) using the Ampli1TM WGA kit according to manufacturer's instructions. Ampli1 kit globally amplifies the genome after generation of defined DNA fragments and adaptor ligation using a single primer to the adaptor sequence [159]. To assess the quality of single cell WGA samples isolated, we performed Quality Control Kit (QC) according to manufacturer's instruction. QC is based on multiplex PCR providing a genome integrity index (GII), defined by the detected PCR bands as a measure for quality of each WGA sample generated from an isolated single cell.

Results

DEPArray calibration setup

Preliminary setup of the microscopy equipment of the DEPArray system was carried out to identify the more efficient antibodies using different set of cells.

A three steps setup was carried out with MDA MB436 cells mixed with PBMC. Cell mixes were stained with 3 different clusters of antibodies. Each cluster was called "tag". The first two-cell type mix was stained with the Epithelial-related tag and CD45, the second with the Stem/Mesenchymal tag and CD45 and the third with the two previous tags and CD45.

The first DEPArray-microscope calibration was performed using only Lymphocytes stained with Grab all-assay. During the Scan step (see Appendix-2), fluorescence channel exposure times were adjusted to reach the best PE and APC channel intensity and obtain images without aspecific signal, and at the same time getting the maximum signal for the FITC channel Figure 1

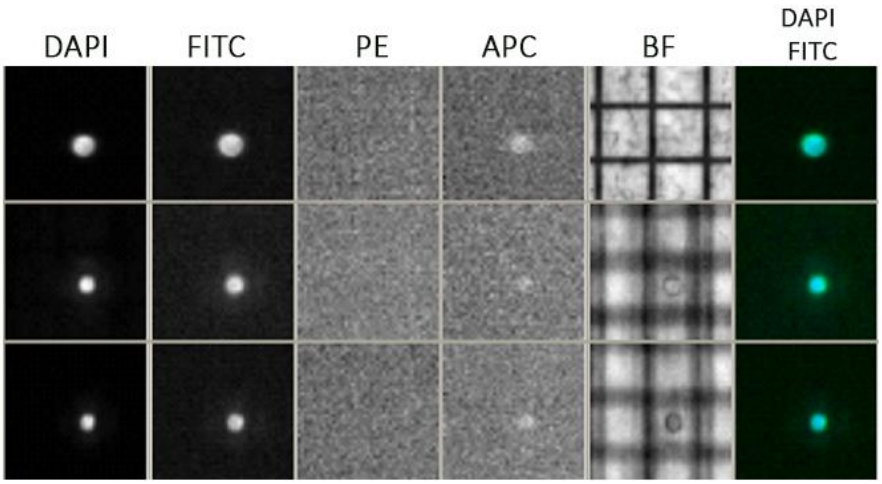


FIGURE 1 DEPARRAY IMAGES OF LYMPHOCYTES. EXAMPLES OF LYMPHOCYTES STAINED WITH GRAB ALL-ASSAY, DAPI CHANNEL IDENTIFY NUCLEUS, FITC CHANNEL IDENTIFY CD45; NO UNSPECIFIC FLUORESCENCE SIGNAL FOR PE (E-TAG) AND APC (MS-TAG) CHANNELS.

A second DEPArray-microscope calibration was performed with mixed MDA MB436 cell line and PBMC stained with only the Epithelial tag of the Grab all assay.

The purpose of this step was to calibrate fluorescence channel settings to maximize signals of the PE channel (E-tag), decrease unspecific signal in the APC channel (MS-tag) and verify the specificity of signals of the FITC channel (CD45) depending from PBMCs [Figure 2].

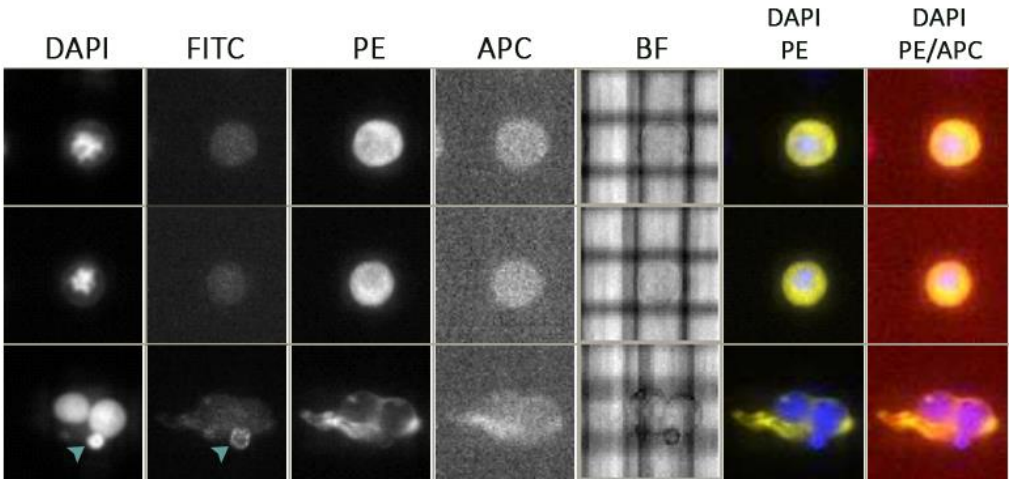


FIGURE 2 DEPARRAY IMAGES OF PBMC MIXED WITH MDA MB 436. STAINED WAS PERFOMED WITH ONLY E-TAG ANTIBODIES AND CD45, MDA MB 436 WERE POSITIVE ONLY FOR E-TAG (PE) NO ASPECIFIC SIGNAL FOR APC CHANNEL (WITHOUT SPECIFIC ANTIBODIES). AQUA ARROWS INDICATE LYMPHOCYTE VISIBLE ON THE FITC CHANNEL. DAPI IDENTIFIES NUCLEUS.

DEPArray-microscope calibration was then performed with MDA MB 436 cells mixed with PBMCs stained with Mesenchymal/Stem tag and CD45 only.

The purpose of this step was to calibrate fluorescence channel settings to maximize signals of the APC channel (MS-tag), decrease unspecific signal of the PE channel (E-tag) and verify the lymphocytes specific signals of the FITC channel (CD45) [Figure 3].

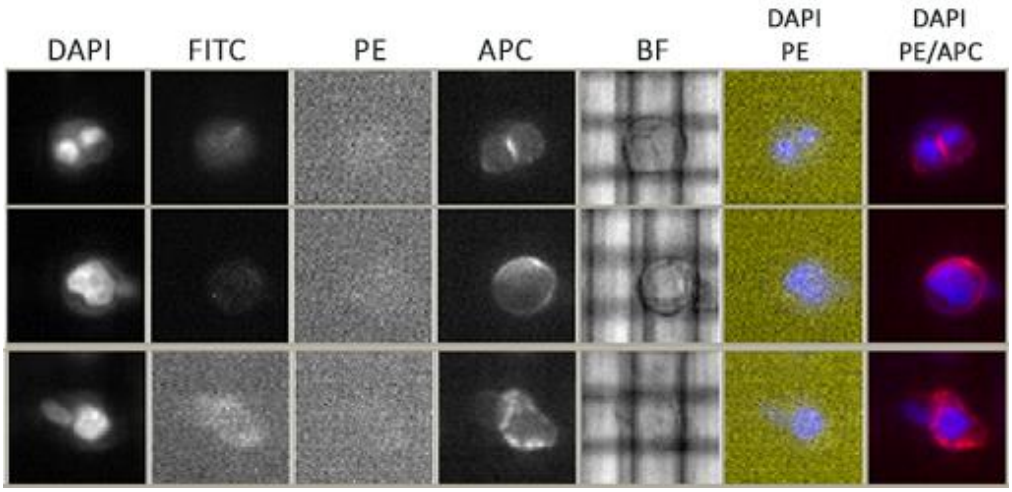


FIGURE 3 DEPARRAY IMAGES OF MDA MB 436 STAINED WITH MS TAG AND CD45. SIGNALS WERE PRESENT ONLY FOR APC CHANNEL (MS-TAG) NO ASPECIFIC SIGNALS FOR BOTH FITC CHANNEL (CD45) ANA PE CHANNEL (E-TAG). DAPI IDENTIFY NUCLEUS

Differently stained cell populations were visible by DEPArray, see dot plots Figure 4. MDA MB 436 cell line was positive for E-tag or MS-tag whereas PBMC population was positive for CD45 only.

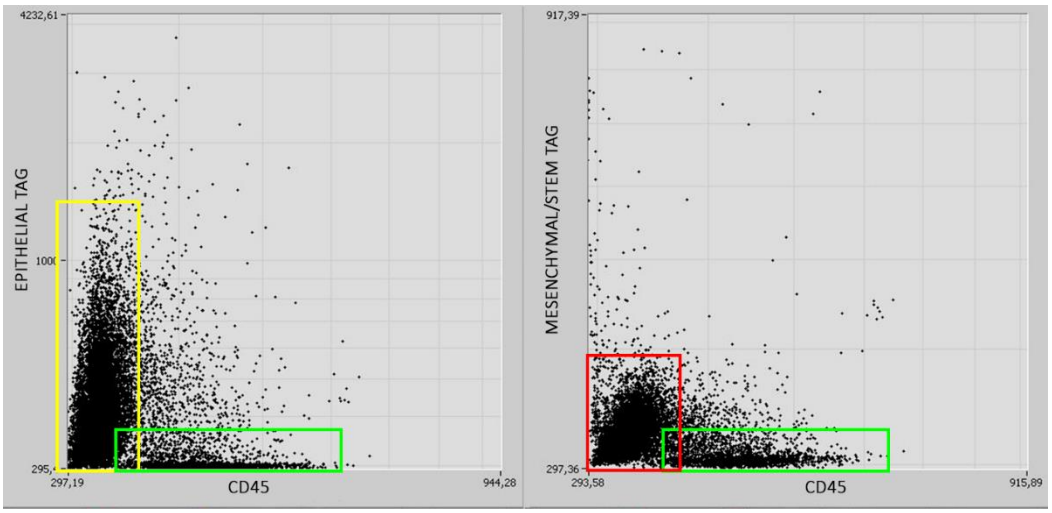


FIGURE 4 DEPARRAY DOTPLOTS. ON THE LEFT, DOTPLOT WITH E-TAG ON THE Y-AXIS AND CD45 ON THE X-AXIS- YELLOW RECTANGLE ENCLOSES MDA MB 436 E-TAG+/CD45-, GREEN RECTANGLE ENCLOSES PBMC E-TAG-/CD45+. ON THE RIGHT, DOTPLOT WITH MS-TAG ON THE Y-AXIS AND CD45 ON THE X-AXIS- RED RECTANGLE ENCLOSES MDA MB 436 MS+/CD45-, GREEN RECTANGLE ENCLOSES PBMC MS-TAG-/CD45+.

The GRAB all-assay

The Grab-all assay sensitivity was tested on cell lines induced in EMT. MCF10A cell line was treated with TGF beta. HME cell line and the stable HME-TWIST1, HME-ZEB1, HME-ZEB2 lines, over-expressing the EMT master transcription factors TWIST, ZEB1, ZEB2 respectively [160]. All the different cell lines were tested for EMT levels and after Grab all assay was performed.

EMT ON MCF10A

Morphological EMT-related changes have been observed in treated MCF10A. Four different stages of induction were selected to investigate morphological changes of EMT transformed MCF10A: before the first treatment, after four days, after six day and after ten days Figure 5. Untreated cells (or before treatment) showed tightly packed clusters with a typical epithelial phenotype, e.g. tight cell-to-cell adhesion. These cells maintained a standard doubling time of 48h. After 4 days of EMT induction, the peculiar epithelial tissue-like structure disappeared and the distinctive cobblestone-like organization was lost and spindle-like phenotype and characteristic fibroblast-like morphology appeared.

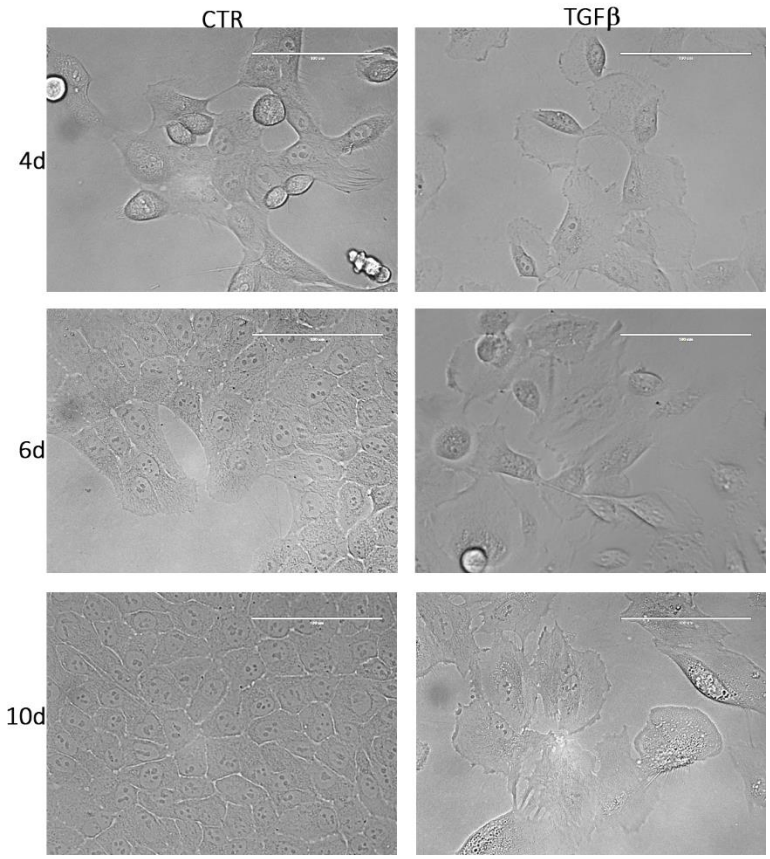


FIGURE 5 MORPHOLOGICAL CHANGES IN MCF10A EMT INDUCED. AFTER 4 DAYS (4D) OF TFG BETA TREATMENT THE COBBLESTONE-LIKE CELLS ORGANIZATION CHANGES, CELL MEMBRANE CRESTS APPEARED AS A FIRST EMT INDICATOR. DURING TGF BETA TREATMENT CELL ORGANIZATION REACHED A SPINDLE-LIKE PHENOTYPE.

GRAB ALL ASSAY ON EMT-INDUCED MCF10A

Assay sensivity evaluation was performed on the four selected times of EMT induction of MCF10A. DEPArray single cell images are shown in Figure 6, DEPArray dot plots representing cells populations in Figure 7.

TGF beta untreated MCF10A cells resulted have any SM-tag positivity.

When morphological changes started to appear (4d) there is a reduction of the mean intensity of cells positive for epithelial tag but only after six days (6d) there is a an appearance of cells weakly positive for SM-tag .

Ten days after the beginning TGFβ-1 treatment (1d0) cells positive for SM-tag are increasing. Hybrid phenotypes (E-tag and MS-tag) and cells displaying no positivity to the E-tag or MS-tag were present at late stages of EMT.

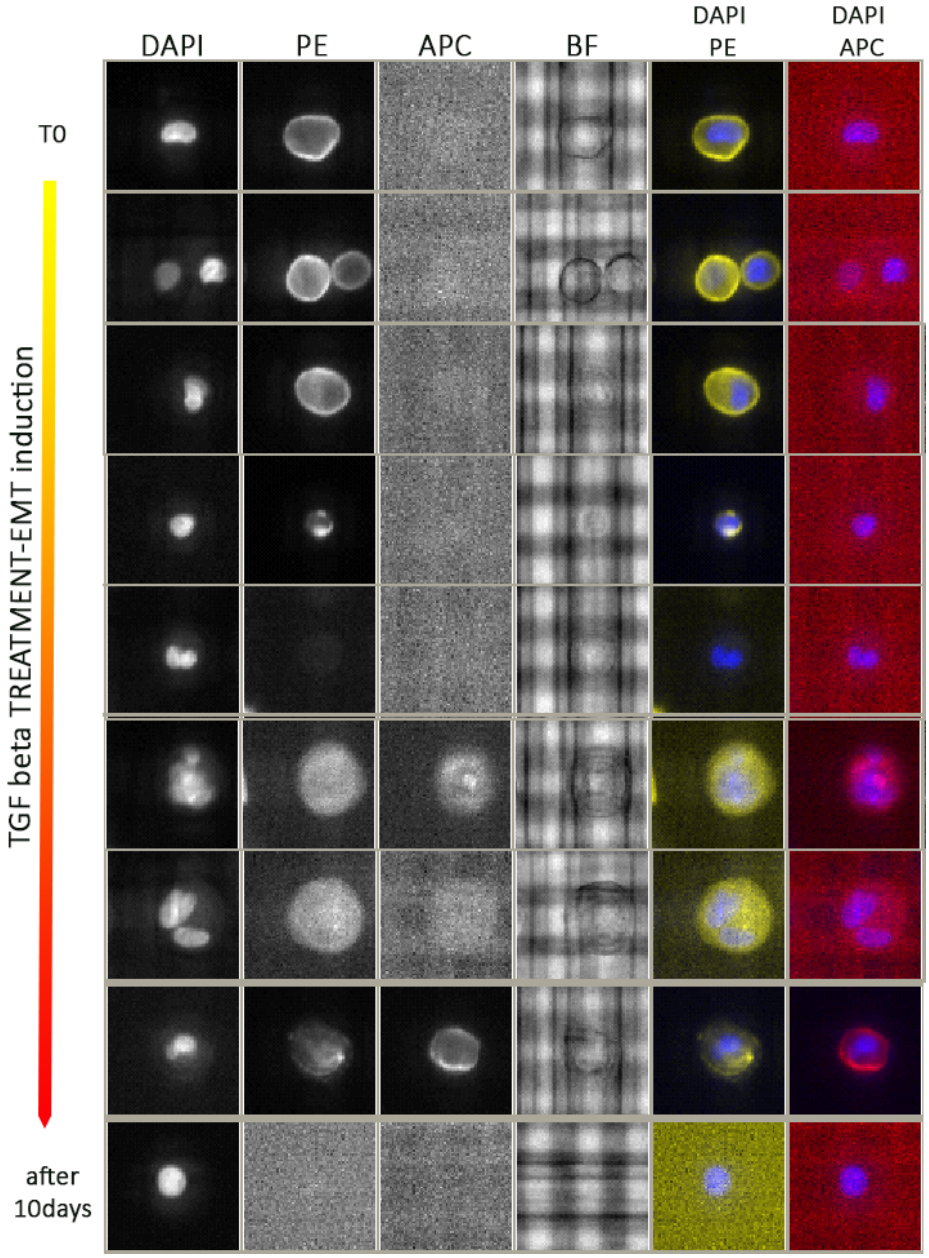


FIGURE 6 DEPARRAY IMAGES OF EMT-MCF10A STAINED WITH GRAB ALL ASSAY. REPRESENTATIVE IMAGES OF MCF10A CELLS AFTER DIFFERENT EXPOSURE TIMES TO TGF BETA (RANGE 0 TO 10 DAYS). IT IS POSSIBLE RECOGNIZE CELLS EXPRESSING ONLY EPITHELIAL MARKERS (E-TAG YELLOW) OR MESENCHYMAL ANTIGENS (MS-TAG, RED) OR HYBRID CELLS (E-TAG/MS-TAG). EPITHELIAL TAGGED CELLS WERE DETECTABLE AFTER SHORT EXPOSURE TIMES, WHEREAS MESENCHYMAL OR HYBRID CELLS WERE OBSERVED ONLY AT EMT LATE STAGES.

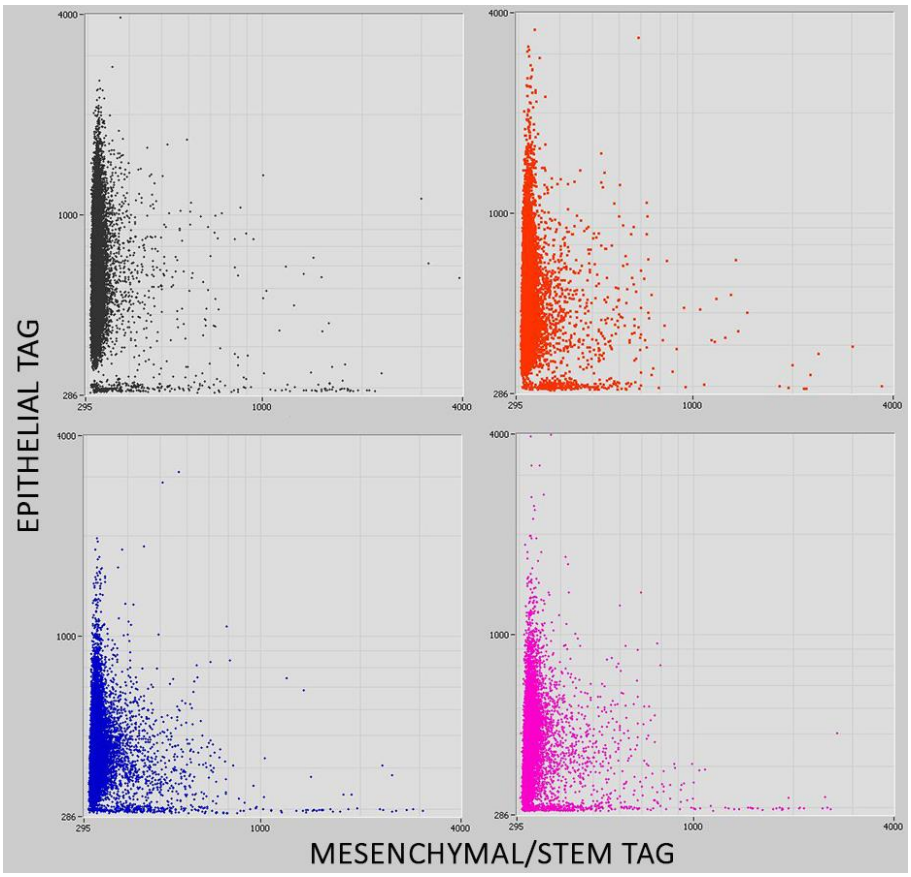


FIGURE 7 DEPARRAY DOTPLOT OF THE FOUR STEPS OF EMT INDUCTION OF MCF 10A. EPITHELIAL TAG ON Y AXIS (PE CHANNEL) STEM/MESENCHYMAL TAG ON X AXIS (APC CHANNEL). UNTREATED MCF10A CELLS ARE REMARKABLY POSITIVE TO THE EPITHELIAL TAG AND NEGATIVE TO THE STEM/MESENCHYMAL TAG [BLACK DOTS], 4D MCF10A CELLS LOOK LIKE LESS POSITIVE TO EPITHELIAL TAG [RED DOTS], 6D [BLUE DOOTS] AND 10D [PURPLE DOTS] LOOK LIKE EVEN LESS POSITIVE/NEGATIVE TO THE EPITHELIAL TAG AND SOME OF THESE CELLS ARE WEAKLY POSITIVE TO THE STEM/MESENCHYMAL TAG

EMT ON HME CELL LINE AND MODIFIED HME-TWIST1, HME-ZEB1, HME-ZEB2

The Grab all assay was performed on models of HME cell line forced in EMT: HME-TWIST1, HME-ZEB1, HME-ZEB2.

Immunofluorescence experiments were conducted on all HME cell lines in order to verify their EMT features using E-cadherin as an epithelial cell adhesion marker and Vimentin as a mesenchymal marker.

Different positivity for the two markers reflected different stages of EMT Figure 8. HME cells were positive only for E-cadherin. The majority of HME-TWIST1 cells had a lower positivity for E-cadherin and while some cells were weakly positive for Vimentin. A more defined EMT-related profile was observed in HME-ZEB2 cells, quite negative for E-cadherin and fairly positive for Vimentin. HME-ZEB1 cells were positive for Vimentin only, showing the most prominent EMT feature.

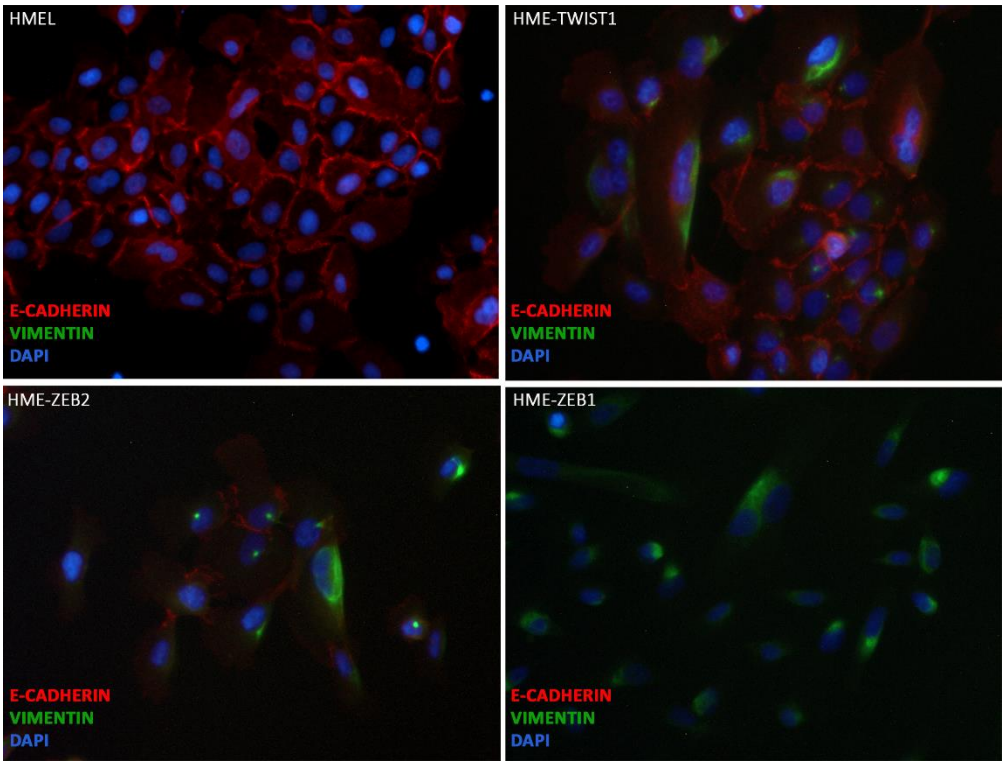


FIGURE 8 EMT FEATURES OF DIFFERENT HME CELLS BY IMMUNOFLUORESCENCE. EPITHELIAL HMEL CELLS WERE E-CAD+/VIM-. HME-TWIST1 CELLS EXHIBIT AN INTERMEDIATE PHENOTYPE E-CAD+-/VIM+- RETAINING A COBBLESTONE MORPHOLOGY. EVEN HME-ZEB2 CELLS DISPLAY AN INTERMEDIATE PHENOTYPE BUT LACKED EPITHELAIL MORPHOLOGY IN FAVOR OF A MORE SPINDLE-SHAPED MORPHOLOGY. ZEB1 EXPRESSION ON HME-ZEB1 WAS SUFFICIENT TO PROMOTE A COMPLETE TRANSDIFFERENTIATION PROCESS, GIVING RISE TO TYPICAL SPINDLE-LIKE CELL MORPHOLOGY, AND A TOTAL LOSS OF E-CADHERIN EXPRESSION AND A TOTAL GAIN OF VIMENTIN EXPRESSION. DAPI (BLUE) IDENTIFY NUCLEUS, VIMENTIN IN GREEN COLOR, E-CADHERIN IN RED COLOR.

EMT features of different HME cells were observed by Grab all assays also, as expected [Figure 9]. HMEL cells were positive only for the E-tag, HME-TWIST1 were E-tag positive but with some cells were weakly positive for the MS-tag.

HME-ZEB2 cells displayed some positivity for the E-tag, combined with a fibroblast-like morphology. Some cells were positive for MS-tag. HME-ZEB1 cells displayed a total negativity for E-tag and some cells were positive for MS-tag.

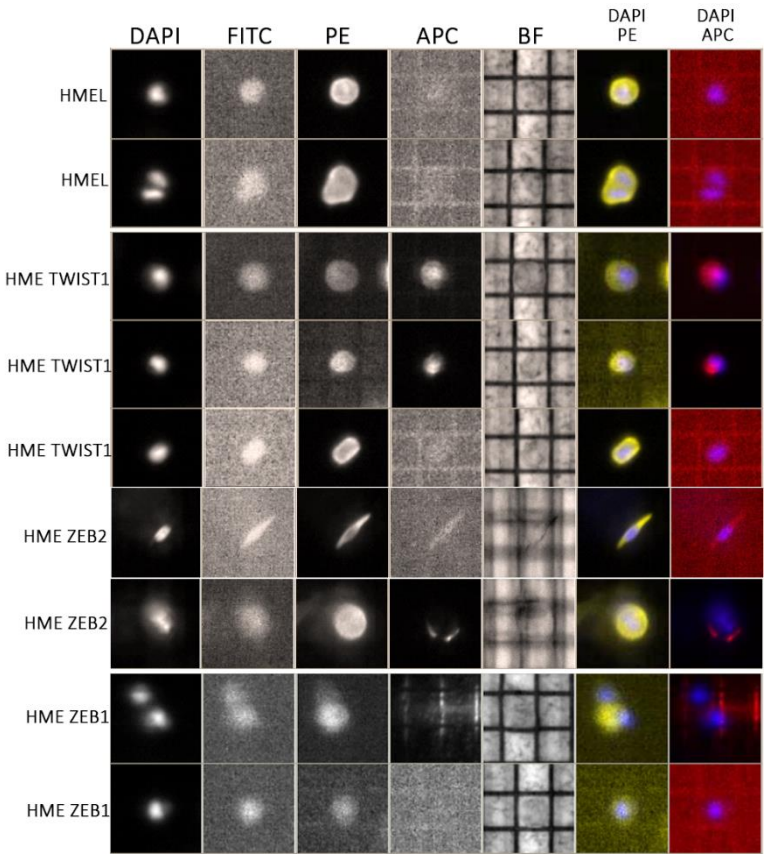


FIGURE 9 DEPARRAY IMAGES REPRESENTING DIFFERENT HME CELLS REFLECTING EMT FEATURES.

Grab all assay specificity on blood of healthy donors.

In order to test the Grab all assay specificity, blood from healthy donors was used. No False positive CTCs was detected.

Grab all assay on blood of esophageal cancer patients.

METASTATIC ESOPHAGEAL CANCER PATIENTS STRATIFICATION BASED ON CTCS ANALYSIS

The number and features of CTCs detected in metastatic esophageal cancer patients are listed in table 1

	1° CTC analysis			2° CTC analysis			3° CTC analysis		
	E-tag	MS-tag	total	E-tag	MS-tag	total	E-tag	MS-tag	total
M1	0	24	24	NE			NE		
M2	2	0	2	NE			NE		
M3	unanalyzable			0	1	1	5	1	6
M4	2	0	2	NE			NE		

TABLE 1 CTCS NUMBER AND FEATURE ON METASTATIC ESOPHAGEAL CANCER PATIENTS. (NE: NOT EVALUATED)

Three out of four patients metastatic at diagnosis (M1, M2, M4) were found to be CTC positive before primary treatment and died before the end of therapy. Patient M3 was not evaluable

before I line chemotherapy, but was tested for CTC evaluation and found to be positive: one MS-tag positive CTC was found; both epithelial CTCs and mesenchymal/stem CTCs were found before II line chemotherapy.

Patients stratification is listed in Figure 10.

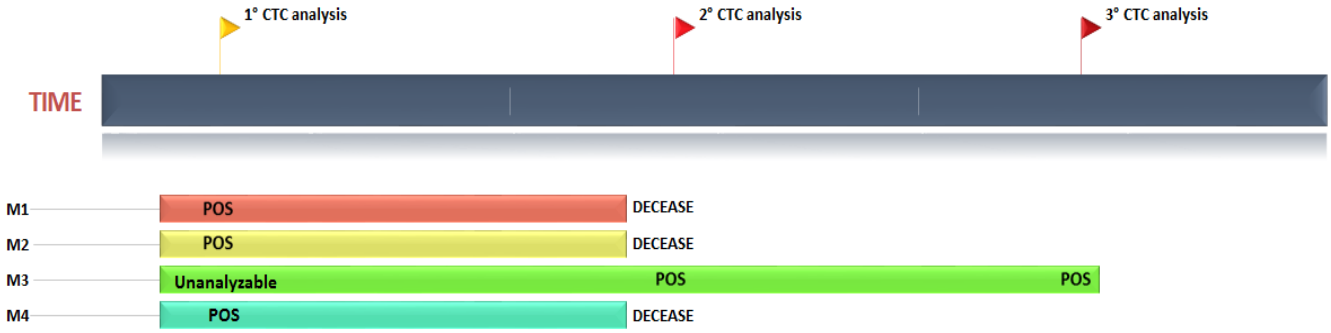


FIGURA 10 METASTATIC PATIENTS STRATIFICATION BASED ON CTCS.

NON-METASTATIC ESOPHAGEAL CANCER PATIENTS STRATIFICATION BASED ON CTCS ANALYSIS

The number and features of CTCs analyzed in non-metastatic esophageal cancer patients are listed in table 2.

	1° CTC analysis			2° CTC analysis			3° CTC analysis		
	E-tag	MS-tag	total	E-tag	MS-tag	total	E-tag	MS-tag	total
NM1	0	0	0	2	0	2		NE	
NM2	0	0	0	0	0	0	0	0	0
NM3	3	1	4		NE			NE	
NM4	0	0	0	6	1	7		NE	
NM5	10	0	10	0	0	0		NE	
NM6	0	0	0	10	0	10		NE	
NM7	6	0	6	0	0	0	4	0	4

TABLE 2 CTCS NUMBER AND FEATURE ON NON-METASTATIC ESOPHAGEAL CANCER PATIENTS. (NE: NOT EVALUATED)

Reported results showed that 4 out of 7 patients without clear evidence of metastases at diagnosis (NM1, NM2, NM4, NM6) did not have detectable CTCs before the beginning of primary treatment.

At the second CTCs analysis, NM1 patient was positive for E-tag CTCs. At that time, a PET analysis showed a lymph-nodes progression disease and patient died during chemotherapy.

NM4 patient resulted positive for second CTCs analysis (E-tag and MS-tag) also. CT scan resulted in the detection of a stable disease. Surgery was then carried out, but patient died about six months later.

At the second CTCs analysis, NM6 was positive for E-tag CTCs. CT analysis showed a partial response to therapy, whereas PET analysis exhibit a metabolic progression disease. At present, NM6 patient is under surgical evaluation.

NM2 patient resulted negative for all the three CTCs analysis. Esophagectomy surgery was carried out and he is currently is on follow-up.

The other 3 non-metastatic patients (NM3, NM5, NM7) were CTCs positive at the first CTC analysis. In NM3 patient were found both E-tag and MS-tag CTCs and patients died before the end of the primary treatment.

NM5 had only E-tag CTCs and resulted negative for subsequent CTCs analysis.

NM7 had E-tag CTCs at first CTCs analysis, no CTCs were found on second CTCs analysis, but was CTCs positive for E-tag CTCs at third CTC analysis. No surgery for patients NM7 was carried out because of a liver tumor relapse.

Patients stratification is listed in Figure 12. Detailed clinical histories of every patients with relatives CTCs status are listed in Appendix-3.

Representative CTCs analyzed by DEPArray platform are shown in Figure 13.

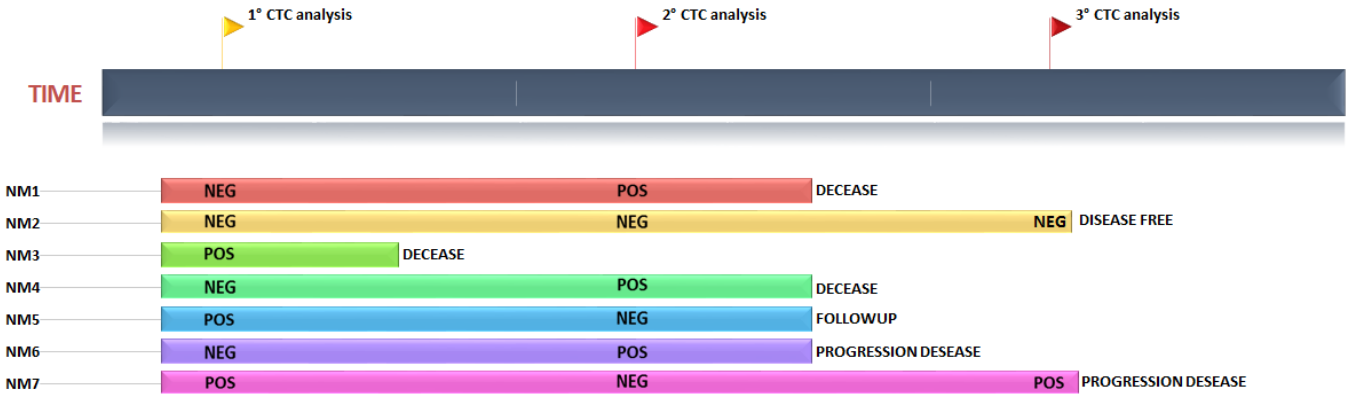


FIGURE 12 NON-METASTATIC ESOPHAGEAL CANCER PATIENTS STRATIFICATION BASED ON CTCS.

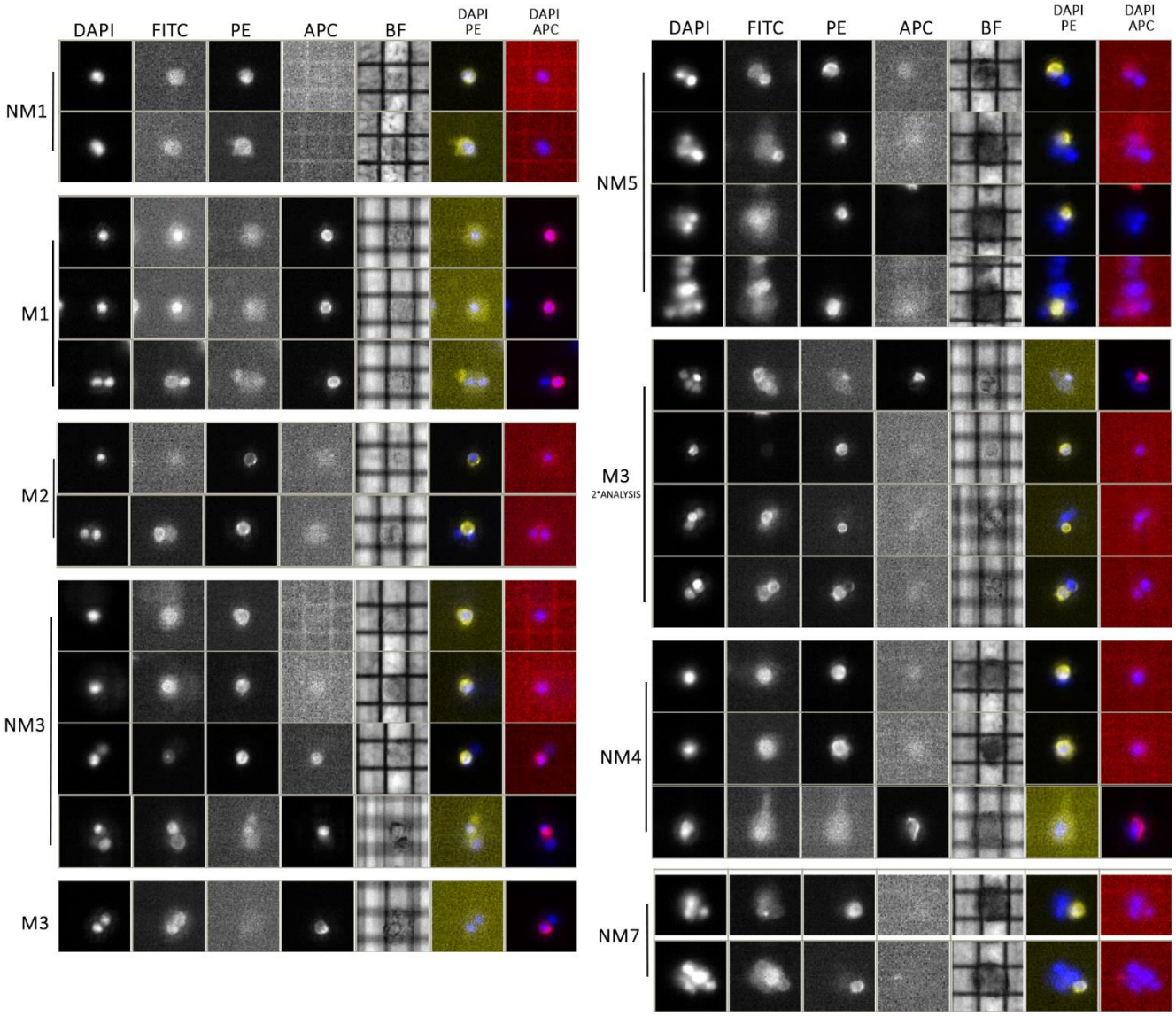


FIGURE 13 DEPARRAY IMAGES OF CTCS ISOLATED FROM ESOPHAGEAL CANCER PATIENTS. SOME CTCS WERE AS A SINGLET, OTHER CELLS WERE CLUSTERED WITH LYMPHOCYTES, INDIVIDUALIZED BY CD45 ANTIBODY. DAPI CHANNEL [PSEUDOCOLOR BLUE] IDENTIFY NUCLEUS, FITC CHANNEL: CD45, PE CHANNEL [PSEUDOCOLOR YELLOW]: E-TAG (EPCAM, E-CADHERIN,PANCK),APC CHENNEL [PSEUDOCOLOR RED]: MS-TAG (N-CADHERIN, CD44V6, ABCG2).

PATIENTS CLINICAL CHARACTERISTICS

Patients clinical characteristics as pathogenic Siewert classification, Histology and differentiation grade are listed in table 3.

PATIENT	STAGING	Histology	GRADE
M1	SIEWERT 3	Squamous carcinoma	G3
M2	SIEWERT 3	Adenocarcinoma	G3
M3	SIEWERT 1	Adenocarcinoma	G3
M4	SIEWERT 3	Intestinal adenocarcinoma	G3
NM1	SIEWERT 3	Adenocarcinoma	G2

NM2	SIEWERT 2	Adenocarcinoma	NE
NM3	SIEWERT 2	Signet ring cell adenocarcinoma	G3
NM4	SIEWERT 2	Signet ring cell adenocarcinoma	G3
NM5	SIEWERT 3	Adenocarcinoma	G2
NM6	NE	Carcinoma	NE
NM7	SIEWERT 1	squamous carcinoma	G2

TABLE 3 PATIENTS MAIN CLINICAL CHARACTERISTICS. NE AS NOT EVALUATED.

FEATURES OF PATIENTS THAT UNDERWENT SURGERY AND CLINICAL F.UP

Characteristics of the 3 patients that underwent surgery are listed in table 4.

After neo-adjuvant chemotherapy NM2 and NM5 patients exhibited a complete tumor regression (T0) Instead, NM4 patient exhibited a T3 staging, underling a moderate effect of neoadjuvant chemotherapy. TNM staging is confirmed by Mandard regression degree.

PATIENT	yTNM	Regression Degree (MANDARD)
NM2	T0N0(0/33)M0 LV0 R0	1
NM4	T3N0(0/8)M0 LV0 R0	3
NM5	T0N0(0/43)M0 LV0 R0	1

TABLE 4 FEATURES OF PATIENTS UNDERWENT SURGERY

Figure 14 shows clinical features of surgery-patients with CTCs status.

Reflecting CTC status, from E-tag CTC-positive to negative, NM5 experienced a complete tumor regression ()(T0) . On the contrary, NM4 experienced only moderate tumor regression (T3) with a subsequent progression disease in parallel with a CTCs status that became positive (E-tag and MS-tag CTCs) after being negative before first treatment. .

NM2 patients was always found to be CTC-negative and experience a complete tumor regression.

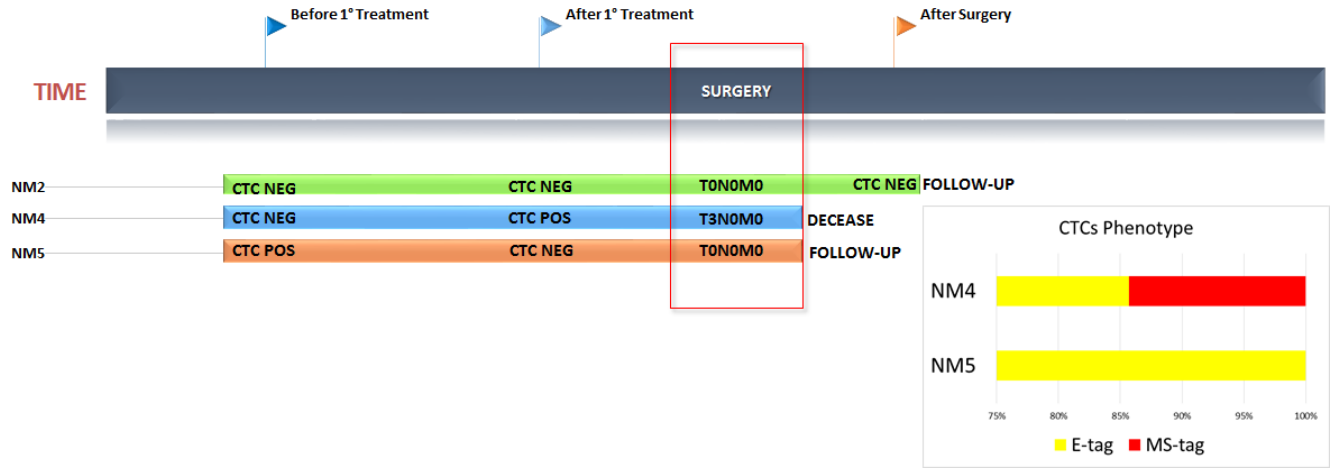


FIGURE 14 PATIENTS SURGERY STATUS VS CTCS STATUS

Whole Genome Amplification on single CTCs

Starting from indications of CTCs status in surgery patients, we performed WGA amplifications of these cells at first. All WGA products of single CTCs (including DEPArray controls) were quality checked by QC kit.

WGAs from single CTCs of NM5 patient showed a low DNA quality as indicated by QC bands, not all samples exhibited any expected bands. Only on 7/13 (53,8%) of CTCs of NM5 patient could be performed a molecular analysis.

Comparable results was obtained on single CTCs of NM4 patient, 7/10 CTCs presented a DNA quality suitable for molecular analysis.

Listed below are QC results of processed samples of NM5 patients CTCs [Figure 15] and NM4 patients CTCs [Figure 16] .

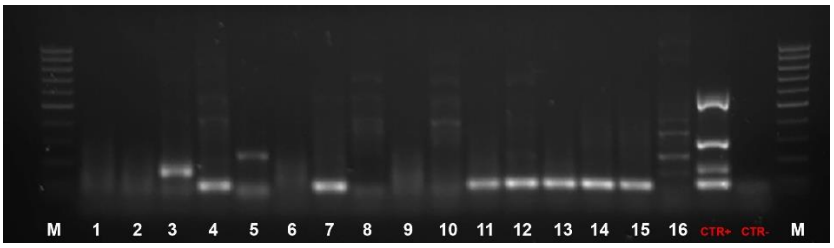


FIGURA 15 QC RESULTS ON WGAS FROM CTCS OF NM5 PATIENTS . 1-12 SINGLE CTCS; 13 CTC WITH LEUCOCYTE, 14 SINGLE LEUCOCYTE, 15 (5) LEUCOCYTES, 16 EMPTY (BUFFER), WGA CTR+/CTR- (RED) M MARKER.

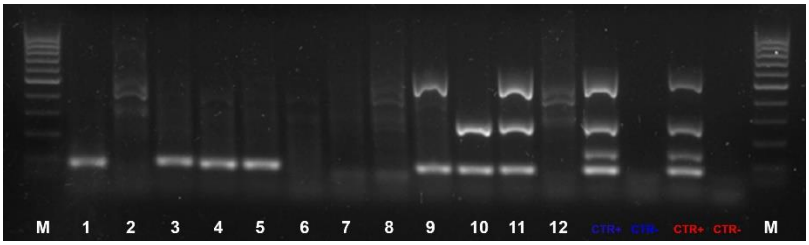


FIGURA 16 QC RESULTS ON WGAS FROM CTCS OF NM4 PATIENT CTCS. 1-11 SINGLE CTCS, 12 (5) LECUCYTES, QC CTR+/- (BLUE), WGA CTR+/- (RED), M MARKER

Discussion

Esophageal cancer is one of the most aggressive tumors, with a long-term survival rates below 15% due to both high frequency of local recurrence and distant metastases. One of the cardinal features of cancer cells is their abnormal mobility [161] that is the key feature of circulating tumor cells. A central tenet of cancer biology has been that cancer cells that leave the primary tumor (CTCs) can then seed metastases in distant organs in a unidirectional process.

In the course of last ten years, few studies investigated the role of CTCs in esophageal cancer. Starting from pioneering studies of mRNA ascribable to CTCs[121,122], through first CTCs count by CellSearch system without any explanation of the association of these cells with cancer [123]. Only recently, the group of Reeh laid the cornerstone of CTCs in esophageal cancer. They showed that CTC positive non-metastatic patients had a significantly shorter overall and relapse-free survival than patients without CTCs [125].

Even though these findings revealed a tight connection between finding CTCs and clinical outcome in esophageal cancer, other works [133,162] have shown that CellSearch is not the perfect assay, hence its FDA clearance does enable completely its use in the clinic and as a stratification marker in clinical trials.

Aberrant activation of epithelial-mesenchymal transition (EMT) has been implicated in carcinogenesis [23,163]. Consistent with this hypothesis, Stoecklein et al reporting that EpCAM expression in early esophageal cancer may vary [126]. They hypothesized that both EpCAM positive and EpCAM negative/low cancer cells sustain metastatic progression during esophageal cancer in a context-dependent manner.

In the absence of any gold standard with which to measure various technologies [see Appendix 1], defining their absolute accuracy, sensitivity, and specificity in detecting CTCs remains a challenge.

To address these theoretical challenges, we optimized enrichment system Oncoquick^{TM} with a multi-antibody assay, named Grab all-assay, with the purpose of identify CTCs with epithelial- and mesenchymal/stem- phenotypes.

The aim of this study was to find and monitor epithelial- and mesenchymal/stem- CTCs to investigate them as potential biomarker for the stratification of non-metastatic patients.

The Grab all-assay compatible with DEPArray system was established to examine tumor cells for expression of three pooled Epithelial proteins (EpCAM, E-Cadherin, Cks) [named E-tag] and three pooled Mesenchymal/Stem proteins (N-Cadherin, CD44v6, ABCG2) [named MS-tag] as well as PBMC marker CD45.

Grab all-assay was first validated in different cell lines to confirm differential expression in epithelial versus mesenchymal cancer cells, and the absence of expression in blood cells that usually contaminate CTC preparations (by CD45 marker).

To do this, we mimicked EMT process by two in vitro models. The first consisted of epithelial cell line MCF10A treated by EMT-master-cytokine TGF beta and the second included HME cell line overexpressing TFs known as EMT regulators (TWIST1, ZEB-2 and ZEB-1).

Different levels of EMT in every cell lines was reflected by different positivity of grab all assay tags in both in vitro models. For example, MCF10A naive was positive only for E-tag, whereas 6-days treated MCF10A tags positivity was reversed (E-tag neg, MS-tag positive). Epithelial HMEL was positive only for E-tag, on the contrary forced expression of either TWIST1 or ZEB1/2 laid HME cells in an EMT transition state and it was reflected on Grab all assay.

The recovery capability of our Oncoquick/DEPArray-based method was assessed as previously reported [164]: a range of 11.6/86% of cell recovery was observed. We next applied Oncoquick™/Grab all assay on samples from three healthy blood donors. The specificity of Grab all-assay was verified since no false positive CTCs were detected.

We next analyzed blood samples from 11 patients at various stages of esophageal cancer (4 metastatic, 7 non-metastatic); blood samplings were achieve before first primary therapy of choice (radio-therapy, radio-chemiotherapy, radio-chemiotherapy), one month after therapy and, when possible, after surgery.

Three out of four patients metastatic at diagnosis (M1, M2, M4) were found to be CTC positive before primary treatment and died before the end of therapy. This data could prompt to describe them as a CTCs-positive-control, because in non-responding metastatic patients CTCs could be seeded from every tumor sites (both primary and metastatic lesions). Clinically this data albeit on a small population, remind the negative prognostic value of CTCs in metastatic cancer [165,166].

M3 patient with an unanalyzable sample at first blood drawn, resulted positive for mesenchymal/stem CTCs during I line chemotherapy. Concomitant CT outcome indicated a partial response at the esophagus lesion but a progression disease in bone lesion. The two analyses were conducted during I line chemotherapy probably bringing out that the selected therapy didn't had any effect on the bone metastasis.

Three months later CTCs analysis was still positive. Most of the CTCs were epithelial rather than mesenchymal/stem. A simultaneous CT scan indicated an esophagus- and bone-stabilization of disease. However, seven months later, the disease still progressed. It possible that CTCs status had indicated an early restarting tumor activity not reflected by CT.

Starting from this hint, we focused on non-metastatic esophageal cancer patients, where biologically CTCs are probably gathered only from primary tumor. Hence, a “more linear" biological system is probable .

Recently the group of Leon Terstappen found that, in early breast cancer setting, pre-surgical CTCs detection suggest a higher tumor relapse[167]. Furthermore CTC detection seems to be a stronger indicator of overall and relapse-free survival than pathological LN stage in non-metastatic esophageal cancer [125].

Despite the small number of patients, which disallow an accurate statistical analysis, an indication of neoadjuvant therapy response would be lead from the comparison of CTC status at basal and after therapy.

One adenocarcinoma patient (NM3) at basal was CTCs positive for both epithelial and mesenchymal/stem phenotypes, CTCs status after therapy wasn't executed due to patient death. NM3 was the only one patient who died before executing second CTC status. His/her esophageal cancer aggressiveness was maybe reflected by mesenchymal/stem CTCs (N-

cadherin/ABCG2/CD44v6) a similar context was reported in five metastatic breast cancer patients [95].

Three patients affected of adenocarcinoma of the esophagus, with no CTCs at basal, after neo-adjuvant treatment were found to be positive for CTCs and all of them had a tumor recurrence [3-12 months from the end of chemotherapy]. One patient affected of esophageal squamous carcinoma had a fluctuating number of CTCs (positive at basal, negative post neo-adjuvant treatment, and positive after 4 months from the end of chemotherapy. CT and PET analysis indicated a partial response. No surgery was performed due to the occurrence of hepatic and lymph nodes metastases concurrent with the last detection of CTCs. Again, monitoring CTC-status more than a single sporadic determination could infer more relevant information.

Noteworthy is the CTC analyses in patients that underwent surgery. Three patients benefited from neo-adjuvant treatments as resulted by CT and PET analysis and then they were underwent surgery.

Clinical stage after chemo-radiation (yTNM) indicated 2/3 patients had a T0 stage reflecting an actual response to therapy. The third patient resulted T3 stage without tumor regression and died one month after surgery.

Examining CTCs status, the T0 patients were CTCs-negative after neo-adjuvant therapy, in particular one was negative even at basal, the other one became CTCs-negative after treatment.

T3 patient was CTCs-negative at basal, but after neo-adjuvant therapy we identified both epithelial and mesenchymal/stem CTCs.

Clinically these results suggest that monitoring CTCs in these cases had traced the actual tumor response to therapies even when CT outcome reported a stable disease. Furthermore, the presence of CTCs into bloodstream post a neo-adjuvant treatment/pre-surgery could lead the possibility of a minimal leukemic-residual cancer.

Molecular analysis of a cancer related panel of genes on whole genome amplification of CTCs are ongoing. Results from these analyses could complete the picture of clinical relevance of CTCs highlighted from this study.

Appendix 1-
Techniques for CTC identification

Various approaches have been developed for CTC isolation from blood. Starting with cell line models, these technologies were primary developed for obtaining multiple performance parameters (i.e. capture efficiency/recovery, enrichment against leukocyte depletion, cell viability, processing speed, blood sample capacity) and then validated through testing with clinical samples. The optimal isolation approach should require a compromise among performance parameters, and the optimal selection is likely to depend on the intended downstream application. Table 1 summarizes the most known technologies to isolate CTCs.

Technology	Method of CTC enrichment	Method of CTC detection	Notes
Antibody-based capture assays			
CellSearch®	EpCAM-coated ferromagnetic beads	Immunocytochemistry for cytokeratin, CD45 and DAPI	FDA approved in advanced breast, prostate and colorectal cancer
MagSweeper	EpCAM-coated magnetic beads enriched using magnetic rod	Microscopic visualization	Live cells can be isolated
GILUPI cell collector	Functionalized EpCAM-coated medical wire	Immunocytochemistry for EpCAM, cytokeratin and DAPI	*In vivo* collection
CTC chip and Herringbone chip	EpCam-coated microposts and chip surface	Immunocytochemistry for cytokeratin, CD45 and DAPI with or without tumour-specific markers, for example, cytokeratins, PSA	Microfluidic microchip technology
CTC iChip®	Magnetic bead capture combined with microfluidic inertial focusing	Immunocytochemistry or RT-PCR	Positive selection by EpCAM or negative selection by CD45 gives a broad population of enriched cells
Ephesia CTC-chip	Functionalized magnetic beads combined with microfluidics	Immunocytochemistry for cell surface and nuclear markers	Flexibility with capture antibody
AdnaTest	Immunomagnetic beads with MUC1-coupled and EpCAM-coupled antibodies	Multiplex RT-PCR for panel of genes (*MUC1*, *HER2* or *EPCAM*)	Cell lysis means quantification of tumour cell number is not possible
IsoFlux	EpCAM-coated magnetic beads combined with microfluidic processing	Immunocytochemistry for cytokeratin, CD45 and Hoechst	Antimouse IgG-coated beads available for user-defined antibody capture
Physical characteristic-based assays			
ISET®	Filtration based on cell size	Immunocytochemistry or FISH	Nonepithelial cells can be isolated
Dean Flow Fractionation	Size-based selection using centrifugal force	Immunocytochemistry for cytokeratin, EpCAM, CD45 and Hoechst	Nonepithelial cells can be isolated
Dielectrophoretic field-flow fractionation	Membrane capacitance	Immunocytochemistry	CTCs selected are viable
Functional assays			
EPISPOT assay	CD45 depletion and short-term culture in plates coated in antibody against MUC-1, PSA or cytokeratin-19	Immunofluorescence secondary antibodies to MUC-1, PSA or cytokeratin-19	Detection of only viable CTCs
CAM	Density gradient centrifugation and cells applied to CAM for short-term culture	Immunocytochemistry for cell-surface markers	Detection of only viable CTCs
Other assays			
ImageStream®	Pre-enrichment by any method of choice	Flow cytometry-based imaging using multi-marker immunofluorescence	Cells can be imaged for up to 10 cell-surface or intracellular markers
High-throughput fluorescent scanning	Red cell lysis and density gradient centrifugation	Immunocytochemistry of cell surface and nuclear markers	Nonepithelial cells can be isolated on a slide
DEPArray™	Requires pre-enrichment step	Fluorescence imaging that enables movement of cells within chip by electric field changes	Isolation of purified single cells for downstream analysis

Abbreviations: CAM, collagen adhesion matrix; CTC, circulating tumour cell; DAPI, 4′,6-diamidino-2-phenylindole; EpCAM, epithelial cell adhesion molecule; FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor 2; MUC1, mucin 1; PSA, prostate-specific antigen; RT-PCR, real-time PCR.

TABLE 1 SELECTED TECHNOLOGIES TO ISOLATE CTCS. MODIFIED FROM DOI 10.1038/NRCLINONC.2013.253

Immuno-affinity systems

Immuno-affinity based CTC isolation takes advantage of highly specific affinity reactions between capture antibodies and target antigens present on cells of interest, and are mainly sub-grouped in: magnetic beads, microfluidic flow, in vivo sampling, leucocyte depletion.

MAGNETIC BEADS

The CellSearch® instrument is the only FDA-cleared technology that is clinically applied for CTC enrichment. Its enrichment process involves the binding of antibody functionalized magnetic beads to the epithelial cell adhesion molecule (EpCAM) antigen on CTCs, and the subsequent isolation of these beads with a magnet. Enumeration of CellSearch®-enriched CTCs has been established as a prognostic marker and predictor of patient outcome in metastatic breast [168], prostate [169], and colon cancers [170]. Alternative immunomagnetic strategies have also been proposed; these similarly rely immuno-targeting of potential CTCs with a magnetic payload followed by binding to a magnetic substrate. For example, MACS® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), or Magnetic Activated Cell Sorting system, is a dedicated instrument that captures cells by immunomagnetic labeling with microbeads tagged to desiderate target (i.e. anti EpCAM or HER2).

AdnaTest® (Adnagen AG, Langenhagen, Germany) is a commercialized series of assays that employs magnetic beads functionalized with cocktails of antibodies specific to either breast, prostate, colon, ovarian or EMT/stem cell markers to improve enrichment. AdnaTest BreastCancer™ coupled with multiplexed RT-PCR based CTC detection has been demonstrated to correlate with patient outcome in metastatic breast [171] and ovarian cancer [172]. The assay was also used to identify a subset of CTCs expressing stem cell and EMT markers in primary breast cancer [129]. In a comparative study AdnaTest BreastCancer™ was positive for 29 of 55 metastatic breast cancer patients compared with the detection of ≥2 CTCs in 26 of 55 patients by CellSearch®[173].

MICROFLUIDIC FLOW

Nagrath, Toner and colleagues developed a microchip consisting of an array of 78,000 silicon micro-pillars functionalized with antibodies targeting EpCAM, allowing the direct processing of whole blood, resulting in a capture efficiency of ≥60% and a final sample purity of about 50% when processing at a throughput of 2.5 mL/hour. This “CTC-chip” was used to enrich and identify CTCs in 115 of 116 tested blood samples from patients with various metastatic cancer types [140,174,175]. To overcome fabrication challenges with the first generation CTC-chip, Stott and Toner et al have reported an improved second generation “herringbone chip” that promoted microfluidic mixing through the generation of microvortices [140]. Here the specific capture antibodies were conjugated to the herringbone-shaped grooves along the bottom surface of the device, and the flow patterns resulted in increased cell to surface contact. This improved capture efficiency to 91.8% using antibodies for EpCAM, and CTCs and microclusters were detected in samples from 14 of 15 prostate cancer patients [176]. More recently, to facilitate the retrieval of CTCs for further analysis, Ozkumur and Toner et al developed a “CTC-iChip” that enables either positive anti-EpCAM CTC selection or leukocyte depletion after an initial size-based enrichment step and hydrodynamic focusing. They reported high capture efficiencies of up to 98.6%, with varying purities in the range of 0.02 – 42%. CTCs were detected in 37 of 42 metastatic cancer patient samples as compared with 29 of 42 using CellSearch® [177].

IN VIVO SAMPLING

GILUPI GmbH developed a novel approach for in vivo sampling by functionalizing a medical wire with EpCAM antibodies (CellCollector™). The medical wire is injected through a cannula into the patient’s cubital vein for a duration of 30 minutes to allow direct continuous sampling of large volumes of blood (1.5 - 3 liters). This approach successfully enriched CTCs in 22 of 24 patients diagnosed with either metastatic breast or lung cancer [178].

LEUKOCYTE DEPLETION

An alternate approach to positive immuno-affinity based CTC selection is to use monoclonal antibodies targeting leukocyte antigens (i.e., CD45, CD14) to deplete cells of hematopoietic origin. Some strategies include antibody labeling of leukocytes for removal through immunomagnetic separation [179,180], or through centrifugation with the RosetteSep™ kit (StemCell Technologies, Vancouver, Canada) [94,181]. These approaches are capable of high recovery rates with minimal disturbance to CTCs, but may achieve relatively low sample purities

Physical properties

Physical properties may be exploited to effectively separate CTCs from peripheral blood cells. The following technologies have been developed based on differences in density, size, deformability and electrical properties.

DENSITY GRADIENT CENTRIFUGATION

Centrifugation is a cheap and efficient method for separating CTCs in the mono-nucleocyte fraction of blood away from erythrocytes and granulocytes based on cell density. Centrifugation with Ficoll-Paque® solution (Pharmacia- Fine Chemicals, Uppsala, Sweden) was used to detect CTCs using an RT-PCR based assay for cytokeratin 20 expression with a resolution of 1 cell/mL of blood in model systems, successfully identifying CTCs in 24 of 58 colorectal cancer patients undergoing surgical resection [182]. OncoQuick® (Grenier BioOne, Frickenhausen, Germany) is a novel technology that incorporates a porous barrier for size-based separation of CTCs in conjunction with density-based centrifugation. Rosenberg et al reported a vastly improved enrichment of 632-fold against leukocytes with OncoQuick® compared to 3.8-fold with Ficoll-Paque® [183]. OncoQuick® , in addition to the DEPArray system[158] , has allowed the identification of CTCs in 21 out of of 40 advanced colon cancer patients.

MICROFILTRATION

Microfiltration operates on the principle of retaining larger CTCs while allowing smaller leukocytes to pass through pores of varying geometries. Vona, Paterlini-Brechot, and colleagues developed the “isolation by size of epithelial tumor cells” (ISET) technique using randomly track-etched polycarbonate filters with 8 µm diameter circular pores for CTC enrichment and cytological detection from fixed blood samples [184]. Track-etched microfilters have been used to enrich and characterize CTCs in studies involving liver cancer [185], melanoma [186], lung cancer [187,188], prostate cancer [64] and various other cancers [187]. These microfilters were demonstrated to be more sensitive than CellSearch®, detecting CTCs in 57 of 60 metastatic patients with breast, prostate and lung cancer compared to 42 of 60 with CellSearch® [189].

DIELECTROPHORESIS

Electrical properties of CTCs may be exploited to discriminate them from blood cells by applying a non-uniform electric field through the phenomenon of dielectrophoresis (DEP). Interdigitated gold electrodes were used by Becker, Gascoyne, and colleagues to isolate leukemia [190] and breast cancer cell lines [190] from spiked healthy donor blood. The application

of an electric field generated by the electrodes attracts tumor cells by positive DEP, while other cells flow past. Upon removal of the electric field the tumor cells can be collected with a capture efficiency of 95% [191]. Based on the success of this method, Huang et al proposed a DEP field flow fractionation approach to allow continuous processing of samples that did not require intermittent application of the electrical field for cell recovery [192]. Another use of DEP to manipulate single cells or small pools of cell is DEPArray system (Silicon Biosystem). This system is able to work on small populations of cells distinguishing them by immunofluorescence and recovering the selected cells performing cell sorting inside a chip where negative DEP cages entrap single cells keeping them floating (see Appendix 2)

Analytic tools for CTCs

Various analytic tools have been deployed for the cellular and molecular characterization of CTCs (Figure 2). These tools are developed in conjunction with CTC enrichment technologies as each downstream application will have its own requirements regarding sensitivity, sample purity, cell viability, and ability to recover cells after enrichment. As any CTC isolation and analysis process applies certain criteria for enrichment and detection, it is important to characterize the false-positive and false-negative rates of the overall CTC process. A common practice to establish a new method is to compare results from duplicate samples with the CellSearch®, that despite its intrinsic problems is still considered a gold standard in CTCs detection

IMMUNOPHENOTYPING

Immuno-staining is the current gold standard technique for CTC detection and enumeration as established by the FDA-cleared CellSearch® system. The defined criteria for identifying CTCs includes (1) positive expression of cytokeratins (CK), a class of intermediate filaments in epithelial cells; (2) negative expression of the leukocyte common antigen, CD45; and (3) positive staining of the nucleus with DAPI [1]. Markers specific to cancer types, such as PSA or PSMA in prostate cancer, have also been used for positive CTC identification [176,193,194].

The lab-on-a-chip based platform, DEPArray, bases the analysis step on immunostaining. Compared with CellSearch system, DEPArray has four/five immunofluorescence channel (Veridex has three/four channels). This improvement is useful for testing a more elaborated immunophenotyping.

Recently, attempts have been made to investigate the expression of other proteins to further characterize CTCs. The presence of stem cell markers, such as CD44+/CD24−, CD133+, ALDH1+, have been identified in CTCs [94,145,171,195,196]. Studies attempting to investigate the EMT status of CTCs have reported high expression levels of mesenchymal markers such as TWIST, AKT2, PIK3α, N-cadherin and vimentin [145,171,188,197]. While the direct clinical relevance of such markers has yet to be established, they are of significant research interest for better characterizing CTCs and understanding their roles in cancer metastasis.

Human epidermal growth factor receptor 2 (HER2) expression status of breast cancer CTCs was determined through IF [198–200]. In prostate cancer, CTCs have been assayed for epidermal growth factor receptor (EGFR) expression [201], as well as androgen receptor (AR) status [202].

FLUORESCENCE IN SITU HYBRIDIZATION

Somatic alterations to gene copy numbers are a hallmark of many cancers, and can be an important prognostic marker. They can further provide valuable predictive information on therapeutic efficacy. Meng et al employed a multicolor fluorescence in-situ hybridization (FISH) assay on CTCs isolated from breast cancer patients to determine the amplification status of the HER2 oncogene [203]. They reported some level of non- concordance between HER2 status determined by the analysis of primary tumors and with their novel CTC assay. Other studies have confirmed this observation with HER2 assays based on IF [199] and RT-PCR [204]. This suggests that a sub- set of patients with initially HER2-negative disease may develop HER2-positive CTCs over the course of progression. CTC analysis provides a minimally-invasive avenue for repeated screening for the development of genomic aberrations. It is interesting to note that CTC HER2 amplification screening has been successfully demonstrated in even primary breast cancer patients [205,206]. In castration-resistant prostate cancer, multicolor FISH has shown potential as a surrogate for status determination of AR and MYC status in CTCs after immunomagnetic enrichment with CellSearch® [201,207].

Oncogenic translocation is an important pathogenic mechanism for some cancers. TMPRSS2-ERG translocation occurs in 30-70% of therapy-naïve prostate cancer patients [208]. Using FISH analysis on CTCs, it has been shown that TMPRSS2- ERG fusion can be identified in CTCs of prostate cancer patients [176,209]. Further, there appears to be a consistency in TMPRSS2-ERG translocation between therapy-naïve prostate cancer from core biopsy specimens and from CTCs at the time of castration-resistance from the same patients [209]. In a recent study using FISH, it has been shown that ALK-rearrangement can be identified in non-small cell lung cancer patients harboring ALK-translocations [210].

There is, therefore, much promise through CTC analysis for minimally-invasive tumor profiling and improved prognosis related to metastatic disease. However, the low sensitivity of current enrichment technologies remains a significant hurdle to routine clinical application. In addition, the reliability and clinical relevance of these FISH-based assays must first be thoroughly validated.

ONCOGENE MUTATION DETECTION AND GENOMIC ANALYSIS

One of the key attractions for isolating CTCs is to enable the detection of cancer gene mutations and genomic aberrations in these metastatic tumor cells that are associated with the worst prognosis for survival, reflect drivers of active disease, and predict drug sensitivity and resistance.

Analysis on colon-CTCs analyzed and isolated with DEPArray system, for the first time, demonstrated heterogeneity between CTCs and primary tumor tissue on KRAS mutation [158].

Using CTCs isolated with microchips, it has been shown that activating EGFR mutations can be effectively detected in patients with non-small cell lung cancer using an allele-specific PCR amplification technique [175]. Further, the EGFR T790M mutation that confers drug resistance can also be detected from patients who have received EGFR kinase inhibitor treatment. While allele-specific PCR is a fast and cost- effective approach to determine specific mutations, prior knowledge of the mutations are necessary for the design of amplicon primer sets.

With the ability to perform single-cell whole genome amplification, it has been shown that one can perform next-generation exome sequencing with CTCs to uncover cancer-specific mutations, both pathogenic and passenger, which are present at very different levels of

prevalence in primary and metastatic colon cancer [211]. Once amplified, the DNA from a single CTC can also be used for array-comparative genomic hybridization analysis to uncover genomic aberrations, such as large deletions and insertions that cannot be detected with exome DNA sequence analysis [211]. Although single-cell DNA sequencing is tedious, such studies confirm the feasibility of applying CTCs to discovery research in clinical oncology studies. Technologies for cancer gene exome analysis such as the Ion Torrent AmpliSeq™ Cancer Panels (Life Technologies, Carlsbad, CA) and the TruSeq® Amplicon-Cancer Panel (Illumina, San Diego, CA) would facilitate the analysis of cancer mutations in CTCs.

GENE EXPRESSION ANALYSIS OF CTCS

While the analysis of mRNA from CTCs presents significant technical challenges due to the molecules labile nature, it is desirable due to: (1) specificity to amplicons that are highly selectively expressed in tumors but not in white blood cells; (2) the ability to detect gene translocations and alternative splice variants; (3) quantitative high-throughput technologies such as multiplex digital PCR and expression arrays for cell profiling; (4) potential application to next generation sequencing analysis.

One study on mRNA analysis explains the low abundance of EpCAM expression associated with increased vimentin in basal-like breast cancer that is poorly captured with EpCAM- based CTC purification schemes[212]. Another study further demonstrate dynamic changes in epithelial and mesenchymal composition in circulating breast cancer cells with mixed probes using RNA in situ hybridization, particularly in CTC clusters[95]. It also suggests a more mesenchymal phenotype for the basal-like molecular subtype of breast cancer. Next generation RNA sequencing has been performed with CTCs isolated from patients with pancreatic cancer using a microfluidic chip [213]. RNA sequencing results revealed the activation of WNT signaling in the CTCs, which may contribute to tumor metastasis. Taken together, while it is very attractive to analyze mRNA for gene expression and pathway profiling, its quantitative analysis requires a high quality source of mRNA that is consistently well preserved.

Appendix 2-DEPArray System

The DEPArray systems is a modulary instrument able to manipulate small cell population by dielectrophoresis force (DEP). It's a semi-automatic lab-on-a-chip composed by a 4-fluorescence (PE, APC, FITC, DAPI) channels optical microscope connected to a camera for the computer-analysis and by a device controller for the DEP-manipulation of single cells.

DIELECTROPHORESIS

Dielectrophoresis (DEP) is a phenomenon in which a dielectric particle is subjected to a non-uniform electric field and to an external force. The particle is polarized by the electric field and the resulting poles exert a force along the fields lines, which can be attractive or repulsive according to the orientation on the dipole. This non-uniform field leads the greatest electric fields to dominate over the other and the particle will move. Due to this characteristic, dielectrophoresis can be used to manipulate, separate and sort different type of particles. Since biological cells have dielectropforetic properties, dielectrophoresis has many medical/research applications.

DEP forces moving particles towards high intensity voltage areas are named positive DEP (pDEP), in the contrary DEP forces moving toward low intensity voltage areas are defined as negative DEP (nDEP). In this way, on varying both the shape of electrode and the excitation of electric field, the DEP forces can be used to entrap, levitate and manipulate particles [214–216].

DEP forces could be employed for at least two purpose: first, streaming electrophoresis where electrokinesis is dominating and trapping regions immobilizing particles are replaced by regions of preferred streaming. Second where DEP act as the dominant force and could be used as trapping, here named trapping DEP. [Transitioning Streaming to Trapping in DC Insulator-based Dielectrophoresis for Biomolecules]

CHIP

As in every Lab-on-a-chip system, the core of the DEPArray system is a disposable cartridge (A300K). The A300K is composed by two main parts: a microfluidic system allowing the loading of sample and the recovery of the targets; the second part is the active area of dielecrophoretic force [Figure 1]. A 640X480 electrodes matrix into the bottom of the "active area" generates the dielectrophoretic cages, whereas on the top a transparent layer is exposed to the microscope objectives (for the cellular analysis). The matrix is able to generate a maximum of $76*10^6$ nDEP cages. A maximum of 30 thousand cells is permitted to have 1 cell per cages (as recommended by the manufacturer).

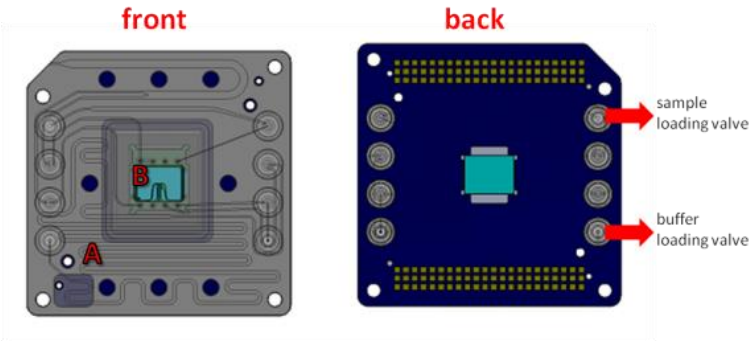


FIGURE 1 DEPARRAY CHIP (A300K). FRONTAL VIEW: MICROFLUIDIC SYSTEM (A) WHERE THE SYSTEM IS ABLE TO LOAD THE SAMPLE THROUGH THE PRESSURE CONTROLLED IN THE VALVES AND TO CARRY THE RECOVERIES IN VOLUMES CHOSEN BY THE OPERATOR; THE ACTIVE AREA OF THE CHIP (B) WHERE SAMPLES ANALYSIS TAKES PLACE. BACK VIEW, CONTROL VALVES, ONE FOR THE LOADING THE SAMPLE AND ONE FOR THE BUFFER LOADING.

The microfluidic system is composed by a polycarbonate cover containing different microcapillaries for samples loading. Together with sample, a controlled volume of a specific buffer must be loaded inside the microfluidic part, this buffer will permit the generation of nDEP cages.

A specific software is designed to control every steps of the calibration, loading, of the sample/chip as well as the DEP cages at different conditions (culture media, different buffers).

The "active area" of the chip is composed by different areas: main chamber, parking chamber, exit chamber each designated for different purpose [Figure 2].

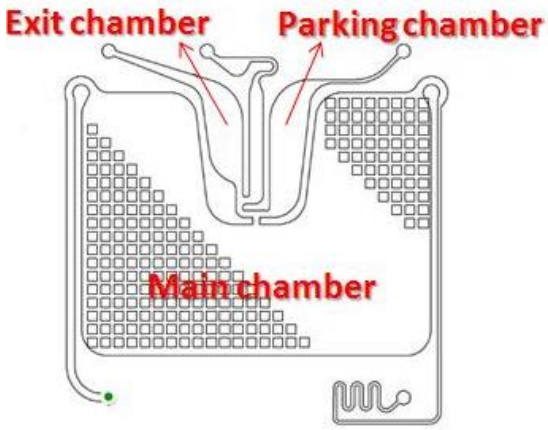


FIGURE 2 ACTIVE AREA OF THE CHIP. THE MAIN CHAMBER IS THE AREA WHERE THE SAMPLE IS LOADED. THE PARKING CHAMBER IS THE AREA USED AS A TEMPORARY PARKING PLACE OF THE CELLS READY TO BE RECOVERED; THE EXIT CHAMBER IS THE AREA WHERE THE CELLS ARE DIRECTED TO THE SINGLE-CELL RECOVERIES

The handling of the single cells take place by a pattern of sinusoidal dielectrophoresis [Figure 3] waves in a on/off scheme of dielectrophoresis cages leading cells to desired directions.

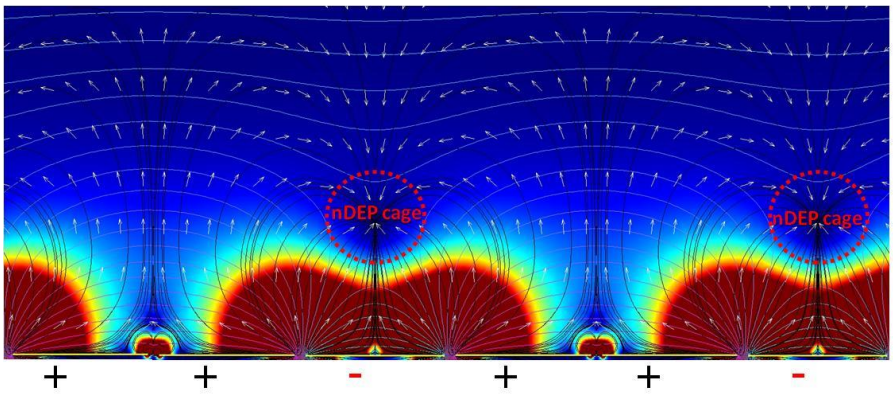


FIGURE 3 NDEP CAGES: NEGATIVE DIELECTROPHORESIS CAN CREATE A "CAGE" WHERE CELLS CAN BE TRAPPED ALLOWING THEIR HANDLING

SOFTWARE

Once A300K cartridge is lodged on the specific support of DEPArray, a series of softwares will be lauched, some of them are operator-free other controlled by operator.

The DEPArray is a platform that allows researchers to decide all the fluorescence settings; after choosing the desired setting, “arrangement softwares” perform: calibration of the optics, Sample Load, Chip Scan, Cropping of the images. Analysis of the sample is carried by the Cell Browser.

The calibration step basically verify the correct arrangement of the chip inside DEPArray. Sample Loading step is composed by three phases: loading of the recovery chamber, parking chamber by buffer/culture media then loading of the main chamber with the sample.

The active area of the chip is scanned by the selected fluorescence channels, in this step pictures of every particles is taken (at 10X magnification). The last step, the cropping, is the processing of every taken picture in order to edit the entire chip, eliminating for example duplicates and assigning to every events an ID number. All these steps are automatic.

The Cell Browser is the analysis software operator dependant. Software interface is divided into analysis panel (where different events visualizations are permitted) and control panel where is possible to view cell pictures selected from analysis panel [Figure 4]. Cell Browser tools allow the in-detail-analysis and if necessary to take new pictures of the target cells/particles with a increased magnification (20x) and different optical settings.

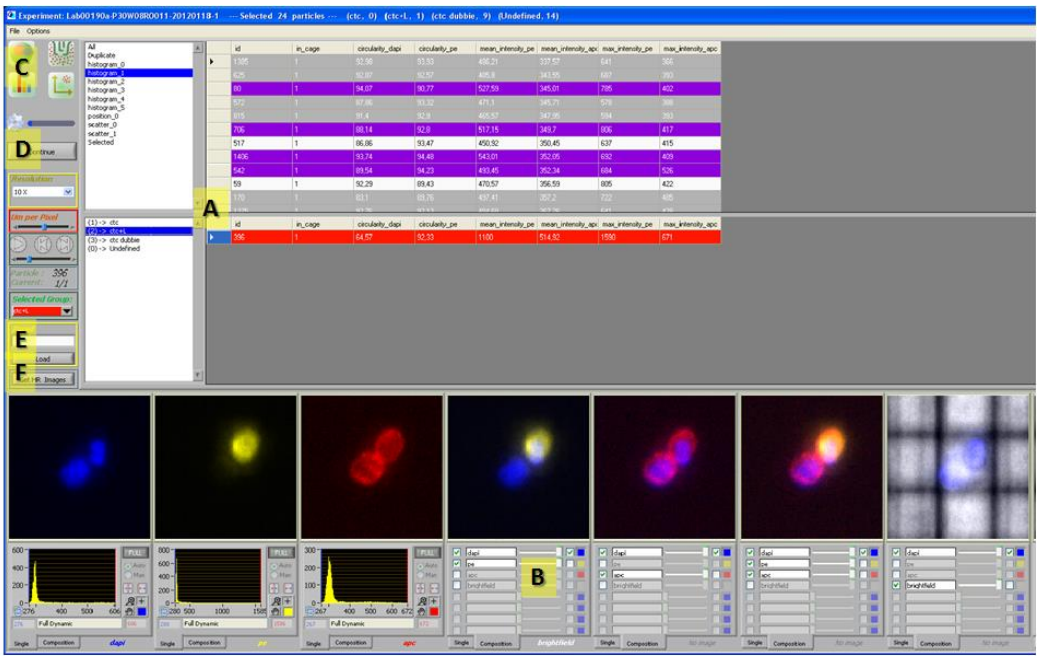


FIGURA 4 CELL BROWSER. THE GRAPHICAL INTERFACE OF THE CELL BROWSER CONSISTS OF ANALYSIS PANEL (A) WHICH LISTS ANALYZED EVENTS IN EACH FLUORESCENCES. CONTROL PANEL (B) DISPLAYING PHOTOGRAPHS FOR EACH SELECTED EVENT. BUTTONS SHOW EVENTS AS SCATTER PLOTS, HISTOGRAMS, SINGLE EVENT LOCALIZATION INTO THE MAIN CHAMBER AS WELL AS ON BY LISTING THEM. THE BUTTON 'CONTINUE' (D) ALLOWS THE SOFTWARE TO LAUNCH CELL DIRECTOR FOR RECOVERY; PANELS OF THE GALLERIES OF IMAGES CAN BE GENERATED (E) AND IT IS POSSIBLE TO RE-CAPTURE IMAGES CHANGING FLUORESCENCE PARAMETERS BY A 20X OBJECTIVE (F).

RECOVERY

Cell Director software is assigned to sort target cells [Figure 5], operators must interact with a graphical user interface which in turn control the active area of the chip. Cell Director designs the electric paths across the main chamber that cells must travel to reach parking chamber, exit chamber and at last be sorted. “Camera Live” tool enables operators to in-streming view step-by-step the route of cells, operators can re-set cells paths in every moments during the travel. One into the exit chamber, cells could be recovered singly with 100% of purity by leaking a established volume of buffer.

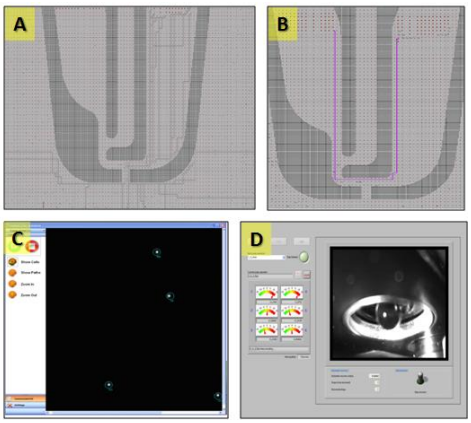


FIGURE 5 CELL DIRECTOR. GRAPHIC INTERFACE WHERE IT IS HIGHLIGHTED THE PATHS THAT CELLS WILL HAVE TO GO THROUGH TO REACH THE PARKING CHAMBER (A) AND THE EXIT CHAMBER (B). CAMERA LIVE SOFTWARE ALLOWING TO MONITOR IN-STREAMING CELLS MOVEMENTS (C). RECOVERY OF THE CELLS

Appendix 3-
Detailed clinical histories of esophageal cancer patients with CTCs analysis.

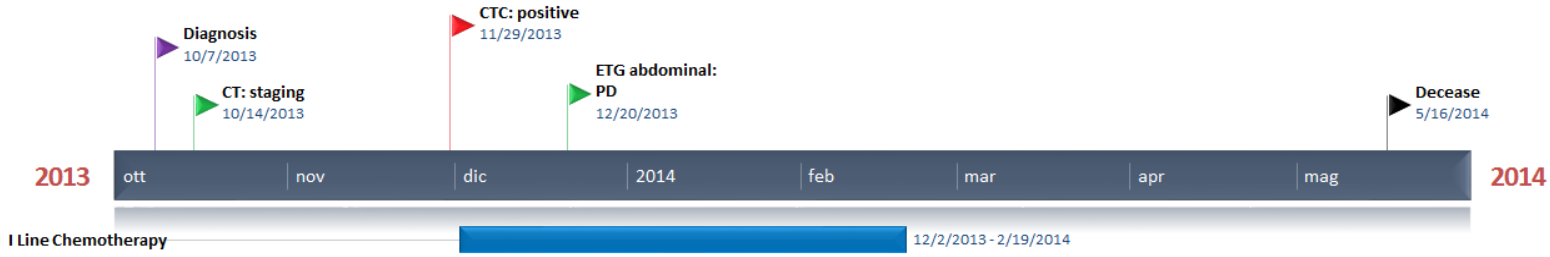
Listed below detailed clinical histories of patients enrolled in this study. Red flags indicate CTCs analysis. Where present, hematoxylin and eosin stains of patients biopsies were kindly given by Dott. Saragoni of the Pathology Unit-Morgagni Pierantoni Hospital , Forlì , Italy.

CT	Computed tomography	**RP**	Partial Responce
PET	Positron Emission Tomography	**SD**	Stable Disease Progression
ETG	Echotomography	**PD**	Disease Complete
EGDS	Esophagoduodenoscopy	**CP**	Response

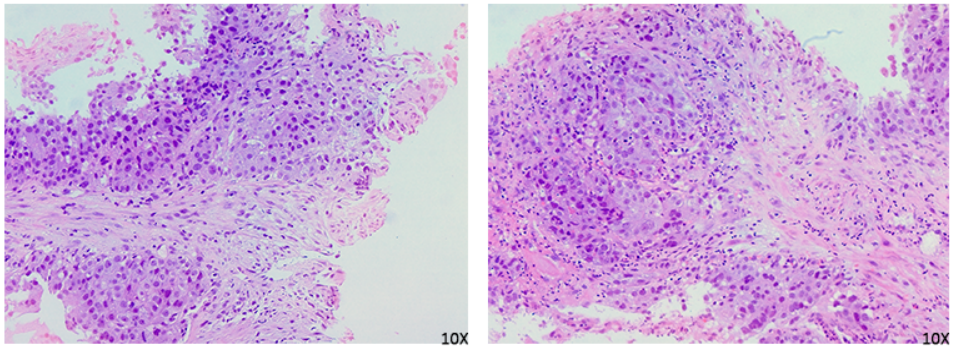
TABLE 2 LIST OF ABBREVIATIONS

Shown below detailed clinical histories of patients enrolled on this study.

M1 PATIENT

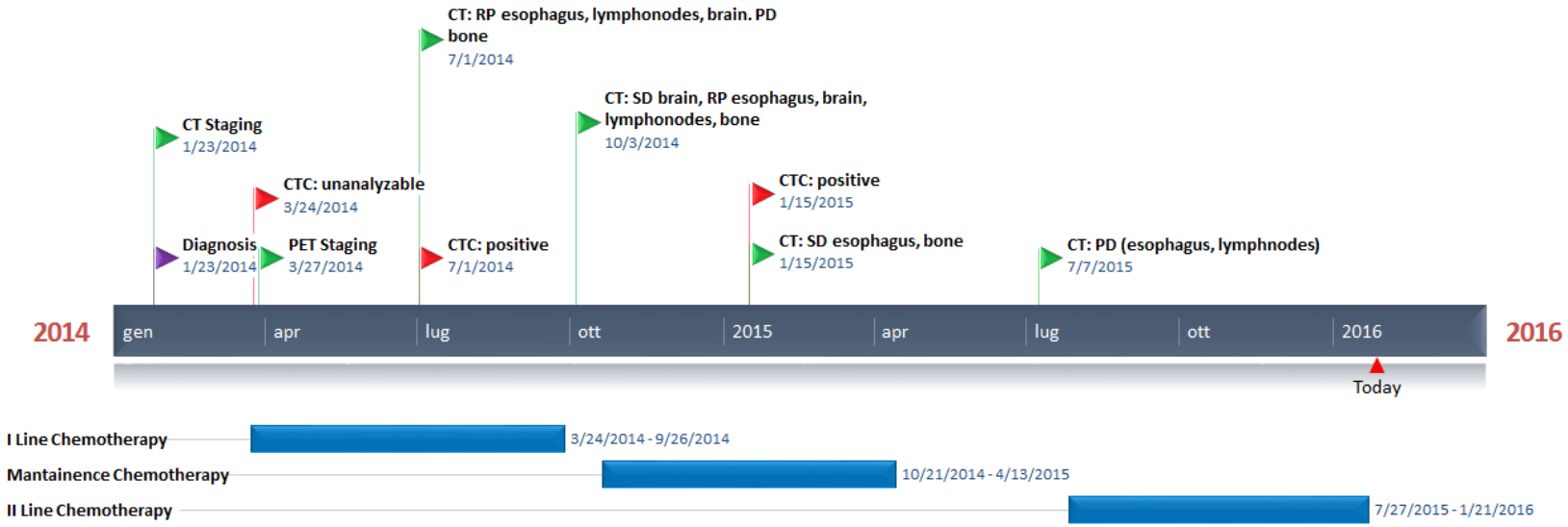


H&E STAINING



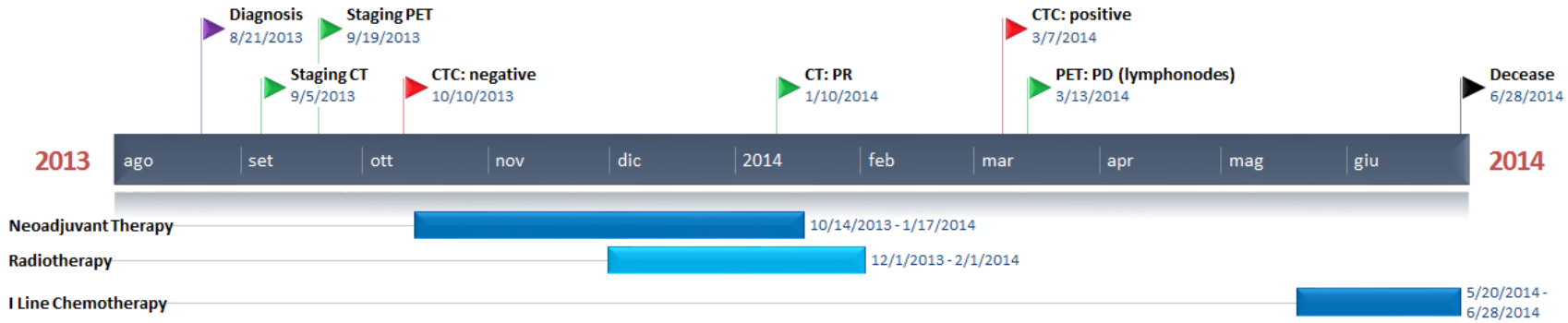
M2 PATIENT

M3 PATIENT



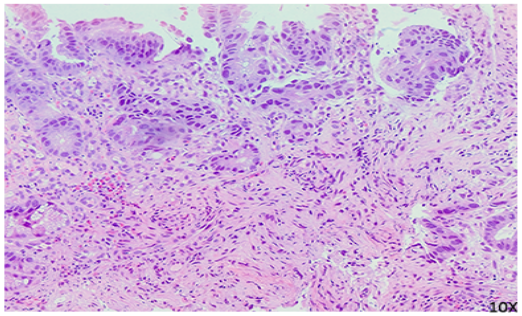
M4 PATIENT

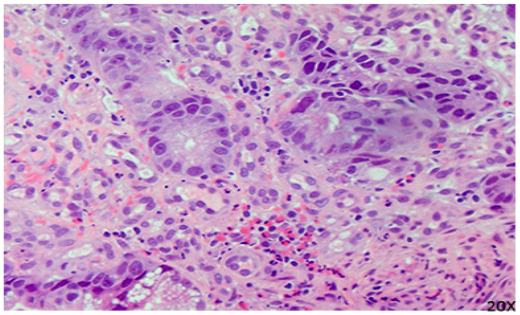
NM1 PATIENT



NM2 PATIENT

H&E STAINING





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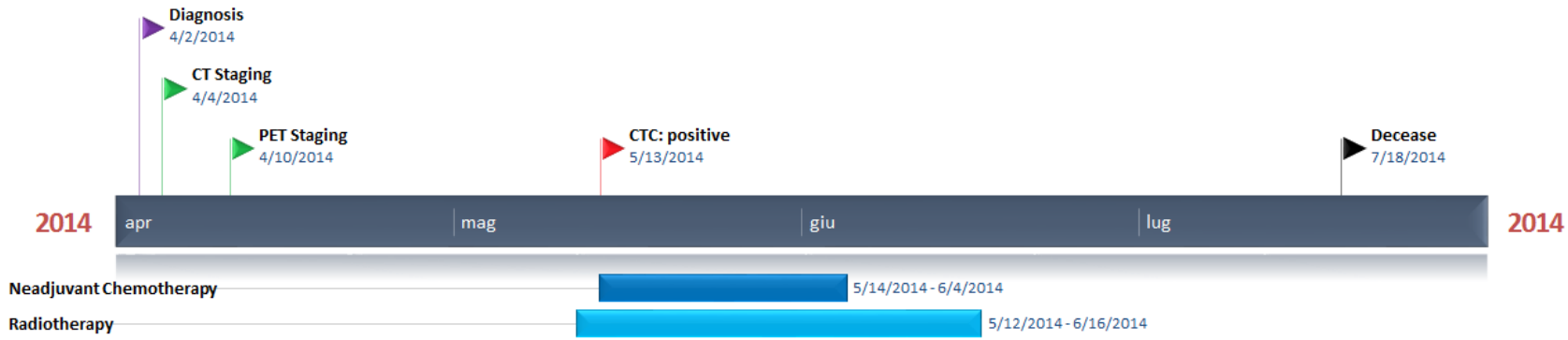
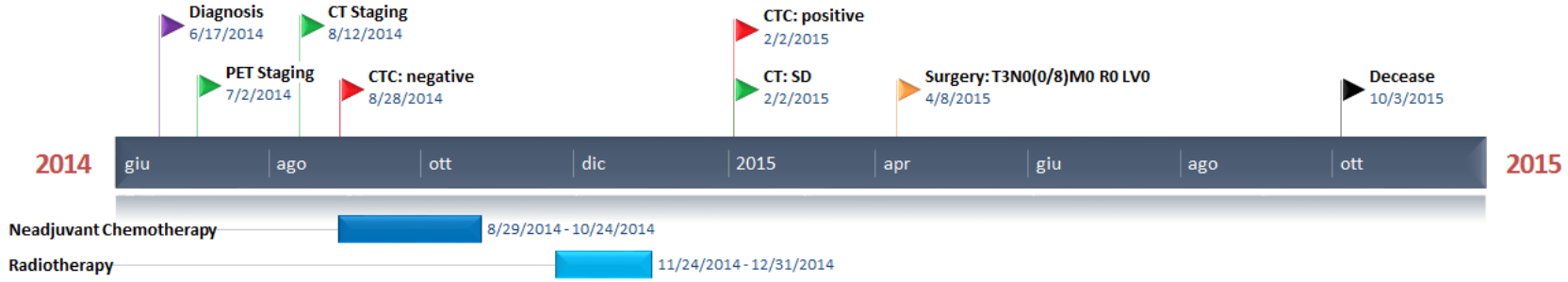
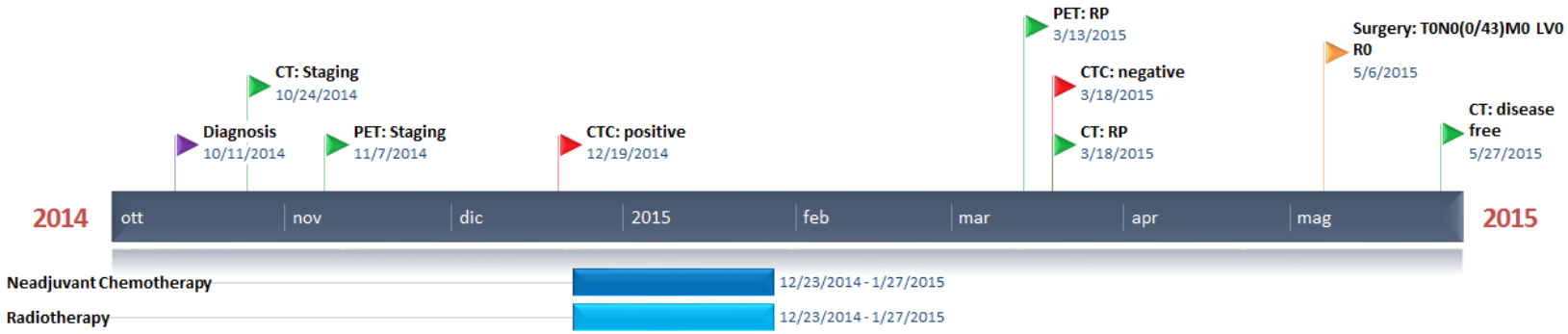


NM4 PATIENT

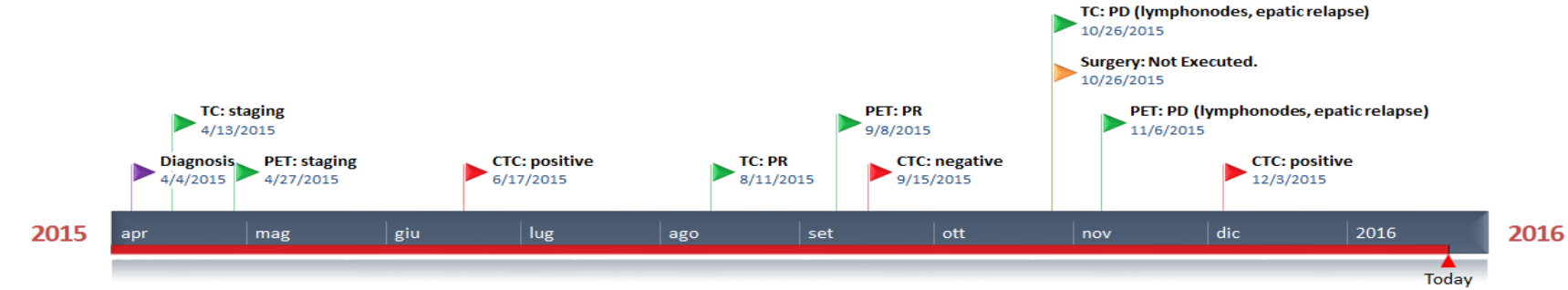


NM5 PATIENT

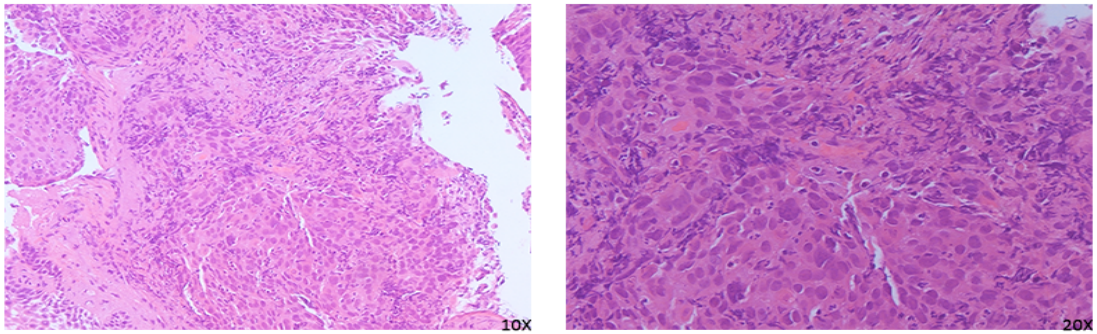


NM6 PATIENT

NM7 PATIENT



H&E STAINING



Appendix-4 Minimal-sample-handling protocol for CTC culture- visiting at Prof. Vescovi Lab.

This pilot study of CTC investigation in non-metastatic esophageal cancer patients put forward that CTC identification and evaluation at different time points during systemic treatment, as a surrogate marker of treatment response as already mentioned in different pathology [168].

Because CTCs are found in circulation as a collectable fraction that is representative of the tumor, they may provide an ideal model to study the biology of the tumor at various intervals before and during treatment [217].

Nonetheless, if CTCs can be isolated from cancer patients as viable cells that can be genotyped and functionally characterized over the course of therapy, they have the potential to identify treatments that most effectively target the evolving mutational profile of the primary tumor[162]

The isolation of viable CTCs is technically challenging: most methods yield low numbers of partially purified CTCs that are fixed before isolation, damaged during the cell purification process, or irreversibly immobilized on an adherent matrix [162].

To lay the basis for address this major challenge, together Professor Vescovi Lab under the supervision of Elena Binda, we applied a minimal-sample-handling protocol for CTC culture from *Scid*-mouse injected with a colon cancer stem cell line (C12).

Cancer stem cells are functionally defined as cells that have enhanced tumor-initiating capacity upon transplantation into a permissive host [68,69,71,218].

C12 is a long-term colon cancer stem cell line established in Prof. Vescovi laboratory from a surgical specimen of primary colon cancer, growing with a specific serum-free stem cell culture medium.

Due to both the spheroid-like growth of C12 and subculturing procedure by dissociating spheres, C12 cell line may bears the self-renewal capacity typical of stem cells [219,220].

Both subcutaneous xenograft and orthotopic xenograft models determined the in vivo tumorigenic potential of C12 cell line.

C12 cells tagged with luciferase gene reporter (C12-luc) were subcutaneously injected into the right flank of Scid/bg mice as referred [221]. After 20/30 days of tumor formation [Fig. 1A], mice were sacrificed, tumor sections were stained with hematoxylin and eosin [Fig.1B]. To confirm the stemness nature of C12-luc cell line, subcutaneous tumor was dissociated and *in vitro* re-cultured. The generated C12-luc showed the identical growth properties of the original C12 derived from patient-tumor [Fig.1C]. The achieving of subsequent generation of tumor and *in vitro* culturing provided the indication that C12 cell line is a *bona fide* colon cancer stem cell.

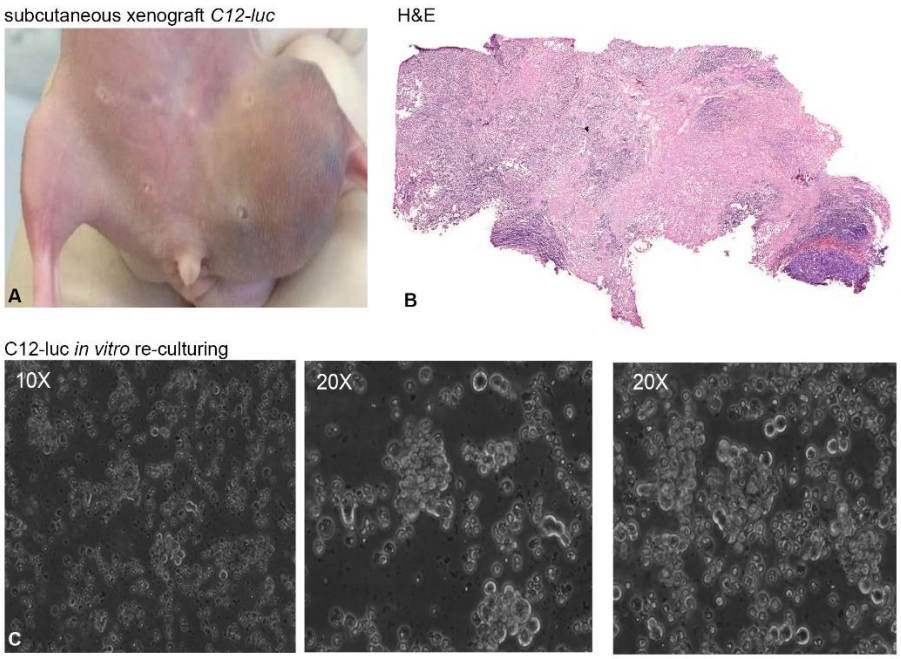


FIGURE 1 C12 CELL LINE STEMNESS VERIFIED BY SUBCUTANEOUS XENOGRAFT. A REPRESENTATIVE IMAGES OF C12 ENGRAFTED SUBCUTANEOUSLY IN SCID-MOUSE. **B** HEMATOXYLIN AND EOSIN OF SUBCUTANEOUS TUMOR SECTION. **C** C12-LUC CELLS RE-CULTURING FROM SUBCUTANEOUS TUMOR, AFTER 15 DAYS CELLS STARTED TO PROLIFERATE IN THE SAME WAY AS THE STARTING C12 CELLS.

Orthotopic xenografts bearing C12-luc cells were performed in order to replicate human disease with a higher fidelity than subcutaneous xenograft. C12-luc cells were injected on the tunica muscularis of the colon at the end of the caecum as by Engleman et al [222]; Tumor growths were well-visible after 20 days after injection as shown by quantitative imaging of luciferase-tagged C12 cells [Fig.2].

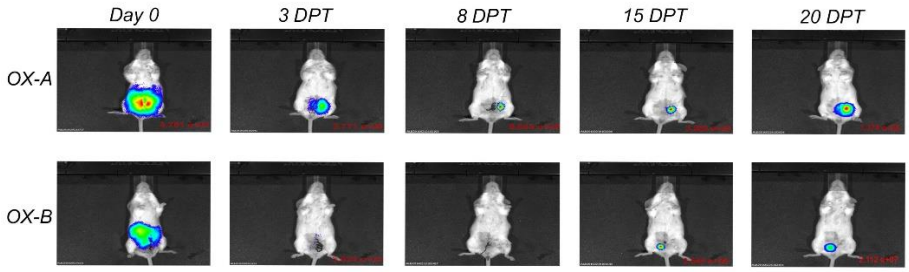


FIGURE 2 ORTHOTOPIC XENOGRAFT OF C12 CELL LINE. TUMOR GROWTH WAS MONITORED BY QUANTITATIVE IMAGING OF LUCIFERASE-TAGGED C12 AND QUANTIFIED BASED ON PHOTON FLUX (PHOTONS PER SECOND PER SQUARE CENTIMETER). AFTER 20 DAYS POST TRANSPLANTATION (DPT) TUMOR MASSES FROM FROM TWO DISTINCT ORTHOTOPIC XENOGRAFTS (OX-A, OX-B) WERE VISIBLE.

Once verified the stemness status of C12 cell line, a controlled number of C12-luc were injected into tail vein of an immunocompromised Scid-mouse.

3/9x10^5 C12 cells were injected into tail vein of three Scid-mouse and let them balanced into the animals circulation for 3/4 hours. Scid-mice were sacrificed by transcardial perfusion (IACUC standard procedure) without fixative solution in order to maintain every cells into the blood available for *in vitro* culturing.

After cutting the right atrium, flowing blood was collected until the fluid (PBS1X) exiting from the right atrium is entirely clear.

The resulting blood samples diluted with a solution of PBS 1X-EDTA underwent to the minimal-sample-handling protocol for CTC culture.

Briefly, after discarded the plasmatic component from the cellular component we performed a gentle erythrocyte lysis until the cell pellets were "colorless".

Enriched samples were then re-suspended and cultured with a selective stem cell culture medium.

About ten days after plating, phase-bright clones reflecting the conventional spheres formed *in vitro* by stem-like cells were detected in all of the three cultures established from the three injected mice [Fig.3A].

Cultured enriched C12 cells shared morphological-growing features with the matched primary C12, suggesting that the ability to form sferoids of the putative cancer stem cells was preserved.

Immunofluorescence tests were performed on a small fraction of enriched C12 cells with an anti-Luc antibody [Fig.3B] as a formal proof of C12 cell line authentication.

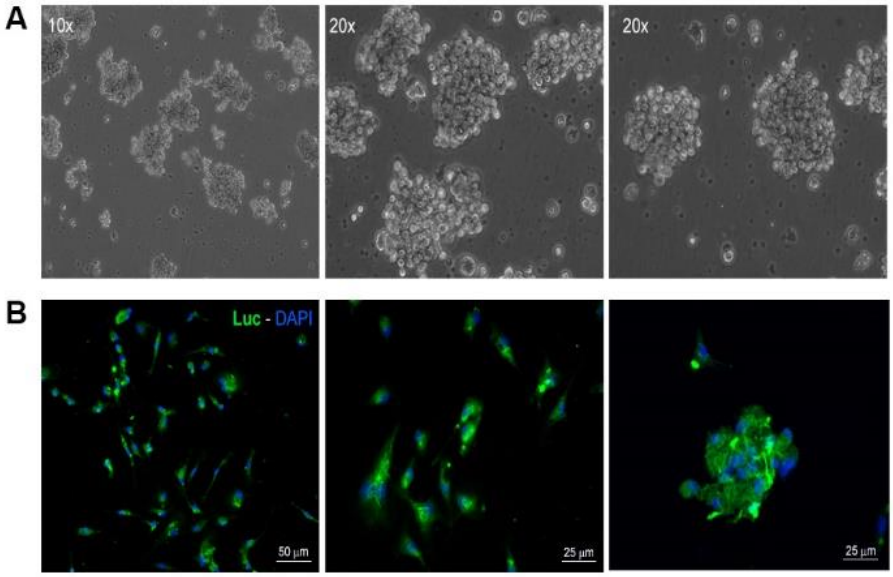


FIGURE 3 CULTURED C12-LUC CELLS FROM WHOLE BLOOD OF TAIL VEIN INJECTED SCID-MICE. A. PHASE-BRIGHT 15 DAYS-CULTURED C12-LUC CELL ENRICHED BY MINIMAL-SAMPLE-HANDLING PROTOCOL FOR CTC CULTURE. ENRICHED CELLS GROWTH IN A SPHEROID-LIKE FASHION AS THE PARENTAL C12-LUC **B.** IMMUNOFLUORESCENCE WITH ANTI-LUC ANTIBODY (GREEN) OF 15 DAYS-CULTURED C12-LUC CELL AS FORMAL PROOF OF CELLS AUTHENTICATION. DAPI (BLUE) COUNTERSTAINED NUCLEUS.

Traditional CTC isolation technologies are limited by extensive cell loss under relatively harsh sample processing conditions that decrease the number and viability of tumor cells circulating in the blood.

During my visiting period at Prof. Vescovi laboratory, we developed a minimal-sample-handling protocol able to get circulating tumor cells in a proper condition for *in vitro* culturing.

The enrichment of the circulating tumor cells with a stem-growth-competence occurred not from the whole blood sample directly, but during the step of cell culture, by culture medium selection.

This new approach minimized both cell lost and cell modifications due to overmuch sample handling; it also opens a window on CTCs culture.

To date orthotopic xenograft model of colon cancer inducted by C12 cell line is ongoing, on this model the protocol for culturing CTCs will be employ. As a future perspective, this protocol will be applied on whole blood from patients affected by cancer.

Bibliography

1. Cristofanilli, M. *et al.* Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med.* **351,** 781–91 (2004).

2. Ashworth, T. R. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *The Medical Journal of Australia* 146 (1869).

3. Shiozawa, Y., Havens, A. M., Pienta, K. J. & Taichman, R. S. The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. *Leukemia* **22,** 941–950 (2008).

4. Fidler, I. J. Cancer biology is the foundation for therapy. *Cancer Biol. Ther.* **4,** 1036–9 (2005).

5. Martin, S. S. & Leder, P. Human MCF10A Mammary Epithelial Cells Undergo Apoptosis following Actin Depolymerization That Is Independent of Attachment and Rescued by Bcl-2. *Mol. Cell. Biol.* **21,** 6529–6536 (2001).

6. Streuli, C. H. & Gilmore, A. P. Adhesion-Mediated Signaling in the Regulation of Mammary Epithelial Cell Survival. *J. Mammary Gland Biol. Neoplasia* **4,** 183–191

7. Wyckoff, J. B., Jones, J. G., Condeelis, J. S. & Segall, J. E. A critical step in metastasis: In vivo analysis of intravasation at the primary tumor. *Cancer Res.* **60,** 2504–2511 (2000).

8. Klein, G. Foulds' dangerous idea revisited: the multistep development of tumors 40 years later. *Adv. Cancer Res.* **72,** 1–23 (1998).

9. Fearon, E. R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **61,** 759–67 (1990).

10. Cairns, J. Mutation selection and the natural history of cancer. *Nature* **255,** 197–200 (1975).

11. Olivier, M. *et al.* The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. *Clin. Cancer Res.* **12,** 1157–67 (2006).

12. Koscielny, S. & Tubiana, M. Parallel progression of tumour and metastases. *Nat. Rev. Cancer* **10,** 156 (2010).

13. WEINBERG, R. A. The many faces of tumor dormancy. *APMIS* **116,** 548–551 (2008).

14. COLLINS, V. P., LOEFFLER, R. K. & TIVEY, H. Observations on growth rates of human tumors. *Am. J. Roentgenol. Radium Ther. Nucl. Med.* **76,** 988–1000 (1956).

15. Friberg, S. & Mattson, S. On the growth rates of human malignant tumors: implications for medical decision making. *J. Surg. Oncol.* **65,** 284–97 (1997).

16. FIDLER, I. J. Selection of Successive Tumour Lines for Metastasis. *Nature* **242,** 148–149 (1973).

17. Klein, C. A. & Hölzel, D. Systemic cancer progression and tumor dormancy: mathematical models meet single cell genomics. *Cell Cycle* **5,** 1788–98 (2006).

18. Baum, B., Settleman, J. & Quinlan, M. P. Transitions between epithelial

and mesenchymal states in development and disease. *Semin. Cell Dev. Biol.* **19,** 294–308 (2008).

19. Hugo, H. *et al.* Epithelial — Mesenchymal and Mesenchymal — Epithelial Transitions in Carcinoma Progression. *J. Cell. Physiol.* 374–383 (2007). doi:10.1002/JCP

20. Thiery, J. P., Acloque, H., Huang, R. Y. J. & Nieto, M. A. Epithelial-mesenchymal transitions in development and disease. *Cell* **139,** 871–90 (2009).

21. Yang, J. & Weinberg, R. a. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev. Cell* **14,** 818–29 (2008).

22. Brabletz, T. To differentiate or not — routes towards metastasis. *Nat. Rev. Cancer* **12,** 425–436 (2012).

23. Kalluri, R. & Weinberg, R. A. The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119,** (2009).

24. López-Nouoa, J. M. & Nieto, M. A. Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. *EMBO Mol. Med.* **1,** 303–314 (2009).

25. Nieto, M. A. The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu. Rev. Cell Dev. Biol.* **27,** 347–76 (2011).

26. Zhang, J. & Ma, L. MicroRNA control of epithelial–mesenchymal transition and metastasis. *Cancer Metastasis Rev.* **31,** 653–662 (2012).

27. Klingbeil, P. & Isacke, C. M. The 'alternative' EMT switch. *Breast Cancer Res.* **13,** 313 (2011).

28. Warzecha, C. C. *et al.* An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *EMBO J.* **29,** 3286–300 (2010).

29. Thiery, J. P. & Sleeman, J. P. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat. Rev. Mol. Cell Biol.* **7,** 131–142 (2006).

30. Hay, E. D. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. *Dev. Dyn.* **233,** 706–720 (2005).

31. Bierie, B. & Moses, H. L. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat. Rev. Cancer* **6,** 506–20 (2006).

32. Bolós, V., Grego-Bessa, J. & de la Pompa, J. L. Notch signaling in development and cancer. *Endocr. Rev.* **28,** 339–63 (2007).

33. Micalizzi, D. S., Farabaugh, S. M. & Ford, H. L. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J. Mammary Gland Biol. Neoplasia* **15,** 117–34 (2010).

34. Evangelista, M., Tian, H. & de Sauvage, F. J. The hedgehog signaling pathway in cancer. *Clin. Cancer Res.* **12,** 5924–8 (2006).

35. Reya, T. & Clevers, H. Wnt signalling in stem cells and cancer. *Nature* **434,** 843–50 (2005).

36. Huber, M. A., Kraut, N. & Beug, H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* **17,** 548–58 (2005).

37. Savagner, P. Leaving the neighborhood: molecular

mechanisms involved during epithelial-mesenchymal transition. *Bioessays* **23,** 912–23 (2001).

38. Thuault, S. *et al.* Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. *J. Cell Biol.* **174,** 175–83 (2006).

39. Zavadil, J. *et al.* Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6686–6691 (2001).

40. Jechlinger, M. *et al.* Autocrine PDGFR signaling promotes mammary cancer metastasis. *J. Clin. Invest.* **116,** 1561–1570 (2006).

41. Dong, M. *et al.* The type III TGF-beta receptor suppresses breast cancer progression. *J. Clin. Invest.* **117,** 206–17 (2007).

42. Eccles, S. a & Welch, D. R. Metastasis: recent discoveries and novel treatment strategies. *Lancet* **369,** 1742–1757 (2007).

43. Karin, M. Nuclear factor-kappaB in cancer development and progression. *Nature* **441,** 431–6 (2006).

44. Huber, M. a *et al.* NF- κ B is essential for epithelial- mesenchymal transition and metastasis in a model of breast cancer progression. *J. Clin. Invest.* **114,** 569–581 (2004).

45. Barrallo-Gimeno, A. & Nieto, M. A. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132,** 3151–61 (2005).

46. Nollet, F., Kools, P. & van Roy, F. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J. Mol. Biol.* **299,** 551–72 (2000).

47. Navarro, P., Lozano, E. & Cano, A. Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells. Possible involvement of plakoglobin. *J. Cell Sci.* **105 (Pt 4,** 923–34 (1993).

48. Cano, A. *et al.* The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2,** 76–83 (2000).

49. Batlle, E. *et al.* The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2,** 84–9 (2000).

50. Nieto, M. A. & Cano, A. The epithelial-mesenchymal transition under control: Global programs to regulate epithelial plasticity. *Semin. Cancer Biol.* **22,** 361–368 (2012).

51. De Craene, B., van Roy, F. & Berx, G. Unraveling signalling cascades for the Snail family of transcription factors. *Cell. Signal.* **17,** 535–47 (2005).

52. Ansieau, S. *et al.* Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell* **14,** 79–89 (2008).

53. Mejlvang, J. *et al.* Direct repression of cyclin D1 by SIP1 attenuates cell cycle progression in cells undergoing an epithelial mesenchymal transition. *Mol. Biol. Cell* **18,** 4615–24 (2007).

54. Rhim, A. D. *et al.* EMT and dissemination precede pancreatic tumor formation. *Cell* **148,** 349–61 (2012).

55. Brabletz, T., Jung, A., Spaderna, S., Hlubek, F. & Kirchner, T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat. Rev. Cancer* **5,** 744–749 (2005).

56. Chaffer, C. L. & Weinberg, R. a. A perspective on cancer cell metastasis. *Science* **331,** 1559–64 (2011).

57. Samavarchi-Tehrani, P. *et al.* Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* **7,** 64–77 (2010).

58. Li, R. *et al.* A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* **7,** 51–63 (2010).

59. Takebe, N. *et al.* Targeting Nothc, HEdgehog, and Wnt Pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol* **12,** 445–464 (2015).

60. Khan, I. N., Al-Karim, S., Bora, R. S., Chaudhary, A. G. & Saini, K. S. Cancer stem cells: a challenging paradigm for designing targeted drug therapies. *Drug Discov. Today* **20,** 1205–16 (2015).

61. Gong, S. *et al.* Regulation of NANOG in cancer cells. *Mol. Carcinog.* **54,** 679–87 (2015).

62. Polyak, K. & Weinberg, R. a. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat. Rev. Cancer* **9,** 265–273 (2009).

63. Polyak, K. & Hahn, W. C. Roots and stems: stem cells in cancer. *Nat. Med.* **12,** 296–300 (2006).

64. Chen, J. *et al.* A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature* **488,** 522–6 (2012).

65. Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432,** 396–401 (2004).

66. Dalerba, P. *et al.* Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 10158–63 (2007).

67. Merlos-Suárez, A. *et al.* The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. *Cell Stem Cell* **8,** 511–524 (2011).

68. O'Brien, C. a, Pollett, A., Gallinger, S. & Dick, J. E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445,** 106–110 (2007).

69. Ricci-Vitiani, L. *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* **445,** 111–115 (2007).

70. Schepers, A. G. *et al.* Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science* **337,** 730–5 (2012).

71. Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. & Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci.* **100,** 3983–3988 (2003).

72. Mani, S. a *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133,** 704–15 (2008).

73. Pece, S. *et al.* Biological and Molecular Heterogeneity of Breast Cancers Correlates with Their Cancer Stem Cell Content. *Cell* **140,** 62–73 (2010).

74. Driessens, G., Beck, B., Caauwe, A., Simons, B. D. & Blanpain, C. Defining the mode of tumour growth by clonal analysis. *Nature* **488,** 527–30 (2012).

75. Malanchi, I. *et al.* Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. *Nature* **452,** 650–3 (2008).

76. Wang, X. *et al.* A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature* **461,** 495–500 (2009).

77. Hermann, P. C. *et al.* Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* **1,** 313–23 (2007).

78. Li, C. *et al.* Identification of pancreatic cancer stem cells. *Cancer Res.* **67,** 1030–7 (2007).

79. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3,** 730–7 (1997).

80. Al-Hajj, M. & Clarke, M. F. Self-renewal and solid tumor stem cells. *Oncogene* **23,** 7274–82 (2004).

81. Müller, a *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410,** 50–56 (2001).

82. Griffiths, E. A., Pritchard, S. A., Welch, I. M., Price, P. M. & West, C. M. Is the hypoxia-inducible factor pathway important in gastric cancer? *Eur. J. Cancer* **41,** 2792–805 (2005).

83. Muller, A. *et al.* Chemokine receptors in head and neck cancer: association with metastatic spread and regulation during chemotherapy. *Int. J. Cancer* **118,** 2147–57 (2006).

84. Pathak, S. K. *et al.* Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies. *Eur. J. Cancer* **41,** 61–70 (2005).

85. Thordur Oskarsson, Eduard Batlle, J. M. Metastatic Stem Cells : Sources , Niches , and Vital Pathways. *Cell Stem Cell* **14,** 306–321 (2014).

86. Ito, S. *et al.* Real-time observation of micrometastasis formation in the living mouse liver using a green fluorescent protein gene-tagged rat tongue carcinoma cell line. *Int. J. Cancer* **93,** 212–7 (2001).

87. Gupta, P. B. *et al.* Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* **146,** 633–44 (2011).

88. Morel, A.-P. *et al.* Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* **3,** e2888 (2008).

89. Gal, a *et al.* Sustained TGF beta exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. *Oncogene* **27,** 1218–30

(2008).

90. James, D., Levine, A. J., Besser, D. & Hemmati-Brivanlou, A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* **132,** 1273–1282 (2005).

91. Bailey, J. M., Singh, P. K. & Hollingsworth, M. A. Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. *J. Cell. Biochem.* **102,** 829–39 (2007).

92. Wang, Z. *et al.* Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res.* **66,** 2778–84 (2006).

93. Fidler, I. J. & Talmadge, J. E. Evidence that intravenously derived murine pulmonary melanoma metastases can originate from the expansion of a single tumor cell. *Cancer Res.* **46,** 5167–71 (1986).

94. Baccelli, I. *et al.* Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat. Biotechnol.* **31,** 539–44 (2013).

95. Yu, M. *et al.* Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* **339,** 580–4 (2013).

96. Mao, W.-M., Zheng, W.-H. & Ling, Z.-Q. Epidemiologic risk factors for esophageal cancer development. *Asian Pac. J. Cancer Prev.* **12,** 2461–6 (2011).

97. Enzinger, P. C. & Mayer, R. J. Esophageal Cancer. *N. Engl. J. Med.* **349,** 2241–2252 (2003).

98. Engel, L. S. *et al.* Population attributable risks of esophageal and gastric cancers. *J. Natl. Cancer Inst.* **95,** 1404–13 (2003).

99. Blot, W. J. & McLaughlin, J. K. The changing epidemiology of esophageal cancer. *Semin. Oncol.* **26,** 2–8 (1999).

100. Lao-Sirieix, P. & Fitzgerald, R. C. Screening for oesophageal cancer. *Nat. Rev. Clin. Oncol.* **9,** 278–87 (2012).

101. Terry, P., Lagergren, J., Ye, W., Nyrén, O. & Wolk, A. Antioxidants and cancers of the esophagus and gastric cardia. *Int. J. Cancer* **87,** 750–4 (2000).

102. Blot, W. J. Continuing Climb in Rates of Esophageal Adenocarcinoma: An Update. *JAMA J. Am. Med. Assoc.* **270,** 1320 (1993).

103. Dawsey, S. M., Lewin, K. J., Liu, F. S., Wang, G. Q. & Shen, Q. Esophageal morphology from Linxian, China. Squamous histologic findings in 754 patients. *Cancer* **73,** 2027–37 (1994).

104. Kountourakis, P., Papademetriou, K., Ardavanis, A. & Papamichael, D. Barrett's esophagus: treatment or observation of a major precursor factor of esophageal cancer? *J. BUON.* **16,** 425–30

105. Parkin, D. M., Bray, F., Ferlay, J. & Pisani, P. Global cancer statistics, 2002. *CA. Cancer J. Clin.* **55,** 74–108

106. Hulscher, J. B., Tijssen, J. G.,

Obertop, H. & van Lanschot, J. J. Transthoracic versus transhiatal resection for carcinoma of the esophagus: a meta-analysis. *Ann. Thorac. Surg.* **72,** 306–13 (2001).

107. Sihvo, E. I. T., Luostarinen, M. E. & Salo, J. A. Fate of patients with adenocarcinoma of the esophagus and the esophagogastric junction: a population-based analysis. *Am. J. Gastroenterol.* **99,** 419–24 (2004).

108. Shahbaz Sarwar, C. M., Luketich, J. D., Landreneau, R. J. & Abbas, G. Esophageal cancer: an update. *Int. J. Surg.* **8,** 417–22 (2010).

109. Izbicki JR, Hosch SB, Pichlmeier U, Rehders A, Busch C, Niendorf A, Passlick B, Broelsch CE, P. K. Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. *N. Engl. J. Med.* **17,** 1188–94 (1997).

110. Vashist, Y. K. *et al.* Disseminated tumor cells in bone marrow and the natural course of resected esophageal cancer. *Ann. Surg.* **255,** 1105–12 (2012).

111. Rice, T. W., Rusch, V. W., Ishwaran, H. & Blackstone, E. H. Cancer of the esophagus and esophagogastric junction: data-driven staging for the seventh edition of the American Joint Committee on Cancer/International Union Against Cancer Cancer Staging Manuals. *Cancer* **116,** 3763–73 (2010).

112. Rice, T. W. *et al.* Worldwide esophageal cancer collaboration. *Dis. Esophagus* **22,** 1–8 (2009).

113. Berry, M. F. Esophageal cancer: staging system and guidelines for staging and treatment. *J. Thorac. Dis.* **6 Suppl 3,** S289–97 (2014).

114. Varghese, T. K. *et al.* The society of thoracic surgeons guidelines on the diagnosis and staging of patients with esophageal cancer. *Ann. Thorac. Surg.* **96,** 346–56 (2013).

115. Daly, J. M. *et al.* Esophageal cancer: results of an American College of Surgeons Patient Care Evaluation Study. *J. Am. Coll. Surg.* **190,** 562–72; discussion 572–3 (2000).

116. Downey, R. J. *et al.* Whole body 18FDG-PET and the response of esophageal cancer to induction therapy: results of a prospective trial. *J. Clin. Oncol.* **21,** 428–32 (2003).

117. Luketich, J. D. *et al.* Role of positron emission tomography in staging esophageal cancer. *Ann. Thorac. Surg.* **64,** 765–9 (1997).

118. Luketich, J. D. *et al.* Evaluation of distant metastases in esophageal cancer: 100 consecutive positron emission tomography scans. *Ann. Thorac. Surg.* **68,** 1133–6; discussion 1136–7 (1999).

119. Krasna, M. J., Reed, C. E., Jaklitsch, M. T., Cushing, D. & Sugarbaker, D. J. Thoracoscopic staging of esophageal cancer: a prospective, multiinstitutional trial. Cancer and Leukemia Group B Thoracic Surgeons. *Ann. Thorac. Surg.* **60,** 1337–40 (1995).

120. Krasna, M. J., Flowers, J. L., Attar, S. & McLaughlin, J. Combined thoracoscopic/laparoscopic staging of esophageal cancer. *J. Thorac. Cardiovasc. Surg.* **111,** 800–6; discussion 806–7 (1996).

121. Kaganoi, J. *et al.* Detection of circulating oesophageal squamous cancer cells in peripheral blood and its impact on prognosis. *Br. J. Surg.* **91,** 1055–1060 (2004).

122. Ito, H. *et al.* Detection and quantification of circulating tumor cells in patients with esophageal cancer by real-time polymerase chain reaction. *J. Exp. Clin. Cancer Res.* **23,** 455–64 (2004).

123. Sclafani, F. *et al.* A Pilot Study Assessing the Incidence and Clinical Significance of Circulating Tumor Cells in Esophagogastric Cancers. *Clin. Colorectal Cancer* **13,** 94–99 (2013).

124. Allard, W. J. *et al.* Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.* **10,** 6897–904 (2004).

125. Reeh, M. *et al.* Circulating Tumor Cells as a Biomarker for Preoperative Prognostic Staging in Patients With Esophageal Cancer. *Ann. Surg.* 1 (2015).

126. Driemel, C. *et al.* Context-dependent adaption of EpCAM expression in early systemic esophageal cancer. *Oncogene* **33,** 4904–15 (2013).

127. Cristofanilli, M. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med.* **351,** 781–91 (2004).

128. Riethdorf, S. *et al.* Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin. Cancer Res.* **13,** 920–8 (2007).

129. Kasimir-Bauer, S., Hoffmann, O., Wallwiener, D., Kimmig, R. & Fehm, T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. *Breast Cancer Res.* **14,** R15 (2012).

130. Liu, S. *et al.* Breast Cancer Stem Cells Transition between Epithelial and Mesenchymal States Reflective of their Normal Counterparts. *Stem Cell Reports* **2,** 78–91 (2013).

131. Zhang, L. *et al.* The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci. Transl. Med.* **5,** 180ra48 (2013).

132. Litvinov, S. V, Velders, M. P., Bakker, H. A., Fleuren, G. J. & Warnaar, S. O. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J. Cell Biol.* **125,** 437–46 (1994).

133. Sieuwerts, A. M. *et al.* Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J. Natl. Cancer Inst.* **101,** 61–66 (2009).

134. Rao, C. G. *et al.* Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. *Int. J. Oncol.* **27,** 49–57 (2005).

135. Khoja, L. *et al.* A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. *Br. J. Cancer* **106,** 508–16 (2012).

136. Gall, T. M. H. & Frampton, A. E. Gene of the month: E-cadherin (CDH1). *J. Clin. Pathol.* **66,** 928–32 (2013).

137. Peinado, H., Portillo, F. & Cano, A.

Transcriptional regulation of cadherins during development and carcinogenesis. *Int. J. Dev. Biol.* **48,** 365–375 (2004).

138. Peinado, H., Quintanilla, M. & Cano, A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J. Biol. Chem.* **278,** 21113–23 (2003).

139. Joosse, S. a. *et al.* Changes in keratin expression during metastatic progression of breast cancer: Impact on the detection of circulating tumor cells. *Clin. Cancer Res.* **18,** 993–1003 (2012).

140. Stott, S. L. *et al.* Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 18392–7 (2010).

141. Allard, W. J. *et al.* Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Non. **10,** 6897–6904 (2005).

142. Hazan, R. B., Qiao, R., Keren, R., Badano, I. & Suyama, K. Cadherin Switch in Tumor Progression. *Ann. N. Y. Acad. Sci.* **1014,** 155–163 (2004).

143. Li, L., Bennett, S. A. L. & Wang, L. Role of E-cadherin and other cell adhesion molecules in survival and differentiation of human pluripotent stem cells. *Cell Adh. Migr.* **6,** 59–70 (2012).

144. Khan, M. A., Chen, H. C., Zhang, D. & Fu, J. Twist: a molecular target in cancer therapeutics. *Tumour Biol* **34,** 2497–2506 (2013).

145. Armstrong, A. J. *et al.* Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. *Mol. Cancer Res.* **9,** 997–1007 (2011).

146. Orian-Rousseau, V. CD44, a therapeutic target for metastasising tumours. *Eur. J. Cancer* **46,** 1271–7 (2010).

147. Tölg, C., Hofmann, M., Herrlich, P. & Ponta, H. Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res.* **21,** 1225–1229 (1993).

148. Orian-Rousseau, V., Chen, L., Sleeman, J. P., Herrlich, P. & Ponta, H. CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev.* **16,** 3074–86 (2002).

149. Jiang, H., Zhao, W. & Shao, W. Prognostic value of CD44 and CD44v6 expression in patients with non-small cell lung cancer: meta-analysis. *Tumour Biol.* **35,** 7383–9 (2014).

150. Chen, Y., Fu, Z., Xu, S., Xu, Y. & Xu, P. The prognostic value of CD44 expression in gastric cancer: a meta-analysis. *Biomed. Pharmacother.* **68,** 693–7 (2014).

151. Chai, L. *et al.* CD44 expression is predictive of poor prognosis in pharyngolaryngeal cancer: systematic review and meta-analysis. *Tohoku J. Exp. Med.* **232,** 9–19 (2014).

152. Hu, B. *et al.* Meta-Analysis of Prognostic and Clinical Significance

of CD44v6 in Esophageal Cancer. *Medicine (Baltimore).* **94,** e1238 (2015).

153. Doyle, L. A. *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15665–70 (1998).

154. Robey, R. W., Polgar, O., Deeken, J., To, K. W. & Bates, S. E. ABCG2: Determining its relevance in clinical drug resistance. *Cancer Metastasis Rev.* **26,** 39–57 (2007).

155. Ross, D. D. & Nakanishi, T. Impact of breast cancer resistance protein on cancer treatment outcomes. *Methods Mol. Biol.* **596,** 251–90 (2010).

156. Tsunoda, S. *et al.* ABCG2 expression is an independent unfavorable prognostic factor in esophageal squamous cell carcinoma. *Oncology* **71,** 251–258 (2006).

157. Breast, H. *et al.* Isolation and Characterization of a Spontaneously Immortalized Isolation and Characterization of a Spontaneously Immortalized Human Breast. *Cancer* 6075–6086 (1990).

158. Fabbri, F. *et al.* Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs. *Cancer Lett.* **335,** 225–31 (2013).

159. Polzer, B. *et al.* Molecular profiling of single circulating tumor cells with diagnostic intention. *EMBO Mol. Med.* **6,** 1371–86 (2014).

160. Morel, A.-P. *et al.* EMT inducers catalyze malignant transformation of mammary epithelial cells and drive tumorigenesis towards claudin-low tumors in transgenic mice. *PLoS Genet.* **8,** e1002723 (2012).

161. Hanahan, D. & Weinberg, R. a. Hallmarks of cancer: the next generation. *Cell* **144,** 646–74 (2011).

162. Yu, M., Stott, S., Toner, M., Maheswaran, S. & Haber, D. a. Circulating tumor cells: approaches to isolation and characterization. *J. Cell Biol.* **192,** 373–82 (2011).

163. Thiery, J. P. Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2,** 442–54 (2002).

164. Maltoni, R. *et al.* Circulating tumor cells in early breast cancer: A connection with vascular invasion. *Cancer Lett.* **367,** 43–48 (2015).

165. Bidard, F.-C. *et al.* Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. *Lancet. Oncol.* **15,** 406–14 (2014).

166. Scher, H. I. *et al.* Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. *Lancet. Oncol.* **10,** 233–9 (2009).

167. Janni, W. *et al.* Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer. *Clin. Cancer Res.* (2016). doi:10.1158/1078-0432.CCR-15-1603

168. Hayes, D. F. *et al.* Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin. Cancer*

Res. **12,** 4218–24 (2006).

169. Danila, D. C. *et al.* Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. *Clin. Cancer Res.* **13,** 7053–8 (2007).

170. Cohen, S. J. *et al.* Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **26,** 3213–21 (2008).

171. Tewes, M. *et al.* Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer: an option for monitoring response to breast cancer related therapies. *Breast Cancer Res. Treat.* **115,** 581–90 (2009).

172. Aktas, B. *et al.* Comparison of estrogen and progesterone receptor status of circulating tumor cells and the primary tumor in metastatic breast cancer patients. *Gynecol. Oncol.* **122,** 356–60 (2011).

173. Andreopoulou, E. *et al.* Comparison of assay methods for detection of circulating tumor cells in metastatic breast cancer: AdnaGen AdnaTest BreastCancer Select/Detect™ versus Veridex CellSearch™ system. *Int. J. Cancer* **130,** 1590–7 (2012).

174. Nagrath, S. *et al.* Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* **450,** 1235–9 (2007).

175. Maheswaran, S. *et al.* Detection of mutations in EGFR in circulating lung-cancer cells. *N. Engl. J. Med.* **359,** 366–77 (2008).

176. Stott, S. L. *et al.* Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer. *Sci. Transl. Med.* **2,** 25ra23 (2010).

177. Ozkumur, E. *et al.* Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. *Sci. Transl. Med.* **5,** 179ra47 (2013).

178. Saucedo-Zeni, N. *et al.* A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *Int. J. Oncol.* **41,** 1241–50 (2012).

179. Lara, O., Tong, X., Zborowski, M. & Chalmers, J. J. Enrichment of rare cancer cells through depletion of normal cells using density and flow-through, immunomagnetic cell separation. *Exp. Hematol.* **32,** 891–904 (2004).

180. Yang, L. *et al.* Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal cells. *Biotechnol. Bioeng.* **102,** 521–34 (2009).

181. He, W. *et al.* Quantitation of circulating tumor cells in blood samples from ovarian and prostate cancer patients using tumor-specific fluorescent ligands. *Int. J. Cancer* **123,** 1968–1973 (2008).

182. Weitz, J. *et al.* Dissemination of tumor cells in patients undergoing surgery for colorectal cancer. *Clin. Cancer Res.* **4,** 343–8 (1998).

183. Rosenberg, R. *et al.* Comparison of two density gradient centrifugation systems for the enrichment of

disseminated tumor cells in blood. *Cytometry* **49,** 150–8 (2002).

184. Vona, G. *et al.* Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells. *Am. J. Pathol.* **156,** 57–63 (2000).

185. Vona, G. *et al.* Impact of cytomorphological detection of circulating tumor cells in patients with liver cancer. *Hepatology* **39,** 792–797 (2004).

186. De Giorgi, V. *et al.* Application of a filtration- and isolation-by-size technique for the detection of circulating tumor cells in cutaneous melanoma. *J. Invest. Dermatol.* **130,** 2440–7 (2010).

187. Hofman, V. J. *et al.* Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method: promises and pitfalls. *Am. J. Clin. Pathol.* **135,** 146–56 (2011).

188. Lecharpentier, A. *et al.* Detection of circulating tumour cells with a hybrid (epithelial/mesenchymal) phenotype in patients with metastatic non-small cell lung cancer. *Br. J. Cancer* **105,** 1338–41 (2011).

189. Farace, F. *et al.* A direct comparison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. *Br. J. Cancer* **105,** 847–53 (2011).

190. Becker, F. F. *et al.* The removal of human leukaemia cells from blood using interdigitated microelectrodes. *J. Phys. D. Appl. Phys.* **27,** 2659–2662 (1994).

191. Becker, F. F. *et al.* Separation of human breast cancer cells from blood by differential dielectric affinity. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 860–4 (1995).

192. Huang, Y., Wang, X. B., Becker, F. F. & Gascoyne, P. R. Introducing dielectrophoresis as a new force field for field-flow fractionation. *Biophys. J.* **73,** 1118–29 (1997).

193. Kirby, B. J. *et al.* Functional characterization of circulating tumor cells with a prostate-cancer-specific microfluidic device. *PLoS One* **7,** e35976 (2012).

194. Wang, Z. P. *et al.* Identification and characterization of circulating prostate carcinoma cells. *Cancer* **88,** 2787–95 (2000).

195. Hou, H. W. *et al.* Isolation and retrieval of circulating tumor cells using centrifugal forces. *Sci. Rep.* **3,** 1259 (2013).

196. Theodoropoulos, P. A. *et al.* Circulating tumor cells with a putative stem cell phenotype in peripheral blood of patients with breast cancer. *Cancer Lett.* **288,** 99–106 (2010).

197. Kallergi, G. *et al.* Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. *Breast Cancer Res.* **13,** R59 (2011).

198. Ignatiadis, M. *et al.* HER2-positive circulating tumor cells in breast cancer. *PLoS One* **6,** e15624 (2011).

199. Pestrin, M. *et al.* Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer

patients. *Breast Cancer Res. Treat.* **118,** 523–530 (2009).

200. Riethdorf, S. *et al.* Detection and HER2 Expression of Circulating Tumor Cells: Prospective Monitoring in Breast Cancer Patients Treated in the Neoadjuvant GeparQuattro Trial. *Clin. Cancer Res.* **16,** 2634–2645 (2010).

201. Shaffer, D. R. *et al.* Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. *Clin. Cancer Res.* **13,** 2023–9 (2007).

202. Lazar, D. C. *et al.* Cytometric comparisons between circulating tumor cells from prostate cancer patients and the prostate-tumor-derived LNCaP cell line. *Phys. Biol.* **9,** 016002 (2012).

203. Meng, S. *et al.* HER-2 gene amplification can be acquired as breast cancer progresses. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 9393–8 (2004).

204. Fehm, T. *et al.* Determination of HER2 status using both serum HER2 levels and circulating tumor cells in patients with recurrent breast cancer whose primary tumor was HER2 negative or of unknown HER2 status. *Breast Cancer Res.* **9,** R74 (2007).

205. Fehm, T. *et al.* Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. *Breast Cancer Res.* **11,** R59 (2009).

206. Wulfing, P. HER2-Positive Circulating Tumor Cells Indicate Poor Clinical Outcome in Stage I to III Breast Cancer Patients. *Clin. Cancer Res.* **12,** 1715–1720 (2006).

207. Leversha, M. A. *et al.* Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. *Clin. Cancer Res.* **15,** 2091–7 (2009).

208. Tomlins, S. A. *et al.* Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* **310,** 644–8 (2005).

209. Attard, G. *et al.* Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. *Cancer Res.* **69,** 2912–8 (2009).

210. Pailler, E. *et al.* Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive non-small-cell lung cancer. *J. Clin. Oncol.* **31,** 2273–81 (2013).

211. Heitzer, E. *et al.* Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. *Cancer Res.* **73,** 2965–75 (2013).

212. Punnoose, E. A. *et al.* Molecular Biomarker Analyses Using Circulating Tumor Cells. *PLoS One* **5,** e12517 (2010).

213. Yu, M. *et al.* RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. *Nature* **487,** 510–3 (2012).

214. Arnold, W. M. & Zimmermann, U. Electro-rotation: development of a technique for dielectric measurements on individual cells and particles. *J. Electrostat.* **21,** 151–191 (1988).

215. Frénéa, M., Faure, S. P., Le Pioufle, B., Coquet, P. & Fujita, H. Positioning living cells on a high-density electrode array by negative dielectrophoresis. *Mater. Sci. Eng. C* **23,** 597–603 (2003).

216. Gascoyne, P. R. C. & Vykoukal, J. Particle separation by dielectrophoresis. *Electrophoresis* **23,** 1973–83 (2002).

217. Shiozawa, Y. *et al.* Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow. *J. Clin. Invest.* **121,** 1298–1312 (2011).

218. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367,** 645–8 (1994).

219. Potten, C. S. & Loeffler, M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110,** 1001–20 (1990).

220. He, S., Nakada, D. & Morrison, S. J. Mechanisms of stem cell self-renewal. *Annu. Rev. Cell Dev. Biol.* **25,** 377–406 (2009).

221. Galli, R. *et al.* Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res.* **64,** 7011–7021 (2004).

222. Tseng, W., Leong, X. & Engleman, E. Orthotopic mouse model of colorectal cancer. *J. Vis. Exp.* 484 (2007). doi:10.3791/484

List of publication and conference poster 2013-2015

Maltoni R*, Fici P*, Amadori D, Gallerani G, Cocchi C, Zoli M, Rocca A, Cecconetto L, Folli S, Scarpi E, Serra P, Fabbri F. Circulating tumor cells in early breast cancer: a connection with vascular invasion, Cancer letters 367 (1), 43-48 (2015)

Barriere G, Fici P, Gallerani G, Fabbri F, Rigaud M. Epithelial Mesenchymal Transition: a double-edged sword. Clinical and translational medicine 4 (1), 14 (2015) (Review)

Barriere G., Fici P., Gallerani G., Fabbri F, Zoli W., Rigaud M. Circulating tumor cells and epithelial, mesenchymal and stemness markers: characterization of cell subpopulations. Annals of Traslational Medicine (2014) novembre vol. 2, No 11: 1-8 (Review)

Gallerani G, Fici P, Valgiusti M, Amadori D, Mattioli S, Fabbri F, "The grab all-assay: a multi-parametric approach to identify heterogeneous circulating tumour cells"Poster Session XII Congress of the European Society for Diseases of the Esophagus (ESDE), November 2014 (Italy).

Gallerani G, Zoli M, Fici P, Bravaccini S, Tumedei M, Delmonte A, Burgio MA, Casadei C, Fabbri F, "EML4-ALK status investigation in CTCs in NSCLC advanced adeno-carcinoma patients: preliminary investigation using an in vivo procedure", Advances in Circulating Tumor Cells (ACTC): From Basic Research to Clinical Practice, September 2014 (Crete)

Gallerani G, Fici P, Amadori D, Zoli W, Fabbri F, A multi-parametric prospect/approach to pinpoint heterogenic circulating tumor cells, Poster session "International Symposium on minimal residual cancer", September 2013 (Paris)

Fabbri F, Fici P, Gallerani G, Ulivi P, Chiadini E, Amadori D, Zoli W. Circulating colon and lung cancer cell heterogeneity. Poster session "International Symposium on minimal residual cancer", September 2013 (Paris)

Fabbri F, Carloni S, Zoli W, Ulivi P, Gallerani G, Fici P, Chiadini E, Passardi A, Frassineti GL, Ragazzini A, Amadori D. Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs. Cancer Lett. 2013 feb 15.