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Review

Circulating tumor cells: Towards mechanical phenotyping of metastasis

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SUMMARY

During cancer progression, metastatic dissemination accounts for ~90% of death in patients. Metastasis occurs upon dissemination of circulating tumor cells (CTC) through body fluids, in particular the bloodstream, and several key steps remain elusive. Although the majority of CTCs travel as single cells, they can form clusters either with themselves (homoclusters) or with other circulating cells (heteroclusters) and thereby increase their metastatic potential. In addition, cancer cell mechanics and mechanical cues from the microenvironment are important factors during metastatic progression. Recent progress in intravital imaging technologies, biophysical methods, and microfluidic-based isolation of CTCs allow now to probe mechanics at single cell resolution while shedding light on key steps of the hematogenous metastatic cascade. In this review, we discuss the importance of CTC mechanics and their correlation with metastatic success and how such development could lead to the identification of therapeutically relevant targets.

INTRODUCTION

The metastatic cascade culminates with cancer cells leaving the primary tumor via body fluids, on their way to vital organs. A plethora of studies now suggest that disseminating tumor cells build on preexisting pre-metastatic niches, which are also under the control of the primary tumor and its ability to secrete pro-metastatic cues, including extracellular vesicles (extensively reviewed by (Ghoroghi et al., 2021)). Upon entering the blood or the lymphatic vasculature, cancer cells become circulating tumor cells (CTCs) (Follain et al., 2020; Lambert et al., 2017) and thereby are subjected to several microenvironmental cues that shape their ability to colonize distant organs. Although metastasis evolves through a series of consecutive steps, tumor cells disseminate very early, even before the primary tumor is fully detectable (Harper et al., 2016; Hosseini et al., 2016; Hu and Curtis, 2020; Hüsemann et al., 2008; Nagrath et al., 2007; Rhim et al., 2012). Accordingly, recent large-scale genomics data from paired primary tumors and metastases revealed low genomic divergence suggesting that metastases commonly derive from a major clone in the primary tumor several years before diagnosis (Hu and Curtis, 2020). Furthermore, CTCs can colonize their tumors of origin in a process known as "tumor self-seeding" (Kim et al., 2009; Lambert et al., 2017). Hence, a holistic approach is being adopted to better understand its complexity (Suhail et al., 2019).

The genomic contribution to tumorigenesis has been well characterized using global "omics" approaches and the influence of the cellular environment on metastatic progression is well established. The biomechanical properties of the tumor and its microenvironments are also important factors in cancer progression (Gensbittel et al., 2021). More recently, the mechanics of body fluids, of CTCs themselves and of other blood or lymph components have proven to be essential for different processes toward the successful secondary tumor seeding (Follain et al., 2020; Gensbittel et al., 2021).

While growing, tumors need oxygen, nutrients, and a way to drain the interstitial fluid, which lead to tumor vascularization through angiogenesis and lymphangiogenesis (Weis and Cheresh, 2011). Cancer cells enter either blood or lymphatics vessels (Aceto et al., 2014; Giampieri et al., 2009) by co-opting resident cells (Pignatelli et al., 2016). The vascular and lymphatic systems present different biophysical properties. Their flow profiles, circulating cells densities, and fluid shear stress (FSS) (i.e., stress generated when a fluid flow moves tangentially to a surface creating deformation in the flow direction) vary among arteries, veins, capillaries, and lymphatics vessels (extensively reviewed by (Follain et al., 2020)). Such fluidic properties have profound impacts on the fate of CTCs. While the high flow velocity and FSS in arteries cause CTCs to face

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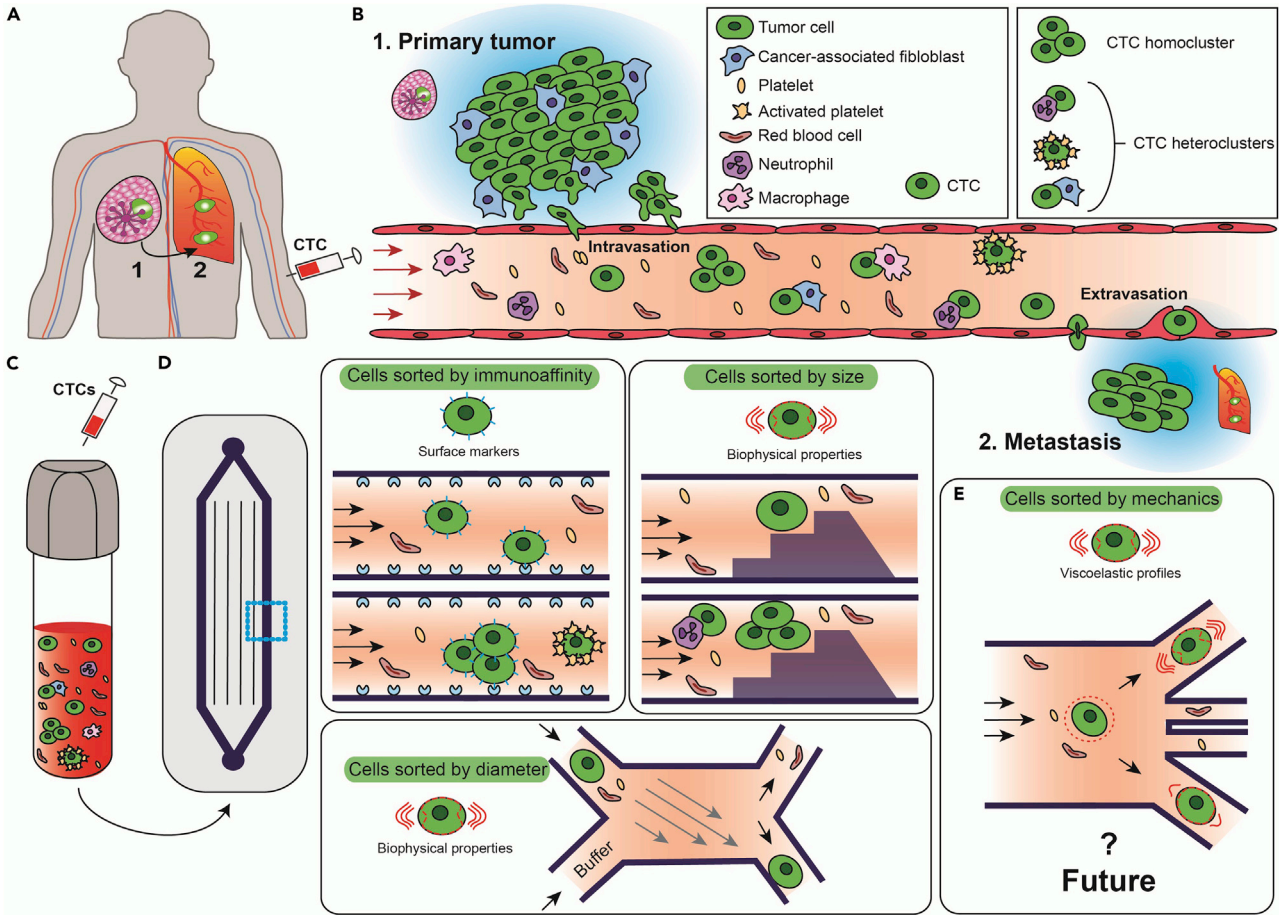

Figure 1. CTCs microfluidic-based isolation approaches

(A) Schematic representation of liquid biopsy performed in a patient with pulmonary metastasis derived from breast primary tumor.

(B) Metastatic cascade representation showing tumor cells leaving the breast tumor and CTCs seeding pulmonary metastasis.

(C) Schematic representation of a cancer patient blood sample analysis to isolate CTCs using microfluidic devices.

(D) Schematic representation of different isolation approaches based on CTCs immunoaffinity, size, or diameter.

(E) Potential clinical use of CTCs viscoelastic properties for isolation and characterization using microfluidic devices.

physical damage leading to cell fragmentation and death (Headley et al., 2016; Regmi et al., 2017), reduced flow profiles in veins favor CTCs intravascular arrest and extravasation (Follain et al., 2018, 2021; Osmani et al., 2019). The lower flow velocity and FSS in lymphatic compared to blood vessels (Dixon et al., 2006) might be less deleterious to CTCs. Interestingly, hematogenous and lymphatic metastasis are not mutually exclusive, after lymph node colonization, a sign of poor prognosis, CTCs can travel to blood vessels (Brown et al., 2018; Pereira et al., 2018).

As patient's blood can be easily collected, liquid biopsies have emerged as a powerful noninvasive tool for prognosis, treatment selection, and monitoring in several cancer types (Keller and Pantel, 2019) (Figures 1A and 1C). Although more work is needed to understand how CTCs mechanics shape tumor metastasis, we and others have proposed that mechanical profiling of CTCs might be relevant as a prognosis biomarker (Alibert et al., 2017; Gensbittel et al., 2021). Thus, a better understanding of CTCs mechanical properties, and the interactions likely to perturb them, will contribute to improve detection accuracy and personalize therapeutic targets design.

In this review, we first detail CTCs characteristics and specificities. We further discuss their ability to form clusters through homo-interactions or with other cell types enhancing their metastatic potential. We finally discuss how such interactions are likely to tune the biomechanical properties of CTCs and their metastatic success. We complement such description with a careful review of some microfluidic platforms that exploit such properties for isolating CTCs and discuss their potential when designing precision medicine strategies.

CTCs in transit : Characteristics, metastatic potential, and isolation methods

Cancer cell dissemination starts as cancer cells acquire invasive potential promoting their escape from the primary tumor (Hanahan and Weinberg, 2011). Epithelial to mesenchymal transition (EMT) of tumor cells, the transdifferentiation process in which cells lose their epithelial characteristics and acquire mesenchymal ones, within the primary site is thought to be a major driver of cancer cell dissemination and thus the generation of CTCs (Caramel et al., 2013). Upon entering the vasculature, and despite disseminating efficiently from the primary tumor (Al-Hajj et al., 2003) (Figure 1B), the number of CTCs forming secondary tumors is very low due to several limiting factors (Cameron et al., 2000). Most of CTCs will die because of hemodynamic forces inducing apoptosis (Headley et al., 2016; Regmi et al., 2017), anoikis (owing to the loss of integrin-mediated adhesion to the extracellular matrix (ECM)) (Kim et al., 2012) or attacked by the immune system (Chen and Mellman, 2017). Surviving CTCs exit the vasculature (extravasate) either by squeezing through endothelial cells (diapedesis) or by co-opting the endothelium (Follain et al., 2018). Estimations suggest that roughly 0.01% of extravasated CTCs invading distant organs effectively form new colonies implying that most of the cells might eventually enter dormancy or die in their new environment (Chambers et al., 2002; Lambert et al., 2017; Luzzi et al., 1998). Interestingly, EMT and WNT signaling upregulation lead to CTCs anoikis resistance improving their survival (Kim et al., 2012; Yu et al., 2012). In addition, mesenchymal to epithelial transition (MET) takes place upon arrival at the secondary site (Beerling et al., 2016; Ocaña et al., 2012; Tsai et al., 2012) suggesting that the plasticity between these two states is a major driver of metastasis success (Beerling et al., 2016; Pastushenko et al., 2018). Recent studies using single-cell lineage tracing of metastatic cancer cells data, including CTCs, support the latter idea. All the cells analyzed occupied a continuum of EMT states and the highest metastatic potential corresponded with hybrid EMT states (Simeonov et al., 2021; Yu et al., 2013). Subsequently, CTC populations are heterogeneous and gene expression levels vary from cell to cell even within the same patient sample (Pixberg et al., 2017; Powell et al., 2012). The CTC subpopulations able to seed secondary tumors are known as metastasis-initiating cells (MICs) or tumor initiating cells (TICs) (Al-Hajj et al., 2003; Baccelli et al., 2013; Ye et al., 2015). Whether they display cancer stem cell (CSC)-like properties or have a regenerative phenotype remains to be elucidated (Ganesh and Massagué, 2021). However, there is consensus regarding CTCs showing hybrid EMT states linked with both adverse disease outcome and drug resistance (Lambert and Weinberg, 2021).

Microfluidic isolation of CTCs

Currently, there are numerous approaches used for CTC isolation that can be broadly divided into two groups (extensively reviewed in (Bankó et al., 2019) and (Lei, 2020)): 1. Biological properties-based (e.g., marker expression) and 2. Physical properties-based (e.g., size/deformability). Belonging to the first group, the CellSearch system is the only FDA-approved device and relies heavily on the expression of epithelial markers such as epithelial cell adhesion molecule (EpCAM) and cytokeratins (Cristofanilli et al., 2004). The advent of microfabrication has led to the appearance of microdevices with precisely controlled flow parameters. It is thus not surprising that CTCs isolation methods were developed using elegant microfluidic-based technologies. One example is the microfluidic-based CTC-chip, which captures CTCs interacting with EpCAM-coated microposts under controlled laminar flow conditions (Nagrath et al., 2007) (Figure 1D). Microfluidic devices contain structures that are comparable to the cell length scale, such as microposts, and allow for the precise control over sample flow increasing the time for cell-antibody contact, thus improving cell capture efficiency. To improve the outcome of CTC-chip, the herringbone-chip (HB-Chip) generates microvortices to increase the interactions between CTCs and the antibody-coated chip surface (Stott et al., 2010). Finally, microfluidic devices also are used to improve the sensitivity and throughput of EpCAM CTC isolation in devices such as the CTC-iChip (Mishra et al., 2020; Ozkumur et al., 2013). The GILUPI Cell Collector, an innovative *in vivo* approach, uses a medical wire functionalized with anti-EpCAM antibodies inserted into the cubital vein of patients overcoming blood volume limitations presents in other CTCs isolation assays (Saucedo-Zeni et al., 2012). However, these isolation methods have led to a selection bias, losing valuable information from CTCs presenting hybrid EMT state markers. Nowadays, new markers such as the actin bundling protein Plastin-3 and the oncofetal chondroitin sulfate which are expressed on epithelial, mesenchymal, and hybrid EMT states CTCs, are being used to refine the detection methods and will play a key role to determine the association between CTCs EMT status and clinical outcome (Agerbæk et al., 2018; Alix-Panabières et al., 2005; Ueo et al., 2015; Yokobori et al., 2013). The second group bypasses the EMT bias relying on biophysical properties of CTCs compared to other blood components (Alibert et al., 2017; Xin et al., 2019). An example is the Parsortix device, a microfluidic based technology that captures CTCs based on physical properties taking advantage of CTCs being bigger and less compressible than other blood cells (Miller et al., 2018; Xu et al., 2015). Other examples of high-

throughput microfluidics capturing CTCs based on size and/or deformability include ClearCell FX (Lee et al., 2018), RUBYchip (Lopes et al., 2021) and CROSS chip (Ribeiro-Samy et al., 2019). A downside of these approaches is that clinically informative tumor cell fragments and extracellular vesicles would be lost. Furthermore, none of these types of devices have been approved for clinical applications. Nevertheless, microfluidic-based separation has the advantage of integrating various biophysical approaches to CTCs isolation such as dielectrophoresis, which separates cells depending on their polarizability (Gascoyne and Shim, 2014), and acoustic techniques, which use the acoustic field to separate CTCs depending on their mechanical properties (Gao et al., 2020).

Better and stronger together : *When CTCs travel as homo-clusters*

Despite being rarer than single CTCs, groups of two or more CTCs known as CTC clusters show higher metastatic potential and relate to worse outcomes in several cancer types (Aceto et al., 2014; Jansson et al., 2016). Lately, the idea of metastasis arising only from single CTCs was challenged by several genomic and intravital imaging studies revealing that metastatic colonies are composed of multiple genetically distinct clones and that polyclonal collective invasion is highly efficient at initiating the early steps of metastasis (Casasent et al., 2018; Cheung et al., 2016). It was thought that CTC clusters were too big to circulate through capillaries to reach distant organs; therefore, arresting fast and leading to the rupture of vessel walls (Wong et al., 2002). However, an innovative microfluidic-based approach designed to mimic human capillary constrictions revealed that clusters are able to pass through narrow capillaries by unfolding into single-file chains which might explain their increased metastatic potential (Au et al., 2016).

Classically, CTC clusters were described as deriving from oligoclonal tumor cell groupings instead of from intravascular aggregation events in breast cancer (Aceto et al., 2014). Recent studies provided molecular basis for CTC cluster formation, before or after intravasation, involving cell adhesion molecules such as CD44 and ICAM-1 (Liu et al., 2019; Taftaf et al., 2021). In addition, the EMT transcription factor Snail induces the expression of Claudin 11 (tight junctional protein) prompting cluster formation in squamous cell carcinoma (Li et al., 2019). Moreover, breast CTC clusters generated *in vitro* show Heparanase induced FAK- and ICAM-1- dependent cell adhesion promoting cell aggregation (Wei et al., 2018). CTCs within the clusters display stable Plakoglobin-dependent, Keratin 14-dependent, and E-cadherin-dependent cell adhesions while traveling in the blood (Aceto et al., 2014; Cheung et al., 2016; Padmanaban et al., 2019) suggesting some of these clusters might retain at least partial epithelial identity. CTCs within the clusters show hybrid EMT states (Pastushenko et al., 2018), with a majority displaying mesenchymal markers (Yu et al., 2013).

Several differences between single CTCs and clusters have been described as responsible for the increased metastatic potential of the clusters. First, although subpopulations of single CTCs are apoptotic or proliferative, neither are observed in CTC clusters implicating anoikis protection, stemness, and higher resistance to cytotoxic drugs (Aceto et al., 2014; Gkountela et al., 2019; Hou et al., 2012; Liu et al., 2019; Taftaf et al., 2021). At the molecular level, anoikis resistance was linked to the higher expression of plakoglobin and Bcl2 (anti-apoptotic) in CTC clusters (Huang et al., 2019; Thangavel et al., 2019). Second, while single CTCs show damaged mitochondria that produce increased reactive oxygen species (ROS) inducing apoptosis, CTCs within clusters switch their cellular metabolism to glycolysis and clear damaged mitochondria limiting ROS and increasing metastatic capacity *in vitro* (Labuschagne et al., 2019). Third, binding sites for stemness- and proliferation-associated transcription factors are hypomethylated in CTC clusters promoting stemness and thus enhancing metastatic success (Gkountela et al., 2019).

On the same line, disaggregation of CTC clusters using several approaches such as CD44 depletion, plakoglobin knockdown, urokinase-type plasminogen activator (thrombolytic agent) injections, and Na^+/K^+-ATPase inhibitors treatment, leads to reduced metastatic potential (Aceto et al., 2014; Choi et al., 2015; Gkountela et al., 2019; Labuschagne et al., 2019; Liu et al., 2019).

Microfluidic isolation of homo-clusters of CTCs

Currently, there are several approaches used for CTC cluster isolation, some like the CellSearch device were designed to capture single CTCs, but can be used to isolate clusters (Hou et al., 2012; Paoletti et al., 2019), although may not fully preserve their integrity and miss clusters presenting hybrid EMT state markers. The Cluster-Chip method provides a more tailored isolation technique, where CTC clusters are captured in specialized bifurcating traps under low-FSS conditions that preserve their integrity (Aceto et al., 2014; Sarioglu et al., 2015). Recently, the Parsortix microfluidic device has shown high efficiency

isolating CTC clusters (99.3% capture rate) avoiding artificial cluster formation because of sample processing (Gkountela et al., 2019) (Figure 1D).

Looking for allies : *Interaction of CTCs with other blood components*

The blood and lymph microenvironment are complex fluidic ecosystems composed of multiple cell types, soluble and encapsulated factors (i.e., extracellular vesicles). The interaction between CTCs and other cell types is pivotal for their metastatic success (Heeke et al., 2019; San Juan et al., 2019) (Figure 1B).

Among the cell types interacting with CTCs are cancer associated fibroblasts (CAFs), which derive from the stroma of the primary tumor and facilitate metastasis (Ao et al., 2015; Ortiz-Otero et al., 2020). CAFs drastically remodel the tumor microenvironment secreting cytokine, chemokines, and growth factors that promote angiogenesis, cancer cells migration, and the evasion of the immune system increasing CTCs viability (Monteran and Erez, 2019).

CTCs also associate with neutrophils enhancing their metastatic potential (Tao et al., 2016). An increase in the neutrophil-to-lymphocyte ratio had been established as a sign of poor prognosis in solid tumors (Templeton et al., 2016); however, the mechanistic explanation behind was unclear. It was recently shown that CTC-neutrophils association leads to the activation of the genetic programs promoting cell cycle progression and providing a likely mechanistic explanation for the enhancement of their metastatic potential (Szczerba et al., 2019). In addition, neutrophils inhibit natural killer (NK) (cytotoxic lymphocytes) function protecting CTCs (Spiegel et al., 2016). Neutrophils can trap CTCs releasing neutrophil extracellular traps (NETs), extracellular DNA webs released in response to inflammatory cues. This interaction relies on the β1-integrin expression in both cell types and can awaken dormant cancer cells leading to metastasis formation (Cools-Lartigue et al., 2013). Interestingly, CTCs attach to arrested neutrophils, use them as a bridge to bind endothelial cells in the liver promoting metastasis (Spicer et al., 2012). The development of *in vitro* microvascular networks (Chen et al., 2013), as part of the novel organ-on-a-chip devices, allows high-resolution time-lapse microscopy to better understand CTCs extravasation mechanisms. Organ-on-a-chip devices consist of microfluidic channels used as scaffolds for organ-specific cells where external artificial cues including biomechanical forces can be applied to mimic the biochemical and biophysical microenvironment of living organs. A combination of *in vivo* (mouse) and *in vitro* (microvascular networks) imaging revealed that neutrophils facilitate CTCs extravasation secreting IL1β and matrix metalloproteinases (Spiegel et al., 2016). Besides, the endothelial glycocalyx can immobilize inflamed neutrophils by self-secreted IL-8 and tumor-derived CXCL-1, leading to neutrophils sequestration with arrested tumor cells and increasing the extravasation potential of the CTCs (Chen et al., 2018).

CTCs can also bind platelets, increasing their metastatic potential (San Juan et al., 2019) and favoring immune evasion by shielding CTCs from NK immunosurveillance (Palumbo et al., 2005). While NK eliminates targets with low expression of major histocompatibility complex (MHC) class I, CTCs coated with platelets displaying high expression of platelet-derived normal MHC class I are spared (Placke et al., 2012). The crosstalk between CTCs and platelets not only modify the tumoral cells, platelets can take up circulating mRNA from CTCs becoming tumor-educated platelets (TEPs) modifying their transcriptome profile (Best et al., 2015). The interaction between CTCs and platelets lead to platelet activation and release of mediators that modify the behavior of the CTCs and surrounding cells (Labelle et al., 2011; Schumacher et al., 2013; Ward et al., 2018). Platelet-derived TGFβ and direct CTC-platelet contact induce EMT in CTCs via TGFβ/Smad and NF-kB pathways activation promoting metastasis. Ablation of platelet-derived TGFβ and NF-kB inhibition in CTCs reduced metastatic success (Labelle et al., 2011). Recently, a direct interaction between platelets and the adhesion G protein-coupled receptor (GPCR) CD97 leading to platelets activation has been uncovered. This interaction leads to granule secretion, including ATP release (Ward et al., 2018) which acts through P2Y2 receptors on endothelial cells rendering the endothelium more permeable thus facilitating CTCs extravasation. Both P2Y2 receptors ablation and ATP release blockade strongly reduce metastasis (Schumacher et al., 2013). Another known interaction occurs between α6β1-integrin on platelets and ADAM9 on CTCs (Mammadova-Bach et al., 2016). A different advantage of CTCs binding platelets is the activation of YAP1 signaling reducing anoikis (Haemmerle et al., 2017). Papa and colleagues developed human modified platelets (platelet decoys) incapable of functional activation and aggregation. Taking advantage of a microfluidic human microvasculature-on-a-chip device, decoys were revealed to interfere with platelet-mediated human breast cancer cell aggregation decreasing CTCs arrest and extravasation (Papa et al., 2019).

Intravital imaging suggested that tumor-associated macrophages (TAMs) proximity to invading tumor cells is essential during intravasation preceding bloodborne dissemination (Harney et al., 2015). TAMs also play a role in immunosurveillance suppression (Gast et al., 2018; Zhang et al., 2017). CTCs and macrophages can fuse creating hybrid cells harboring epithelial/tumoral and hematopoietic properties (Gast et al., 2018). These cells are found in the peripheral blood of patients with several cancer types and correlates with bad prognosis and enhanced metastatic potential (Gast et al., 2018; Zhang et al., 2017). In addition, TAMs secrete interleukin 6 (IL6) that promote EMT increasing mesenchymal CTC ratio and inhibit the tumor suppressor miR-506-3p. This inhibition indirectly results in chemokine ligand 2 (CCL2) production that promotes macrophage recruitment. Blockage of IL6 or CCL2 broke this loop reducing mesenchymal CTCs number and macrophage migration, respectively (Wei et al., 2019).

Microfluidic isolation of CTC hetero-clusters

Isolation of CTC hetero-clusters is a challenging task. In particular, platelets-coated CTCs are extremely difficult to trap because of masking or downregulation of surface epitopes. However, recent advances in microfluidics devices allowed the design of an efficient approach to trap platelets-coated CTCs using platelets as a ubiquitous cell-surface marker. This two-steps microfluidic strategy first depletes free platelets by size, second isolates platelets-coated CTCs using the herringbone CTC chip which induces microvortices. Moreover, this method has proven efficient to capture CTC-leukocyte clusters (Jiang et al., 2017; Stott et al., 2010). Another microfluidic-based method that has been efficiently used to isolated hetero clusters, specifically CTC-neutrophils, is the Parsortix device (Szczerba et al., 2019) (Figure 1D).

Mechanoadaptation : *CTCs force their way to metastasis*

Mechanical cues have a broad impact on cancer progression (Kumar and Weaver, 2009) from tumor growth and vascularization to invasion and metastasis dissemination through body fluids. Indeed, during their hematogenous journey CTCs experience considerable levels of FSS (Follain et al., 2020) that are deleterious for the majority of them (Regmi et al., 2017; Xin et al., 2019). Interestingly, using a microfluidic circulatory device mimicking the FSS achievable during intense exercise, Regmi and colleagues show an increase in breast CTCs destruction. Cell death occurred through necrosis within 4 h of flow and by apoptosis between 16 and 24 h of flow (Regmi et al., 2017). One mechanism promoting FSS-induced apoptosis is mediated by the tumor necrosis factor apoptosis-inducing ligand (TRAIL) which binds to death receptors expressed on the surface of CTCs in a force-dependent and time-dependent manner (Mitchell and King, 2013). To unravel whether clusters behave similarly to single CTCs under FSS, Marella and colleagues developed a multichannel microfluidic device able to reproduce different FSS characterizing the human circulatory system (capillaries, veins, and arteries) that showed clusters disaggregation under high FSS (Marrella et al., 2021).

In vitro work suggested that CTCs are more resistant to FSS than expected because of a transient response triggered by plasma membrane damage that relies on extracellular calcium and actin cytoskeletal dynamics (Barnes et al., 2012). The ability to repair plasma membrane damages caused by FSS and the maintenance of their nuclear integrity via Lamin A/C are essential for CTCs survival (Mitchell et al., 2015). Furthermore, FSS prompts cytoskeletal mechanoadaptation that mediates plasma membrane reparations (Moose et al., 2020) and activated the fluid mechanosensor atonal bHLH transcription factor 8 (ATOH8) which promoted glycolysis-dependent CTC survival (Huang et al., 2020). Similarly, CTC clusters under FSS develop their own survival strategies like the generation of stable cell aggregates resistant to anoikis in an E-cadherin-dependent manner. Interestingly, CTCs clusters are also able to regulate their cortical actin-myosin dynamic achieving higher stability under FSS (Maeshiro et al., 2021). Hetero-cluster formed between CTCs and CAFs enhance FSS resistance via intercellular contact and soluble derived factors. Moreover, CAFs conserve the proliferative capability of CTCs subjected to high FSS (Ortiz-Otero et al., 2020). Likewise, platelets shield platelets-coated CTCs from FSS-derived damage (Labelle et al., 2011). Of note, FSS seems to regulate the kinetics and molecular mechanisms of platelets binding CTCs, low FSS revealed higher adhesion efficiency (Mccarty et al., 2002). After treatment with S-nitrosocaptopril that blocks platelets-CTCs interaction, naked CTCs were exposed to the hostile bloodstream environment increasing their elimination because of FSS and immunosurveillance (Lu et al., 2019).

Although, the effects of FSS on CTCs mechanics are not fully understood, recent works suggest a possible role for FSS-induced EMT enhancing CTCs survival and metastatic capabilities (Choi et al., 2019; Xin et al., 2019; Yu et al., 2021). Upon FSS, CTCs changed their morphology becoming elongated, expressed EMT markers, and reduced F-actin assembly and stiffness (Xin et al., 2019). The resulting soft CTCs displayed

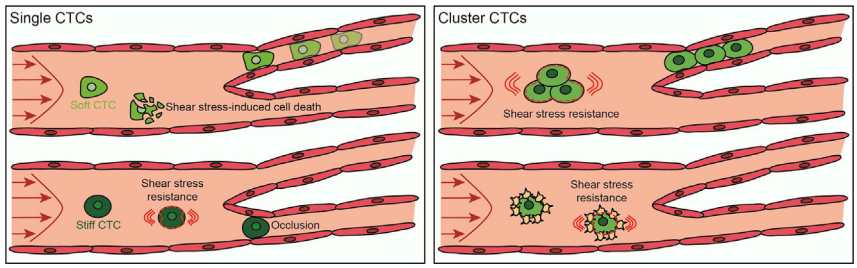

Figure 2. Biomechanics and early intravascular behavior of CTCs

Schematic representation of single CTCs, soft and stiff, and homo-clusters and hetero-clusters biomechanical properties.

higher chemotherapy resistance (Xin et al., 2019). The Jun N-terminal kinase (JNK) signaling was reported as a mediator between FSS and EMT in CTCs. Flow-activated JNK signaling favors and maintains mesenchymal status that suppresses the proapoptotic p53. Correspondingly, JNK silencing or p53 overexpression decreases CTCs survival (Xin et al., 2020). FSS-dependent nuclear translocation of YAP induces SNAIL1 expression which also triggers EMT (Yu et al., 2021). Interestingly, EMT activation might provide new properties to CTCs such as stemness and the enhanced survival potential usually associated with CSCs or TICs. These properties depend on ROS/nitric oxide (NO) generation and suppression of extracellular signal-regulated kinase (ERK)/glycogen synthase kinase (GSK)3β (Choi et al., 2019). The relationship between FSS and ROS production was further confirmed using a microfluidic chip with five culture areas (for different concentrations) and a shear stress gradient inside each area (Lo et al., 2013).

Upon EMT induction major structural changes in the CTCs cytoskeleton are expected. Cell stiffness is regulated not only by its progression through the cell cycle, but also by malignant transformation (Guck et al., 2005; Hosseini et al., 2021). While it remains unclear whether there is an optimal viscoelastic profile that would allow tumor cells to reach every step of the metastatic cascade, we recently proposed that the key to metastatic success could be their ability to adjust their mechanics to the situation (Gensbittel et al., 2021). Atomic force microscopy used to quantify the rheology (time-dependent deformation under the stress applied) of the tumor cells actin cortex revealed that EMT modulated it in a cell cycle-dependent manner, softening it in interphase and stiffening it in mitosis (Hosseini et al., 2021). Currently, the consensus is that tumor cells are softer and more deformable than healthy cells (Alibert et al., 2017; Kumar and Weaver, 2009) and these differences in cytoskeleton, thus in cell morphology and deformability can be used to isolate CTCs (Guck et al., 2005; Rosendahl et al., 2018; Toepfner et al., 2018). Optical deformability measured using a microfluidic optical stretcher, a two-beam laser trap optimized to serially deform single suspended cells by optically induced surface forces, grants label-free recognition of pathological cells monitoring subtle changes (Guck et al., 2005). Recent advances in microfluidics allow high-throughput real-time deformability cytometry to perform label-free mechanical characterization (Rosendahl et al., 2018), and is likely to provide meaningful CTC mechanics information. In addition, single cell morpho-rheological analysis (MORE) performs the characterization of blood cells in a continuous capillary-like flow, quantifying morphological parameters such as cell shape, size, aggregation, and rheological information leading to accurate identification of CTCs showing a strong potential for clinical application (Toepfner et al., 2018). Considering that different CTC subpopulations derived from diverse cancer types show differential sensitivities to FSS consistent with their biophysical properties (e.g., increased CTCs stiffness lead to increase cell death) (Hope et al., 2021); the biomechanical characterization of specific CTCs might provide a valuable prognostic tool to adapt treatment strategies in real time (Figure 2).

Eventually, CTCs need to arrest inside the vessels and then extravasate, leaving the vasculature, to reach a new niche to form a metastasis. Blood flow modulates both processes of intravascular CTC arrest and endothelial remodeling during extravasation. *In vivo* imaging in the zebrafish, together with microfluidic characterization of in-flow CTC behavior, revealed that CTCs arrest occurs in vessels with permissible flow profiles, similar to the low perfusion areas where human supratentorial brain metastasis develop preferentially (Follain et al., 2018). Microfluidic devices proved to be a key to dissect CTCs intravascular arrest revealing a two-steps process: first, CTCs stop using low-energy fast-activated adhesion receptors forming transient adhesions; second, high-energy slow-activated adhesion receptors stabilize the adhesions. However, only the receptors involved in stable adhesions are required for extravasation and metastasis (Osmani et al., 2019). *In vitro* microvascular networks also showed that CTCs glycocalyx, particularly

hyaluronic acid, mediates the adhesion to the endothelium binding CD44 (Offeddu et al., 2021). Regarding the possible role of CTC mechanical properties during intravascular arrest, novel computational models provided a possible explanation to why softer and smaller CTCs adhere more efficiently to the endothelium, whereas stiffer CTCs need 0.05 s longer to adhere which might be explained because of the smaller contact area between the CTC and the vessel (Anvari et al., 2021). These models also shed light into why platelets-coated CTCs arrest faster. Platelets reduce time and distance of CTCs circulation by increasing the forces exerted on the endothelium which leads to stronger adhesions and boosting endothelial VEGF expression promoting extravasation. Moreover, platelets protect CTCs from deformation, improving their survival (Anvari et al., 2021). *In vitro* assays support the modeling showing that platelets-coated CTCs present higher probability to adhere to endothelial cells under low FSS, as expected, this advantage is loss under high FSS (Burdick and Konstantopoulos, 2004). However, this remains to be demonstrated *in vivo*.

The extravasation process is also modulated by blood flow, *in vivo* imaging in the zebrafish showed it can be delayed by impairing flow (Follain et al., 2018). Microfluidic devices used to obtain the transcriptomics profile of endothelial cells cultured under capillary-like FSS revealed an upregulation of vascular endothelial growth factor receptor (VEGFR) signaling. Zebrafish treated with VEGFR inhibitors, including FDA-approved anti-angiogenic Sunitinib, presented impaired flow-mediated endothelial remodeling thus reducing CTCs extravasation (Follain et al., 2021). At the CTCs level, extravasation is enhanced by FSS-dependent matrix metalloproteinases (MMP) upregulation (Qazi et al., 2013) and hyaluronic acid secretion that will bind to endothelial CD44 glycocalyx (Offeddu et al., 2021). Furthermore, degradation of the CTCs glycocalyx and MMP inhibition decreased extravasation (Offeddu et al., 2021; Qazi et al., 2013). These examples highlight the relevance of using microfluidic devices associated with *in vivo* validation, to dissect biomechanical factors involved in key steps of the metastatic cascade.

PERSPECTIVES

Considering that metastasis is the most important cause of death in cancer patients, a deeper understanding of CTCs, in all aspects, is essential to develop innovative clinical strategies. From a fundamental point of view, providing accurate measurements and description of cellular mechanics during the metastasis cascade will provide a valuable resource to understand better how primary tumors develop life threatening metastases. Therefore, we think that one of the next challenges is the design of microfluidic platforms that allow robust and reproducible usages of isolated CTCs (clustered or not). In addition, relevance of cellular mechanics in human pathology requires validation in more realistic situations such as human samples. One should thus assess cellular mechanics from primary tumor cells derived from patients, at various steps of the disease. Such an approach could (i) confirm that cellular compliance scales with metastasis and (ii) affirm the potential use of cell mechanics as a diagnosis and prognosis tool. Recently, genomic studies have allowed to revisit CTCs' identity and revealed that CTCs display plastic hybrid-EMT states, which contribute to their adaptability, survival, and ultimate metastatic success. Classically, the characterization of CTCs based on epithelial markers biases the subpopulations isolated for study and thus our understanding of their properties. Similarly, because metastatic potential scales with the ability that CTCs have to cluster, significant efforts should be put towards the development of efficient isolation methods that not only enrich this specific CTC subpopulation, but also preserve their physical/structural integrity allowing deeper characterization. The development of new microfluidic-based CTCs isolation methodologies represents an important breakthrough to achieve a complete characterization of single- and clustered-CTCs. Furthermore, the need to rely on different CTC properties to bypass the EMT-status bias, highlight the interest of biophysical properties. Changes in the mechanical properties of cells often correlate with their pathophysiological states. However, clinical relevance requires measurement throughputs of > several 1000 cells in a few seconds. Such throughput has recently been achieved using real-time deformability cytometry (RT-DC) (Otto et al., 2015) and paves the way for exploiting mechanical phenotyping for cancer diagnostic applications (Figure 1E). In conclusion, the creation of high throughput microfluidic-based and mechanical properties-based isolation devices to collect the different CTCs subpopulation; together with the design of tailored CTC cluster isolation methods, will bring us closer to winning the battle against metastasis.

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DECLARATION OF INTEREST

The authors declare no competing interests.

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