Review

A fatal affair: Circulating tumor cell relationships that shape metastasis

Lauren L. Ozimski,[1,2] David Gremmelspacher,[1,2] and Nicola Aceto[1,*]

SUMMARY

Circulating tumor cells are metastatic precursors in several cancer types. Their biology and clinical utility are subject to numerous investigations, yet one aspect that is often neglected is their entanglement with the tumor microenvironment, namely the cross talk with stromal and immune cells and their relationships with other tumor-derived components such as circulating tumor DNA and extracellular vesicles in circulation. We will focus our short review specifically on these aspects, i.e., providing some examples of the liaison that circulating tumor cells have with stromal or immune cells and illustrating their relationship with other circulating tumor derivatives such as circulating tumor DNA and extracellular vesicles.

INTRODUCTION

Despite continuous advances in cancer research and therapy, metastasis still accounts for the majority of cancer-related deaths. Circulating tumor cells (CTCs) are cancer cells that have shed from a solid tumor lesion and entered into the bloodstream, acting as seeds that can potentially spread to distant sites and cause new metastatic lesions (Aceto et al., 2015; Yu et al., 2011). Therefore, these cells are crucial to understanding the roots of blood-borne metastasis and are consequently of great biological interest. In peripheral blood samples, CTCs are extremely rare compared to blood cells, and, in most patients with advanced cancer, we can typically expect to find between 0 and 10 cancer cells per 10 mL of blood. Nevertheless, despite their rarity, it is believed that CTCs are hierarchically organized, with only very few of them being able to survive the bloodstream, extravasate, and complete the metastatic process. CTCs have been detected in many different epithelial cancers, including breast, prostate, lung, colon, and pancreatic cancer, and also in cancers that do not express markers of epithelial origin, such as glioblastoma multiforme or melanoma (Aceto et al., 2015; Alix-Panabières and Pantel, 2014).

While the majority of CTCs travel alone in the peripheral circulation and their presence is associated with a poor clinical outcome (Cristofanilli et al., 2004; Wang et al., 2017), some have been shown to navigate the bloodstream as multicellular aggregates (CTC clusters) (Aceto et al., 2014). These CTC clusters (Heeke et al., 2019; Noman et al., 2014) can be found as aggregates of more than one cancer cell, i.e., homotypic CTC clusters, or in combination with other cell types such as immune or stromal cells, i.e., heterotypic CTC clusters (Aceto et al., 2015; Duda et al., 2010; Heeke et al., 2019; Szczerba et al., 2019). CTC clusters have been shown to be endowed with a higher metastatic potential compared to single CTCs and have been associated with worse clinical outcome in patients with multiple cancer types (Aceto et al., 2014; Murlidhar et al., 2017; Wang et al., 2017; Zhang et al., 2017a). Thus, CTCs are not only of biological importance for a better understanding of metastasis and its Achilles' heels but also are valuable in the clinical setting to pinpoint patients with a poorer prognosis. While CTCs are yet to reach their full potential in the clinic, recent studies have shown encouraging data toward their utility in early stages of localized prostate cancer (Broncy and Paterlini-Bréchot, 2019) and how they could serve as a helpful early treatment monitoring tool in breast cancer (Schochter et al., 2019).

Importantly, CTCs are not the only cancer-derived material contained in liquid biopsies—these contain other elements such as extracellular vesicles (EVs), micro-RNAs (miRNAs), or circulating cell-free tumor DNA (ctDNA), each of which can be of clinical relevance (Gold et al., 2015). Firstly, ctDNA refers to the fraction of cell-free DNA (cfDNA) that is shed into circulation by tumor cells, thus reflecting the mutational landscape of their primary tumor. CtDNA can be highly fragmented and, especially in early stages of disease, only represents a small fraction of the total cfDNA, hampering accurate ctDNA detection (Mouliere et al., 2018). Exosomes are defined as a subset of EVs of smaller size, ~30–150 nm (Kalluri and LeBleu, 2020;

[1]Department of Biology, Institute of Molecular Health Sciences, Swiss Federal Institute of Technology (ETH) Zurich, 8093 Zurich, Switzerland

[2]These authors contributed equally

*Correspondence: nicola.aceto@biol.ethz.ch

https://doi.org/10.1016/j.isci.2021.103073

Whiteside, 2016), originating from the endosomal compartment and released by viable cells into the bloodstream via exocytosis (Cocucci and Meldolesi, 2015; Kalluri and LeBleu, 2020).

Technologies for the identification and characterization of tumor-derived components in liquid biopsies have been developed extensively, yet vary substantially regarding the principles that characterize them. For example, CTCs are typically isolated with antigen-dependent or antigen-independent technologies. The former relies on the presence of specific biomarkers for CTC capture, while the latter utilizes other features such as size or deformability (Ferreira et al., 2016). The most well-known example of antigen-dependent isolation is the CellSearch system, which isolates EpCAM-positive CTCs and is currently the only FDA-cleared test for monitoring patients with metastatic breast, prostate, and colorectal cancer (Swennenhuis et al., 2016). Additionally, epitope-independent, size-based platforms have also become a popular method in recent years, allowing not only the capture but also the harvest of live CTCs (Miller et al., 2018). However, despite this variety of CTC isolation technologies, there are still numerous limitations. With many methods depending on specific biomarkers, the isolation efficiency may be compromised due to the heterogeneity of CTCs, but more importantly, there may be technology-driven biases, in this case, promoting the enrichment of high-antigen expressing CTCs. The same occurs with other types of technology, such as those that are size-based and that favor the enrichment of larger CTCs (Castro-Giner and Aceto, 2020). Isolation of ctDNA is considered less technically challenging, with several commercial kits available, which have been reviewed and compared extensively elsewhere (Diefenbach et al., 2018; van der Leest et al., 2020; van Dessel et al., 2019). However, while most ctDNA studies opt for the manual QIAamp (QA) platform, there is still limited consensus or standardization regarding the use of these techniques on patient samples, which will be an important aspect moving forward in the clinical setting (van der Leest et al., 2020). As for EVs, such as exosomes, there are also various isolation methods based on different principles, including precipitation-based methods or commercially available columns (Patel et al., 2019). Currently, also in this case, the main limitation is that protocols are not standardized and are instead selected based on the downstream applications. We speculate that introduction of standard operating procedures (SOPs) for liquid biopsy sampling, biomarker extraction, handling, and storage protocols will be a critical step for the extensive use of CTCs, ctDNA, and exosomes in the clinic. Importantly, the clinical utility of CTCs, ctDNA, and exosomes not solely depends on their inherent biological properties but also on the chosen method of analysis. For instance, ctDNA interrogation based on targeted sequencing of a panel of selected cancer genes may not be feasible for interrogation of cancers with very low mutational burden as provided in most pediatric cancers (Peneder et al., 2021). Thus, we argue that a multi-analyte interrogation in combination with thoroughly deliberated downstream analysis methods based on individual conditions may provide the most successful outcomes for cancer detection, disease monitoring, and therapy management.

Together, as a number of increasingly precise technologies emerge, our understanding of the features that characterize tumor-derived components in liquid biopsies improves. It is unquestionable, however, that several unresolved "mysteries" still frame the liquid biopsy field, some of which are summarized below. In particular, these revolve around the role of each of these tumor-derived components in relation to each other and to the tumor microenvironment (TME) and to which extent can they be exploited to gain fundamental insights about disease progression and vulnerabilities.

UNRESOLVED MYSTERIES IN THE CTC FIELD
Mutational heterogeneity and correlation with the primary tumor

Tumors generally consist of multiple clonal subpopulations with distinct genotypic and phenotypic signatures. This observation is frequently referred to as intratumor heterogeneity, and it is the consequence of mutational alterations as well as epigenetic, transcriptional, and post-translational modifications, resulting in spatial and temporal differences. Intratumor heterogeneity is believed to be a major contributor to cancer progression and inherent resistance to therapy, for instance, through the expansion of rare, pre-existing drug-tolerant subpopulations (Dagogo-Jack and Shaw, 2018; Diaz et al., 2012; Stanta and Bonin, 2018). Selective advantages of single cells within a large tumor are able to give rise to dominant clones in a clinically relevant time (Waclaw et al., 2015); however, very little is known about the exact molecular drivers, as well as the spatial arrangements involved in this process. Molecular characterization of drug-tolerant clones may provide valuable insights into the mechanisms of drug resistance and enable identification of distinct biological features to specifically target them.

Intratumor heterogeneity makes comprehensive tumor profiling a challenging task. Molecular data obtained from classical tissue biopsies may only represent a small part of the entire tumor complexity and not necessarily the most relevant part. Approaches to better recapitulate intratumor heterogeneity (or to capture highly relevant tumor subclones) have shifted the attention toward tumor-derived material that can be detected in blood circulation (Ignatiadis et al., 2021; Parikh et al., 2019). While it is unlikely that tumor-derived components in liquid biopsies may represent the entire tumor heterogeneity, information obtained from CTCs is possibly related to highly aggressive tumor subclones (i.e. capable to survive, proliferate, and migrate out of the tumor), as recently demonstrated in non-small cell lung cancer (Chemi et al., 2019). Future efforts will be needed in large patient cohorts to determine to which extent the information derived from tumor-derived components in liquid biopsies captures tumor heterogeneity but, most importantly, to which extent it captures disease progression-relevant tumor subclones.

Early vs. late metastasis seeding

A comprehensive understanding of the metastatic cascade not only requires investigation of the cellular cross talk and molecular drivers involved in this process but also reveals the timing of the occurring events. Temporal dynamics of primary tumor progression, CTC shedding, extravasation at distant sites, dormancy of micro-metastatic lesions, and outgrowth to overt, detectable metastasis are poorly understood.

Considering the occurrence of the first cancer-initiating mutation, metastasis seeding can be seen as a relatively late event. Estimations suggest that it takes about 10 years for a healthy cell to gain driver mutations and to obtain fitness to start forming a malignant tumor (Hu and Curtis, 2020). Based on mathematical models, it was concluded that many patients with breast cancer had metastatic occurrences before the primary tumor becomes clinically detectable. Interestingly, this model also provides evidence for a long-lasting dormancy of disseminated tumor cells prior to metastatic outgrowth (Hanin and Pavlova, 2016). More specifically, comparing genomic profiles of paired primary tumors and metastasis in patients with breast, colorectal, and lung cancer, it was estimated that metastatic seeding occurs 2–4 years prior to primary tumor diagnosis (Hu et al., 2020). Consistent with these observations, animal models report early dissemination of tumor cells in breast cancer xenografts (Harper et al., 2016; Hosseini et al., 2016).

Controversial models have emerged though, trying to describe the spatiotemporal behavior of tumor cell dissemination (Hu and Curtis, 2020). Supported by observations that only a very small subset of disseminated tumor cells successfully seed metastases, the minor clone linear progression model states that only small subclones within the primary tumor undergo genetic alterations that confer fitness to seed metastasis and that this happens relatively late in primary tumor evolution. However, if disseminated tumor cells would derive from rare subpopulations only, metastases should possess private driver mutations that would not be detectable by bulk sequencing of the primary tumor. Instead, analysis of paired primary tumors and metastases among patients with breast, colorectal, and lung cancer revealed that the majority of driver mutations in metastasis correspond to their primary tumor (Hu et al., 2020). This emphasizes the major clone linear progression model, arguing that metastasis derives from early dissemination of a dominant clone with selective advantages, resulting in genetic and phenotypic similarities between metastasis and the primary tumor. The parallel progression model on the other hand suggests a very early dissemination of tumor cells, followed by independent evolution and acquisition of private driver mutations in primary tumor and metastasis, resulting in genomic divergence (Klein, 2009). Although early metastatic spread of tumor cells seems reasonable in certain tumor types (Harper et al., 2016; Hosseini et al., 2016; Hu et al., 2019, 2020), frequently matched mutational profiles in paired primary tumors and metastasis (Hu and Curtis, 2020) fail to entirely support this model.

For a comprehensive discussion on the spatial and temporal dynamics of cancer progression, we also need to consider the idea of metastasis-to-metastasis dissemination. It seems plausible that tumor cells do not irreversibly lose their invasive and migratory properties once reaching secondary tumor sites, suggesting that tumor cell spread does not stop there. In fact, in a phylogenetic analysis of metastatic prostate cancers, it was recently revealed that metastasis-to-metastasis seeding is a very common event. Performing whole-genome sequencing of samples obtained from primary tumors and corresponding metastases of ten patients with prostate cancer, the clonal relationship between different tumor sites for each patient was determined. Multiple metastases matched the mutational profiles of each other more than the profile of the primary tumor, with a higher similarity of metastases in close spatial proximity (Gundem et al., 2015). In a similar approach, comparing single-nucleotide variant (SNV) and copy number alteration (CNA)

profiles in primary tumors and associated metastases from autopsied patients with breast cancer, supporting evidence was provided for the cross-seeding of cancer cells from metastatic precursors to new metastatic sites in a clinically relevant time frame, suggesting metastasis-to-metastasis dissemination as a dominant route of cancer progression in patients with breast cancer after primary tumor resection (Brown et al., 2017).

Together, while certainly a more detailed understanding of the temporal dynamics that characterize cancer progression is needed, it is likely that timing of the events will depend on a variety of factors including mutational profile, fitness of the immune system, and presence of specific TME-related signals. In this context, it is conceivable that individual tumors growing in individual patients are characterized by their own individual temporal dynamics, some of which comprising dissemination events that occur early and some others where dissemination is a late event.

Which primary tumor cells become CTCs

The spatial relationship of CTCs with the primary tumor and the detailed mechanisms leading to CTC intravasation are still poorly characterized. Previously, we mentioned that local differences in the TME contribute to intratumor heterogeneity in multiple ways. It seems obvious that these differences could also result in unequal intravasation potential from the primary tumor, indicating the possibility that CTC may more likely represent certain subclones with enhanced ability to spread.

One example is provided by intratumor hypoxia, shown to play a role in the spread of cancer. The term hypoxia refers to a reduction in normal oxygen levels in the tumor tissue. It is a general hallmark of neoplastic tissues across various cancer types, whereby uncontrolled proliferation and reduced blood supply lead to reduced oxygen availability (Bhandari et al., 2019; Harris, 2002). As a result of aberrant vascularization and variable oxygen demand from changes in tumor cell metabolism, oxygen levels vary among different tumor subregions, ranging from well-oxygenated to anoxic and necrotic areas. Although hypoxia is generally toxic to cells, tumor cells in hypoxic regions are in some cases capable to survive and even develop selective advantages (Harris, 2002), including an enhanced metastatic potential (Brizel et al., 1996). Hypoxia has been often suggested to induce an epithelial-to-mesenchymal transition (EMT) *in vitro* (Chen et al., 2010; Copple, 2010; Lei et al., 2013; Lou et al., 2015; Sahlgren et al., 2008; Yang et al., 2008); however, recent evidence suggests that the majority of single CTCs in breast cancer xenografts derive from normoxic tumor regions (Donato et al., 2020). In contrast, it was shown that spontaneous hypoxia occurrence *in vivo* leads to cell-cell junction upregulation and intravasation of clustered CTCs with increased metastatic ability (Donato et al., 2020).Together, uneven occurrence of hypoxia in particular tumor regions may be one of the signals involved in cancer cell intravasation and formation of CTCs with aggressive phenotypes.

Further studies are required in this context, investigating the spatial and molecular features that demarcate cells within the tumor with high propensity to intravasate. We speculate that these may include a variety of factors and vary across cancer types. Identification of those signals that dictate the entry of cancer cells in the bloodstream may be a fundamental new way to block metastasis at its start.

Interaction of CTCs with other components

The entire tumor mass does not only consist of heterogeneous cancer cell populations but also comprises several other elements, such as endothelial cells, cancer-associated fibroblasts (CAFs), infiltrating immune cells, extracellular matrix (ECM) components, and other secreted factors, summarized as the TME. The maintenance, progression, and response to therapy of a primary or metastatic cancerous lesion are not only determined by genetic alterations in tumor cells but also in many ways rely on the influence of the TME in an intercorrelated manner (Junttila and de Sauvage, 2013).

The accomplishment of metastasis requires the execution of a multistep process, including intravasation of single or clustered CTCs in the bloodstream, survival within circulation, extravasation, and establishment of a proliferative distant lesion. Accumulating evidence vigorously suggest that the environmental cross talk of tumor cells with the TME is not limited to the primary tumor. In fact, there is considerable evidence supporting a role for non-neoplastic cells in each and every step of the metastatic cascade (Hurtado et al., 2020; Kitamura et al., 2015). These include CAFs (Duda et al., 2010; Liu et al., 2016), different types of immune cells (Sprouse et al., 2019; Szczerba et al., 2019), and platelets (Gay and Felding-Habermann, 2011; Labelle et al., 2011). However, we can assume that immune and stromal partners of CTCs will depend on the status of the
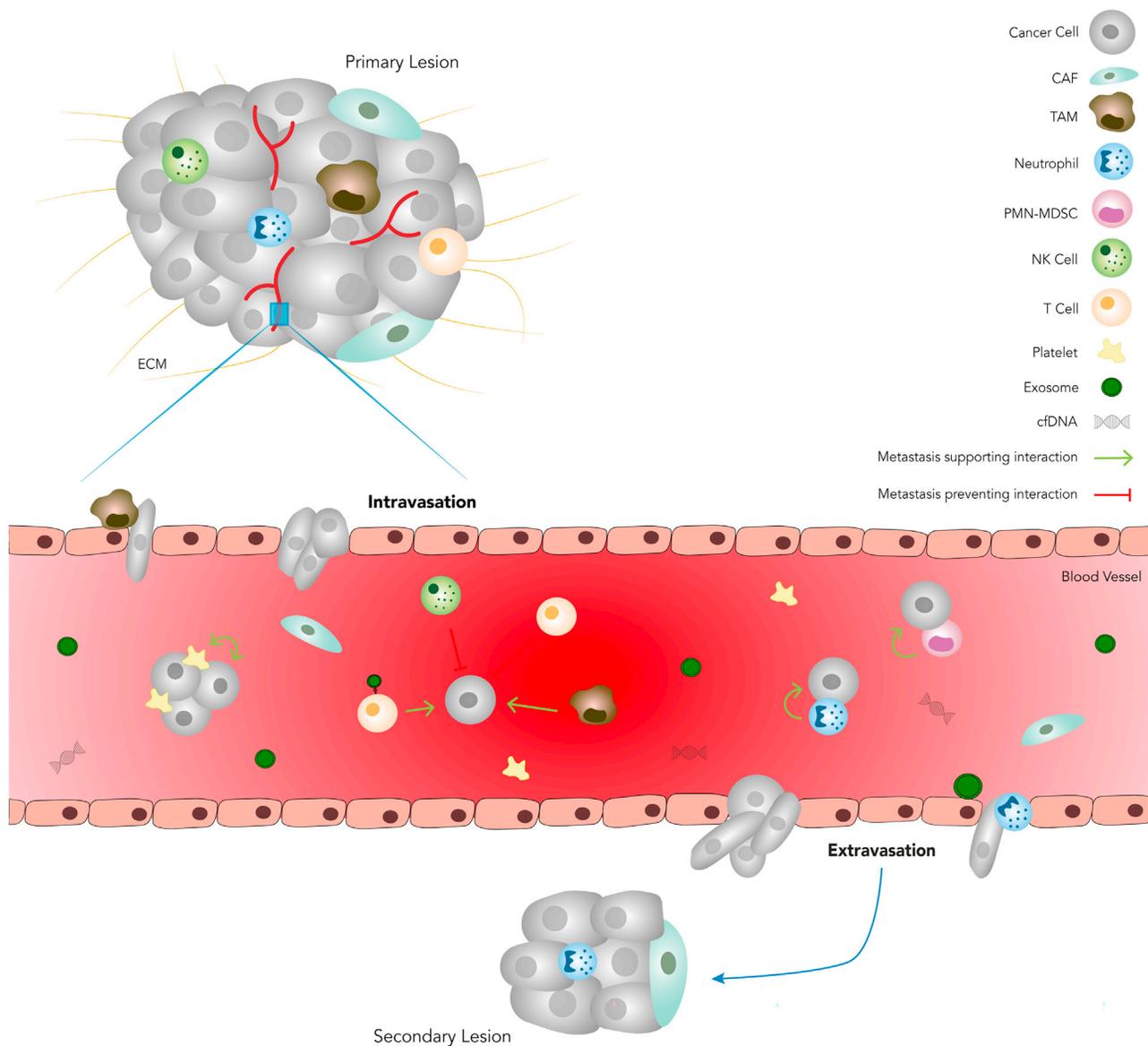
Figure 1. Relationship between CTCs and the tumor microenvironment during metastasis

Schematic representation of the metastatic cascade and the interactions between CTCs and other elements that impact this process. Both immune and stromal cells impact metastasis at the primary site and promote cancer cell intravasation. Once in the bloodstream, CTC survival and ability to metastasize is influenced by other cell types. Finally, CTCs can carry stromal cells to the secondary site, creating a favorable metastatic niche. CAF, cancer-associated fibroblast; ECM, extracellular matrix; PMN-MDSC, polymorphonucler myeloid-derived suppressor cells; TAM, tumor-associated macrophage; cfDNA, cell-free DNA.

TME at the intravasation site; thus, further studies are needed to reveal all possible interaction partners involved in various cancer types and their metastases. In addition to other cell types, CTCs could also interact with different tumor-derived components in circulation. This idea has encouraged studies to investigate how these elements interact with each other and potentially promote metastasis in an intercorrelated manner. These relationships (Figure 1) and their clinical importance will be discussed in more detail in the following sections.

RELATIONSHIP BETWEEN CTCS AND OTHER CELL TYPES

The crucial role of the TME in tumor biology was impressively demonstrated through the clinical success of immune checkpoint inhibitors (ICIs) in several cancer types, including melanoma and non-small cell lung

cancer (NSCLC) (Brahmer et al., 2012; Hodi et al., 2010; Larkin et al., 2015; Powles et al., 2014; Rosenberg et al., 2016). ICIs aim to stimulate the cytotoxic activity of the immune system against cancer cells by blocking the inhibitory signal in T cells, particularly mediated through the PD1/PD-L1 or CTLA-4/CD80/86 axis (Waldman et al., 2020). However, selecting patients that are likely to benefit from ICI therapy still remains a challenge. Expression of PD-L1 in tumor biopsies does not necessarily correlate with response to ICI, as recently shown in melanoma (Hodi et al., 2018; Larkin et al., 2019). Prevailing limitations in the evaluation of PD-L1 protein expression in tumor biopsies may have a considerable impact on this. In contrast, PD-L1 expression analysis in CTCs holds promise to be a complementary biomarker to predict responses to ICI therapy and, through longitudinal liquid biopsy sampling, could enable monitoring of disease dynamics and allow rapid therapy decisions (Kloten and Krahn, 2019).

It is now known that many different cell types may influence the dissemination of cancer cells from the primary site. In this context, CAFs are versatile players that secrete a great number of cytokines, growth factors, and other molecules that influence metastasis initiation. It has been found that CAFs can promote metastasis by modifying extracellular matrix stiffness, via the increase of N-cadherin expression, inducing epithelial-mesenchymal transition, via paracrine secretion of transforming growth factor β (TGF-β), and even via recruiting of tumor-associated macrophages (TAMs) through cytokines such as CCL-2 (Hurtado et al., 2020). These TAMs have also been reported as promoters of an aggressive microenvironment that fosters an invasive phenotype in small-cell lung cancer in vitro and directly promote intravasation in other cancers including glioma and breast cancer, where they initiate a paracrine signaling loop that involves tumor-derived colony-stimulating factor 1 (CSF-1) and macrophage-derived epidermal growth factor (EGF (Hamilton and Rath, 2017). Additionally, in non-small-cell lung cancer and inflammatory breast cancer, CTC levels have been found to correlate with the degree of immune surveillance, especially in regard to certain immune cell types. Specifically, the number of CTCs was inversely correlated to CD8-positive peripheral T lymphocytes in patients with breast cancer and to CD3-positive, CD4-positive, and CD8-positive peripheral T lymphocytes in patients with NSCLC (Mego et al., 2016; Ye et al., 2017). However, the responsible biological mechanisms of CTC and T lymphocyte interactions are yet to be fully unraveled. This correlation was also found for NK cells in NSCLC (Ye et al., 2017), which is not surprising considering their cytotoxic effects on CTCs (Dianat-Moghadam et al., 2021).

Once in the bloodstream, CTCs have been found in cooperation with different cell types including platelets, neutrophils, macrophages, myeloid-derived suppressor cells (MDSCs), and even CAFs. Firstly, it has been shown that platelets are "educated" by CTCs and share a reciprocal relationship with them in which both cell types influence each other (Heeke et al., 2019). It is thought that platelets assist CTCs in multiple ways including anoikis prevention, by providing integrin signaling and immune escape, e.g., via TGF-β secretion that suppresses cytolytic natural killer cells. However, the molecular mechanisms supporting these processes are still to be fully characterized, including the events leading to the activation of platelets by CTCs (Heeke et al., 2019). In addition to direct contact, platelets also produce cytokines that influence the CTC phenotype, such as an increase of tissue factor (TF) and P2Y12 receptor activity (Heeke et al., 2019). Moreover, neutrophils have been shown to promote CTC extravasation on multiple occasions, either via an interaction with the endothelial cells of vessel walls, mediated by intercellular adhesion molecule-1 (ICAM-1), or via the formation of neutrophil extracellular traps in an IL-8 and β1-integrin-dependent manner (Heeke et al., 2019). CTC-neutrophil clusters have also been studied in patients with breast cancer and murine models, revealing an even closer relationship mediated by vascular cell adhesion molecule-1 (VCAM-1) and leading to increased proliferation and metastatic ability of CTCs that are partnered with neutrophils within the bloodstream (Szczerba et al., 2019). Another study has revealed that polymorphonuclear (PMN) MDSCs, which are related to neutrophils and monocytes, directly interact with CTCs promoting their dissemination and metastatic potential via a Nodal-Notch1-Jagged1 signaling axis in response to PMN-MDSC-secreted reactive oxygen species (Sprouse et al., 2019). Further, it has also been suggested that macrophages not only interact with CTCs via multiple cytokines but also fuse with them forming "tumacrophages" that can better evade immune detection (Zhang et al., 2017b). More on the functional side, it has been shown that CTCs are able to carry their partner cells to the metastatic site, creating a favorable environment and facilitating the growth of metastatic lesions, as is the case for CAFs that can increase the efficiency of lung metastasis (Duda et al., 2010).

Enhanced knowledge on the versatile entanglements involved in assisting CTCs on their way to metastasis have encouraged studies to investigate how these interactions could be subject to pharmacologic

intervention in order to suppress metastasis. For instance, acetylsalicylic acid (aspirin) is a well-established anti-platelet agent, leading to reduced platelet activation through inhibition of thromboxane A2 (TXA2) synthesis via cyclooxygenase-1 (COX-1) (Schafer, 1995). The "Add-Aspirin" trial is currently investigating the effects of daily administration of low- (100 mg) and medium-dose (300 mg) aspirin on disease recurrence and survival after primary therapy in common non-metastatic solid tumors (Coyle et al., 2016). More studies, monitoring the effects of selected agents on CTC intravasation, CTC count, and CTC extravasation will be required in the future to assess the full potential of targeting CTC interactions with non-malignant cells. For example, studies investigating the direct effect of ICIs on CTCs could provide interesting insights in the abilites of ICIs to suppress metastasis in a direct fashion. Development and validation of well-designed *in vitro* models, preclinical models, and rational clinical designs are urgently needed in this regard.

Together, it is becoming increasingly clear that the TME and in particular immune and stromal cells are a key component during metastatic cancer progression. A comprehensive and quantitative definition of these partnerships during cancer dissemination is required to define a better picture of metastasis biology and most importantly of its vulnerabilities.

RELATIONSHIP BETWEEN CTCS, EXOSOMES, AND CTDNA

We now appreciate how secretion of exosomes is a common event across most cells and tissues with critical roles in health and disease (Osaki and Okada, 2019; Raposo and Stoorvogel, 2013; Whiteside, 2016). Exosomes mediate cellular communication either through direct interaction with target proteins, hereby activating signaling pathways, or by fusing with the recipient cell membrane and leading to the release of the cargo into the cytoplasm (Guo et al., 2019). A mechanism was suggested whereby exosomes released by primary tumor cells induce signaling pathways in recipient cells, leading to enhanced metastatic potential (Fu et al., 2018). In this study, they created an *in vitro* model where they observed that hepatocellular carcinoma (HCC)-derived exosomes, containing SMAD Family Member 3 (SMAD3) protein and mRNA, induce ROS increase in HCC cells through enhanced TGF-β/SMAD3 signaling, resulting in facilitated cell adhesion. They also reported delivery of bioactive materials from tumor-derived exosomes to CTCs *in vivo*, again resulting in enhanced cell viability and cell adhesion. To evaluate the clinical significance of these findings, they compared levels of SMAD3-containing exosomes in human peripheral blood and found high levels of SMAD3-containing exosomes correlated with a reduced disease-free survival of patients with HCC (Fu et al., 2018). In addition to directly enhancing the fitness of CTCs to adhere to the endothelium, extravasate, and form metastatic lesions, tumor-derived exosomes may also influence metastasis and CTC viability in an indirect manner. It was recently shown that tumor-derived exosomes carrying PD-L1 have the ability to induce immune suppression by binding PD-1 expressed on T cells (Theodoraki et al., 2018). This might not only facilitate immune system evasion in the primary tumor but also elsewhere, i.e. including CTCs.

Several reports have also suggested the ability of tumor-derived exosomes to influence metastasis by preparing a pre-metastatic niche for organotropic CTC seeding (Hood et al., 2011; Hoshino et al., 2015; Peinado et al., 2012). It was revealed that tumor-derived exosomes show distinct integrin expression patterns, determining preferred sites of exosomal uptake (Hoshino et al., 2015). They linked exosomal integrins α6β4 and α6β1 with lung metastasis, while exosomal integrin $\alpha_v\beta_5$ was associated with liver metastasis in mouse models. Targeting these integrins *via* genetic knockout reduced exosome uptake, resulting in decreased metastasis in lung and liver, respectively. Mechanistically, following uptake in recipient cells, tumor-derived exosome integrins activate proto-oncogene tyrosine-protein kinase Src and proinflammatory S100 gene expression to promote migration and inflammation, i.e. to "prepare" the pre-metastatic niche. It was also demonstrated that melanoma-derived exosomes are able to transfer the oncoprotein c-Met to bone marrow progenitor cells, resulting in increased c-Met signaling and enhanced motility in recipient cells. Motile bone marrow progenitor cells then facilitate metastasis through formation of a suitable microenvironment in the pre-metastatic niche (Peinado et al., 2012). Further, transfer of cancer-secreted miRNAs, abundantly found in tumor-derived-exosomes, may also play a pivotal role in promoting metastasis. It was reported that overexpression of miR-105 in non-metastatic cancer cells induces vascular permeability in distant organs by efficiently destroying tight junctions and integrity of these natural barriers against metastasis, i.e. facilitating CTC extravasation (Zhou et al., 2014). Interestingly, studies have identified hypoxia as a key regulator for exosome-mediated support of angiogenesis and vascular leakage (King et al., 2012; Kucharzewska et al., 2013). More generally, however, exosomes represent a very heterogeneous population

and most likely reflect features of the cancerous lesion they originate from. Given this, not surprisingly, other studies suggest the ability of tumor-derived exosomes to inhibit tumor cell metastasis by increasing the number of lung-patrolling monocytes, recruiting NK cells, and inducing macrophage differentiation and phagocytosis (Plebanek et al., 2017). Exosomes and their characterization certainly deserve great attention in the future, as they could provide very useful information along disease progression and along the analysis of other tumor-derived components.

Differently from exosomes, ctDNA has already gained a prominent role in the clinical setting. ctDNA levels typically increase hand-in-hand with tumor burden (Mouliere et al., 2018). Correlation of ctDNA abundance with poor prognosis highlights ctDNA as an accurate biomarker for tumor progression (Bratman et al., 2020; Vandekerkhove et al., 2021). Studies also demonstrated the feasibility of ctDNA analysis for accurate and sensitive assessment of treatment response and monitoring of residual disease (McDonald et al., 2019; Tie et al., 2016). The huge potential of ctDNA analysis for early diagnostic purposes was recently demonstrated. Using a blood test based on the simultaneous assessment of mutations in cell-free DNA and levels of circulating protein markers, investigators were able to detect cancer types such as the ovary, liver, stomach, pancreas, and esophagus with sensitivities ranging from 69 to 98% and a specificity of more than 99% (Cohen et al., 2018).

Unlike CTCs, which per definition represent living descendants of a tumor lesion and thus rather reflect invasive and migratory tumor subclones, the majority of ctDNA derives from dying cells, i.e. apoptotic and necrotic tumor areas (Diaz and Bardelli, 2014). Therefore, highly proliferative and drug-resistant genetic information may be underrepresented and ctDNA may rather be highly representative of tumor regions exposed to insufficient oxygenation or strong immune system cytotoxic activity. While genetic information obtained from ctDNA may not reflect the most invasive tumor subclones, a complementary analysis of ctDNA alongside exosomes and CTCs in liquid biopsies could provide a more comprehensive picture of the tumor profile and constitute a precious tool for monitoring disease dynamics.

Altogether, while CTCs, exosomes, and ctDNA represent *per se* highly valuable tumor-derived components, they are also characterized by inherent differences. These fundamental differences, mostly dictated by the biological processes that lead to their generation, impact on the information that is available from each of these components, arguing that a more comprehensive analysis of liquid biopsies might positively influence the diagnosis and treatment of cancer.

CONCLUDING REMARKS

The metastatic spread of cancer is a complex, multi-step process that involves a variety of factors. These factors are entangled with each other and define disease aggressiveness but also therapeutic opportunities. Investigations of the TME have clearly revealed a prominent role for non-neoplastic cells in shaping the metastatic cascade in various cancer types, and it is also apparent how various tumor-derived components obtained from liquid biopsies may provide complementary tools to investigate cancer biology in a minimally invasive fashion. Integration of all these components with the latest tools (e.g. artificial intelligence, integrative biology) and together with the analysis of large patient cohorts will be key for exploiting the resources that a liquid biopsy may offer, aiming to improve diagnosis, stratification, and treatment of cancer.

AUTHOR CONTRIBUTIONS

L.L.O., D.G., and N.A. designed and wrote the manuscript. All authors reviewed and edited subsequent drafts and gave final approval for submission.

DECLARATION OF INTERESTS

N.A. is a paid consultant for companies with an interest in liquid biopsy. All other authors declare no conflict of interest.

REFERENCES

Aceto, N., Bardia, A., Miyamoto, D.T., Donaldson, M.C., Wittner, B.S., Spencer, J.A., Yu, M., Pely, A., Engstrom, A., Zhu, H., et al. (2014). Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 158, 1110–1122. https://doi.org/10.1016/j.cell.2014.07.013.

Aceto, N., Toner, M., Maheswaran, S., and Haber, D.A. (2015). En route to metastasis: circulating tumor cell clusters and epithelial-to-mesenchymal transition. Trends Cancer. https://doi.org/10.1016/j.trecan.2015.07.006.

Alix-Panabières, C., and Pantel, K. (2014). Challenges in circulating tumour cell research. Nat. Rev. Cancer 14, 623–631. https://doi.org/10.1038/nrc3820.

Bhandari, V., Hoey, C., Liu, L.Y., Lalonde, E., Ray, J., Livingstone, J., Lesurf, R., Shiah, Y.-J., Vujcic, T., Huang, X., et al. (2019). Molecular landmarks of tumor hypoxia across cancer types. Nat. Genet. 51, 308–318. https://doi.org/10.1038/s41588-018-0318-2.

Brahmer, J.R., Tykodi, S.S., Chow, L.Q.M., Hwu, W.-J., Topalian, S.L., Hwu, P., Drake, C.G., Camacho, L.H., Kauh, J., Odunsi, K., et al. (2012). Safety and activity of anti–PD-L1 antibody in patients with advanced cancer. New Engl. J. Med. 366, 2455–2465. https://doi.org/10.1056/NEJMoa1200694.

Bratman, S.v., Yang, S.Y.C., Iafolla, M.A.J., Liu, Z., Hansen, A.R., Bedard, P.L., Lheureux, S., Spreafico, A., Razak, A.A., Shchegrova, S., et al. (2020). Personalized circulating tumor DNA analysis as a predictive biomarker in solid tumor patients treated with pembrolizumab. Nat. Cancer 1, 873–881. https://doi.org/10.1038/s43018-020-0096-5.

Brizel, D.M., Scully, S.P., Harrelson, J.M., Layfield, L.J., Bean, J.M., Prosnitz, L.R., and Dewhirst, M.W. (1996). Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res. 56, 941–943.

Broncy, L., and Paterlini-Bréchot, P. (2019). Clinical impact of circulating tumor cells in patients with localized prostate cancer. Cells 8, 676. https://doi.org/10.3390/cells8070676.

Brown, D., Smeets, D., Székely, B., Larsimont, D., Szász, A.M., Adnet, P.-Y., Rothé, F., Rouas, G., Nagy, Z.I., Faragó, Z., et al. (2017). Phylogenetic analysis of metastatic progression in breast cancer using somatic mutations and copy number aberrations. Nat. Commun. 8, 14944. https://doi.org/10.1038/ncomms14944.

Castro-Giner, F., and Aceto, N. (2020). Tracking cancer progression: from circulating tumor cells to metastasis. Genome Med. 12, 31. https://doi.org/10.1186/s13073-020-00728-3.

Chemi, F., Rothwell, D.G., McGranahan, N., Gulati, S., Abbosh, C., Pearce, S.P., Zhou, C., Wilson, G.A., Jamal-Hanjani, M., Birkbak, N., et al. (2019). Pulmonary venous circulating tumor cell dissemination before tumor resection and

disease relapse. Nat. Med. 25, 1534–1539. https://doi.org/10.1038/s41591-019-0593-1.

Chen, J., Imanaka, N., Chen, J., and Griffin, J.D. (2010). Hypoxia potentiates Notch signaling in breast cancer leading to decreased E-cadherin expression and increased cell migration and invasion. Br. J. Cancer 102, 351–360. https://doi.org/10.1038/sj.bjc.6605486.

Cocucci, E., and Meldolesi, J. (2015). Ectosomes and exosomes: shedding the confusion between extracellular vesicles. Trends Cell Biol. 25, 364–372. https://doi.org/10.1016/j.tcb.2015.01.004.

Cohen, J.D., Li, L., Wang, Y., Thoburn, C., Afsari, B., Danilova, L., Douville, C., Javed, A.A., Wong, F., Mattox, A., et al. (2018). Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 359, 926–930. https://doi.org/10.1126/science.aar3247.

Copple, B.L. (2010). Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-β-dependent mechanisms. Liver Int. 30, 669–682. https://doi.org/10.1111/j.1478-3231.2010.02205.x.

Coyle, C., Cafferty, F.H., Rowley, S., MacKenzie, M., Berkman, L., Gupta, S., Pramesh, C.S., Gilbert, D., Kynaston, H., Cameron, D., et al. (2016). ADD-ASPIRIN: a phase III, double-blind, placebo controlled, randomised trial assessing the effects of aspirin on disease recurrence and survival after primary therapy in common non-metastatic solid tumours. Contemp. Clin. Trials 51, 56–64. https://doi.org/10.1016/j.cct.2016.10.004.

Cristofanilli, M., Budd, G.T., Ellis, M.J., Stopeck, A., Matera, J., Miller, M.C., Reuben, J.M., Doyle, G.V., Allard, W.J., Terstappen, L.W.M.M., and Hayes, D.F. (2004). Circulating tumor cells, disease progression, and survival in metastatic breast cancer. New Engl. J. Med. 8, 351.

Dagogo-Jack, I., and Shaw, A.T. (2018). Tumour heterogeneity and resistance to cancer therapies. Nat. Rev. Clin. Oncol. 15. https://doi.org/10.1038/nrclinonc.2017.166.

Dianat-Moghadam, H., Mahari, A., Heidarifard, M., Parnianfard, N., Pourmousavi-Kh, L., Rahbarghazi, R., and Amoozgar, Z. (2021). NK cells-directed therapies target circulating tumor cells and metastasis. Cancer Lett. 497, 41–53. https://doi.org/10.1016/j.canlet.2020.09.021.

Diaz, L.A., and Bardelli, A. (2014). Liquid biopsies: Genotyping circulating tumor DNA. J. Clin. Oncol. 32. https://doi.org/10.1200/JCO.2012.45.2011.

Diaz, L.A., Williams, R.T., Wu, J., Kinde, I., Hecht, J.R., Berlin, J., Allen, B., Bozic, I., Reiter, J.G., Nowak, M.A., et al. (2012). The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 486, 537–540. https://doi.org/10.1038/nature11219.

Diefenbach, R.J., Lee, J.H., Kefford, R.F., and Rizos, H. (2018). Evaluation of commercial kits for purification of circulating free DNA. Cancer Genet. 228–229, 21–27. https://doi.org/10.1016/j.cancergen.2018.08.005.

Donato, C., Kunz, L., Castro-Giner, F., Paasinen-Sohns, A., Strittmatter, K., Szczerba, B.M., Scherrer, R., di Maggio, N., Heusermann, W., Biehlmaier, O., et al. (2020). Hypoxia triggers the intravasation of clustered circulating tumor cells. Cell Rep. 32, 108105. https://doi.org/10.1016/j.celrep.2020.108105.

Duda, D.G., Duyverman, A.M.M.J., Kohno, M., Snuderl, M., Steller, E.J.A., Fukumura, D., and Jain, R.K. (2010). Malignant cells facilitate lung metastasis by bringing their own soil. Proc. Natl. Acad. Sci. U S A 107, 21677–21682. https://doi.org/10.1073/pnas.1016234107.

Ferreira, M.M., Ramani, V.C., and Jeffrey, S.S. (2016). Circulating tumor cell technologies. Mol. Oncol. 10, 374–394. https://doi.org/10.1016/j.molonc.2016.01.007.

Fu, Q., Zhang, Q., Lou, Y., Yang, J., Nie, G., Chen, Q., Chen, Y., Zhang, J., Wang, J., Wei, T., et al. (2018). Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer. Oncogene 37, 6105–6118. https://doi.org/10.1038/s41388-018-0391-0.

Gay, L.J., and Felding-Habermann, B. (2011). Contribution of platelets to tumour metastasis. Nat. Rev. Cancer 11, 123–134. https://doi.org/10.1038/nrc3004.

Gold, B., Cankovic, M., Furtado, L.v., Meier, F., and Gocke, C.D. (2015). Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility?: a report of the association for molecular pathology. J. Mol. Diagn. 17, 209–224. https://doi.org/10.1016/j.jmoldx.2015.02.001.

Gundem, G., van Loo, P., Kremeyer, B., Alexandrov, L.B., Tubio, J.M.C., Papaemmanuil, E., Brewer, D.S., Kallio, H.M.L., Högnäs, G., Annala, M., et al. (2015). The evolutionary history of lethal metastatic prostate cancer. Nature 520, 353–357. https://doi.org/10.1038/nature14347.

Guo, Y., Ji, X., Liu, J., Fan, D., Zhou, Q., Chen, C., Wang, W., Wang, G., Wang, H., Yuan, W., et al. (2019). Effects of exosomes on pre-metastatic niche formation in tumors. Mol. Cancer 18. https://doi.org/10.1186/s12943-019-0995-1.

Hamilton, G., and Rath, B. (2017). Circulating tumor cell interactions with macrophages: implications for biology and treatment. Transl. Lung Cancer Res. 6, 418–430. https://doi.org/10.21037/tlcr.2017.07.04.

Hanin, L., and Pavlova, L. (2016). A quantitative insight into metastatic relapse of breast cancer. J. Theor. Biol. 394, 172–181. https://doi.org/10.1016/j.jtbi.2016.01.014.

Harper, K.L., Sosa, M.S., Entenberg, D., Hosseini, H., Cheung, J.F., Nobre, R., Avivar-Valderas, A., Nagi, C., Girnius, N., Davis, R.J., et al. (2016). Mechanism of early dissemination and metastasis in Her2+ mammary cancer. Nature *540*, 588–592. https://doi.org/10.1038/nature20609.

Harris, A.L. (2002). Hypoxia — a key regulatory factor in tumour growth. Nat. Rev. Cancer *2*, 38–47. https://doi.org/10.1038/nrc704.

Heeke, S., Mograbi, B., Alix-Panabières, C., and Hofman, P. (2019). Never travel alone: the crosstalk of circulating tumor cells and the blood microenvironment. Cells *8*, 714. https://doi.org/10.3390/cells8070714.

Hodi, F.S., Chiarion-Sileni, V., Gonzalez, R., Grob, J.-J., Rutkowski, P., Cowey, C.L., Lao, C.D., Schadendorf, D., Wagstaff, J., Dummer, R., et al. (2018). Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. Lancet Oncol. *19*, 1480–1492. https://doi.org/10.1016/S1470-2045(18)30700-9.

Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., et al. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. New Engl. J. Med. *363*, 711–723. https://doi.org/10.1056/NEJMoa1003466.

Hood, J.L., San, R.S., and Wickline, S.A. (2011). Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res. *71*, 3792–3801. https://doi.org/10.1158/0008-5472.CAN-10-4455.

Hoshino, A., Costa-Silva, B., Shen, T.-L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., di Giannatale, A., Ceder, S., et al. (2015). Tumour exosome integrins determine organotropic metastasis. Nature *527*. https://doi.org/10.1038/nature15756.

Hosseini, H., Obradović, M.M.S., Hoffmann, M., Harper, K.L., Sosa, M.S., Werner-Klein, M., Nanduri, L.K., Werno, C., Ehrl, C., Maneck, M., et al. (2016). Early dissemination seeds metastasis in breast cancer. Nature *540*, 552–558. https://doi.org/10.1038/nature20785.

Hu, Z., and Curtis, C. (2020). Looking backward in time to define the chronology of metastasis. Nat. Commun. *11*, 3213. https://doi.org/10.1038/s41467-020-16995-y.

Hu, Z., Ding, J., Ma, Z., Sun, R., Seoane, J.A., Scott Shaffer, J., Suarez, C.J., Berghoff, A.S., Cremolini, C., Falcone, A., et al. (2019). Quantitative evidence for early metastatic seeding in colorectal cancer. Nat. Genet. *51*, 1113–1122. https://doi.org/10.1038/s41588-019-0423-x.

Hu, Z., Li, Z., Ma, Z., and Curtis, C. (2020). Multi-cancer analysis of clonality and the timing of systemic spread in paired primary tumors and metastases. Nat. Genet. *52*, 701–708. https://doi.org/10.1038/s41588-020-0628-z.

Hurtado, P., Martínez-Pena, I., and Piñeiro, R. (2020). Dangerous liaisons: circulating tumor cells (CTCs) and cancer-associated fibroblasts (CAFs). Cancers *12*, 2861. https://doi.org/10.3390/cancers12102861.

Ignatiadis, M., Sledge, G.W., and Jeffrey, S.S. (2021). Liquid biopsy enters the clinic — implementation issues and future challenges. Nat. Rev. Clin. Oncol. *18*, 297–312. https://doi.org/10.1038/s41571-020-00457-x.

Junttila, M.R., and de Sauvage, F.J. (2013). Influence of tumour micro-environment heterogeneity on therapeutic response. Nature *501*, 346–354. https://doi.org/10.1038/nature12626.

Kalluri, R., and LeBleu, V.S. (2020). The biology, function, and biomedical applications of exosomes. Science *367*, eaau6977. https://doi.org/10.1126/science.aau6977.

King, H.W., Michael, M.Z., and Gleadle, J.M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. BMC Cancer *12*, 421. https://doi.org/10.1186/1471-2407-12-421.

Kitamura, T., Qian, B.-Z., and Pollard, J.W. (2015). Immune cell promotion of metastasis. Nat. Rev. Immunol. *15*, 73–86. https://doi.org/10.1038/nri3789.

Klein, C.A. (2009). Parallel progression of primary tumours and metastases. Nat. Rev. Cancer *9*, 302–312. https://doi.org/10.1038/nrc2627.

Kloten, L., and Krahn, S. (2019). Circulating tumor cell PD-L1 expression as biomarker for therapeutic efficacy of immune checkpoint inhibition in NSCLC. Cells *8*, 809. https://doi.org/10.3390/cells8080809.

Kucharzewska, P., Christianson, H.C., Welch, J.E., Svensson, K.J., Fredlund, E., Ringner, M., Morgelin, M., Bourseau-Guilmain, E., Bengzon, J., and Belting, M. (2013). Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. Proc. Natl. Acad. Sci. U S A *110*, 7312–7317. https://doi.org/10.1073/pnas.1220998110.

Labelle, M., Begum, S., and Hynes, R.O. (2011). Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell *20*, 576–590. https://doi.org/10.1016/j.ccr.2011.09.009.

Larkin, J., Chiarion-Sileni, V., Gonzalez, R., Grob, J.J., Cowey, C.L., Lao, C.D., Schadendorf, D., Dummer, R., Smylie, M., Rutkowski, P., et al. (2015). Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. New Engl. J. Med. *373*, 23–34. https://doi.org/10.1056/NEJMoa1504030.

Larkin, J., Chiarion-Sileni, V., Gonzalez, R., Grob, J.-J., Rutkowski, P., Lao, C.D., Cowey, C.L., Schadendorf, D., Wagstaff, J., Dummer, R., et al. (2019). Five-Year survival with combined nivolumab and ipilimumab in advanced melanoma. New Engl. J. Med. *381*, 1535–1546. https://doi.org/10.1056/NEJMoa1910836.

Lei, J., Ma, J., Ma, Q., Li, X., Liu, H., Xu, Q., Duan, W., Sun, Q., Xu, J., Wu, Z., and Wu, E. (2013). Hedgehog signaling regulates hypoxia induced epithelial to mesenchymal transition and invasion in pancreatic cancer cells via a ligand-independent manner. Mol. Cancer *12*. https://doi.org/10.1186/1476-4598-12-66.

Liu, L., Liu, L., Yao, H.H., Zhu, Z.Q., Ning, Z.L., and Huang, Q. (2016). Stromal myofibroblasts are associated with poor prognosis in solid cancers: a meta-analysis of published studies. PLoS One *11*, e0159947. https://doi.org/10.1371/journal.pone.0159947.

Lou, X.L., Sun, J., Gong, S.Q., Yu, X.F., Gong, R., and Deng, H. (2015). Interaction between circulating cancer cells and platelets: clinical implication. Chin. J. Cancer Res. *27*, 450–460. https://doi.org/10.3978/j.issn.1000-9604.2015.04.10.

McDonald, B.R., Contente-Cuomo, T., Sammut, S.-J., Odenheimer-Bergman, A., Ernst, B., Perdigones, N., Chin, S.-F., Farooq, M., Mejia, R., Cronin, P.A., et al. (2019). Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer. Sci. Transl. Med. *11*, eaax7392. https://doi.org/10.1126/scitranslmed.aax7392.

Mego, M., Gao, H., Cohen, E.N., Anfossi, S., Giordano, A., Sanda, T., Fouad, T.M., de Giorgi, U., Giuliano, M., Woodward, W.A., et al. (2016). Circulating tumor cells (CTC) are associated with defects in adaptive immunity in patients with inflammatory breast cancer. J. Cancer *7*, 1095–1104. https://doi.org/10.7150/jca.13098.

Miller, M.C., Robinson, P.S., Wagner, C., and O'Shannessy, D.J. (2018). The Parsortix™ Cell Separation System—a versatile liquid biopsy platform. Cytometry A *93*, 1234–1239. https://doi.org/10.1002/cyto.a.23571.

Mouliere, F., Chandrananda, D., Piskorz, A.M., Moore, E.K., Morris, J., Ahlborn, L.B., Mair, R., Goranova, T., Marass, F., Heider, K., et al. (2018). Enhanced detection of circulating tumor DNA by fragment size analysis. Sci. Transl. Med. *10*, eaat4921. https://doi.org/10.1126/scitranslmed.aat4921.

Murlidhar, V., Reddy, R.M., Fouladdel, S., Zhao, L., Ishikawa, M.K., Grabauskiene, S., Zhang, Z., Lin, J., Chang, A.C., Carrott, P., et al. (2017). Poor prognosis indicated by venous circulating tumor cell clusters in early-stage lung cancers. Cancer Res. *77*, 5194–5206. https://doi.org/10.1158/0008-5472.CAN-16-2072.

Noman, M., Messai, Y., Muret, J., Hasmim, M., and Chouaib, S. (2014). Crosstalk between CTC, immune system and hypoxic tumor microenvironment. Cancer Microenviron. *7*, 153–160. https://doi.org/10.1007/s12307-014-0157-3.

Osaki, M., and Okada, F. (2019). Exosomes and their role in cancer progression. Yonago Acta Med. *62*, 182–190. https://doi.org/10.33160/yam.2019.06.002.

Parikh, A.R., Leshchiner, I., Elagina, L., Goyal, L., Levovitz, C., Siravegna, G., Livitz, D., Rhrissorrakrai, K., Martin, E.E., van Seventer, E.E., et al. (2019). Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. Nat. Med. *25*, 1415–1421. https://doi.org/10.1038/s41591-019-0561-9.

Patel, G.K., Khan, M.A., Zubair, H., Srivastava, S.K., Khushman, M., Singh, S., and Singh, A.P. (2019). Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream

applications. Sci. Rep. 9, 5335. https://doi.org/10.1038/s41598-019-41800-2.

Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., García-Santos, G., Ghajar, C.M., et al. (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat. Med. 18, 883–891. https://doi.org/10.1038/nm.2753.

Peneder, P., Stütz, A.M., Surdez, D., Krumbholz, M., Semper, S., Chicard, M., Sheffield, N.C., Pierron, G., Lapouble, E., Tötzl, M., et al. (2021). Multimodal analysis of cell-free DNA whole-genome sequencing for pediatric cancers with low mutational burden. Nat. Commun. 12, 3230. https://doi.org/10.1038/s41467-021-23445-w.

Plebanek, M.P., Angeloni, N.L., Vinokour, E., Li, J., Henkin, A., Martinez-Marin, D., Filleur, S., Bhowmick, R., Henkin, J., Miller, S.D., et al. (2017). Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche. Nat. Commun. 8, 1319. https://doi.org/10.1038/s41467-017-01433-3.

Powles, T., Eder, J.P., Fine, G.D., Braiteh, F.S., Loriot, Y., Cruz, C., Bellmunt, J., Burris, H.A., Petrylak, D.P., Teng, S., et al. (2014). MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 515, 558–562. https://doi.org/10.1038/nature13904.

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. J. Cell Biol. 200. https://doi.org/10.1083/jcb.201211138.

Rosenberg, J.E., Hoffman-Censits, J., Powles, T., van der Heijden, M.S., Balar, A.V., Necchi, A., Dawson, N., O'Donnell, P.H., Balmanoukian, A., Loriot, Y., et al. (2016). Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. The Lancet 387, 1909–1920. https://doi.org/10.1016/S0140-6736(16)00561-4.

Sahlgren, C., Gustafsson, M.v., Jin, S., Poellinger, L., and Lendahl, U. (2008). Notch signaling mediates hypoxia-induced tumor cell migration and invasion. Proc. Natl. Acad. Sci. U S A 105, 6392–6397. https://doi.org/10.1073/pnas.0802047105.

Schafer, A.I. (1995). Effects of nonsteroidal antiinflammatory drugs on platelet function and systemic hemostasis. J. Clin. Pharmacol. 35, 209–219. https://doi.org/10.1002/j.1552-4604.1995.tb04050.x.

Schochter, F., Friedl, T.W.P., deGregorio, A., Krause, S., Huober, J., Rack, B., and Janni, W. (2019). Are circulating tumor cells (CTCs) ready for clinical use in breast cancer? An overview of completed and ongoing trials using CTCs for clinical treatment decisions. Cells 8, 1412. https://doi.org/10.3390/cells8111412.

Sprouse, M.L., Welte, T., Boral, D., Liu, H.N., Yin, W., Vishnoi, M., Goswami-Sewell, D., Li, L., Pei, G., Jia, P., et al. (2019). PMN-MDSCs enhance CTC metastatic properties through reciprocal interactions via ROS/Notch/Nodal signaling. Int. J. Mol. Sci. 20, 1916. https://doi.org/10.3390/ijms20081916.

Stanta, G., and Bonin, S. (2018). Overview on clinical relevance of intra-tumor heterogeneity. Front. Med. 5. https://doi.org/10.3389/fmed.2018.00085.

Swennenhuis, J.F., van Dalum, G., Zeune, L.L., and Terstappen, L.W.M.M. (2016). Improving the CellSearch® system. Expert Rev. Mol. Diagn. 16, 1291–1305. https://doi.org/10.1080/14737159.2016.1255144.

Szczerba, B.M., Castro-Giner, F., Vetter, M., Krol, I., Gkountela, S., Landin, J., Scheidmann, M.C., Donato, C., Scherrer, R., Singer, J., et al. (2019). Neutrophils escort circulating tumour cells to enable cell cycle progression. Nature 566, 553–557. https://doi.org/10.1038/s41586-019-0915-y.

Theodoraki, M.-N., Yerneni, S.S., Hoffmann, T.K., Gooding, W.E., and Whiteside, T.L. (2018). Clinical significance of PD-L1 + exosomes in plasma of head and neck cancer patients. Clin. Cancer Res. 24, 896–905. https://doi.org/10.1158/1078-0432.CCR-17-2664.

Tie, J., Wang, Y., Tomasetti, C., Li, L., Springer, S., Kinde, I., Silliman, N., Tacey, M., Wong, H.-L., Christie, M., et al. (2016). Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci. Transl. Med. 8, 346ra92. https://doi.org/10.1126/scitranslmed.aaf6219.

van der Leest, P., Boonstra, P.A., ter Elst, A., van Kempen, L.C., Tibbesma, M., Koopmans, J., Miedema, A., Tamminga, M., Groen, H.J.M., Reyners, A.K.L., and Schuuring, E. (2020). Comparison of circulating cell-free DNA extraction methods for downstream analysis in cancer patients. Cancers 12, 1222. https://doi.org/10.3390/cancers12051222.

van Dessel, L.F., Vitale, S.R., Helmijr, J.C.A., Wilting, S.M., van der Vlugt-Daane, M., Oomen-de Hoop, E., Sleijfer, S., Martens, J.W.M., Jansen, M.P.H.M., and Lolkema, M.P. (2019). High-throughput isolation of circulating tumor DNA: a comparison of automated platforms. Mol. Oncol. 13, 392–402. https://doi.org/10.1002/1878-0261.12415.

Vandekerkhove, G., Lavoie, J.-M., Annala, M., Murtha, A.J., Sundahl, N., Walz, S., Sano, T., Taavitsainen, S., Ritch, E., Fazli, L., et al. (2021). Plasma ctDNA is a tumor tissue surrogate and enables clinical-genomic stratification of metastatic bladder cancer. Nat. Commun. 12, 184. https://doi.org/10.1038/s41467-020-20493-6.

Waclaw, B., Bozic, I., Pittman, M.E., Hruban, R.H., Vogelstein, B., and Nowak, M.A. (2015). A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity. Nature 525, 261–264. https://doi.org/10.1038/nature14971.

Waldman, A.D., Fritz, J.M., and Lenardo, M.J. (2020). A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat. Rev. Immunol. 20, 651–668. https://doi.org/10.1038/s41577-020-0306-5.

Wang, C., Mu, Z., Chervoneva, I., Austin, L., Ye, Z., Rossi, G., Palazzo, J.P., Sun, C., Abu-Khalaf, M., Myers, R.E., et al. (2017). Longitudinally collected CTCs and CTC-clusters and clinical outcomes of metastatic breast cancer. Breast Cancer Res. Treat. 161, 83–94. https://doi.org/10.1007/s10549-016-4026-2.

Whiteside, T.L. (2016). Tumor-derived exosomes and their role in cancer progression. Adv. Clin. Chem. 74, 103–141. https://doi.org/10.1016/bs.acc.2015.12.005.

Yang, M.-H., Wu, M.-Z., Chiou, S.-H., Chen, P.-M., Chang, S.-Y., Liu, C.-J., Teng, S.-C., and Wu, K.-J. (2008). Direct regulation of TWIST by HIF-1α promotes metastasis. Nat. Cell Biol. 10, 295–305. https://doi.org/10.1038/ncb1691.

Ye, L., Zhang, F., Li, H., Yang, L., Lv, T., Gu, W., and Song, Y. (2017). Circulating tumor cells were associated with the number of T lymphocyte subsets and NK cells in peripheral blood in advanced non-small-cell lung cancer. Dis. Markers 2017, 1–6. https://doi.org/10.1155/2017/5727815.

Yu, M., Stott, S., Toner, M., Maheswaran, S., and Haber, D.A. (2011). Circulating tumor cells: approaches to isolation and characterization. J. Cell Biol. 192, 373–382. https://doi.org/10.1083/jcb.201010021.

Zhang, D., Zhao, L., Zhou, P., Ma, H., Huang, F., Jin, M., Dai, X., Zheng, X., Huang, S., and Zhang, T. (2017a). Circulating tumor microemboli (CTM) and vimentin+ circulating tumor cells (CTCs) detected by a size-based platform predict worse prognosis in advanced colorectal cancer patients during chemotherapy. Cancer Cell Int. 17, 6. https://doi.org/10.1186/s12935-016-0373-7.

Zhang, Y., Zhou, N., Yu, X., Zhang, X., Li, S., Lei, Z., Hu, R., Li, H., Mao, Y., Wang, X., et al. (2017b). Tumacrophage: macrophages transformed into tumor stem-like cells by virulent genetic material from tumor cells. Oncotarget 8, 82326–82343. https://doi.org/10.18632/oncotarget.19320.

Zhou, W., Fong, M.Y., Min, Y., Somlo, G., Liu, L., Palomares, M.R., Yu, Y., Chow, A., O'Connor, S.T.F., Chin, A.R., et al. (2014). Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. Cancer Cell 25, 501–515. https://doi.org/10.1016/j.ccr.2014.03.007.