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Review

Circulating tumour cells: The Good, the Bad and the Ugly

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ABSTRACT

This review is an overview of the current knowledge regarding circulating tumour cells (CTCs), which are potentially the most lethal type of cancer cell, and may be a key component of the metastatic cascade. The clinical utility of CTCs (the "Good"), includes their diagnostic, prognostic, and therapeutic potential. Conversely, their complex biology (the "Bad"), including the existence of CD45+/EpCAM+ CTCs, adds insult to injury regarding their isolation and identification, which in turn hampers their clinical translation. CTCs are capable of forming microemboli composed of both non-discrete phenotypic populations such as mesenchymal CTCs and homotypic and heterotypic clusters which are poised to interact with other cells in the circulation, including immune cells and platelets, which may increase their malignant potential. These microemboli (the "Ugly") represent a prognostically important CTC subset, however, phenotypic EMT/MET gradients bring additional complexities to an already challenging situation.

1. The Good: The clinical utility of CTCs

Circulating tumour cells (CTCs) are cancer cells that have been shed or migrated from the primary tumour into circulation and are a critical component of the metastatic cascade, which is responsible for 90% of cancer-associated deaths [1]. Following detachment from the primary tumour and intravasation into the bloodstream, these migratory cells undergo epithelial-mesenchymal transition (EMT) and interact with other cell types within the circulation, eventually adhering to endothelial membranes and extravasating to distant organs where they form secondary tumours [2]. Representing as few as 1 cell in 10^7 white cells/ 10 mL of blood, there are inherent difficulties in isolating and characterising these tumour cells [3]. The intrinsic value of CTCs, however, lies in their potential clinical utility as a non-invasive biomarker for assessing patient disease at various stages. Acting not only as a diagnostic marker but as a marker for assessing patient prognosis and a means of assessing and monitoring treatment response and identifying tumour surface markers and mutations which may be therapeutically actionable. In the diagnostic setting, CTCs are particularly attractive as they are readily detectable in blood and may precede limits of detection of conventional clinical diagnostic imaging procedures such as computed tomography, magnetic resonance imaging, and positron emission tomography where tumour lesions may already be considerably larger before they are detectable with sufficient accuracy [4]. CTC isolation therefore may allow for earlier detection and treatment and prevent disease progression. This is especially important for fast-progressing cancers. CTCs are detectable in a multitude of cancer types including breast, prostate, ovarian, lung, liver, pancreatic, colorectal, gastric and melanoma [5–10] which is quite advantageous over other biomarkers which may be quite cancer-specific or even variable within single cancer types due to genotypic or phenotypic variation. Although their use in early detection remains controversial [4]. There is

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some debate as to how early in the disease process CTCs disseminate from the primary tumour and whether this is a continuous process or occurs in waves or in response to certain stimuli. Although various *in-vivo* studies suggest CTC dissemination is an early event [4]. Their relative scarcity, fragility and the difficulty in their isolation also hinders the clinical translation of CTCs as a routine diagnostic [4].

The reliable detection and characterisation of CTCs, however, is crucially important for the management of patients with solid tumours, especially breast cancer where they have been shown to have significant prognostic value [9]. CTCs were even included in the 8th edition of the AJCC TNM staging system for breast cancer [11]. Various technologies exist for the isolation of CTCs utilising either surface marker expression or exploiting differences in physical properties of CTCs to separate them from other blood components [12]. CTCs generally express epithelial markers such as Epithelial Cell Adhesion (EpCAM) and lack markers specific to normal blood leukocytes such as CD45 [13]. The detection of >5 EpCAM+ CD45- morphologically viable CTCs in 7.5 mL of blood (CellSearch®) correlates with both reduced progression-free survival (PFS) and reduced overall survival (OS) in patients [9,14]. This clinical threshold also appears to distinguish patients with malignant disease, as CTC events are typically rare in benign patients and healthy controls [9,14]. The prognostic potential of CTCs has also been demonstrated across the cancer space in a large number of meta-analyses (Table 1) and pooled analyses [5–10] with CTC counts >5 again usually indicating inferior survival in patients. Steady or increasing counts over the course of treatment, CTC aggregation and the underlying molecular phenotype of CTCs also similarly have strong prognostic value [4,15–19].

CTCs represent a heterogeneous population of cells whose phenotype may be molecularly distinct from the primary tumour and provide a perhaps more up-to-date sample which due to ease of access may allow for real-time monitoring and detection of actionable therapeutic targets. Their presence in the circulation and phenotypic differences may render them resistant/diminish the effects of radiation therapy regimens which are localised to the primary tumour as well as targeted therapies which are based on the surface expression of specific markers detected in the primary tumour. As a major source of secondary metastasis and associated mortality, their clearance is of the utmost importance and their persistence throughout the course of treatment has been shown to be a major source of relapse and treatment failure in breast, lung and potentially other cancers as well [20–28]. However, they may harbour mutations or surface markers not detected in the primary tumour which may be therapeutically actionable [29,30]. HER2 for example is frequently found to be expressed on CTCs isolated from breast cancer patients and their presence and frequency is associated with poor clinical outcome and response to anti-HER2 therapy [31,32]. Furthermore, the clinical utility is not limited only to breast cancer, HER2 can also be detected on CTCs from ovarian, colorectal and other tumours [33–36]. In ovarian cancer interestingly HER3 a dimerisation partner of HER2 has been found to play a key role in haematogenous metastasis to the omentum where it interacts with binding partner NRG-1 [37]. Targeted therapies which inhibit HER2 or its dimerization such as trastuzumab, lapatinib or pertuzumab may help prevent metastatic spread and secondary site colonisation in patients with HER2+ CTCs. Patients may display HER2+ CTC clones even if their primary tumour was HER2 negative [38]. However, there are some limitations to the detection of HER2 on CTCs such as differences in the methods of detection. HER2 on CTCs is typically detected using immunofluorescence whereas tumour biopsies and resections are examined using immunohistochemistry and in situ hybridisation which have different sensitivities as well as defined clinical cut-offs [39].

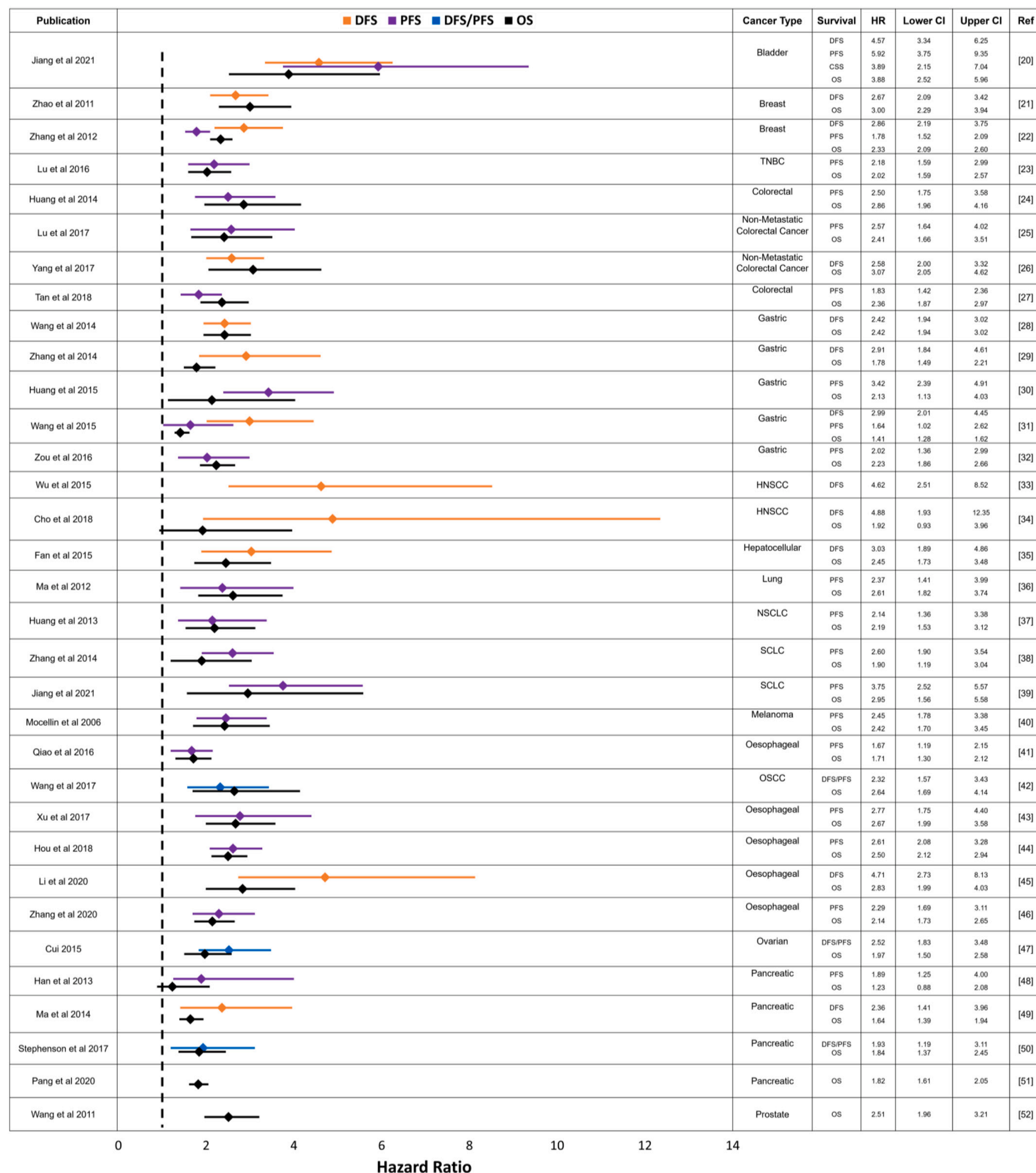
CTCs may also be useful as a tool to assess treatment resistance and to identify novel resistance mechanisms and biomarkers that aid treatment personalisation and clinical options for overcoming therapeutic resistance. In one NSCLC study [40], Pailler et al assessed the mutational profile of CTCs from patients at disease progression treated with ALK inhibitor Crizotinib. Crizotinib-resistant patients displayed mutations in ALK-independent pathways such as RTK-KRAS and TP53 pathways were predominantly identified in the CTCs of crizotinib-resistant patients. An interesting study by Scher et al [41] found that nuclear expression of AR-V7 in metastatic castration-resistant prostate cancer was a treatment-specific biomarker and was associated with superior survival in patients treated with taxane therapy over ARS-directed therapy. CTCs have been also been shown to upregulate various immune checkpoint molecules including B7-H3, PD-1, PD-L1, PD-L2, and CTLA-4 which may render them susceptible to checkpoint inhibitor therapy [42,43]. PDL1 in particular is frequently found to be expressed on CTCs from various solid tumours including breast, prostate, hepatocellular, urothelial, head and neck, melanoma, gastric and colorectal cancer. PDL1 may become upregulated in CTCs undergoing EMT or as a result of interaction with platelets and utilised as a means of immune escape [44]. PDL1 expression on CTCs has shown been to correlate with poor survival and resistance to therapy. However PDL1 status on CTCs appears to predict response to immune checkpoint therapies such as nivolumab and pembrolizumab [45–53]. Clearly, CTCs have vast potential as theranostic markers. The detection or re-emergence of CTCs post-treatment may also be an early indicator of disease recurrence and surface marker expression and neoantigen profile may even be altered post-therapy [54]. CTCs may also offer a route for pre-clinical drug screening, through *ex-vivo* culture as cell lines or spheroids or expansion in *in-vivo* models [20,55].

2. The Bad: The liquid biopsy heterogeneity problem

Detection and enumeration of CTCs represents a clinically useful and non-invasive diagnostic biomarker which could aid patient clinical management and decision-making. The fact that CTCs can also be detected in a multitude of cancer types makes them a relatively “Good” marker for assessing the illicit effects of metastatic disease in patients. The “Bad” thing about CTCs, however, is the fact that their detection may be limited by assessing EpCAM and CD45 alone as CTCs represent a more diverse population of cells. This phenotypic diversity presents a major challenge for clinicians and researchers for the enumeration, characterisation and fundamental understanding of the underlying biology of these important cancer cells. Traditional surface marker-based detection systems such as the FDA-approved CellSearch® device have shown clinical utility as a diagnostic/prognostic test. However, there are inherent limitations with this approach for isolation, with for example populations of dual EpCAM and CD45 positive and EpCAM negative CTCs and other atypical circulating cells having been described [56–60]. There are various hypotheses and explanations as to why these abnormal entities are detected, in many cases authors may be describing the same phenomena but under slightly different contexts.

The most common explanation for the lack of surface expression of epithelial markers by CTCs is their ability to undergo EMT [61–63]. These cells are undetectable by systems such as CellSearch® and may be present in as many as 35% of metastatic breast cancer patients and over 60% of patients with brain metastasis [14,64–66]. These CTCs often display enhanced metastatic seeding capabilities, greater resistance to chemotherapy, appear to be particularly enriched for stemness markers and can remain dormant in circulation for extended periods [65,67–69]. In breast cancer, CTCs with a CD44+CD24- antigenic profile have been frequently described [60]. These mesenchymal CTCs may also induce immune escape mechanisms such as elevating the expression of CD47 which through binding of SIRP-α on immune cells inhibits phagocytic clearance [70]. Although it has also been postulated that CTCs that exhibit an intermediate or quasimesenchymal state are required for successful metastatic dissemination as true mesenchymal CTCs may not be able to efficiently establish metastatic disease [71]. CTCs that display this intermediate quasimesenchymal phase due to their enhanced cellular plasticity are more likely to be able to undergo MET and extravasate to access distant tissue target sites [72,73]. Whether CTCs have an inherent capacity to switch to a mesenchymal phenotype or if

Table 1
Forest plot and summary table of meta-analyses showing the prognostic association between CTCs and survival in different cancer types.



Publication	Cancer Type	Survival	HR	Lower CI	Upper CI	Ref
Jiang et al 2021	Bladder	DFS	4.57	3.34	6.25	[20]
		PFS	5.92	3.75	9.35	
		CSS	3.89	2.15	7.04	
		OS	3.88	2.52	5.96	
Zhao et al 2011	Breast	DFS	2.67	2.09	3.42	[21]
		OS	3.00	2.29	3.94	
Zhang et al 2012	Breast	DFS	2.86	2.19	3.75	[22]
		PFS	1.78	1.52	2.09	
		OS	2.33	2.09	2.60	
Lu et al 2016	TNBC	PFS	2.18	1.59	2.99	[23]
		OS	2.02	1.59	2.57	
Huang et al 2014	Colorectal	PFS	2.50	1.75	3.58	[24]
		OS	2.86	1.96	4.16	
Lu et al 2017	Non-Metastatic Colorectal Cancer	PFS	2.57	1.64	4.02	[25]
		OS	2.41	1.66	3.51	
Yang et al 2017	Non-Metastatic Colorectal Cancer	DFS	2.58	2.00	3.32	[26]
		OS	3.07	2.05	4.62	
Tan et al 2018	Colorectal	PFS	1.83	1.42	2.36	[27]
		OS	2.36	1.87	2.97	
Wang et al 2014	Gastric	DFS	2.42	1.94	3.02	[28]
		OS	2.42	1.94	3.02	
Zhang et al 2014	Gastric	DFS	2.91	1.84	4.61	[29]
		OS	1.78	1.49	2.21	
Huang et al 2015	Gastric	PFS	3.42	2.39	4.91	[30]
		OS	2.13	1.13	4.03	
Wang et al 2015	Gastric	DFS	2.99	2.01	4.45	[31]
		PFS	1.64	1.02	2.62	
		OS	1.41	1.28	1.62	
Zou et al 2016	Gastric	PFS	2.02	1.36	2.99	[32]
		OS	2.23	1.86	2.66	
Wu et al 2015	HNSCC	DFS	4.62	2.51	8.52	[33]
Cho et al 2018	HNSCC	DFS	4.88	1.93	12.35	[34]
		OS	1.92	0.93	3.96	
Fan et al 2015	Hepatocellular	DFS	3.03	1.89	4.86	[35]
		OS	2.45	1.73	3.48	
Ma et al 2012	Lung	PFS	2.37	1.41	3.99	[36]
		OS	2.61	1.82	3.74	
Huang et al 2013	NSCLC	PFS	2.14	1.36	3.38	[37]
		OS	2.19	1.53	3.12	
Zhang et al 2014	SCLC	PFS	2.60	1.90	3.54	[38]
		OS	1.90	1.19	3.04	
Jiang et al 2021	SCLC	PFS	3.75	2.52	5.57	[39]
		OS	2.95	1.56	5.58	
Mocellin et al 2006	Melanoma	PFS	2.45	1.78	3.38	[40]
		OS	2.42	1.70	3.45	
Qiao et al 2016	Oesophageal	PFS	1.67	1.19	2.15	[41]
		OS	1.71	1.30	2.12	
Wang et al 2017	OSCC	DFS/PFS	2.32	1.57	3.43	[42]
		OS	2.64	1.69	4.14	
Xu et al 2017	Oesophageal	PFS	2.77	1.75	4.40	[43]
		OS	2.67	1.99	3.58	
Hou et al 2018	Oesophageal	PFS	2.61	2.08	3.28	[44]
		OS	2.50	2.12	2.94	
Li et al 2020	Oesophageal	DFS	4.71	2.73	8.13	[45]
		OS	2.83	1.99	4.03	
Zhang et al 2020	Oesophageal	PFS	2.29	1.69	3.11	[46]
		OS	2.14	1.73	2.65	
Cui 2015	Ovarian	DFS/PFS	2.52	1.83	3.48	[47]
		OS	1.97	1.50	2.58	
Han et al 2013	Pancreatic	PFS	1.89	1.25	4.00	[48]
		OS	1.23	0.88	2.08	
Ma et al 2014	Pancreatic	DFS	2.36	1.41	3.96	[49]
		OS	1.64	1.39	1.94	
Stephenson et al 2017	Pancreatic	DFS/PFS	1.93	1.19	3.11	[50]
		OS	1.84	1.37	2.45	
Pang et al 2020	Pancreatic	OS	1.82	1.61	2.05	[51]
Wang et al 2011	Prostate	OS	2.51	1.96	3.21	[52]

Studies include pooled survival data from multiple cancer subtypes and isolation devices, with the majority of studies utilising CellSearch® technology. TNBC – triple-negative breast cancer, HNSCC – head and neck squamous cell carcinoma, NSCLC – non-small cell lung cancer, SCLC – small cell lung cancer, OSCC – oesophageal squamous cell carcinoma.

the tumour microenvironment acts as a selection system for cells with this capacity is an important question in CTC biology.

The expression of CD45 by CTCs may also be related to EMT or it may possibly be due to tumour cell interactions with immune cells and platelets either in the primary tumour microenvironment prior to entry into systemic circulation or while in transit [18,74]. EMT can trigger the induction of immune-related surface markers such as PDL1 so it may be an attempt at immune invasion or immune mimicry [75]. Other immune-related surface markers including PD1 [76], FOXP3 [77], CD68 [78] and CD163 [79] have also been reportedly detected on tumour cells. However, very little is known currently about the actual physical mechanism of how CTCs come to acquire a CD45+ phenotype. Are CTCs that come into contact with or that travel with immune cells simply imprinted with CD45 or other immune cell antigens or can membrane fragments remain from apoptosed immune cells which are engulfed by the tumour cells either in the primary tumour microenvironment or in circulation. CTCs may also actively exchange membrane components during intercellular contact with other cell types, via the release of exosomes, or through the extension of cellular projections such as nanotubes, cytonemes, epithelial bridges or other specialised filipodic structures [74,80,89–95,81–88]. A recent study by Gutwillig et al [96] found that tumour cells could transiently hide within other cancer cells and by doing so, resist immunotherapy leading to tumour relapse. Other cell-in-cell phenomena such as emperilosis, entosis and cell cannibalism have also been described previously which could explain their acquisition of immune cell antigens [97].

Conversely, these EPCAM+ CD45+ cells may in fact be immune cells or other cell types which have somehow developed a tumour cell phenotype. This may be due to previous interactions with tumour cells and potentially be a method of immune cell subversion or immune cell hijacking by tumour cells in an attempt to alter the phenotype of immune cells towards a more tumour-sparing phenotype [98]. This could also serve as a means of sequestering/hoarding scarce metabolic resources in the tumour microenvironment and effectively act as a means of sabotaging their competition [99,100]. Phagocytic engulfment is also likely another key reason for the description of immune cells with cancer cell phenotypes which are described in different contexts such as Cancer-Associated Macrophage-Like Cells (CAMLs) and Tumacrophages. CAMLs express CD45 and epithelial markers such as cytokeratins and EpCAM and are described as enlarged (25-300 μm) highly differentiated cells with multiple nuclei and contain phagocytosed tumour matter [57]. They are thought to form by the fusion of tumour-associated macrophages, which have phagocytosed dying tumour cells and then re-entered circulation with the phagocytosed fragments. Adams and colleagues [58] reported the presence of these fusion hybrid cells in blood samples tested using cell sieve microfiltration technology in 93% of malignant breast cancer patients, and 26% of patients with benign disease; but they were not detected in healthy controls. They also reported the presence of CAMLs bound to CTCs in 10% of patients with metastatic cancer [59]. CAMLs while perhaps not being tumourigenic themselves may help facilitate the immunoevasion and metastatic seeding of CTCs. Tumacrophages [101] similar to CAMLs have been described although only in a single publication as CD163+ macrophages, which have acquired epithelial markers such as EpCAM and cytokeratins following engulfment of apoptotic cancer cells; these cells then also display both enhanced proliferation and migration phenotypes. Phagocytosis and subsequent horizontal transfer of DNA by apoptotic bodies is thought to reprogram these cells into cancer stem cells. The detection of such macrophages harbouring tumour cell transcripts in blood samples isolated from breast, endometrial, pancreatic, ovarian and colorectal tumours [101] indicates that this is a common phenomenon.

Another explanation for the acquisition of immune cell traits by tumour cells or vice versa is due to cellular fusion or the true merger of the two cell types. Indeed, cellular fusion events are not a unique phenomenon and are widely observed throughout biology [102–105]. Fusion hybrid cells have also been reported in the literature by various groups and are thought to represent true cellular fusion events between tumour cells and leukocytes [56,57,105–107]. Very little is known however about the underlying mechanisms governing this bizarre cell fusion event between leukocytes and tumour cells. Proponents of the leukocyte-tumour cell fusion theory argue that the inheritance of leukocyte genes would provide tumour cells with many cellular traits we typically associate with CTCs and metastasis such as enhanced motility, angiogenesis, organ tropism, immune signalling, expression of matrix degradation and remodelling proteins, hypoxia signalling and even multidrug resistance [108]. Gast and colleagues [56] demonstrated that the presence of such cells in peripheral blood samples also correlated with both stage of disease and reduced OS in a small cohort of pancreatic ductal adenocarcinoma patients. Clawson and colleagues [109] similarly were reportedly able to isolate, and culture dual EpCAM+ CD45+ hybrid cells from enriched CTC fractions from patients with melanoma. In a subsequent study Clawson and colleagues [110] isolated hybrid CTC populations from the peripheral blood of patients with colorectal and pancreatic tumours, where metastases had formed in multiple organs. It is feasible that by de-differentiating, cancer cells may be able to adopt multiple cellular phenotypes and use this as a mechanism for selective advantage to combat and adapt to multiple cellular environments.

Clearly, marker positivity or negativity alone is insufficient for CTC determination. Many currently utilised positive selection technologies based on EpCAM/CD45 such as CellSearch® will automatically exclude many of the above-described atypical phenotypes of CTCs, which likely have extremely important prognostic relevance for cancer patients. Even with label-free enrichment devices using microfluidics such as Parsortix® or ClearCell® FX1 or other filtration-based technologies such as ScreenCell®, may likely discount these cells during downstream evaluation using immunofluorescence and/or be mistakenly labelled as leukocytes. Therefore a more comprehensive understanding of the molecular taxonomy of CTCs is warranted to expose the CTC treasure. It is, however, good that we have begun to recognise the critical importance of CTCs and their morphological variants for both cancer surveillance and clinical intervention. The importance of different CTC phenotypes and architecture is discussed as a reference point throughout the review.

3. The Ugly: CTC safety in numbers

The true ugliness of CTCs, however, lies in their ability to form monoclonal or polyclonal homotypic clusters with other CTCs or heterotypic clusters with platelets and immune cells. These large CTC clusters also termed microemboli which can consist of two cells or be considerably larger and consist of 50 or more cells [18] are thought to become entrapped within blood vessels downstream of the primary tumour unlike single CTCs that can continue to migrate in the peripheral circulation [18,111] (Fig. 1). Aceto and colleagues [18] identified that CTC clusters within xenograft mouse models were able to extravasate from the bloodstream at triple the rate of singlets. Although representing only a minute subset of CTC events (2-5%) they exhibit a 20-50-fold higher potency for developing metastatic lesions. They are therefore a particularly lethal component of the metastatic cascade and their presence, which is detectable in multiple cancer types is associated with reduced survival rates, response to therapy and poor clinical outcome [16,18,119–122,19,112–118]. CTC clusters may also seed venous thromboembolism, which is a major cause of cancer-associated mortality [123]. (See Figs. 2 and 3.)

Cluster formation appears to promote CTC aggressiveness by enhancing CTC proliferative capacity, resistance to anoikis and damage from the circulatory shear forces [18,115,124]. The large size of clusters likely enhances tethering to the endothelium and promotes their extravasation as opposed to single cells which will only be capable of extravasating in postcapillary venules where reduced vessel diameter and blood flow would permit them to make close contact with the

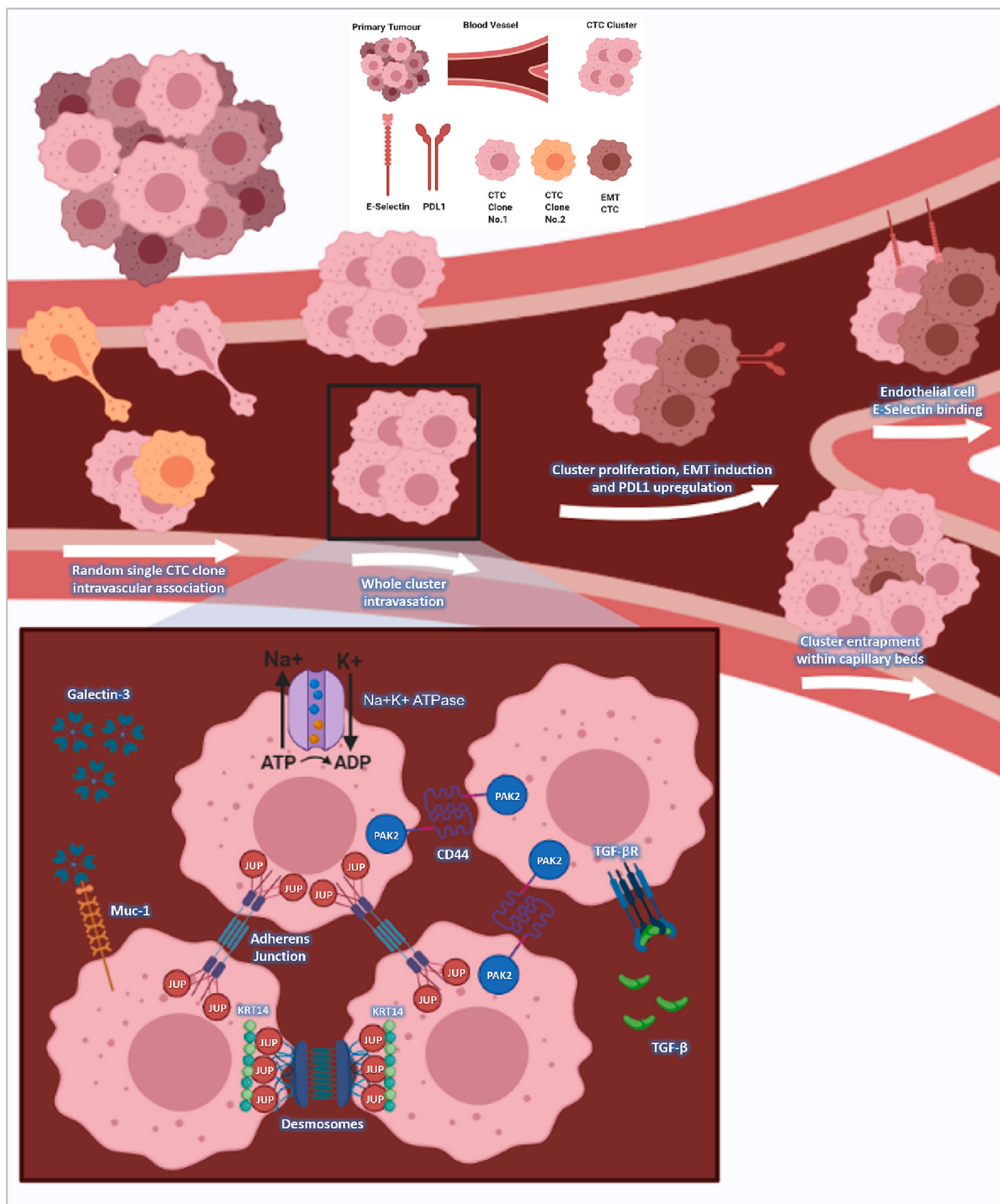


Fig 1. CTC cluster formation enhances CTC extravasation and lethality. CTC clustering is mediated by plakoglobin (JUP) a component of desmosomes and adherens junctions [136] and homotypic interactions between CD44 molecules between adjacent cells which activate the downstream serine-threonine protein kinase p21 protein (Cdc42/Rac)-activated kinase 2 (PAK2) [137]. Na+K+ ATPase also appears to be crucial to the tight association of cells within clusters while circulating Galectin-3 can promote cell aggregation through the binding of Muc-1 [129]. CTC clusters form by various mechanisms including the release of whole intact clusters into the bloodstream or can form through the intravascular aggregation of single CTCs. Intact CTCs within clusters can undergo proliferation, EMT and upregulate PDL1 to suppress the immune response. Increased CD44 expression also enhances extravasation through binding of E-selectin on endothelial cell membranes. Large clusters can become entrapped within capillary beds, another factor which likely enhances their extravasation.

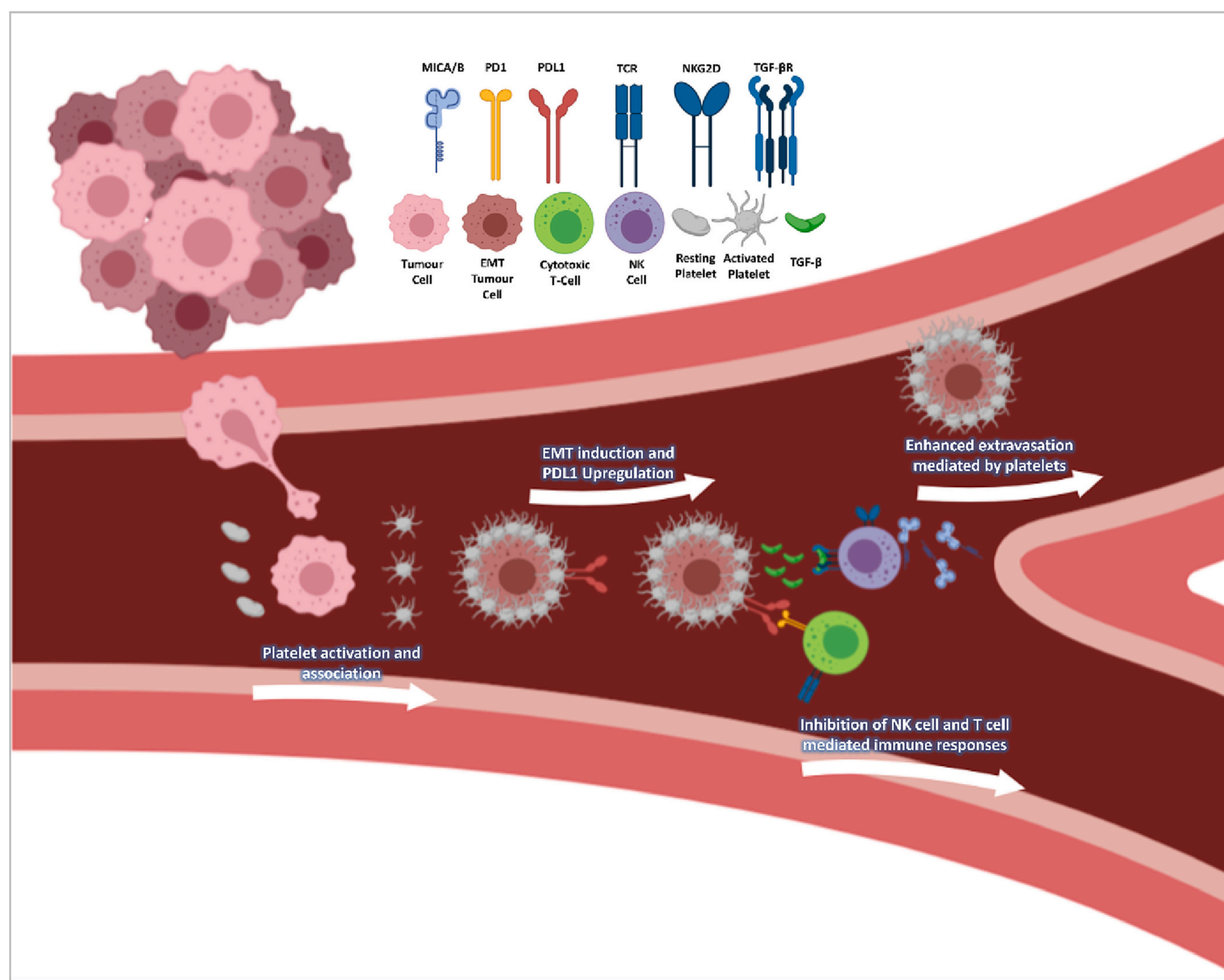


Fig. 2. Platelet Cloaking promotes CTC dissemination. Activated platelets adhere to CTCs in circulation, induce EMT and upregulate PDL1 resulting in suppression of T-cell cytotoxicity. Platelet cloaking and platelet-derived TGF-β also suppress NK cell-mediated cytotoxicity by inducing cleavage of NKG2D soluble ligands MICA & MICB and downregulation of other NK cell ligands and their corresponding receptors. Platelet cloaking may also prevent T-Cell Receptor (TCR) recognition by T-cells further promoting immunoevasion[138,148].

endothelium. CTC clusters have also been found to display enhanced expression of EMT/stemness markers including CD44, OCT4, SOX2, Nanog and SIM3A and display enhanced expression of cell junction proteins such as plakoglobin and E-Cadherin. Other markers such as KRT14, PAK2 and MUC1 have also been shown to also contribute to CTC aggregation [18,125–129]. The aggressiveness of CTC clusters may also be further promoted by hypoxia which has been shown to enhance cell proliferation and promote mitophagy in cell clusters limiting ROS production by damaged mitochondria, which accumulate in cells following detachment [130]. Furthermore, hypoxia also appears to promote Warburg metabolism and reliance on glycolysis for ATP production rather than oxidative phosphorylation [131]. Disruption of this metabolic switch has been shown to diminish cluster formation, enhance levels of ROS production and decrease metastatic seeding abilities of tumour cells *in-vivo*. [132]. Therapeutic targeting of CTC clusters and CTC aggregation may represent a viable strategy for inhibiting metastatic disease [114,133–135].

CTC interactions with platelets and other immune cells are thought to aid CTC immunoevasion and dissemination as reviewed by our group previously [138]. In various studies examining the transcriptional profiles of CTCs and CTC clusters, transcripts for various platelet-associated genes have been identified, indicating a key role for platelets in CTC biology [18,139,140]. In the case of platelets, membrane transfer from platelets to CTCs known as “platelet cloaking” is thought to allow CTCs to evade the immune system and promote CTC survival plasticity and eventual extravasation by facilitating attachment to extracellular matrices on endothelial cells [141]. Evidence suggests that molecular exchange between interacting platelets and CTCs also promotes a more stem-like phenotype in CTCs, which likely further enhances their metastatic potential and ability to survive circulatory shear forces [142,143]. Targeting the interaction between platelets and CTCs with therapies such as aspirin may help prevent and treat cancer metastasis [144–147].

CTCs are also frequently observed in circulation associated with neutrophils and this interaction likely promotes tumour immune evasion and enhances trafficking to distal sites due to the inherent abilities of neutrophils to extravasate. Neutrophils can also interact directly with platelets and both cell types may act in concert to promote thrombus formation and CTC- associated lethality [138]. Szczerba and colleagues [74] reported that the presence of even a single CTC-neutrophil cluster was associated with significantly worse prognosis. Neutrophils also contribute to metastasis through the release of

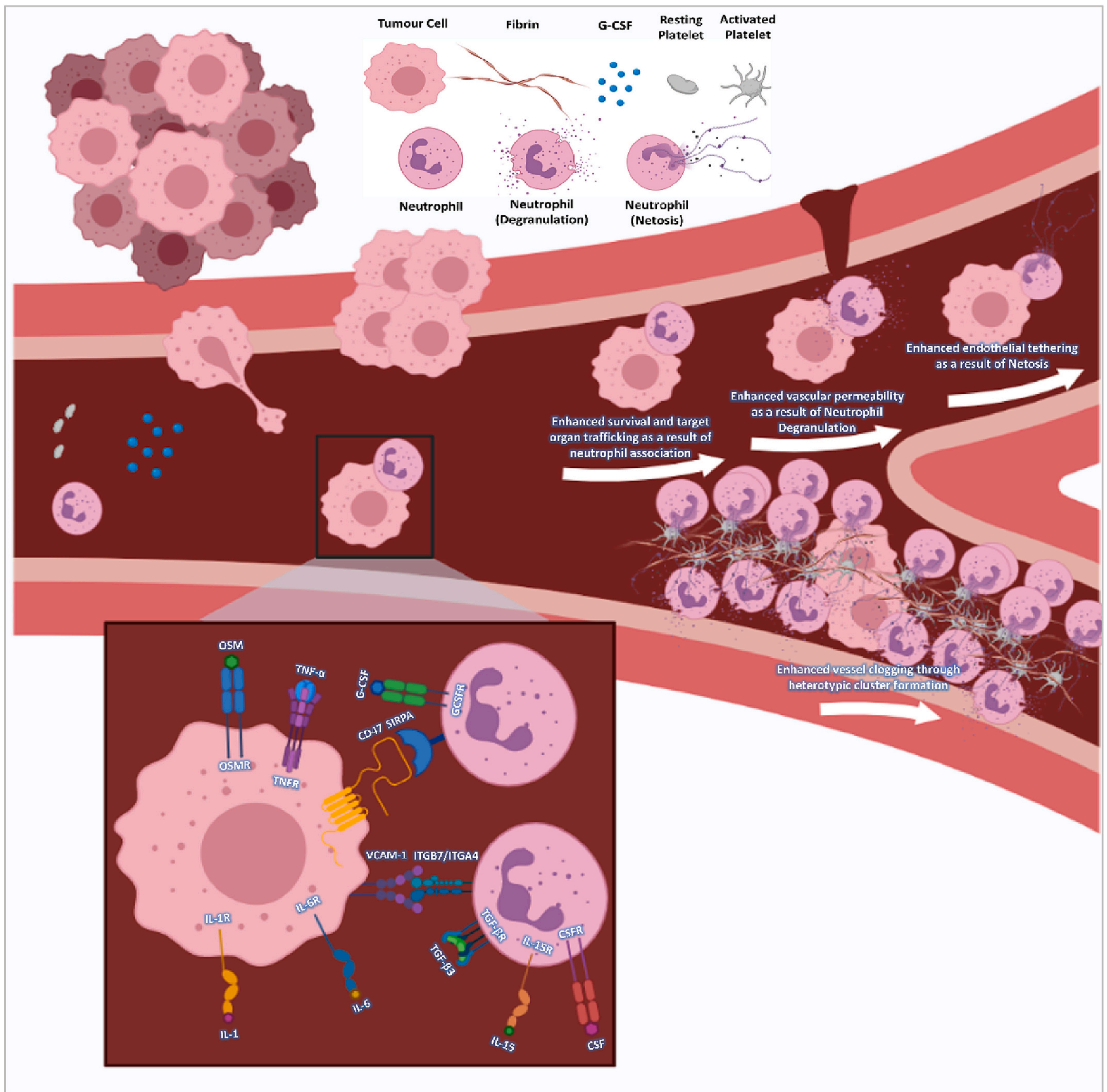


Fig. 3. Association between Neutrophils and CTCs. The association between neutrophils and CTCs is a crucial component of the metastatic cascade. The release of soluble factors from the primary tumour such as G-CSF recruits neutrophils, where the association of neutrophils with tumour cells likely aids their extravasation into the bloodstream. Engagement between tumour cells and neutrophils is facilitated by cell adhesion molecules such as VCAM-1 which bind to neutrophil surface integrins. Molecules such as CD47 on tumour cells likely also protects them from neutrophil-mediated lysis acting as a "don't kill me signature". Once associated neutrophils can aid the trafficking of CTCs to target sites, protecting them from immune destruction and circulatory shear forces and enhancing their adherence to endothelial membranes. CTCs have been shown to enhance the proliferation and circulating half-life of neutrophils. The release of neutrophil granules which contain various factors including matrix remodelling enzymes may aid CTC extravasation by enhancing vascular permeability. While induction of neutrophil extracellular traps may further help adherence to endothelial membranes and also trap CTCs in capillary beds.

neutrophil extracellular traps (NETs), which can promote mitochondrial biogenesis and tumour cell proliferation, migration and invasive capabilities [149,150]. Furthermore, elevated levels of surrogate markers of NETs, such as HMGB1 and MPO-DNA complexes, have been shown to correlate with disease relapse in patients [151]. All of these studies highlight the hugely important role of neutrophils and their associated NETs in promoting CTC dissemination. Surrogate markers of NETs such as MPO-DNA complexes have also shown great utility as diagnostic biomarkers. Targeting the interactions between Neutrophils, NETs and CTCs may improve patient outcomes.

4. Discussion

CTCs are a very heterogeneous population of circulating cells and exhibit a dynamic array of cell surface markers. Significant efforts have been made to capture, enumerate and ultimately understand the

complex biology of these cells. There is an urgent need to better characterise these rare cells and utilise technologies that capture the phenotypic diversity of CTCs or indeed multiple devices that detect distinct populations of CTCs may be needed when evaluating the patient. Label-free enrichment devices may capture a greater diversity of CTCs within patients but may be prone to higher rates of false-positive detection and contamination from circulating leukocyte populations. Methods utilising surface markers such as EpCAM and CD45 may not detect populations of CTCs undergoing EMT or containing more stem-like phenotypes that are potentially more aggressive. CD45 negative selection also excludes potential CTCs with a dual positive phenotype. There are inherent limitations to these methods due to the complex biology of CTCs and currently, there are no biomarkers which clearly and definitively identify CTCs. The potential for CTCs to acquire leukocyte surface markers and vice versa as well as the frequent association between CTCs and immune cells further hampers efforts to isolate and characterise them. Clearly, there is a need to improve methods for the validation of CTCs to increase diagnostic accuracy and reproducibility.

New emerging technologies such as those which allow the molecular analysis of CTCs at single-cell resolution offer great promise and could potentially resolve issues surrounding the cellular heterogeneity of circulating cell populations. Ultimately transcriptomic analysis of circulating cells is not only necessary but essential to reconcile issues surrounding the identity of CTCs and their cellular heterogeneity and to clearly distinguish them from potentially contaminating leukocyte populations. Transcriptome analysis of these cells may also improve current efforts analysing CTCs for treatment monitoring as well as providing insight into the cellular processes underlying tumour metastasis and potentially providing new therapeutic targets for exploitation in future clinical trials.

Current methods of characterising CTCs either at the protein level such as IHC, single-cell western blotting or other methods or at the gene level such as PCR and FISH, are currently constrained due to both the rarity of CTCs in circulation and due to limitations on the number of molecular markers that can be analysed. Sequencing approaches may therefore be required to overcome these issues. Low-pass sequencing is becoming an increasingly common method for the detection of CTCs due to its increased affordability and due to the low amounts of input DNA required and has great potential to be utilised as a part of clinical investigations [152–154]. Advances in scRNAseq technology may also help to improve CTC characterisation and provide a more diverse array of potential markers, which may more clearly distinguish CTCs from contaminating leukocytes and further clarify issues surrounding the identities of dual positive circulating cells and those undergoing EMT. An interesting study by Cheng and colleagues [155] utilised the scRNAseq technology Hydroseq, which pairs single cells within microwells with barcoded mRNA capture beads. In this study, they were able to capture and sequence 666 CTCs from 21 metastatic breast cancer patients following pre-enrichment. Utilising this technology, they were able to clearly distinguish CTCs from potentially contaminating leukocytes and were able to capture the inter-patient and intra-patient diversity of CTCs and distinguish between populations of epithelial, quasi mesenchymal and mesenchymal CTCs. Hydroseq and similar technologies provide an interesting platform for the characterisation of CTCs. Massively parallel sequencing of isolated single cells has distinct advantages over other approaches to scRNAseq, which have been used in many CTC studies to date. Single-cell manipulation methods, which have been utilised throughout the CTC space, have relied on capillary suction and dielectrophoretic microfluidics to dispense target cells to different tubes for downstream processing. Although this methodology has been successfully utilised to generate single-cell profiles, such approaches are laborious, low throughput, and difficult to scale up. Studies have also relied on immunofluorescent staining of markers to guide cell picking limiting their application for CTC heterogeneity studies. Massively paralleled single-cell sequencing approaches may also allow for greater standardisation of CTC sequencing and validation of CTCs in future studies. Single-cell analysis of increased numbers of CTCs will likely also allow for more comprehensive profiling of the phenotypic diversity of CTCs within individual patients and intra-patient heterogeneity. Furthermore, profiling of increased numbers of cells at single-cell resolution may help overcome issues such as CTC frailty and allelic dropout improving the quality of generated data sets across studies.

5. Conclusion

With the increasing integration of CTC assessment into clinical workflows such as staging and treatment and progression monitoring, researchers and clinicians must be aware of the current difficulties in analysing CTCs. Analysis of this diverse population of cells has many potential challenges and pitfalls. As our understanding of CTCs and their diversity improves it will likely lead to improvements in diagnostic accuracy and new therapies which can cut through the ugliness of CTC biology leading to improvements in patient survival.

Author contributions

Conceptualisation: MB, BM, MPW, JOL, SOT, CM, DB, RB & SS
Writing - original draft: MB, BM, MPW, JOL, SOT, CM, DB, RB & SS
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Declaration of Competing Interest

The authors declared no conflicts of interests

Data availability

No data was used for the research described in the article.

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