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Circulating Tumor Cells – An Overview of the Current Progress and Clinical Perspectives

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Abstract

Circulating tumor cells (CTCs) are cancerous cells that can detach from the primary tumor and circulate through the blood vessels until they reach a tissue or an organ and initiate metastasis. It is worth noting that in many types of cancer, the presence of CTCs in blood samples either independently or in clusters is considered a poor prognostic marker. This is because it indicates a lower overall survival (OS), a poorer progression-free survival (PFS), and a higher potential for metastasis. Characterising circulating tumor cells (CTCs) and monitoring their numbers can provide crucial information in managing cancer progression. Therefore, CTCs can be extremely useful in therapeutic monitoring, allowing doctors to follow treatment efficacy and make certain adjustments depending on their quantification.

Research on CTCs as a liquid biopsy analyte has seen significant advancements, leading to the translational use of CTCs as potential biomarkers. However, low concentration and the lack of standardised detection methods make it challenging to detect CTCs.

We offer an overview of the various phenotypic changes of CTCs and the epithelial-mesenchymal transition (EMT) process that promotes the spread of cancerous cells. We also explore the biomarkers that characterise CTCs, as well as the primary isolation techniques. Finally, we highlight the clinical perspectives of CTCs and their relevance in monitoring cancer progression and response to treatment. Thus, we believe that the study of CTCs can provide a deeper understanding of the metastasis process, which could ultimately lead to improved patient outcomes.

Keywords

circulating tumor cells, CTCs, cancer, circulating tumor DNA, ctDNA, epithelial-mesenchymal transition, metastases

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1. Background

The process of cancer metastasis is well recognised as the leading cause of mortality on a global scale and continues to be a significant obstacle in the pursuit of effective cancer treatments (Asrani et al., 2019).

Circulating tumor cells (CTCs) play a crucial role in the growth and dissemination of tumors, since they can endure the physiological challenges within the bloodstream and afterward migrate to distant locations, where they could lead to new tumor formations (Rupp et al., 2022).

The concept of CTCs was first documented in 1869 by Ashworth, who reported the presence of "certain cells" in the bloodstream of a patient with metastatic disease. These cells had morphological characteristics like those seen in the primary tumor (Lin et al., 2021).

CTCs possess unique characteristics that differentiate them from primary tumor cells. These characteristics include undergoing an epithelial-mesenchymal transition (EMT) that enables them to detach from the primary tumor, enter the bloodstream, form clusters of CTCs, and exhibit stem cell-like properties that increase their capacity to initiate metastasis (Lin et al., 2021; Pantel and Speicher, 2016). Therefore, comprehending the molecular processes behind CTC dissemination will provide a new perspective on the concept of metastatic disease with significance for the treatment approach.

Considering that CTCs are a noninvasive, accessible tool that may overcome tumor heterogeneity, various studies have been conducted over the last decade to investigate effective detection methods (Ju et al., 2022).

However, since these cells are exceedingly rare (1-10 per millilitre of whole blood) and may have significant differences in the expression of surface biomarkers, several challenges have been noted regarding their detection and isolation (Alvarez Cubero et al., 2017; Brasó-Maristany et al., 2020; Gires & Stoecklein, 2014).

Herein, we provide a summary of the biological milieu of CTCs, as well as an analysis of the current and emerging CTC detection techniques, with emphasis on their potential as biomarkers for metastatic disease.

2. Biological features and markers of CTCs

CTCs are cancerous cells that have undergone a series of cellular transformations through the process of EMT. This process shifts their phenotype from epithelial to mesenchymal. Cells that possess epithelial characteristics are able to form intercellular junctions through apical and basolateral polarity and have strong adhesion properties. The mesenchymal phenotype is defined by low adhesion, high motility, increased resistance to apoptosis, and invasiveness by inducing stem cell-like characteristics in the cancerous cells (Christiansen and Rajasekaran, 2006; Polyak and Weinberg, 2009).

Different markers differentiate between the

The three most well-known families of antigen on CTCs

epithelial markers			mesenchymal markers			stemness markers		
Marker	**Cancer type**	**Ref.**	**Marker**	**Cancer type**	**Ref.**	**Marker**	**Cancer type**	**Ref.**
E-cadherin	PCa mBC	Armstrong et al., 2011; Pal et al., 2015	N-cadherin	CRPrC mBC	Armstrong et al., 2011	ALDH1	BC mCRC NSCLC	Alix-Panabières and Pantel, 2014; Hanssen et al., 2016; Ning et al., 2018
EpCAM	Epithelial tumors	Alix-Panabières and Pantel, 2014	Vimentin	mCRPrC BC	Kallergi et al., 2011; Lindsay et al., 2016; Satelli et al., 2016	CD44	BC	Alix-Panabières and Pantel, 2014
Cks	Epithelial tumors	Alix-Panabières and Pantel, 2014	Twist1	BC	Kallergi et al., 2011	GD2, GD3 and GD1a**	NB	van der Haar Àvila et al., 2023
ZO	BlC	Chen et al., 2020	Akt and Pi3K	mCRC NSCLC	Hanssen et al., 2016; Ning et al., 2018	ABC proteins***	LC PC RB	Barriere et al., 2014
ESPR1	CRC	Fagoonee et al., 2017	ZEB1	BC	Kalinkova et al., 2021			
			FGFR2IIIc, Mena, p120 catenin*	OSCC	Lyu and Cheng, 2022; Osada et al., 2019			
			PLS3	CRC	Yokobori et al., 2013			

epithelial and mesenchymal phenotypes. In order to characterise an epithelial phenotype, several markers have to be overexpressed in the cancerous cells, such as epithelial cellular adhesion molecule (EpCAM), E-cadherin, cytokeratins (CKs) and epithelial splicing regulator1 (ESPR1). A mesenchymal phenotype is characterised by the overexpression of other markers, such as N-cadherin and vimentin (Barriere et al., 2014).

The transforming growth factor-β (TGF-β) released from platelets mediates EMT in cancerous cells. CTC clusters that undergo a mesenchymal transformation can originate from a single cell that proliferates into a cluster and has undergone EMT or from CTC clusters that have already gone through EMT (Yu et al., 2013).

In relation to different tumor localization, ***Table 1*** provides an overview of the three most well-known families of antigens expressed on CTCs.

Processes in which distinct epithelial markers are replaced with proteins found primarily in mesenchymal phenotypes, such as the E-cadherin to N-cadherin switch, have been associated with cancer progression and metastasis (Barriere et al., 2014). In tumor cells, the downregulation of the cell-cell adhesion molecule E-cadherin results from somatic mutations, chromosomal deletions, proteolytic cleavage, and the suppression of the E-cadherin gene (*CDH1*) promoter (Onder et al., 2008). Transcription factors such as Slug, Snail, and Twist, and a state of hypermethylation are involved in silencing *CDH1*. The basic helix-loop transcription factor Twist interferes with E-cadherin and mesenchymal marker-mediated cell adhesion. Furthermore, the abnormal expression of Twist promotes EMT. Invasive breast cancer and diffuse gastric cancer were associated with a higher expression of Twist and mutations of the *CDH1* gene (Monster et al., 2022; J. Yang et al., 2004).

EMT can also be induced by the TGF-β, miR-34 and miR-200 families. TGF-β mediates SNAIL1/2 and ZEB1/2, which are factors in the E-cadherin to N-cadherin switch, by downregulating E-cadherin expression. SNAIL1 increases the translation of ZEB. ZEB1/2 inhibits the expression of miR-200, while miR-200 impairs the translation of ZEB1/2. Similarly, SNAIL and miR-34 also go through a so-called 'double negative feedback loop' (Tian et al., 2013).

However, the phenotype of a CTC cannot be strictly classified as mesenchymal since *the EMT process can be reversible*, resulting in a mesenchymal-epithelial transition (MET) or attaining an intermediate state as a hybrid epithelial/mesenchymal (E/M) phenotype. The mixed phenotype allows the CTCs to form clusters and easily detach from the primary tumor site, exhibiting both epithelial and mesenchymal properties. Once the cancerous cells have reached a tissue or an organ and have begun seeding, they switch back to an epithelial phenotype to adhere to the surrounding area and develop into overt metastases (Jolly, 2015; Loh et al., 2019). (***Figure 1***).

CTCs shed from the primary or metastatic tumor and can easily enter the leaky blood vessels, as the tumor vascular supply is hypoxic, has a higher interstitial fluid pressure and favours angiogenesis (Amintas et al., 2020; Carmeliet and Jain, 2011; Liu et al., 2021).

To generate a metastatic spread, CTCs have to survive unfavourable factors in the bloodstream, such as anoïkis (detachment-induced cell death) and shear stress. Clusters show a higher resistance to these factors than a single CTC and 23-50 times increased metastatic potential (Aceto et al., 2014; Amintas et al., 2020; W.-C. Wang et al., 2018). Inside the blood vessel, a CTC's half-life depends on how it circulates: independently or in clusters.

An independent CTC has a half-life of approximately 25-30 minutes, while the clusters have a shorter half-life of 6-10 minutes (Aceto et al.,

Table 1:
CTC markers

EpCAM – Epithelial cellular adhesion molecule,
CKs – Cytokeratins,
ZO – Zonula occludens,
ESPR1 – Epithelial splicing regulator 1,
ZEB1– Zinc finger E-box binding homeobox 1,
PLS3 – Plastin-3,
ALDH1 – Aldehyde dehydrogenase-1,
BC – breast cancer,
m – metastatic,
BlC – bladder cancer,
CRC – colorectal cancer,
CRPrC – castration resistant prostate cancer,
NSCLC – non-small cell lung cancer,
OSCC – oral squamous cell carcinoma,
NB – neuroblastoma,
LC – lung cancer,
PC – pancreatic cancer,
RB – retinoblastoma,
FDA – Food and Drug Administration,
*** Alternative splicing proteins,**
**** Gangliosides,**
***** xenobiotic extrusion pump proteins.**

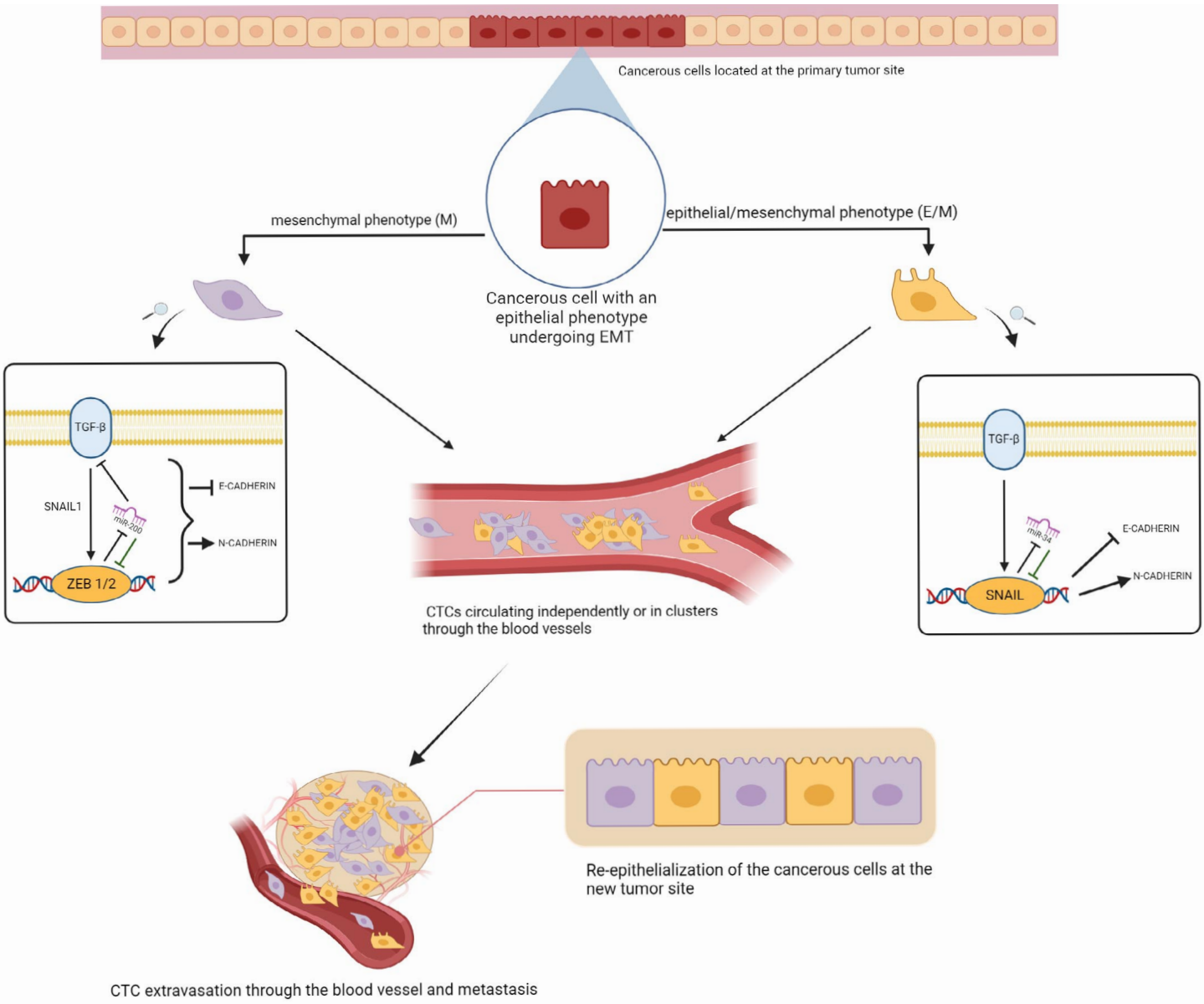

Figure 1
Epithelial – Mesenchymal Switch
The EMT process may be reversible, resulting in a mesenchymal-epithelial transition (MET) or an intermediate phenotype – epithelial/mesenchymal (E/M). TGF-β induces EMT and partial EMT by mediating ZEB1/2 and SNAIL. The hybrid CTCs can separate from the primary tumour and form clusters. After the CTCs have seeded and invaded a tissue/organ, they shift to an epithelial phenotype to adhere to the surrounding tissue.

(Created with BioRender.com).

2014; Micalizzi et al., 2017). The "seed and soil" theory by Stephen Paget suggests that cancer cells that have metastasized have the ability to selectively choose where they "seed" and form a new tumor in a specific organ, which represents the "soil" (Langley & Fidler, 2011). This selective process depends on various factors which can be classified as either intrinsic or extrinsic. The extrinsic factors are those that are secreted by the tumor and the "soil". These factors are mainly related to the extracellular matrix remodelling and the hypoxic conditions. On the other hand, intrinsic factors refer to the stemness of the cancerous cell, the "seed", and the shifting process between EMT and MET (Q. Liu et al., 2017). Moreover, it has been proposed that CTCs can seed the primary tumor through a theory known as the "tumor self-seeding" hypothesis, which suggests that various factors attract the CTCs. According to this theory, cytokines IL-6 and IL-8 promote self-seeding, and leaky abnormal blood vessels allow the CTCs to re-enter the tumor (Kim et al., 2009; Langley and Fidler, 2011).

3. Methods for isolating CTCs

CTCs have shown considerable promise in facilitating the comprehension of diseases and treatment responses. Consequently, new methods are being developed to identify, isolate, and quantify CTCs from blood samples. Two categories of CTC separating techniques

have been proposed depending on the surface-specific antigens expressed on the cells or the intrinsic physical properties (e.g., size and surface charge) (Hu et al., 2022). It is also possible to integrate both methods, as they are not competitive (Nikolaos G et al., 2020). A summary of the current methods of CTC isolation is presented in ***Table 2*** (adapted after Costa & Davila-Ibanez) (Costa and Dávila-Ibáñez, 2020).

Isolating and identifying the various subpopulations of CTCs while simultaneously excluding the background contamination of blood cells is an absolute need for an optimal CTC detection technology (Costa and Dávila-Ibáñez, 2020). Given that CTCs are rare, they must first be enriched in order to be identified (Alvarez Cubero et al., 2017). Following acquisition, CTCs are phenotypically characterised via immunocytochemistry, immunofluorescence, or other methods (Nagrath et al., 2016).

3.1. Label-independent detection

Isolation of viable CTCs with high capture efficiency is possible without the use of fluorescent labels via physical factor-based methods ("label-free methods" or "epitope-independent methods") (Bankó et al., 2019). This method, as opposed to immunoisolation-based techniques, permits the isolation of cells exhibiting epithelial and mesenchymal characteristics (Costa and Dávila-Ibáñez, 2020), avoiding the error of variable antigen expression seen in CTCs (Harouaka et al., 2013). In addition, because they do not rely on chemical interactions, this method is less aggressive and thus increases cell viability (Costa and Dávila-Ibáñez, 2020). ***Table 2*** summarises the current approaches for CTC isolation based on physical variables.

By utilising physical property-based isolation, CTCs can be differentiated from other cells in peripheral blood via their electrical properties, deformability and cell size (Bankó et al., 2019; Kurniali et al., 2023). Nevertheless, it is advisable to exercise prudence when interpreting cell size measurements, as a substantial disparity exists between those derived from cell volume measurement in suspension by flow cytometry and those determined by microscopy on a two-dimensional surface (Harouaka et al., 2013). One plausible factor contributing to the variability in CTC size could also be its transition from an active state to a dormant one (Harouaka et al., 2013). Numerous studies assessing elastic properties in tumor cells have shown that the more deformable the cells, the greater their propensity to metastasize. This idea is supported by a decrease in F-actin concentration during malignant transformation (Z. Liu et al., 2013). Finally, it is essential to note that CTCs possess a distinct surface charge, distinguishing them from other cells. This characteristic enables the use of dielectrophoresis, a method of isolating cells based on their response to electric field gradients (Costa and Dávila-Ibáñez, 2020).

Physical property-based technologies function by entrapping CTCs within a device to generate an enriched population while discarding blood cells. However, the obstruction of mechanical microfilters and microfluidic systems and the adhesion of peripheral blood cells to the filter surface constitute the primary challenge of these approaches. Moreover, the cells can be damaged due to the high fluid pressure inside the filters (Bankó et al., 2019; Tretyakova et al., 2022).

3.2. Label-dependent detection

Different cell surface markers can be used to distinguish between tumor and blood cells. CTCs, for example, do not express CD45, a white blood cell (WBC) differentiation marker, but they do express epithelial markers such as CKs and EpCAM (Habli et al., 2020; Patriarca et al., 2012). Thus, label-dependent detection relies upon antibodies selectively binding on CTCs surface antigens (Kurniali et al., 2023). There are two ramifications for this strategy. One would be the positive selection, a widely used technique for CTC detection focusing on tumor-associated antigens expressed by the cells. In contrast, the other approach would be the negative selection, which involves magnetically depleting the leukocytes from the sample using CD45, thus not limited by CTCs markers (Costa and Dávila-Ibáñez, 2020) (Table 2).

One of the most widely used immunoisolation-based systems is Menarini Silicon Biosystems' CellSearch®. It is the only technique the Food and Drug Administration (FDA) approved for isolating and detecting CTCs in metastatic cancer. This platform intends to separate CTCs of epithelial origin CD45–ve, EpCAM+ve, and cytokeratin (CK 8, 18, and 19)+ve (Kumar, 2020). However, a loss

Biophysical properties		Immuno-affinity	
Technology	**Methods**	**Technology**	positive selection Delivers high purity High cell recovery with specific markers (Rupp et al., 2022)
RareCyte® LeukApheresis, Ficoll-Paque™	density utilises centrifugation (Habli et al., 2020)	CellSearch® FDA approved	
ISET®, MetaCell®, ScreenCell®, Celsee Genesis system	filtration utilises membranes with varying pore sizes for the isolation of CTCs (Hao et al., 2018)	AdnaTest	
ParsortixTM, ClearCell® FX1, CTC-Chip, LiquidBiopsy®, Target Selector™, IsoFlux, HBCTC-Chip, CytoTrapNano™	microfluidics used in the case of size-based separation (Dong et al., 2013)	Dynabeads	
ApoStream®, DEPArrayTM	dielectrophoresisu ses a non-uniform electric field to polarize cells (Gabriel et al., 2016)	CellCollector®	
DEPArrayTM, VyCAP, Celsee Genesis system	single cell analysis image-based isolation of single CTCs (Di Trapani et al., 2018)	RosetteSep™	negative selection Not limited based on CTC surface markers (Rupp et al., 2022)

Table 2
Current methods for CTCs isolation
(adapted after Costa & Davila-Ibañez)

of system sensitivity is noted with the decrease of EpCAM and other epithelial markers when CTCs undergo EMT (Rushton et al., 2021).

Clinical perspectives

The presence of CTCs and circulating tumor materials (CTMat) in the blood indicates a poor prognosis, although the number of CTCs in the blood vessels is very low, with approximately 1 CTC to 10^9 white blood cells. Daily, approximately 3.2×10^6 cells per gram of tumor tissue disseminate from the tumor site, but only approximately half remain viable. As a result of the physical shear stress in the bloodstream, as well as other factors, the cellular membranes of the other half of CTCs are ruptured, and the circulating tumor material is released (Bailey and Martin, 2019; Mendelaar et al., 2021).

Although there is still evidence to support the clinical value of CTCs, a high count has been correlated with a poor prognosis in several malignancies, particularly in breast, lung, and prostate cancer (Vasseur et al., 2021).

Wang and colleagues published an interesting analysis that enrolled 128 mBC patients. The authors show that clustered CTCs have a higher metastatic potential than single CTCs and that cancer progression is also linked to the size of the CTC cluster. A higher number of CTCs in blood samples (≥5 CTCs/7.5 ml) and the presence of CTC clusters is linked with a lower progression-free survival (PFS), lower overall survival (OS), and with a higher metastatic potential (C. Wang et al., 2017). Conversely, a prospectively designed retrospective translational study (SWOG S0500) states that after initiating first-line chemotherapy, the number of CTCs per 7.5 mL of whole blood may be more helpful regarding prognosis than the size of the CTC clusters (Paoletti et al., 2019). Smerage et al., conducted a phase III randomised study on 595 patients with mBC that demonstrated strong prognostic value of CTCs. The authors showed that the lack of decrease in the CTC level after the first course of chemotherapy is associated with a poorer overall prognosis (Smerage et al., 2014).

In non-metastatic colorectal cancer, a meta-analysis of 12 studies that comprise 2363 patients determines that the CTC presence detected by RT-PCR in peripheral blood is linked with a lower disease-free survival (DFS) and overall survival (OS), metastasis in the adjacent lymph nodes, and vascular invasion of cancerous cells. However, the size of the tumor was not correlated with the presence of CTCs (C. Yang et al., 2017). Another meta-analysis of 6 studies that includes 1713 patients with colorectal cancer notes that circulating-tumor DNA (ctDNA) released from cancerous cells acts as a marker of prognosis and that higher values correlates with a lower DFS (Fan et al., 2023).

Thus, cancer relapse could potentially be identified as minimal residual disease (MRD) by the presence of ctDNA in the liquid biopsy. This idea is supported by a meta-analysis conducted in 2022 that revealed a strong correlation between the pathological response and the clearance of ctDNA.

Therefore, to assess MRD and guide different lines of cancer therapies, there is a pressing need for future improvements in liquid-biopsy optimization techniques for detecting CTCs, ctDNA, methylation, and miRNAs (Shen et al., 2022). To summarise, many aspects should be taken into consideration when evaluating the clinical significance of CTCs. These include the number of CTCs, whether they are clustered or solitary, the type of cancer, the size of CTC clusters, and the response to treatment (Paoletti et al., 2019; Smerage et al., 2014).

Conclusions

Liquid biopsy and circulating markers such as CTCs have demonstrated significant potential in assessing the prognosis of cancer patients and monitoring their response to therapy.

Adding CTC and ctDNA to the currently available tools to detect and classify tumors, whether metastatic disease exists or not, will be a significant step towards personalised medicine. Furthermore, understanding the underlying resistance mechanisms to various cancer treatments is crucial in improving patient outcomes. CTCs can play a significant role in this area, as these cells can provide insights into tumor progression and help identify potential treatment options. Ultimately, this knowledge can lead to more effective therapies and increased chances of survival for cancer patients. It is also important to note that evaluating oncological patients using CTCs has the added benefits of being relatively inexpensive and minimally invasive.

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List of Symbols and Abbreviations

ALDH1 – aldehyde dehydrogenase-1
BC – breast cancer
BlC – bladder cancer
CDH1 – E-cadherin gene
CKs - cytokeratins
CRC – colorectal cancer
CRPrC – castration resistant prostate cancer
CTCs – circulating tumor cells
CTMat – circulating tumor materials
ctDNA – circulating tumor DNA
DFS – disease-free survival
EMT – epithelial-mesenchymal transition
EpCAM – epithelial cellular adhesion molecule
ESPR1 – epithelial splicing regulator-1
FDA – Food and Drug Administration
LC – lung cancer
m – metastatic
MET – mesenchymal-epithelial transition
MRD – minimal residual disease
NB – neuroblastoma
NSCLC – non-small cell lung cancer
OS – overall survival
OSCC – oral squamous cell carcinoma
PFS – progression free survival
PC – pancreatic cancer
PLS3 – Plastin-3
RB – retinoblastoma
TGF-β – transforming growth factor-β
WBC– white blood cells
ZEB1 – Zinc finger E-box binding homeobox-1
ZO – Zonula occludens

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