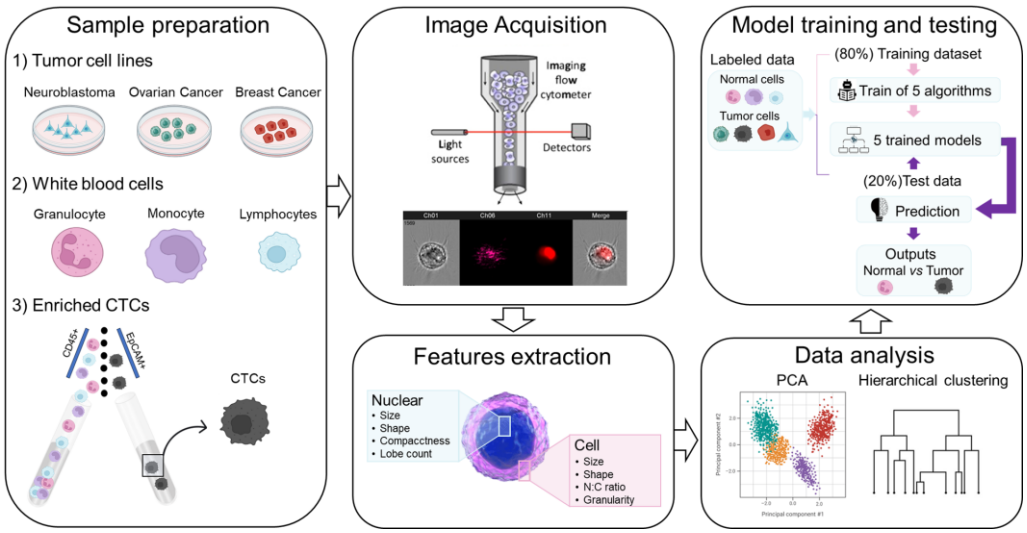
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXVII CYCLE

Annalaura Montella

Morphological biomarkers for early diagnosis in cancer



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Morphological biomarker for early diagnosis in cancer

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Candidate
Annalaura Montella

2021-2024

STATEMENT

With this I declare that this thesis is the outcome of my personal work and contains, to the best of my knowledge, no material that has been previously published by myself or by any another person, except where appropriately referenced in the thesis.

Any contribution made by others is explicitly acknowledged in the thesis.

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List of Abbreviations

AFP	alpha-fetoprotein
AIOM	Associazione italiana di oncologia medica
AIRTUM	Associazione italiana registri tumori
AML	acute myeloid leukemia
AUC	area under the ROC curve
BC	breast cancer
BF	brightfield
CA-125	cancer antigen-125
CART	Classification and Regression Trees
CD45-	CD45 negative
CD45+	CD45 positive
CEA	carcinoembryonic antigen
cfDNA	cell-free DNA
CKs	cytokeratins
COPD	chronic obstructive pulmonary disease
CRC	colorectal cancer
CSCs	cancer stem cells
CT	computed tomography
CTCs	circulating tumor cells
DEF	Dean Flow Fractionation
DEP	Dielectrophoresis
DT	decision tree
DTCs	disseminated tumor cells
E RL	enrichment procedure with RBCs lysis
E w/o RL	enrichment procedure without RBCs lysis
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecules
EpCAM-	EpCAM negative

EpCAM+	EpCAM positive
EVs	extracellular vesicles
FC	Flow cytometry
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FPR	false positive rate
HCG	human chorionic gonadotropin
HGF	hepatocyte growth factor
IFC	imaging flow cytometry
IGF	insulin-like growth factor
ISET	Isolation by Size of Epithelial Tumor Cells
ISTAT	Italian National Institute of Statistics
ISx	Imagestream Imaging flow cytometer
KNN	K-Nearest Neighbors
LB	liquid biopsy
MACS	Magnetic-activated Cell Sorting
MET	mesenchymal-epithelial transition
miRNA	microRNA
ML	machine learning
MNCs	mononuclear cells
MRD	minimal residual disease
MRI	magnetic resonance imaging
N:C	nuclear-to-cytoplasmic
NB	neuroblastoma
NK	natural killer
NSCLC	non-small-cell lung cancer
PASSI	Progressi nelle aziende sanitarie per la salute in Italia
PB	peripheral blood
PCa	prostate cancer
PCA	Principal Component Analysis

PCM	phase-contrast map
PD-1	programmed death-1
PET	positron emission tomography
PSA	prostate-specific antigen
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
QPI	quantitative phase imaging
RBCs	red blood cells
RCC	renal cell carcinoma
RI	refractive index
RMS	root mean square
ROC	receiver operating characteristic
ROC	receiver operating characteristic
RT	root temperature
SD	standard deviation
SHAP	SHapley Additive exPlanations
SSC	side scatter
TGFβ	transforming growth factor-beta
TLRs	toll-like receptors
TPR	true positive rate
TrkB	ropomyosin-related kinase B
VEGF	vascular endothelial growth factor
WBCs	white blood cells
XGBoost	Extreme Gradient Boosting

ABSTRACT

Circulating tumor cells (CTCs) offer a minimally invasive approach for early cancer diagnosis and therapeutic responses monitoring, while also capturing intratumoral heterogeneity. However, their scarcity in the bloodstream and the absence of universal tumor-specific biomarkers pose significant challenges for their accurate detection and classification, particularly for label-dependent strategies. An emerging focus in liquid biopsy research involves leveraging morphological biomarkers to distinguish CTCs from normal blood cells, especially using label-independent approaches that integrate advanced imaging and machine learning technologies.

This study evaluates the potential of combining morphological parameters to differentiate CTCs from white blood cells (WBCs). Cellular and nuclear morphology of tumor cell lines (neuroblastoma, ovarian, and breast cancer) and WBCs were analyzed using the Amnis ImageStreamX Mk II flow cytometer, a high-specific, single-cell, label-dependent system. Following DRAQ5 staining, at least 1,000 single cells per type were assessed using IDEAS software, which measured a total of 34 morphological features related to size, shape, and inner complexity. Principal component analysis (PCA) and hierarchical clustering revealed distinct tumor cell line clusters separated from WBCs.

Further analysis of CTCs enriched from the peripheral blood of breast cancer patients—via CD45-positive cell depletion, EpCAM-positive cell sorting, and DRAQ5 staining—confirmed that CTCs shared similar morphological clustering patterns with tumor cell lines, distinct from WBCs. These findings suggest that nuclear and cellular morphology, along with inner complexity, represent promising biomarkers for CTCs detection.

Integrating these morphological features with machine learning (ML) algorithms allowed the robust classification of tumor and normal cells, confirming that the synergic use of these characteristics and advanced detection technologies has the potential to improve CTCs identification, uncovering

subpopulations missed by label-dependent methods, and enhancing early cancer detection, monitoring, and treatment strategies.

1. BACKGROUND

1.1 The cancer "problem" and the importance of early cancer diagnosis

In Italy cancer represents the second leading cause of mortality, with a total of 174,511 deaths recorded in 2022 by Italian National Institute of Statistics (ISTAT) (ISTAT 2022) (**Figure 1**) and over 395,000 (208,000 in men and 187,000 in women) new cases diagnosed in 2023 (AIRC 2023).

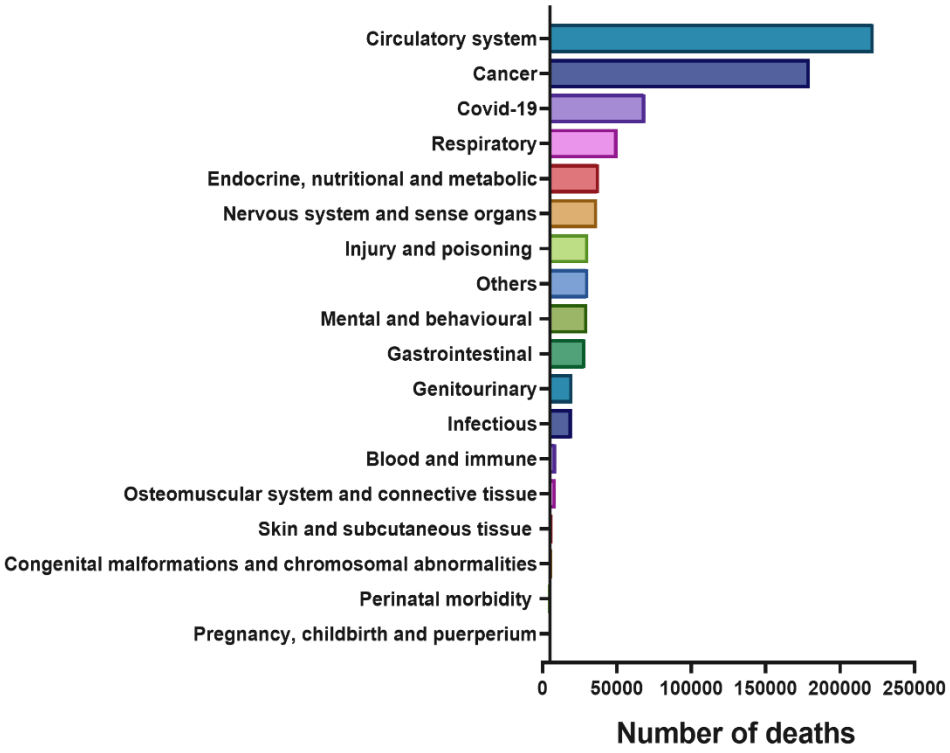

Figure 1 Causes of death in Italy. The histogram shows the most frequent causes of death occurred during 2021 in the Italian population. *Data extracted on 12th March 2024 11:40 UTC (GMT) from ISTAT.*

Despite the increasing number of newly diagnosed individuals registered each year, the survival rate of cancer patients is improving both for men and women. Overall, men's survival has increased by 54% in 2005–2009 against 51% in 2000–2004, 46% in 1995–1999, and 39% in 1990–1994. On the other hand, women's survival has reached 63% against 60%, 58%, and 55% in the

same time periods (Cazzolla Gatti 2022). Moreover, according to the report "I numeri del cancro in Italia 2023" published jointly by AIRTUM (Associazione Italiana Registri Tumori), AIOM (Associazione Italiana di Oncologia Medica) and Fondazione AIOM e PASSI (Progressi nelle Aziende Sanitarie per la Salute in Italia) almost 270,000 deaths for cancer were avoided in 2007-2019 compared to those expected (based on data collected in 2003-2007) (AIRC 2023).

Despite the meaningful improvements in 5-year overall survival of patients and the significant progresses in our understanding regarding this malignancy, cancer remains a major public health problem worldwide with still too many patients without effective cures (Zeng 2015, Dal Maso 2019).

One of the main reasons behind the high rate of cancer mortality and morbidity is the late diagnosis (Phallen 2017, Adashek 2021), which leads to the identification of tumors e in the most aggressive and the least responsive to therapeutic treatments stages (**Figure 2**).

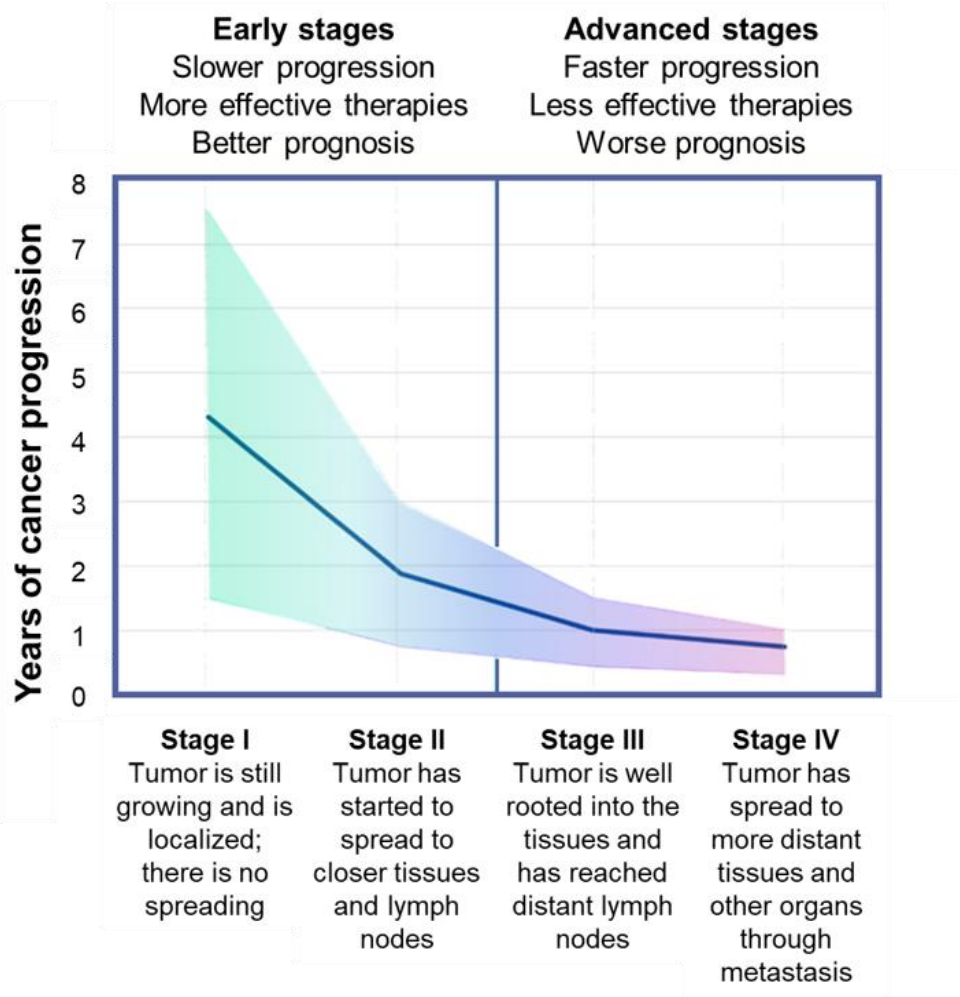

Figure 2 Cancer progression during years. *Adapted from Connal S, et. al. Liquid biopsies: the future of cancer early detection. J Transl Med. 2023.*

In particular, cancers are typically classified in stages indicating how far the disease has spread: Stage 0 (in situ), I, II, III, and IV. Localized cancer (Stages 0-II) remains confined to the original site, while regional cancer (Stages II-III) has spread to nearby tissues or lymph nodes. Metastatic cancer (Stage IV) invades other regions of the body that are distant from the original site of the disease. Since tumor growth accelerates and the time between stages shortens as the disease progresses, higher-stage cancers are more challenging to treat (Hubbell 2021, Connal 2023). Indeed, at late stages, surgery is less effective, radiotherapy is often needed, and chemotherapy becomes more toxic.

Delays in diagnosis lead to poorer patient outcomes, while medical costs (including those for medication, home care, clinical visits, and hospitalization) rise sharply as the cancer progresses (Iragorri 2021). Indeed, it is estimated that the cost of treating patients with Stage III or IV cancer increases dramatically compared to those at earlier stages (Mariotto 2020, Connal 2023).

The diagnosis of late-stage tumors shows a significant impact also on patients' prognosis, decreasing the 5-years relative survival by more than 50% (**Figure 3**) (Siegel 2022, Connal 2023).

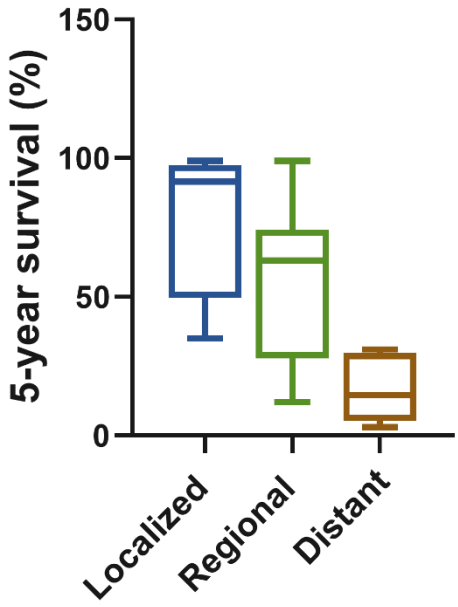

Figure 3 5-years relative survival by stage at diagnosis. *Data adapted from Mariotto AB, et. al. Medical Care Costs Associated with Cancer Survivorship in the United States. Cancer Epidemiol Biomarkers Prev. 2020.*

In this scenario, the early detection of cancer can significantly reduce the death rate and medical costs of patients, enhancing the possibility to select feasible options of treatment and survival rate.

1.2 Liquid biopsy

To date, clinically validated biomarkers for accurate diagnosis and patient management are not always available in the early stages of diseases.

Common tumor markers in the blood, such as carcinoembryonic antigen (CEA), human chorionic gonadotropin (HCG), prostate-specific antigen (PSA), alpha-fetoprotein (AFP), and cancer antigen-125 (CA-125), often lack accuracy and effectiveness, as levels of these proteins can be unexpectedly elevated also in healthy individuals (Ramsey 2015, Murray 2016, Dunn 2017, Lou 2017, Sikaris 2011). On the other hand, early detection based on imaging methods, which has shown to be effective over the years, presents limitations. Imaging techniques such as computed tomography (CT) and/or a magnetic resonance imaging (MRI) scan are relatively expensive and often lead to a high rate of false positives (Schnitzer 2023). For instance, cases of overdiagnosis due to false positives were observed in breast and lung cancer screenings using mammography and low-dose (CT). (Loberg 2015, Blandin Knight 2017). Another example is colonoscopy that, despite its effectiveness in removing precancerous polyps, carries risks of infection and perforation (Griffin-Sobel 2017). Other approaches, such as sputum cytology, stool-based molecular tests, and pap smears, are limited to specific cancer types and often face poor patient compliance (Blandin Knight 2017, Griffin-Sobel 2017, Kessler 2017). In summary, new reliable methods are needed for the early detection of multiple types of cancer.

When a slow-growing tumor is detected, additional and sometimes invasive procedures are applied. To date, the "gold standard" to diagnose tumor and determine the specific cancer type is tissue biopsy. Although this clinically validated procedure provides tumor histological diagnosis and staging and useful results to guide therapy, it presents several drawbacks including difficulties in acquiring enough quantity of high quality tumor samples and failure in reflecting tumor heterogeneity, detecting metastasis at distant sites and in monitoring the treatment response (Perakis 2017, Siravegna 2017, Lone 2022). In this scenario, the rise of liquid biopsy (LB) has introduced a new generation of clinical tools, offering promising potential for early cancer detection. Conied for the first time in 2010, the term “liquid biopsy” indicates a new diagnostic method for targeting

of tumor biomarkers inside body fluids (Pantel 2010). Although different body fluids like mucosa, pleural effusions, urine, cerebrospinal fluid and saliva can be analyzed by this technique, LB mostly involves blood sampling (Pantel 2010). Indeed, the National Cancer Institute defines LB as "A test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood" (NIH).

Compared to conventional surgical tissue biopsy, LB provides more benefits in terms of cost, time, reproducibility and reliability (**Figure 4**) (De Rubis 2019).

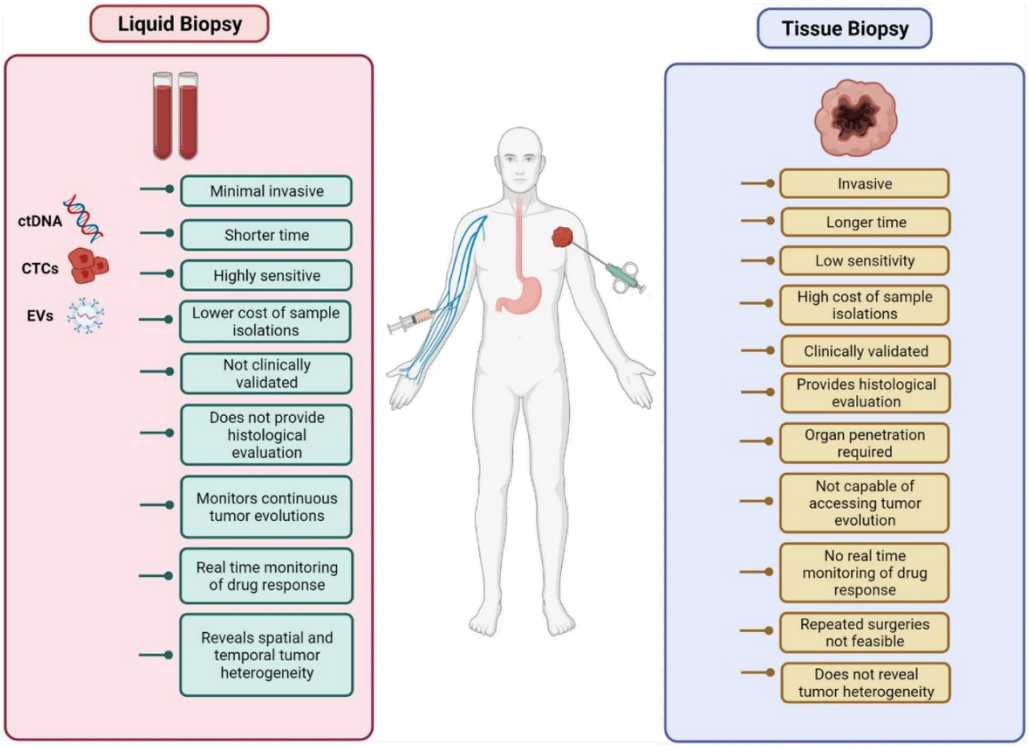

Figure 4 Traditional tissue biopsy versus liquid biopsy. The schematization highlights the advantages of liquid biopsies achieved over the past decade compared to tissue biopsy. *Adapted from Lone et al. Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments. Mol Cancer. 2022.*

This technology offers a safer and less invasive alternative to obtain information about the tumor cells and can be useful in the diagnosis and management of various types of cancer, especially for those cases where traditional surgical biopsies are not feasible due to the high risk involved in the

procedure (Crowley 2013). LB can be applied to monitor the progression of the disease and to detect any potential recurrence of cancer after treatment. Moreover, differently from surgical biopsies, tumor spatial and temporal heterogeneity does not represent a problem for LB (Gilson 2022).

By using LB techniques, different circulating biomarkers can be detected and characterized from both plasma and cellular fraction of blood samples (Alix-Panabieres 2014). Indeed, different components which constitute useful markers for cancer diagnosis and prognosis are released from cancer cells in peripheral blood (PB) of patients: circulating tumor cells (CTCs), cell-free DNA (cfDNA), extracellular vehicles (EVs) and circulating microRNA (miRNA) (**Figure 5**) (Caputo 2023).

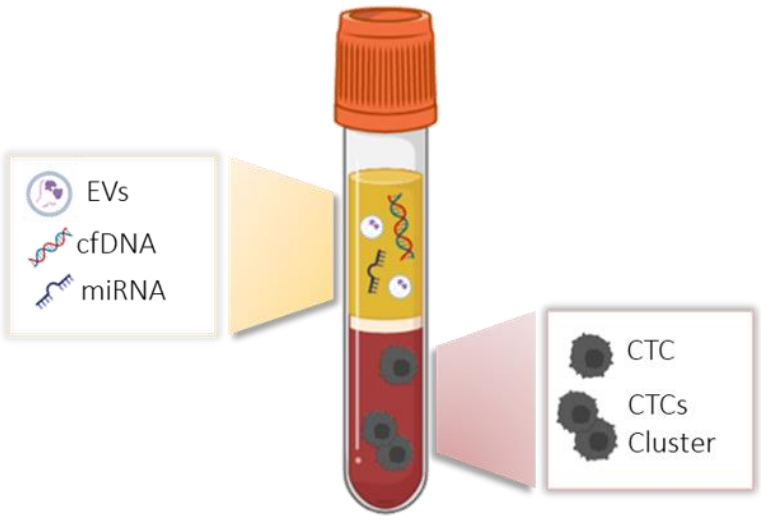

Figure 5 Liquid biopsy biomarkers. In the plasma or serum of a cancer patient PB tumor-derived EVs, cfDNA and miRNA can be detected. CTCs and CTCs clusters can be identified in the cellular fraction. *Created with BioRender.com.*

1.2.1 Circulating tumor cells

CTCs were identified for the first time in PB of a metastatic cancer patient in 1869 by the pathologist Thomas Ashworth, which supposed that these cells arise from the cancer tissue (Lin 2021).

CTCs play a critical role in the metastatic progression of several human cancers (Majidpoor 2021) and represent very rare cancer cells which disseminate from primary tumor site through the circulatory system to distant sites (**Figure 6**) (Feng 2022).

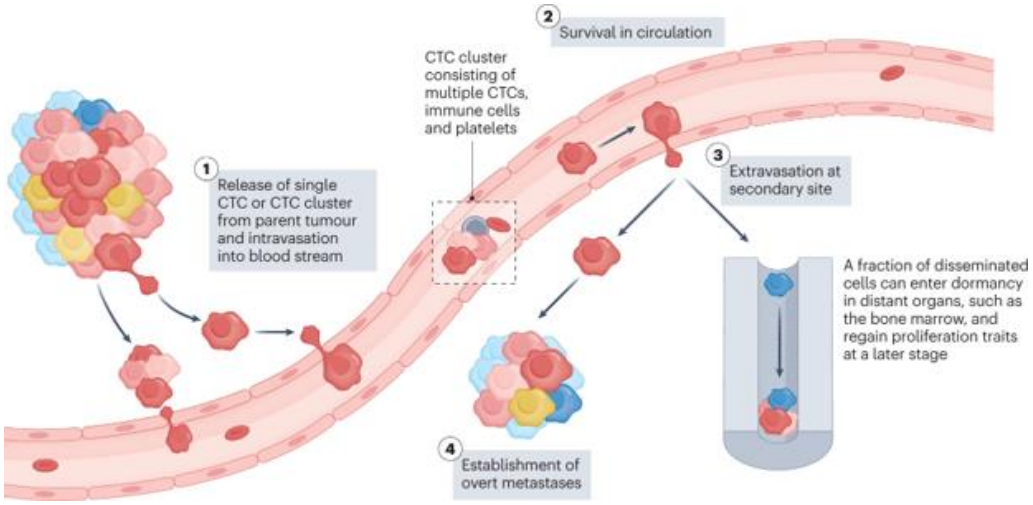

Figure 6 Key steps by which CTCs lead to metastasis formation. Step 1: Dissemination into circulation; Step 2: CTCs survive in the bloodstream; Step 3: Extravasation at secondary sites; Step 4: Formation of distant metastasis. *Adapted from Lawrence R, et. al. Circulating tumour cells for early detection of clinically relevant cancer. Nat Rev Clin Oncol. 2023.*

The first step for CTCs to disseminate into the blood stream is the access to proximal vessels, both in active and passive manner (Joosse 2013, Joosse 2015).

In active intravasation, CTCs exhibit motility and migrate independently, penetrating the basal lamina and crossing the endothelial layer (Feng 2022). With this end, CTCs undergo changes in morphology and molecular characteristics, becoming more aggressive and invasive. This dynamic process, known as the epithelial-mesenchymal transition (EMT), is crucial for cancer metastasis and it is also observed during embryogenesis and wound healing (Feng 2022). During EMT, CTCs switch from an epithelial to a mesenchymal phenotype by downregulating epithelial markers like epithelial cell adhesion molecules (EpCAM), cytokeratins (CKs), and E-cadherin, and upregulating mesenchymal markers such as N-cadherin and vimentin. EMT is regulated by both intracellular and extracellular factors. When triggered by extracellular

signals such as transforming growth factor-beta (TGFβ), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and fibroblast growth factor (FGF), EMT activates transcription factors like TWIST, SNAIL, SLUG and ZEB1, which represent master regulators sustaining mesenchymal traits (Tam and Weinberg). As a result, the tightly connected epithelial cells loosen their junctions and lose attachment to the extracellular matrix, gaining the ability to detach from the tumor. Importantly, EMT activation also produces intermediate cells known as cancer stem cells (CSCs), which exhibit features between epithelial and mesenchymal states (Dongre 2019).

In contrast, passive intravasation occurs when CTCs are transported into blood circulation by external forces (Fornvik 2013).

The tumor vasculature and microenvironment play a crucial role in supporting CTCs survival. A key feature of cancer is angiogenesis, where carcinomas secrete vascular endothelial growth factor (VEGF), promoting the formation of new blood vessels to supply oxygen and nutrients (Hanahan 2011).

Once CTCs enter the bloodstream, they face a hostile environment and must undergo complex adaptive processes to survive. Although tumor cells that have gone through EMT are more resistant to the shearing forces, oxidative stress, and collisions with other blood components, the majority of them are still vulnerable due to the loss of adherence to the extracellular matrix (Mitchell 2013). Without essential matrix proteins, CTCs floating in suspension may undergo anoikis, a type of programmed cell death triggered by detachment from the matrix (Han 2021). During this process, pro-apoptotic proteins such as Bak and Bax suppress anti-apoptotic proteins like Bcl-XL, Bcl-W, and Mcl-1. To survive under these conditions, CTCs must bypass the death receptor pathway of caspase by activating tropomyosin-related kinase B (TrkB) and the caspase-8 inhibitor FLIP, while blocking the mitochondrial pathway by upregulating Bcl-2 family proteins (Simpson 2008).

Another major threat to CTCs survival is represented by the cells of the immune system. However, CTCs employ several mechanisms to evade immune detection. For example, they can upregulate the "don't eat me" signal CD47 to protect themselves from macrophages and dendritic cells. Additionally, CTCs express PD-L1 to avoid immune destruction. In particular, PD-L1 forms a complex with programmed death-1 (PD-1) that suppresses T-cell activity. Downregulation of toll-like receptors (TLRs) on macrophages and natural killer (NK) cells further hinders immune surveillance (Mohme 2017). Moreover, CTCs can enhance survival by forming clusters and reducing their time in the bloodstream. Although CTCs can exploit multiple strategies to escape the immune system, they are rare (typically fewer than 10 cells in 10 mL of blood) and have a short half-life, with clusters lasting less than 10 minutes and single cells surviving for about 30 minutes.

CTCs that survive in the bloodstream reach branch points in vessels or small capillaries, where they extravasate through the endothelial walls to establish metastases in a new microenvironment (Reymond 2013). After extravasation, mesenchymal-like CTCs are still motile but not proliferative (Celia-Terrassa 2012). To facilitate metastasis formation, CTCs must reverse the EMT through a process called mesenchymal-epithelial transition (MET), in which their epithelial characteristics are re-established. During MET, epithelial markers are upregulated while mesenchymal traits are suppressed, highlighting the remarkable epithelial-mesenchymal plasticity of CTCs (Lu 2019). Additionally, primary tumors and target organs release cytokines that upregulate E-selectin on endothelial cells, a phenomenon that supports CTCs penetration through vessel walls (Hiratsuka 2011). Once left the bloodstream, CTCs cross the basal lamina, reach the stroma, and begin to grow locally, forming secondary tumors.

Upon extravasation, CTCs display gene expression heterogeneity depending on the cancer type. For instance, CTCs in breast cancer can express Her2, Notch1, EGFR, and HSPE but lack EpCAM, and this profile is often associated with brain metastasis (Zhang 2013). Additionally, EGFR ligand HBEGF and

COX2 have been identified as key regulators in metastatic models that specifically target the brain (Bos 2009). This suggests that analyzing the gene expression patterns of CTCs may help to predict their metastatic destination.

Among extravasated CTCs, a subpopulation migrates to the bone marrow, where they can remain dormant for decades before metastases become detectable. These cells are known as disseminated tumor cells (DTCs) (Hen 2020). Many cancers, such as breast, lung, and colorectal cancers, often metastasize to the bone, making the bone marrow a reservoir for DTCs (Kang 2013). Dormancy is thought to be linked to the initial EMT stage, allowing DTCs to evade detection and remain inactive until a specific stimulus triggers their proliferation (Bragado 2013).

1.2.2 Circulating tumor cells in early cancer diagnosis

CTCs were initially believed to be a hallmark of advanced disease and their potential in diagnosing early-stage cancers was not considered.

Additionally, the technology to detect such rare cells was limited at the time (Alix-Panabieres 2021). However, emerging evidence suggests that local invasion of cancer cells can happen quickly, indicating that CTCs detection might precede clinical cancer diagnosis (Massague 2016, Rhim 2012).

Several clinical studies have explored the potential of CTCs for early cancer detection using blood samples from patients with confirmed cancer diagnoses (Lawrence 2023). In this context, several studies highlight the presence of CTCs in patients with early-stage (stage I–IIIA) breast cancer (BC) (Riethdorf 2017, Bidard 2018, Trapp 2019, Shao 2022). As an instance, in a cohort of 102 patients with treatment-naive BC, 117 patients with benign breast disease and 64 women without cancer, CTCs detection showed a specificity of 93.8% (Shao 2022). In another group of BC patients examined prior surgical treatment, more than one CTC was identified in 20% of patients with stage I disease, 26.8% with stage II, and 26.7% with stage III disease (Krol 2021). Similarly, CTCs have been detected in 8 out of 10 patients with known non-metastatic colorectal cancer

(CRC), and in 1 out of 5 donors without known cancer (20%) (Gupta 2019). Among 36 non-metastatic prostate cancer (PCa) patients, 5 (14%) had detectable CTCs, including 1 patient with a circulating tumor micro emboli (Loh 2014). Moreover, CTCs were detected in over 50% of patients with localized PCa (Xu 2017, Xu 2020). CTCs were found also in 49% of patients with stage I non-small-cell lung cancer (NSCLC), a detection rate similar to that observed in stages II–IV (48%, 48%, and 52%, respectively) (Hofman 2012). Additionally, a meta-analysis of 18 prospective studies demonstrated that CTC positivity is a promising biomarker for predicting poor overall survival in early-stage NSCLC (HR 3.53, 95% CI 2.51–4.95; $P < 0.00001$) (Wankhede 2022), underscoring CTCs' potential to identify aggressive cancers and guide new treatment strategies. In pancreatic cancer, CTCs were detected in 60% of patients with stage II disease, with CTC positivity distinguishing pancreatic ductal adenocarcinoma from non-adenocarcinoma pancreatic conditions with a sensitivity of 75% and specificity of 96.3% (Ankeny 2016).

In a prospective study aimed at predicting biopsy-based diagnoses in 98 individuals with suspected PCa, CTCs detection showed a strong correlation with clinically significant cancer biomarkers (Xu 2020). The study cohort included patients with PCa symptoms, elevated serum prostate-specific antigen (PSA) levels, and/or abnormal digital rectal exams. Combining CTCs positivity with PSA levels allowed to predict the biopsy-confirmed diagnosis of clinically significant PCa (Xu 2020). In another study involving 265 asymptomatic individuals with risk factors for various cancers (such as family history, lifestyle factors, or medication usage), 49.8% had detectable CTCs (Ried 2017). Follow-up tests within 10 months revealed early cancerous lesions in 20% of CTC-positive individuals, and positron emission tomography (PET) scans of prostate-specific membrane antigen identified early-stage prostate cancer in 50% of men with normal serum PSA levels but detectable CTCs (Ried 2017). In a separate study of 168 patients with chronic obstructive pulmonary disease (COPD), five patients with detectable CTCs were later diagnosed with NSCLC within 1–4

years, and all had resectable tumors with no recurrence at 12 months after surgery (Ilie 2014).

Altogether, these studies highlight the potential of CTCs for early cancer detection and diagnosis, although further research and technical improvements are needed. Several ongoing clinical trials are exploring CTCs usage in early cancer diagnosis, including the PROLIPSY trial for PCa (NCT04556916), and similar trials for BC (NCT03511859), NSCLC (NCT02380196), CRC (NCT05127096), and pancreatic cancer (PANCAID). Positive results from these trials could pave the way for CTCs in early cancer detection.

1.2.3 Technologies for CTCs isolation and enrichment

Due to the low number (around 1–10 CTCs among ~6×10^6 white blood cells (WBCs), ~2×10^8 platelets and ~4×10^9 red blood cells (RBCs) per ml of blood) and heterogeneity of CTCs in peripheral blood, developing robust and efficient methods for their isolation and enrichment is highly challenging (Allard 2004). Over the past decade, several commercial CTCs capture technologies have emerged. These techniques can generally be categorized into label-dependent and label-independent methods based on their recognition of cell surface markers (**Figure 7**).

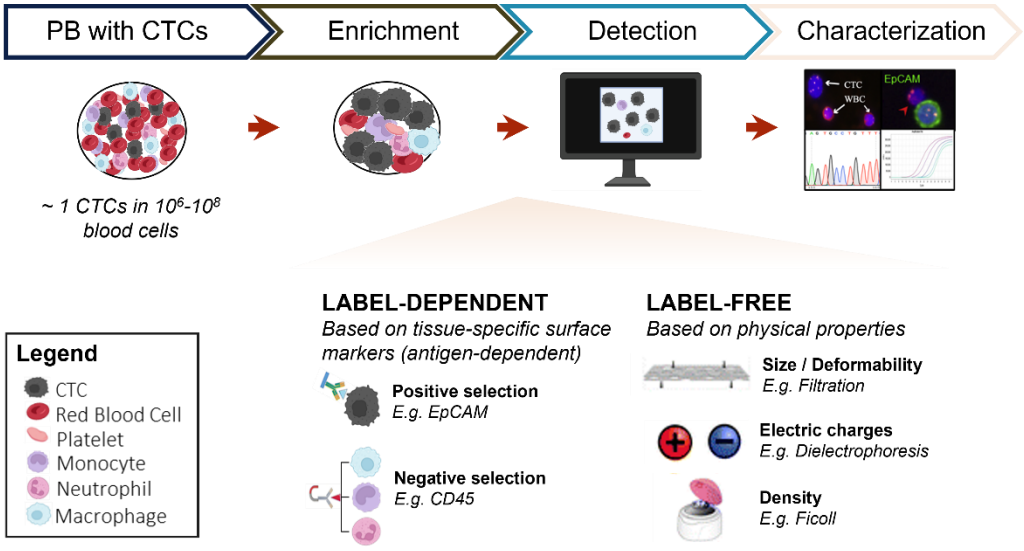

Figure 7 Process to CTCs isolation. *Created with BioRender.com.*

1.2.3.1 Label-dependent technologies

Label-dependent technologies rely on immunoaffinity to identify and capture CTCs based on specific surface markers that differentiate them from blood cells.

1.2.3.1.1 CTCs positive selection methods

The most common method for CTCs isolation is positive selection, where antibody-conjugated magnetic microbeads target cancer-associated markers absent in WBCs (Deng 2008). For example, EpCAM and CKs are exposed on CTCs surface but not on WBCs, which expressed the leukocyte common antigen CD45.

CTCs were first isolated in 1998 using a label-dependent strategy based on cytometry (Pachmann 2001). After two years, the first specific-CTCs searcher, CellSearch®, was commercially released and approved for CTCs enumeration by U.S. Food and Drug Administration (FDA) in 2019. To date, this platform is widely regarded as the gold standard for CTCs detection in metastatic cancers (Munoz-Arcos 2023, Zapatero 2020, Gorges 2016). CellSearch® uses ferrofluid nanoparticles coated with epithelial markers to enrich EpCAM-positive (EpCAM+) CTCs, which are then confirmed through immunostaining for CK8, CK18, and CK19, and the absence of CD45 (Swennenhuis 2016). However, this system only relies on epithelial marker expression, which may not capture CTCs that have downregulated EpCAM during EMT (Gabriel 2016). For this reason, many methods have been developed that combine anti-EpCAM antibody together with different types of other antibodies for the detection of different tumor markers to isolate a wider spectrum of CTCs types (Wu 2015). For instance, the positive enrichment system AdnaTest® uses immunomagnetic beads with antibodies against epithelial antigens in combination with tumor-associated markers detection. To isolate CTCs from PCa patients, the system exploits the usage of reverse transcription and PCR primers to evaluate the expression of prostate-specific markers, such as PSA, prostate-specific

membrane antigen (PSMA), etc. (Todenhofer 2012). To date, CellSearch® and AdnaTest® have been used to detect CTCs in several types of cancers such as breast, ovarian, prostate, and colon cancer (Danila 2016, Muller 2012). Although these systems based on CTCs positive selection offers high efficiency, reproducibility and the possibility to capture CTCs heterogeneity, they have several drawbacks.

Indeed, the detection of CTCs from some tumor types is challenging due to the lack of epithelial differentiation or a universal and specific cell-surface marker (Hernandez-Yanez 2012, Yang 2009). In this regard, renal cell carcinoma (RCC) typically exhibits low expression of epithelial markers, making it difficult to capture these CTCs using traditional labeling techniques (Cappelletti 2020). In neuroblastoma (NB), the standard positive label-dependent enrichment method used for CTCs detection in epithelial cancers is not efficient (Beiske 2009). Moreover, due to the significant heterogeneity that characterizes CTCs in patients with NB, the use of antibodies against specific markers, such as anti-GD2 and anti-CD56, fails to capture all cell types (Liu 2023).

Additionally, most fluorescence imaging techniques rely on antibody-based fluorescent probes, which depend on the overexpression of specific proteins on cell membranes. However, this overexpression is often unstable and varies based on cancer type and patient (Mikulova 2011). Additionally, issues like photobleaching and phototoxicity arise shortly after exposure to fluorescent light sources, and the selection of appropriate fluorophores requires specialized expertise (Toseland 2013). Fluorescence staining can also compromise cell viability and, as a consequence, their cultivation and further analysis (Wang 2020).

1.2.3.1.2 CTCs negative selection methods

To date, it is well known that lower performance in CTCs isolation is obtained when native antigens are not preserved (Zamay 2017). For this reason,

systems based on negative selection of CTCs are developed. Negative selection represents a strategy for CTCs enrichment in which the other cells in the blood, such as WBCs, RBCs, platelets, etc., are discarded with the use of specific antibodies against their cell-surface proteins. The prominent markers selected include CD45/CD66b (granulocytes), CD235a (RBCs), CD41/CD61 (platelets), CD4/CD8 (lymphocytes), CD11b/CD14 (macrophages) and CD34 (hematopoietic progenitors/endothelial cells) (Yoon 2007, Lone 2022). Among the label-dependent systems based on CTCs negative-selection, RosetteSep™ (StemCell Technologies) employs tetrameric antibody complexes (targeting CD45, CD66b, and glycophorin) to capture WBCs and RBCs. After the depletion of these cells, CTCs are immediately ready for downstream analysis. This system is already used in various cancers such as BC, PCa, and CRC (Habli 2020, Buscail 2019). This test employs tetrameric antibody complexes that capture WBCs and RBCs, targeting CD45, CD66b, and glycophorin. The identification and characterization of CTCs are then performed using flow cytometry. From the same manufacturer, EasySep™ Depletion Kit (StemCell Technologies) enables the isolation of CTCs directly from human whole blood through immunomagnetic negative selection, without the need for density gradient centrifugation or RBC lysis (Wang 2016). One of the main limitations of negative selection methods is the potential contamination by other blood components that do not express CD45, such as endothelial cells. Since these cells are CD45-negative (CD45-), similar to CTCs, they can be included in the isolated sample, leading to inaccurate results. Additionally, there is an increased risk of losing CTCs during the removal of WBCs. This can reduce the overall efficiency and sensitivity of CTC detection, compromising the reliability of the technique for clinical or diagnostic purposes (Yang 2023). Other devices, such as Magnetic-activated Cell Sorting (MACS) and CTC-iChip can perform both positive and negative selection to detect CTCs. The MACS system allows the immunomagnetic isolation of CTCs with anti-pan CK antibody (Effenberger 2018). CTC-iChip, instead, represents an integrated microfluidic CTC capture

platform enabling CTCs detection by the combination of multiple properties including immuno-magnetic beads selection and cell size-based separation, exploiting both biological and physical proprieties (Ozkumur 2013).

1.2.3.2 Label-independent technologies

Considering the high variability of epithelial markers types and levels expressed by CTCs across different cancer stages, alternative isolation techniques based on biophysical properties have been developed. These methods identify CTCs by their size, density, and electrical properties.

1.2.3.2.1 Size-based isolation methods

Several well-established size-based isolation techniques include microfluidic chips, membrane filters, and hydrodynamic methods which are able to isolate larger CTCs (mean diameter – 15.6 µm) from smaller blood components (WBCs, diameter range of 7-15 µm) without relying on tumor cell surface markers (Mohamed 2009, Dolfi 2013). Size-based enrichment methods for isolating CTCs include techniques such as Isolation by Size of Epithelial Tumor Cells (ISET) and the Dean Flow Fractionation (DEF) technique (Ma 2013, Hou 2013). ISET, one of the earliest size-based approaches, captures CTCs by filtering blood through a polycarbonate Track-Etch membrane with 8-µm pores. This method has been shown to detect CTCs in 75% of patients (80 out of 106) with advanced-stage III/IV lung adenocarcinoma (Ilie 2017). DEF technique employs inertial microfluidics for size-based CTC isolation. This spiral microfluidic chip separates cells based on size, with smaller blood cells following Dean vortices along the inner and outer walls, while larger CTCs are subjected to stronger inertial forces, causing them to accumulate along the microchannel's inner wall (Hou 2013). This method offers rapid, non-selective, label-free CTC separation. However, a drawback is the difficulty in distinguishing monocytes from CTCs in blood samples. Although size-based filtration is generally simple and reliable, a major drawback is its low recovery

efficiency due to the increase of filtration resistance. Moreover, in many instances, CTCs are almost the same size as WBCs, leading to significant losses when using size-based exclusion techniques. As a result, up to half of the CTCs can be missed during the isolation process, reducing the overall effectiveness and accuracy of the methods (Alunni-Fabbroni 2010).

1.2.3.2.2 Density-based methods

Introduced by Dr. Seal in 1959, density gradient centrifugation is a standard method for separating cells based on their density (Seal 1959). During centrifugation, cells are distributed along the gradient according to their sedimentation coefficients (Jacob 2017). In particular the blood separates into distinct layers with RBCs at the bottom, followed by granulocytes, a buffy coat (containing mononuclear cells like CTCs) and plasma (Davidson 1988). Several commercial separation media have been developed, including Ficoll-Paque (GE Healthcare), Percoll (GE Healthcare), Lymphoprep (STEMCELL Technologies), and OncoQuick (Greiner Bio-One). Ficoll-Paque, a mixture of high molecular weight sucrose polymers and sodium diatrizoate, is widely used to isolate peripheral blood mononuclear cells (MNCs) (Hou 2013). However, due to the cytotoxic nature of Ficoll, cell aggregates may form, causing the loss of some tumor cells which migrate to the bottom of the medium. Percoll, another density gradient medium composed of colloidal silica particles, has lower cytotoxicity and a wider density gradient range compared to Ficoll (Davidson 1988). OncoQuick improves these methods by placing a permeable device within the separation media, allowing RBCs and WBCs to pass through based on their buoyancy during centrifugation, while retaining CTCs (Lagoudianakis 2009). Both OncoQuick and Ficoll-Paque show similar tumor cell recovery rates of 70–90%, but OncoQuick significantly reduces the number of captured blood MNCs—by 632-fold—compared to Ficoll-Paque (Gertler 2003, Rosenberg 2002). This reduction in blood MNCs simplifies subsequent immunocytochemical detection, maintaining cell viability for further culturing

without additional processing. However, a major limitation of this technique is its inability to capture all plasma after centrifugation, which can lead to potential CTCs loss. Indeed, CTCs might also move into the plasma fraction or form aggregates that fall to the bottom of the gradient (Danova 2011).

1.2.3.2.3 Deformability-based methods

CTCs can also be enriched based on their physical property of deformability. Previous studies have shown that the deformability differences between WBCs and CTCs is much more pronounced (with CTCs being more deformable than WBCs) compared to the variability in deformability among CTCs themselves (Shaw Bagnall 2015). This finding suggests that deformability could serve as a useful indicator for distinguishing tumor cells from WBCs. For this reason, an innovative technique that combines size exclusion and deformability to isolate and characterize CTCs has been developed by Celsee Diagnostics (Riahi 2014). This device is characterized by a parallel network of fluidic channels containing 56,320 capture chambers. Larger tumor cells are retained in these chambers, while smaller blood cells, such as RBCs and most WBCs, are allowed to pass through. One limitation of this method is the potential loss of smaller CTCs (Gogoi 2016). However, this microchannel device allows for the rapid label-free capture of CTCs, which can then be characterized using further in-depth analysis using various antibodies (Gogoi 2016).

1.2.3.2.4 Electric-charge-based methods

Dielectrophoresis (DEP) offers highly accurate CTCs isolation by exploiting differences in cells' electrical properties, determined by their composition, morphology, and phenotype (Becker 1995). This method leverages differences in surface charge and polarizability to enrich CTCs, minimizing cell damage during capture. The underlying principle is that tumor cells tend to have a more negative surface charge compared to WBCs (Li 2020). A prominent example of this technology is the DEPArray™ system, a cutting-edge technology designed

with a 320 × 320 grid of electrodes, which precisely guides each CTC into an individual spherical cage (Di Trapani 2018). This system is particularly effective for isolating rare cells and detecting single CTCs.

1.2.3.2.5 Microfluidic-based methods

In recent years, advancements in microfluidic technology have made it possible to manipulate tiny volumes of fluid on micro- and nano-scales. A key component of this technology is the microfluidic chip, which serves as the primary platform for various applications. Due to its small size, precise control of flow rate and transparent structure, microfluidic chips have become widely used for the separation and enrichment of CTCs. One notable example is the Parsortix system, a microfluidic device that captures CTCs based on their larger size and higher deformability compared to other blood cells (Miller 2018). This system can process 7.5 mL of whole blood in approximately two hours. In particular, the Parsortix has a trapezoidal shape that gradually narrows, trapping larger CTCs (preventing their deformation) while allowing smaller blood cells to pass through (Miller 2018). The system also extends the separation channel to allow counterflow, facilitating further CTC analysis. However, a major limitation of this method is the challenge of removing WBCs that are similar in size to CTCs. Another microfluidic system, ClearCell® FX1 (Clearbridge Biomedics), does not require sample preprocessing and minimizes the risk of losing CTCs. This device uses inertial and centrifugal forces to guide smaller RBCs and WBCs toward the channel's outer wall, while larger CTCs are directed toward the inner wall, enabling the recovery of CTCs from 1 mL of blood in just 10 minutes (Lee 2018). Nevertheless, smaller CTCs may still flow along the outer wall of the channel, and some WBCs may end up in the CTC fraction. One of the challenges with microfluidic systems lies in the complexity of blood, which contains a variety of components such as plasma, RBCs, platelets, and proteins, which make it more than three times viscous than water. As a result, it is difficult to manage interactions between cells, especially in

samples with high cell concentrations, leading to reduced sorting efficiency. Additionally, the method relies solely on the physical characteristics of cells, which can sometimes result in chip blockages or false-positive CTCs identification (Li 2020).

1.3 Morphological differences between tumor and normal cells

The morphological differences between tumor and normal cells represent the manifestation of the molecular and genetic alterations underlying cancer progression (Baba 2007). During the transformation of a non-cancerous cell into a malignant one, cell size and shape became larger and irregular. These changes are due to different mechanisms that take place in the non-malignant cell such as alteration in cytoplasm. For instance, an accumulation of ribosomal and messenger RNA makes the cytoplasm basophilic, with a reduced cytoplasmic volume often accompanied by the presence of vacuoles (Baba 2007). The granular endoplasmic reticulum (ER) shows fragmentation and degranulation, indicating a loss of connection with mitochondria. This is associated with free ribosomes and polysomes increasing, which reflects enhanced protein production for cell growth. Mitochondria shrink as the tumor develops, but they can also show variability in size, sometimes becoming unusually large. The Golgi apparatus is poorly developed in malignant cells, particularly in those that have lost differentiation (Baba 2007). Cancer cells undergo metabolic reprogramming, favoring glycolysis over oxidative phosphorylation even in the presence of oxygen (the Warburg effect). Their cytoskeleton becomes disorganized, which helps invasiveness and reduces cell adhesion, facilitating metastasis. Unlike normal cells, which regulate division based on external signals, cancer cells proliferate uncontrollably. Tumor cells also decrease cell-to-cell adhesion and communication, allowing them to detach and invade other tissues. Additionally, they promote angiogenesis to ensure an adequate blood supply for growth (Baba 2007).

For cancer diagnosis, pathologists typically assess nuclear structure of tumor cells using brightfield microscopy on cells stained with reagents like Papanicolaou, Hematoxylin and Eosin, Giemsa, or Romanowski stains. Additionally, computer-assisted nuclear morphometry methods, which utilize conventionally stained slides imaged by brightfield microscopy to analyze nuclear features such as size, shape, and chromatin texture have been developed (Millot 2020, Pienta 1991). In addition, tumor cells often display considerable variation in nuclear shape and size, in numbers and sizes of nucleoli, while normal cells maintain a more consistent structure (**Table 1**) (Zink 2004).

Table 1 Principal differences in nucleus between normal and tumor cells. *Data extracted from Nandini DB. J Mol Biomark Diagn. 2017, S:2. DOI: 10.4172/2155-9929.S2-026.*

Nuclear Features	Normal cell	Tumor cell
Number	one per cell	multinucleation and presence of micronuclei
Area and perimeter	regular	Increase
Shape	round to oval	irregular and elliptical with coarse heterochromatin aggregates
Chromatin	even distribution and finely granular	irregular clumps with variable size, shape and sharp pointed
Margin	regular	irregular
Nucleoli	one or two inconspicuous nucleoli	become prominent, more numerous, enlarged and irregular
N:C ratio	maintain a roughly constant ratio of nuclear volume to cell volume	the ratio increases due to genome size and ploidy

Nuclear size and shape variations in cancer cells were firstly described in 1860 examining sputum from a cancer patient (Lim 2016). To date, it is well established that cancer cell nuclei are typically enlarged, with disorganized chromatin and prominent nucleoli.

Clinicians have recognized that nuclear enlargement is a key morphological feature of malignant cells. Indeed, across species and cell types, normal cells typically maintain a relatively constant ratio between nuclear volume and cell volume (Fischer 2020). In these cells, nuclear size generally scales with cell size, preserving what is commonly referred as the nuclear-to-cytoplasmic (N:C) ratio, defined as the ratio of the nucleus's cross-sectional area to that of the cytoplasm (Sung 2014). An increase of N:C ratio represents a commonly used parameter in tumor staging and grading (Su Lim 2015). The exact pathophysiologic mechanisms driving nuclear enlargement in malignant cells remain largely

unclear, but could be connected with an increased amount of chromatin (Denais 2014, Jevtic 2014).

Differently from a regular and ellipsoid shape observed in normal cell nuclei, irregular nuclear edges are a hallmark of many cancers (Zink 2004). A key role in determining nuclear shape is played by the nuclear lamina, a protein layer made up of proteins from the lamin A/C and lamin B genes, which lies at the interface between chromatin and the inner nuclear membrane (Goldman 2002). The nuclear lamina interacts directly with chromatin and is involved in transcriptional repression through the formation of heterochromatin at the nuclear periphery (Zastrow 2004, Brown 1997). Changes in nuclear shape and lamina structure could disrupt chromatin organization and gene expression, potentially promoting malignant transformation. In this regard, small-cell lung carcinoma is characterized by malleable nuclei prone to crushing during biopsy, possibly due to the absence of lamin A/C proteins (Broers 1993). Irregular nuclear shapes, along with the loss of heterochromatin aggregates, are hallmark features of papillary thyroid carcinoma and represent key diagnostic indicators of this cancer (Zink 2004). In papillary thyroid carcinoma, irregular nuclear shapes and loss of heterochromatin are distinctive diagnostic features, often associated with the expression of the RET/PTC oncoprotein (Fischer 1998). The constitutive activation of RET kinase, resulting from a fusion involving its gene, drives tumorigenesis and induces both nuclear shape changes and chromatin dispersal (Fischer 2003). Indeed, transgenic mice expressing RET/PTC and developing thyroid tumors at early age, show nuclear shape irregularities and heterochromatin loss (Russo 2011).

1.4 Cell and nuclear morphological differences between CTCs and WBCs

CTCs and WBCs exhibit distinct differences in cell size and shape, nuclear morphology, and nucleus-to-cytoplasm ratios (**Figure 8**).

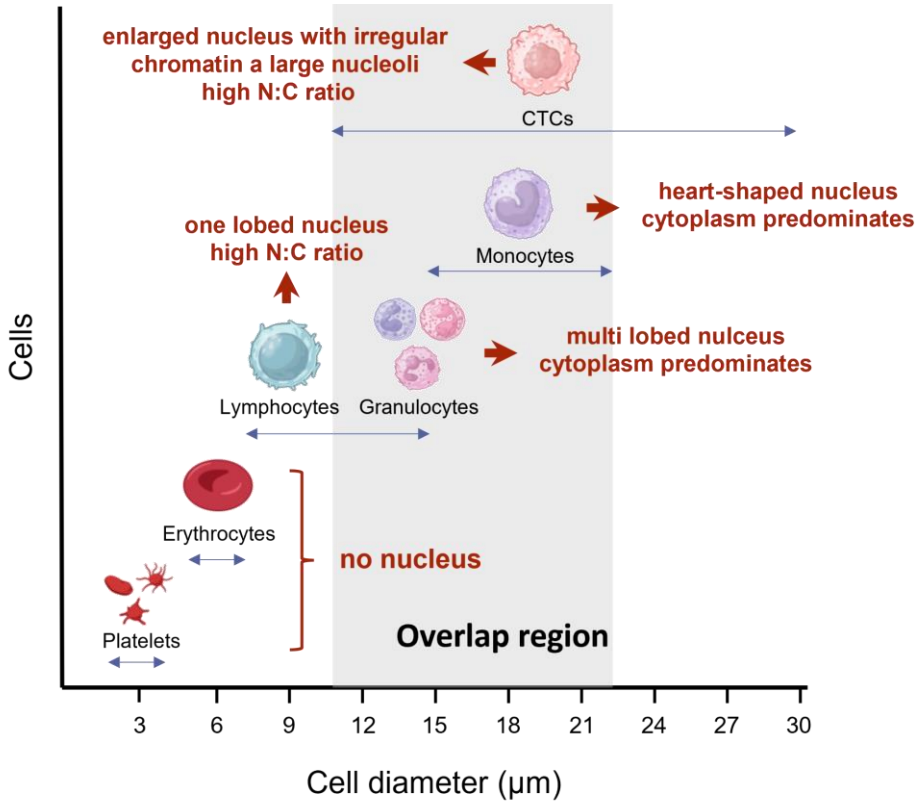

Figure 8 Morphological differences between cell and nucleus of CTCs and WBCs. *Created with BioRender.com.*

In particular, CTCs cell size typically range from 15 to 30 um in diameter, which can change among different patients and cancer types (Peyman Rostami 2019, Coumans 2013). Instead, WBCs are characterized by a heterogeneous population of cells with different ranges in cell sizes. Lymphocytes and monocyte, with a diameter of approximately 7 to 15 um and 16 to 22 um respectively, are considered the smallest and the greatest cells among WBCs (Chiu 2019). Cell size of granulocytes ranges from 10 to 15 um (Tigner 2024). Although CTCs represent a cell type larger than WBCs, there is an overlapping region in terms of cell diameter between these two groups. Considering cell shape, CTCs exhibited significant shape variability with many cells having a more elongated shape (Park 2014). Despite the presence of longer cell shapes, some studies report that there is no significant difference between CTCs and

WBCs, while other reported that WBCs are more circular (roundness = 1.8) compared to CTCs (roundness = 1.5) obtained from patients with different types of cancer (Shashni 2018, Ligthart 2013).

In CTCs' nucleus is possible to identify abnormal patterns typically seen in tumor cells (e.g., pleomorphism, nonuniform margins, unusually large size), with a N:C ratio of approximately 1:1 to 1:2 (Park 2014, Adams 2015). Instead, WBCs show different N:C ratio among their subpopulations. In lymphocytes, the nucleus occupies about 90% of the cell volume, resulting in a high nucleus-to-cytoplasm ratio of 9:1 (Schmid-Schonbein 1980). Monocytes exhibit a nucleus of about 50-70% of the cell volume, leading to a nucleus-to-cytoplasm ratio of around 1:1 (Schmid-Schonbein 1980). In granulocytes, the nucleus occupies approximately 50-60% of the cell volume, with a ratio of about 1:2 (Schmid-Schonbein 1980). CTCs and WBCs also differ in the shape of their nuclei. In particular, it has been observed that CTCs are characterized by a nuclear shape often irregular and pleomorphic, which may include multilobular configurations (Adams 2015). On the other hand, lymphocytes exhibit a nucleus generally round or slightly jagged (Prinyakupt 2015). Monocytes and granulocytes can show multilobed nuclei. In particular, the nucleus of monocytes is typically kidney-shaped or oval, with one or two lobes, while granulocytes can have 2 to 5 lobes connected by thin strands of chromatin (Prinyakupt 2015).

1.5 Single-cell imaging techniques for cell characterization

Currently, pathologists examine the tissue structure and cellular morphometry, including the nucleus size and N:C ratio, to ascertain a cancer diagnosis. The gold standards for assessing morphological features of tumor cells is the histological evaluation of tissue samples obtained from suspected cancerous areas through optical microscopy (S. Reddy 1996). The measurements are based on a single sectional image, which does not accurately represent all histopathologic features of the ex vivo slice. Moreover, the measurement of nucleus size and N:C ratio through optical microscopy images can produce

unreliable subjective results and not objectively quantifiable values. In this regard, it is well-documented that inter-observer variability occurs among pathologists when using optical microscopy images to calculate the N:C ratio (Chekanov 1989, Zhang 2016, Vaickus 2015). Furthermore, this process requires several days to complete, is low-throughput, and unable to fully capture three-dimensional (3D) cell structure, which is essential for precise diagnostics (Hillman 2000).

These limitations have driven the development of alternative methods for N:C ratio quantification. Emerging technologies like digital image analysis using HALO software in urine cytology, immunofluorescence microscopy combined with ImageJ, and 3-D multiphoton microscopy have been utilized for N:C ratio assessment (McIntire 2019, Khan 2016, Huang 2019). However, all of these techniques require extended imaging and analysis times for individual cells.

1.5.1 Flow cytometry techniques

Flow cytometry (FC) addresses the issue of slow analysis but sacrifices the ability to directly measure cell morphology. FC can analyze large cell populations by measuring emitted fluorescence, transmitted light, and scattered light from each cell, offering speed, automation, and objectivity (Balasubramanian 2009). While conventional FC overcome optical microscopy in speed and automation, it is unable to provide absolute values for cell or nucleus size (Zuba-Surma 2011). Recent advances in cytometry have introduced imaging flow cytometry (IFC), conventional FC with high-resolution microscopy, enabling high-resolution imaging of single cells in suspension (Zuba-Surma 2011, Nandakumar 2011). Imaging cells in suspension is beneficial, as it simulates biological cells in flow, such as those found in blood (Pinto 2019, More 2020). IFC software also offers tools for rapid image capture and analysis of cell size, shape, and intensity patterns through feature-based image acquisition strategies (Han 2016, Doan 2018). This technology has been applied to various fields, including radiation biodosimetry, autophagy analysis,

and cellular heterogeneity studies (Beaton 2013, Beaton-Green 2016, Rajan 2015, Ponomarev 2011). Moreover, IFC is already used to assess the differences between normal and cancerous cell lines and in CTCs detection (Sebastian 2021). Among the IFC systems, Imagestream Imaging flow cytometer (ISx) is applied to detect and morphologically characterize CTCs in different types of cancer (Dent 2016, Ogle 2016, Merugu 2020, Debnath 2023, Liu 2018). The exploiting of ISx in 42 NB patients led to the detection of around 1-264/mL CTCs in 26 patients, highlighting the presence of a higher number of CTCs at diagnosis (1–264/mL) compared with relapse (1–39/mL) due to clearance of CTCs from the blood by chemotherapy and likely earlier detection of relapsed disease (Merugu 2020). In hepatocellular carcinoma, CTCs detection and characterization using the expression of different cell surface (EpCAM, CK, AFP and CD45) and nuclear (DRAQ5) markes revealed that the enumeration and cell area of CTCs may be used as a biomarker for early detection of this cancer and guiding treatment (Debnath 2023).

Despite IFC technologies shows a highly potential use in clinical practice, they are limited to 2D imaging, leaving a gap for methods that can capture the 3D structure of single cells and their organelles, which is an ideal approach for N:C ratio characterization (Doan 2018, Moore 2019). Furthermore, IFC data analysis remains challenging due to the large number of cells acquired, the usage of manual gating techniques in which a series of two-dimensional plots were generated to visualize data and apply hierarchical gates to identify specific cell populations (Hu 2021). One of the main advantages of manual gating is that it allows researchers to incorporate prior knowledge, such as the function of protein markers and the developmental relationships between cell populations, into the analysis. However, it struggles with high-dimensional cytometry data, as two-dimensional plots often fail to capture the complexity of the data's structure (Hu 2021). Additionally, manual gating can introduce human bias and is prone to human error, particularly in clinical settings, where it also suffers from slow processing speeds (Hu 2021).

1.5.2 Combining FC with machine learning

Numerous computational tools have been developed to automate various steps of cytometry data analysis, including quality control, batch normalization, data visualization, cell population identification and sample classification (Monaco 2016, Schuyler 2019, Van Gassen 2020, Amir el 2013, Qiu 2011, Hu 2018, Dorfman 2016, Bruggner 2014, Hu 2020). These tools employ a wide range of computational methods, from rule-based algorithms to machine learning (ML) models. In particular, ML represents a set of computational and statistical methods that can learn patterns from data (training data) with minimal human input to make predictions or inferences (Wang 2020). These algorithms are broadly categorized into four types: supervised, unsupervised, semi-supervised, and reinforcement learning (Sarker 2021).

Data visualization is often the first step in data analysis and it can have a profound influence on the subsequent interpretation of high-dimensional data, representing a significant challenge in the context of FC. Dimensionality reduction techniques are essential in this context as they improve human interpretability, reduce computational costs, and help avoid overfitting by simplifying models (Hu 2021). Among the numerous algorithms proposed to facilitate dimensionality reduction, principal component analysis (PCA) stands out as a well-known unsupervised learning technique. PCA is a mathematical method that transforms a set of correlated variables into a set of uncorrelated ones known as principal components (Jolliffe 2016). This process is visually represented by projecting the original features in multi-dimensional space onto a lower-dimensional plane, which can significantly simplify data visualization and analysis (Sarker 2020). Two methods are established that combine PCA with FC: the Automated Population Separator (APS) and Flow cytometric Orthogonal Orientation for Diagnosis (FLOOD) (Costa 2010, Jansen 2016). By exploiting APS, 205 patients with small B-cell chronic lymphoproliferative disorders were classified into specific World Health Organization disease groups with an

efficiency > 85% (Costa 2010). On the other hand, the application of FLOOD in multicolor FC led to the discrimination of surface marker profiles specific for immune responders and no responders to lipopolysaccharides stimulation (Jansen 2016).

Another significant challenge in the FC fields is the identification of specific cell types. In this scenario, supervised and unsupervised ML methods are often used to analyze cell populations within biological samples (Sarker 2021). Among supervised learning methods, common classification algorithms include:

- Logistic Regression: a statistical model that accomplishes binary classification by predicting the probability of an outcome, event, or observation (Nick 2007).
- K-Nearest Neighbors (KNN): a non-parametric, supervised learning algorithm that relies on proximity (based on Euclidean distance) to classify or predict the grouping of individual data points (Zhang 2016).
- Classification and Regression Trees (CART): a decision tree algorithm that is used for both classification and regression tasks (Hu 2022).
- Random Forest: a classification technique made up of multiple decision trees used for both classification and regression tasks (Becker 2023). This approach minimizes overfitting and improves prediction accuracy compared to CART (Becker 2023).
- eXtreme Gradient Boosting (XGBoost): an ensemble learning algorithm that implements decision trees using a sequential approach known as gradient boosting (Silva 2022). Known for its efficiency and scalability, XGBoost performs well on large datasets and provides interpretable results (Moore 2022).

In FC, by exploiting these systems is possible to annotate known cell populations and to identify novel cell subsets from high-dimensional cytometry data (Hu 2021). For instance, a strategy based on logistic regression (CytoDx) is applied on real-world datasets using cytometry data without cell gating to predict clinical outcomes, such as the response to influenza vaccine (Hu 2019). In

myelodysplastic neoplasms' diagnosis, random forest is used to detect cells expressing a combination of cell surface markers, leading to the identification of primary myelofibrosis with high sensitivity and specificity (Zhang 2024). Similarly, single-cell XGBoost classifiers trained using 98 acute myeloid leukemia (AML) cell populations and 30 minimal residual disease (MRD) negative samples obtained from multiparameter FC, effectively differentiated AML cell populations from normal cells (Shopsowitz 2024).

Among unsupervised methods, clustering is a fundamental unsupervised machine learning technique that focuses on identifying and grouping related data points within large datasets (Jiawei Han 2012). This method organizes a collection of objects such that items within the same cluster are more similar to one another than to those in different clusters. Among clustering methods, hierarchical clustering is one common approach that seeks to create a hierarchy of clusters represented in a tree structure (Guess 2002). Hierarchical clustering of FC data was used to classify chondrosarcoma samples based on similarities in surface marker profiles with known cell types (Diaz-Romero 2010). In this way, the author observed that the primary chondrosarcoma cells grouped into two distinct clusters: one with mesenchymal stem cells and another with fibroblasts, highlighting the potential of hierarchical clustering in flow cytometry to classify cancer samples based on marker expression (Diaz-Romero 2010).

2. AIMS

Over the last decades, the detection of CTCs in the blood cells' background represents a challenge in the field of liquid biopsy. Although some label-dependent and independent technologies led to important clinical progresses in early cancer detection, a gold standard for CTCs identification has not been developed to date. This is mainly due to the paucity of these cells in the peripheral blood of cancer patients, the lack of a universal cell surface marker able to capture all types of CTCs in the same individual and the physical similarities between them and white blood cells.

In this scenario, the aim of this project is to identify a combination of cell and nuclear morphological biomarkers which can universally discriminate CTCs from blood cells, leveraging both label-dependent and independent systems. In addition, we aim to develop a machine learning classifier able to distinguish between tumor and normal cells based on the selected morphological parameters. These features will be used to fine-tune an innovative label-free technology based on high-throughput tomographic imaging to identify and morphological characterize tumor cells and specific organelle inside them. Moreover, our morphological parameters and machine learning classifier will be integrated with this cutting-edge technology to dichromate tumor cells from blood cells. In this way, the successful identification of CTCs through this strategy will provide a technique that offers significant advantages in disease screening programs by reducing the burden of invasive diagnostic tests at advanced tumor stages. Moreover, this approach will significantly enhance the sensitivity of current liquid biopsy-based molecular diagnostics, thus holding great clinical potential.

6. CONCLUSIONS

The present study investigates the use of cellular and nuclear morphological features to identify CTCs within WBCs, to improve the current LB technology for early cancer diagnosis.

What emerges is that just one parameter is not enough to distinguish cancer cells, but the combination of multiple characteristics, such as N:C ratio, cell and nuclear shape and inner complexity, can allow to overcome this crucial challenge in LB field, representing promising biomarkers for CTCs detection. Since this huge amount of data requires ML systems to handle it in order to achieve accurate cell classification, we fed our morphological data at single cell level about CTCs, WBCs and tumor cell lines to ad hoc ML algorithms. Our findings highlighted that XGBoost represent the most reliable and robust model to correctly classify both tumor and normal cells.

Prospectively, further validation is necessary by expanding the dataset to include a higher number and different types of CTCs. This will enable the generation of a comprehensive database of CTCs morphological features which can improve the capture of all CTCs among different tumor types and inside a single patient. Moreover, with the help of ML algorithms this data can allow also the detection of CTCs which not express known tumor surface markers.

In conclusion, the results of this pilot study can pave the way for the development of highly reliable CTCs capture systems. This strategy not only promises significant advancements in disease screening by reducing reliance on invasive diagnostic methods but also enhances the sensitivity of liquid biopsy-based molecular diagnostics, offering substantial clinical potential for cancer detection and monitoring.

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