REVIEW ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Circulating Cell Free DNA (ccfDNA): Is It the Future of Breast Cancer Diagnosis?

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Abstract

Recently, liquid biopsies have emerged as a very promising diagnostic technique with a number of benefits over traditional invasive techniques. Novel approaches to Circulating cell-free nucleic acid (cfDNA) detection in blood have provided new, rapid, convenient, minimally invasive, and sensitive biomarkers for cancer diagnosis, prognosis, and monitoring of response to therapy. Circulating cfDNA, also known as circulating tumor nucleic acid (ctDNA), are one of several significant biomarkers that may be obtained from liquid biopsies. They are widely studied as promising biomarker candidates in the diagnosis and prognosis of cancer. Circulating cfDNA analysis may reveal details on the mutation profile of tumor cells. Many of these markers may be useful to clinicians in choosing therapies and in patient follow-up. Therefore, a new avenue in personalized medicine is represented by DNA-based diagnostics. cfDNA application in breast cancer is expected to be employed in ordinary clinical practice. Classification and treatment choices for breast cancer are now determined by tumor analysis; however, cfDNA may be examined from a straightforward, non-invasive blood sample and contain many characteristics of the original tumor. cfDNA may play a role as novel biomarkers in both early-stage and metastatic breast cancer for screening, diagnosis, recurrence detection after surgery, follow-up, detection of mutations, prognosis, and treatment monitoring.

Keywords: Cell-free DNA (cfDNA); Breast Cancer (BC); Diagnostic Biomarker; Prognostic Biomarker.

1. Introduction

Breast cancer (BC) is the most common cancer in women worldwide and the primary cause of cancer-related mortality in women **(Deng et al., 2025; Elhawary et al., 2025)**. BC is a complex heterogeneous disease with an estimated 2,001,140 new cases and 611,720 cancer deaths in 2024 **(Messeha et al., 2024)**. The WHO's International Agency for Research on Cancer expects that by the year 2030, there will be approximately 21.7 million new cancer cases and 13 million cancer deaths worldwide **(Kong et al., 2023)**. In Egypt, the incidence rate of BC is 48.8/100,000, accounting for 32% of all women's cancers **(Azim et al., 2023)**. For many exogenous and endogenous factors, such as drug resistance, failure of curative management, and poor quality of life, as well as metastasis, BC develops and leads to high mortality among women **(Łukasiewicz et al., 2021)**. Also, a high percentage of cancer patients are diagnosed at

advanced stages **(Koo et al., 2020)**. Conventional BC biomarkers such as CEA, CA125, CA15-3, CA19-9, and other tumor markers are of low diagnostic and prognostic value in breast cancer **(Gaughran et al., 2020)**, with limited assessment for BC prediction, progression, and monitoring treatment response and recurrence. As a result, there is an urgent need for new biomarkers with high sensitivity and accuracy for breast cancer prediction, as well as with a clinical value in tumor diagnosis, prognosis, and treatment monitoring **(Alix-Panabières & Pantel, 2021; Heidrich et al., 2021)**.

Recently, there has been significant interest in using cfDNA for breast cancer detection and treatment. It is crucial for the early detection, assessment of efficacy, and tracking of breast cancer metastases and recurrence **(Sant et al., 2022)**. Consequently, cfDNA is regarded as a novel tumor molecular diagnostic technique with a wide range of potential uses **(Song et al., 2022)**. cfDNA shows stability and is easily extracted from human plasma, serum, and other bodily fluids. As a result, cfDNA can be used as a non-invasive biomarker for breast cancer that has numerous clinical uses as a prognostic, predictive, and diagnostic tool **(Gianni et al., 2022; Sant et al., 2022)**.

Liquid biopsies that employ the cfDNA circulating in human fluids are a common area of study in disease research. Liquid biopsy refers to a blood test that looks for signs of cancer by analyzing plasma's circulating tumor cells and cell-free tumor DNA **(Allen, 2024)**. This test illustrates the potential of these technologies in cancer care. The majority of liquid biopsy investigations evaluate tumor-specific changes such as point mutations, deletions and insertions, translocations, amplification, and epigenetic modifications using cutting-edge technology like real-time PCR, next-generation sequencing, or more focused approaches **(Raval & Bhattacharya, 2025)**.

Despite being the gold standard for diagnosis and therapy selection, surgical biopsy has many drawbacks. In addition to being invasive, it can only provide a static and spatially constrained image of the disease at the time of the surgical process **(Zaffino et al., 2020)**. On the other hand, cfDNA has the capacity to detect tumor heterogeneity and enables real-time longitudinal cancer monitoring in addition to being noninvasive, quick, and inexpensive **(Wen et al., 2022)**.

Although the amounts reported in various research varied, cancer patients often have higher cfDNA concentrations than healthy people. These findings prompted thorough quantitative and qualitative studies of cfDNA in BC patients **(Jongbloed et al., 2021)**. According to numerous studies, cfDNA concentrations are fairly high in benign cancer and extremely high in malignant cancer. Also, tumor size, the extent of tumor infiltration, illness progression, survival, and stage were associated with plasma DNA levels. As well as numerous investigations on the capacity of cfDNA to identify minimal residual illness following surgery or treatment have been conducted in various cancer types, indicating its great prognostic and recurrence prediction abilities **(Jiang et al., 2020; Gale et al., 2022; Faulkner et al., 2023)**.

Since tumor-associated DNA frequently enters the bloodstream in cancer patients, screening for plasma or serum DNA may reveal details on the genetic and epigenetic profiles linked to the onset, progression, and response to treatment of breast cancer **(Vlataki et al., 2023)**. Molecular characterization of circulating DNA can provide significant tumor features pertinent to the selection of targeted therapy for specific patients, and quantitative measurement of circulating DNA can indicate tumor burden **(Reichert et al., 2023)**. Personalized medicine also may be advanced by circulating DNA analyses that yield predictive and prognostic data. So, periodic evaluation can be used to assess the course of the disease and the effectiveness of treatment because cfDNA varies with disease status during therapy. Combining cfDNA detection with a panel of traditional cancer markers used for screening programs appears to be the most promising use case **(Szilágyi et al., 2020)**.

2. Circulating cell-free DNA (cfDNA)

Circulating cfDNA has gained significant attention in the oncologic field seventy years after its discovery, and its plasma genotyping is revolutionizing cancer treatment **(Khurram et al., 2024)**. Circulating cfDNA fragments were first discovered in the bloodstream by French scientists Mandel and Métais in 1948 **(Mandel & Metais, 1948)**. Later, in 1977, an increased serum level of cfDNA was observed by Leon et al. in the blood circulation of breast cancer patients **(Leon et al., 1977)**. cfDNA circulates freely and can be measured in the bloodstream and several other body fluids **(Khurram et al., 2024)**, such in urine **(Li et al., 2020; Santos et al., 2022)**. So, it may be

used as a non-invasive marker surrogate for tissue biopsies when proposed to be secreted from tumor tissue sites **(Addanki et al., 2022)**.

Circulating cfDNA is the degraded pieces of DNA released by cells into the blood plasma **(Grabuschnig et al., 2020)**. Its molecular detection may be useful for early diagnosis, prognostic monitoring, and treatment efficacy tracking in several tumors, such as lung, breast, colorectal, and ovarian cancer **(Chen et al., 2022)**. In healthy subjects, the detected level of cfDNA is low due to the rapid clearance of dead cells from circulation by phagocytosis **(Hu et al., 2021)**. On the other hand, the primary reason for cfDNA buildup in blood during tumor development is increased DNA turnover and release by cancerous cells **(Sánchez-Herrero et al., 2022; Stejskal et al., 2023)**. It has been found that the cfDNA level in blood plasma or serum is related to the tumor burden, so their measurement may be utilized to assess the tumor load and biological activity **(Bryzgunova et al., 2021; Bronkhorst et al., 2022; Stejskal et al., 2023)**.

Research on cancer patients has revealed that cfDNA concentration in their bloodstream is noticeably higher than that of healthy individuals **(Bu et al., 2020; Bryzgunova et al., 2021)**. cfDNA is present in a variety of bodily fluids in humans, and its levels fluctuate in response to conditions like inflammation, cancer, and tissue damage **(de Miranda et al., 2021; Zhang & Li, 2023)**. According to the majority of quantitative studies conducted to date, in cancer patients (breast cancer, lung cancer, colorectal cancer, and lymphoma), the amount is six times greater in plasma or serum than in healthy individuals **(Telekes & Horváth, 2022; Tivey et al., 2022)**. This suggests that cfDNA can be utilized to assess tumor therapy outcomes and patient prognoses **(Cisneros-Villanueva et al., 2022)**.

2.1. Origin of circulating cell-free DNA

Fragments of cfDNA originate in blood due to several causes. However, the main three sources of cfDNA in the blood are apoptosis, necrosis, and active cell secretion **(Grabuschnig et al., 2020; Heitzer et al., 2020; Hu et al., 2021)**. Tumor metastasis has also been shown to cause a significant amount of lengthy DNA fragments to be released into the peripheral circulation, but the fact that its origin is unclear **(Sant et al., 2022) (Fig. 1)**. Apoptosis is one of the three primary sources of cfDNA in the blood. The term "apoptosis" was used to describe the programmed cell death. It is employed to get rid of undesirable cells in the early stages of growth. In adults, apoptosis is utilized to eliminate cells that are irreparably damaged from the body **(Heitzer et al., 2020)**. Extracellular DNA concentration has been found to rise in vitro when tumor cells undergo induced apoptosis. This implies that cfDNA might be a byproduct of cell death. Research on cfDNA fragments from apoptosis has revealed that the cfDNA length in cancer patients ranged between 160 and 180 bp or an integral multiple of that, which is comparable to the electrophoresis results of DNA from apoptotic cell nucleosomes **(Heitzer et al., 2020; Zhu et al., 2023)**.

Cell necrosis or release upon phagocytosis is the second source of cfDNA. When a cell is injured chemically or physically, it swells and loses its membrane integrity, which causes intracellular material to leak out. This is known as necrosis. Hypoxia, inadequate blood flow, or immune cell phagocytosis and killing can all contribute to local necrosis during tumor growth. Released DNA fragments due to necrosis are extremely varied and primarily lengthy (>1000 bp). Long-chain DNA fragments are released since there is no particular chromatin digestion due to the sudden membrane rupture. When cfDNA from the donated organ is identified, it has shown promise in assessing the degree of necrosis **(Delmonico et al., 2023)**.

The majority of cfDNA in healthy, normal people originates from apoptotic cells; very few come from necrotic cells. Necrotic cells, however, release the majority of the cfDNA in cancer patients **(Hu et al., 2021)**.

Furthermore, primary tumors, peripheral blood-circulating tumor cells, and distant metastases are some of the causes that cause the release of cfDNA into the circulation **(Addanki et al., 2022)**. According to reports, the amounts of long fragment DNA and cfDNA in peripheral blood may be utilized to measure the biological activity of tumors and indirectly reflect variations in the tumor burden **(Xu & Liu, 2020; Underhill, 2021)**.

2.2. Types of circulating cell-free DNA

There are several different types of cfDNA, including virtosomes, nucleosomes, vesicle-bound DNA, and loose DNA fragments **(Fig. 1) (Aarthy et al., 2015; Pös et al., 2020)**.

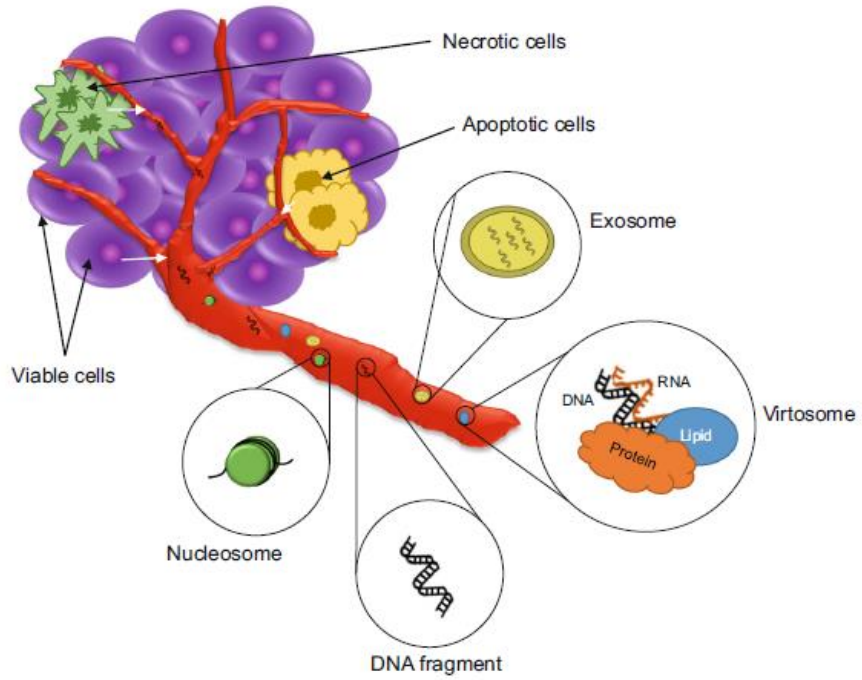

Fig. 1. Origin and types of cell free DNA (cfDNA). The release of DNA into the bloodstream can occur via cell death, such as necrosis (green) or apoptosis (yellow), or it can be secreted by living cells (purple). Unbound DNA, nucleosomes, vesiclebound DNA, and virtosomes are all examples of cell-free DNA **(Aarthy et al. 2015).**

1.2.1. Free DNA fragments

Free DNA fragments are unattached to surfaces or other molecules. DNases in blood break them down after they are released **(Grabuschnig et al., 2020)**. Nevertheless, DNase concentrations are low in cancer, and DNase inhibitors have also been found, which may help explain why cfDNA levels are rising. It has been reported that the half-life of circulating DNA is less than two hours **(Kustanovich et al., 2019)**.

1.2.2. Nucleosomes

Nucleosomes are complexes made up of DNA encircled by an octamer of histone proteins **(Raman, 2022)**. One hundred base pairs of linker DNA connect nucleosomes to one another. Chromatin is broken down into mono- and oligo-nucleosomes during apoptosis, then packaged into vesicles and expelled from the cell before being taken up by nearby cells. High rates of cell death after chemotherapy or radiation therapy for cancer cause the phagocytic system to become saturated, which raises the number of nucleosomes in the blood **(Khier & Gahan, 2021; Rapoport & Anderson, 2019)**. Elevated cfDNA concentrations were associated with higher nucleosome concentrations; this has been connected to breast cancer spread and disease progression **(Gianni et al., 2022; Yang et al., 2021)**.

1.2.3. Vesicle-Bound DNA

DNA found in exosomes, microparticles, and apoptotic bodies are examples of vesicle-bound DNA. Cell blebbing during apoptosis results in the formation of apoptotic bodies. Following their packaging in these entities, nucleic acids are expelled from the cell for phagocyte ingestion. Numerous cell types produce tiny vesicles called exosomes. Mutated nucleic acid fragments found in exosomes secreted by tumor cells may aid in the development and metastasis of primary and metastatic malignancies **(Logozzi et al., 2019)**.

1.2.4. Virtosomes

Only living cells may release virtosomes, which are complexes of freshly generated DNA, RNA, proteins, and lipids **(Grabuschnig et al., 2020)**. Nucleases cannot easily degrade the nucleic acids unless pretreated with proteinase or lipase. After being created in the nucleus, the DNA travels to the cytoplasm, where it is joined by freshly made lipid, protein, and RNA before being expelled from the cell. It is simple for virtosomes to infiltrate recipient cells and alter their biology. Therefore,

cancer cells' secreted virtosomes have the potential to infiltrate and change healthy cells **(Witz et al., 2024)**.

2.3. Clearance of circulating cell-free DNA

Extrusion processes are not the only explanation for high blood levels of cfDNA; clearance-related variables may also play a role. The concentration of cfDNA in healthy human blood circulation is kept low because macrophages themselves remove it **(Alix-Panabières & Pantel, 2021)**. However, the cfDNA content of breast cancer patients rises when the amount of DNA produced by the necrosis and proliferation of cancer cells in these individuals surpasses the capacity of macrophages to remove it. It also could be that the high amount of DNA fragments released from cancer cells may exceed the maximum level that could be cleared by macrophages at a particular time. So, their concentration in BC patients becomes elevated **(Stejskal et al., 2023)**.

2.4. Biochemical analysis of circulating cell-free DNA

Circulating cfDNA is easily extracted from the peripheral blood of patients, making it readily accessible **(Mack et al., 2022; Tivey et al., 2022)**. It is being investigated as a possible biomarker for cancer through their concentration measurements and the identification of genetic changes such as mutations, methylation, and microsatellite variations. Tracking these metrics in routinely drawn blood samples would provide insight into the course of the illness and the effectiveness of treatment **(Wen et al., 2022)**. It is possible to separate cfDNA from patient blood, plasma, stool, and urine **(Barták et al., 2019)**.

2.4.1. Pre-analytical consideration

Some of the challenges in molecular analysis of cfDNA are low yield, high fragmentation, and the possibility of genomic DNA contamination. In order to maximize the amount of isolated material in this situation, it is crucial to optimize and follow appropriate laboratory practices throughout the pre-analytical phase **(Fig. 2) (Delmonico et al., 2020; Song et al., 2022)**.

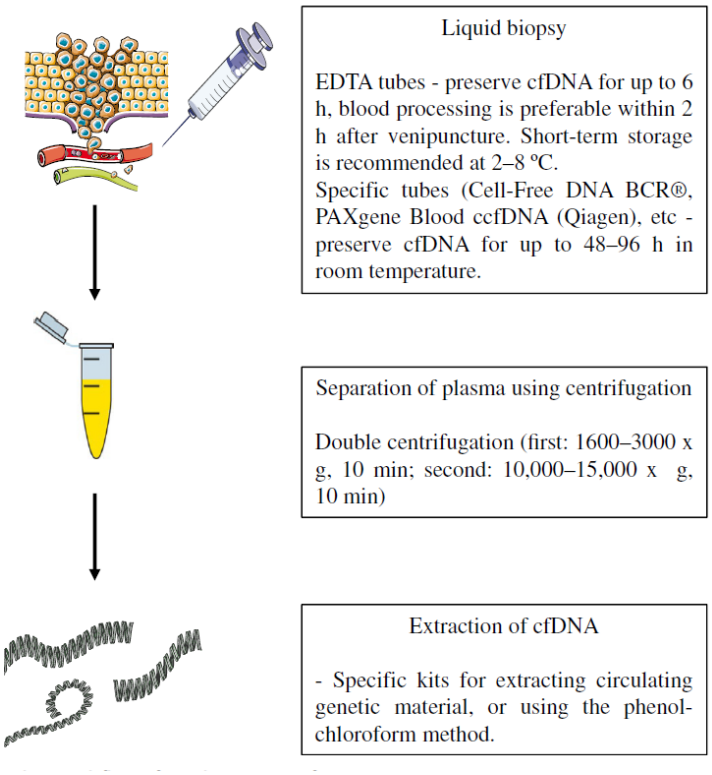

Fig. 2. Blood collection, plasma isolation, storage, and extraction of cfDNA **(Delmonico et al., 2020).**

2.4.2. Methods of detection

Detection and cfDNA quantification are still challenging. Their short duration and low quantities in bodily fluids are the main causes of determination issues. However, a number of methods have been established to quantify cfDNA in different forms of cancer. Also, point mutations, microsatellite changes, chromosomal changes (inversion and deletion), and promoter sequence hypermethylation can all be detected at the DNA level **(Hirahata et al., 2022)**. After isolation, total cfDNA can be determined using fluorescence-based techniques like UV spectrometry or PicoGreen staining, or quantitative real-time PCR using dual tagged fluorescent/quencher probes or intercalating dyes like SYBR green for detection **(Fig. 3) (Bohers et al., 2021)**.

2.4.2.1. Quantification

Numerous methods, have been used to quantify cfDNA, including fluorescence-based techniques like PicoGreen labeling and UV spectrometry, as well as the more sensitive quantitative polymerase chain reaction (PCR; SYBR Green or TaqMan) of repetitive elements or housekeeping genes. The main repeating unit of DNA organization in chromatin, circulating nucleosomes, has often been measured using enzyme-linked immunosorbent assays (ELISA) **(Chin et al., 2019; Hirahata et al., 2022)**.

2.4.2.2. Genetic analyses

Microsatellite-based fluorescence PCR has been used frequently to evaluate microsatellite instability. PCR assays unique to each allele have been used to investigate cfDNA mutations and copy number **(Bos et al., 2021)**. However, more sensitive methods like PAP (pyrophosphorolysis-activated polymerization) or BEAMing (beads, emulsion, amplification, and magnetics), as well as digital genomic technologies modified with deep or next-generation sequencing DNA, are being used more for the detection of rare mutations. These technologies have been covered in full elsewhere **(Bronkhorst & Holdenrieder, 2023; Foda et al., 2023)**.

2.4.2.3. Epigenetics methylation

Analysis of epigenetic methylation by treating extracted cfDNA with sodium bisulphite, which changes unmethylated cytosines into uracil, allows abnormally methylated cytosines inside CpG dinucleotides to be identified. Recently, methods like quantum dot-based fluorescence resonance energy transfer (QD-FRET), methylation-specific PCR, and methyl-BEAMing have been modified for the detection of methylated cfDNA **(Hirahata et al., 2022)**.

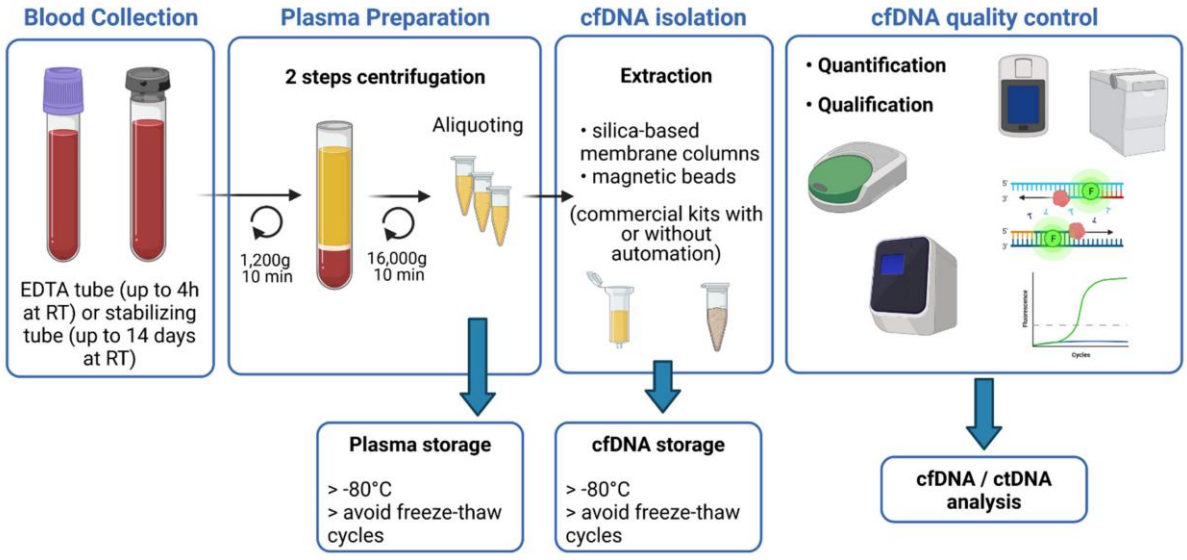

Fig. 3. Biochemical analysis of cfDNA **(Bohers et al. 2021)**

2.5. Clinical applications of circulating cell-free DNA

Currently, the usage of circulating cfDNA as a biomarker is investigated in several applications involving screening and prediction, treatment and recurrence monitoring, therapeutic resistance detection, and minimal residual disease of cancer **(Jiang et al., 2020; Tao et al., 2024) (Fig. 4)**.

3. Conclusion

Circulating cfDNA has attracted a lot of interest in the early diagnosis, evaluation, and monitoring of breast cancer metastasis and recurrence. Thus, due to cfDNA's relative stability and its easy extraction, it can be used as a new test for non-invasive molecular diagnosis of different types of cancer, in addition to conventional biomarkers. Previous studies have found that the blood concentration of patients with colorectal, lung, or breast cancer or lymphoma is significantly higher than that of healthy people. Other research showed that cfDNA analysis in the blood is likely to provide valuable information in breast cancer. This promises that cfDNA can be used as a routine biomarker for BC diagnosis, prognosis, and treatment evaluation.

Prospective large-scale clinical studies are needed for further understanding and evaluation of cfDNA's role and clinical applications in cancer diagnosis, prognosis, and monitoring.

Conflict of interest

None of the authors have any conflicts of interest.

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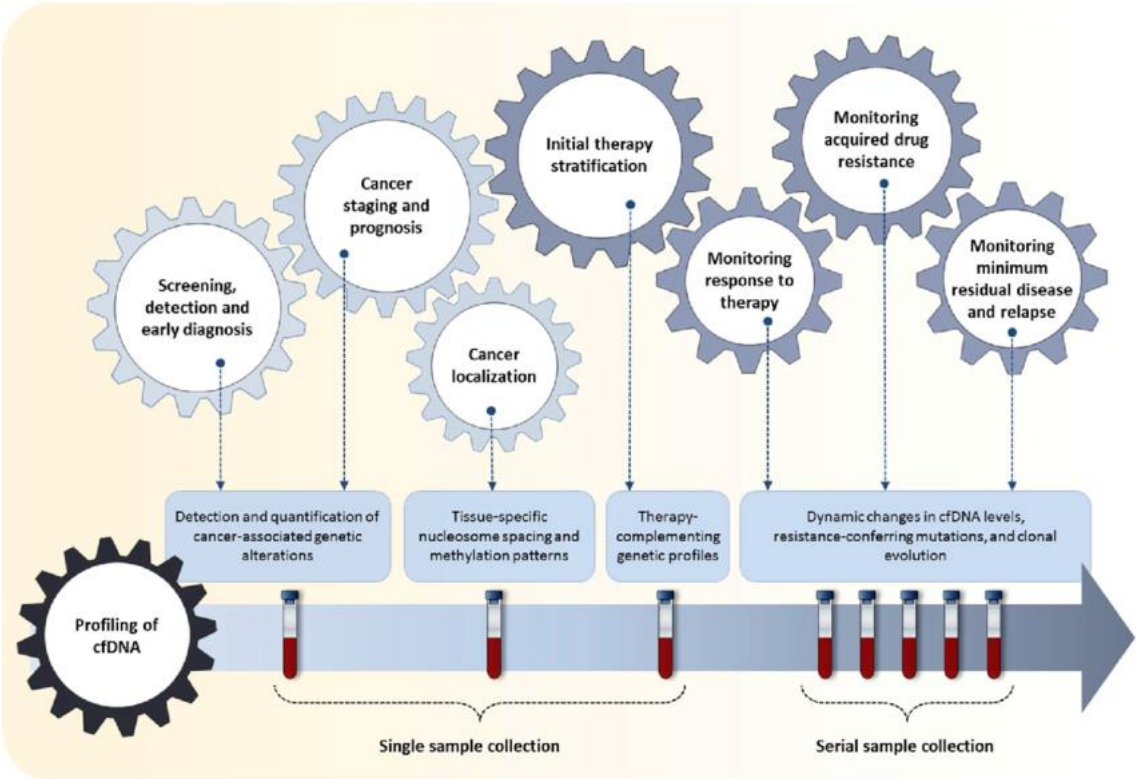

Fig. 4. Application of cfDNA in cancer detection and management **(Bronkhorst et al., 2019)**

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