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REVIEW

Circulating Tumor DNA And Its Potential Applications for Assessing Effectiveness of Neoadjuvant Drug Therapy in the Breast Cancer Patients

Tatiana M. Zavarykina[1,2,a*], Irina V. Pronina[1,2], Polina S. Mazina[1,2], Svetlana V. Khokhlova[2], and Gennady T. Sukhikh[2]

[1]*N. M. Emanuel Institute of Biochemical Physics, Russian Academy of Science, 119334 Moscow, Russia*

[2]*B. I. Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology, Ministry of Health of the Russian Federation, 117997 Moscow, Russia*

[a]*e-mail: tpalievskaya@yandex.ru*

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Abstract—The review discusses main characteristics and analytical features of the circulating tumor DNA (ctDNA), which accounts for a minor fraction of the cell-free DNA (cfDNA) in cancer patients. Currently, ctDNA is considered to be a promising biomarker for assessing treatment efficacy, prognosis, and disease monitoring in oncology, including breast cancer (BC). A significant proportion of BC patients receive neoadjuvant drug therapy, effectiveness of which largely determines necessity and extent of subsequent treatment. Determination of ctDNA could be the most sensitive method for evaluating response to neoadjuvant therapy, as it enables real-time monitoring of molecular changes during the treatment, prediction of therapeutic response, and assessment of recurrence risk. This approach could become an additional tool for personalization of BC therapy.

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INTRODUCTION

Breast cancer (BC) is the most common and socially significant malignancy among women, requiring substantial economic resources for the patient treatment and rehabilitation. Development of cancer, among other factors, is associated with accumulation of somatic mutations in the tumor, particularly mutations in the driver genes, which play a major role in malignant transformation and serve as targets for targeted therapies. Presence of tumor-associated disturbances in the DNA isolated from blood plasma has been reported in various types of cancer, including breast cancer [1], lung cancer [2, 3], colorectal cancer [4, 5], prostate cancer [6], gastric cancer [7], ovarian cancer [8], and other malignancies [9-13]. Detection of the circulating tumor DNA (ctDNA), which carries genetic alterations unique to the tumor, is a basis for a new form of liquid biopsy that is being integrated into the clinical practice. Since most molecular aberrations identified in the plasma ctDNA reflect genetic changes present in the primary tumor, the ctDNA analysis serves as a convenient predictive and prognostic biomarker, as well as a tool for monitoring disease progression [14-16].

DEVELOPMENT OF THE CIRCULATING TUMOR DNA CONCEPT

The existence of extracellular nucleic acids was first demonstrated in the mid-20th century, when Mandel and Métais (1948) [17] detected and isolated extracellular nucleic acids from human blood plasma. In 1966, Tan et al. [18] performed quantitative analysis of the cell-free DNA (cfDNA) levels in blood samples

* To whom correspondence should be addressed.

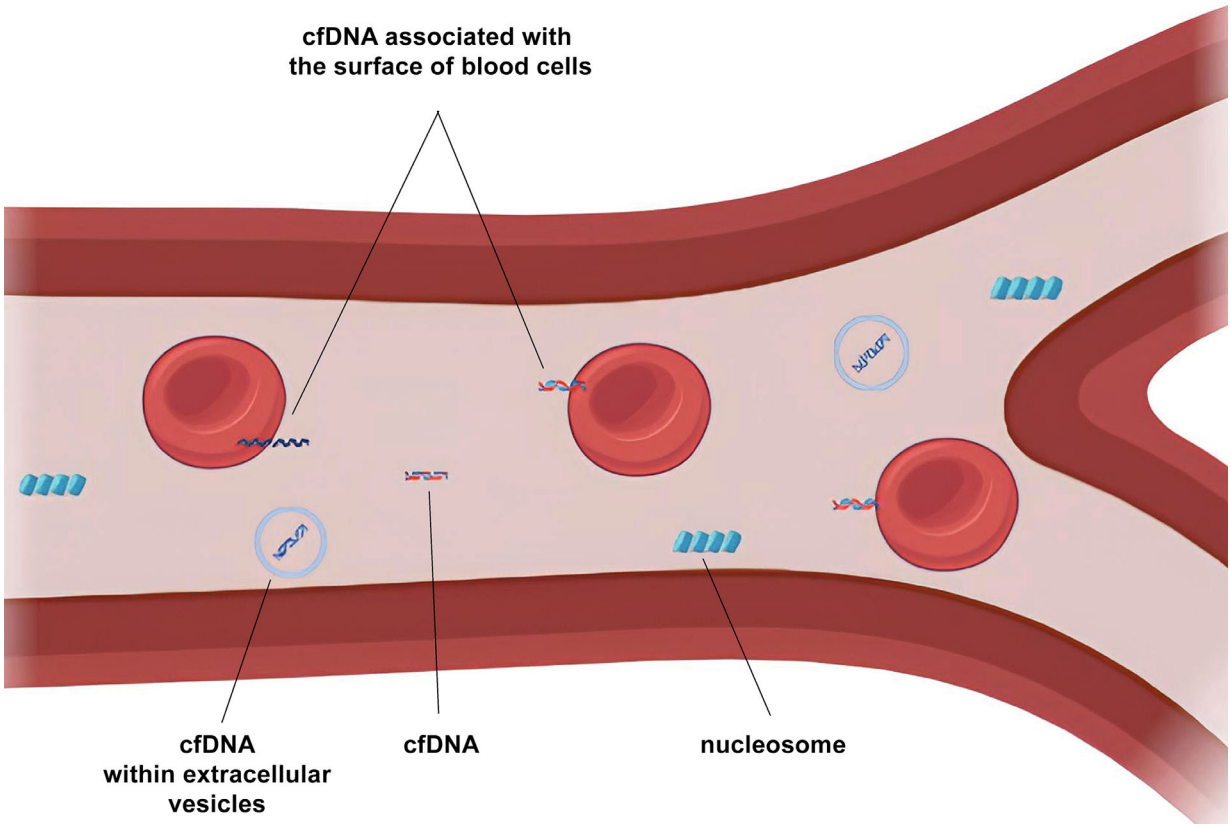

Fig. 1. Forms of cell-free DNA (cfDNA) in the bloodstream.

from the patients with systemic lupus erythematosus. They were the first to confirm the hypothesis that the disease-specific cfDNA can circulate in the bloodstream. Elevated cfDNA levels in the samples of patient serum provided prospects of its molecular profiling for prediction and management of autoimmune disorders. Prognostic potential of cfDNA for assessing cancer treatment efficacy was first demonstrated in 1977. In the study by Leon et al. [19], total cfDNA concentration in the serum of the patients with various cancers was measured and found to be significantly higher than in the healthy individuals. Furthermore, the cfDNA concentration correlated with the tumor response to neoadjuvant therapy, showing decrease in the patients who responded positively to radiation treatment [19]. These findings suggested that quantitative cfDNA analysis could be employed to evaluate efficacy of anticancer therapies, including comparison of different treatment regimens. In 1999, Silva et al. [20] detected ctDNA in the plasma of patients with BC, and its presence showed a statistically significant correlation with the clinicopathological characteristics of the patients.

Today, ctDNA is being actively investigated as a potential biomarker for cancer diagnosis and as a minimally invasive tool for assessing treatment efficacy, prognosis, and disease monitoring in oncology.

CHARACTERISTICS OF ctDNA

There are various types of free-circulating nucleic acids distinguished depending on the origin, such as genomic DNA, mRNA, viral DNA and RNA, microRNA, etc. Cell-free DNA (cfDNA) represents fragmented DNA molecules circulating in biological fluids. It can exist as a single- or double-stranded DNA with fragment lengths ranging from 120 to 21,000 bp (on average 160-180 bp). In most cases, cfDNA circulates in the bloodstream as part of nucleosomes – macromolecular complexes of histones and DNA [21] – or within vesicles [22] (Fig. 1). In these forms, cfDNA is protected from nuclease degradation and from eliciting immune responses [22]. In addition, cfDNA can be bound to the surface of blood cells, such as erythrocytes, through specific membrane proteins.

In the healthy individuals, cfDNA originates from several physiological processes, including apoptosis, phagocytosis, DNA release from neutrophils, and enucleation of erythroid precursors [23]. Plasma cfDNA is actively secreted by leukocytes (55%), erythroid precursors (30%), and lymphocytes (12%) [24]. Concentration of free-circulating cfDNA in the healthy individuals typically ranges from 1 to 10 ng/mL [25, 26]. Due to the rapid nuclease degradation, half-life of free-circulating cfDNA varies between 15 min and 2 h,

which makes it suitable for use in modern medical monitoring approaches [27].

In cancer patients, a fraction of cfDNA consists of ctDNA, which accounts for approximately 0.01-1.0% of the total cfDNA. Concentration of ctDNA depends on the tumor stage and localization, ranging from 5 to 1500 ng/mL [25]. ctDNA could be released into the bloodstream from the primary tumor cells, distant metastatic nodes, or circulating in the peripheral blood tumor cells [28, 29]. Detection of tumor-specific mutations in cfDNA allows ctDNA to be identified within the total pool of circulating nucleic acids. Both qualitative and quantitative mutation composition of ctDNA vary not only among the cancer types but also between the patients with the same diagnosis. For example, in the case of metastasis, ctDNA could comprise more than 10% of cfDNA, whereas at early stages of cancer or during minimal residual disease (MRD) monitoring, its fraction could be below 0.1% [30]. Although cfDNA is rapidly degraded in bloodstream, a comparative study employing digital PCR (dPCR) approach, targeted and whole-genome sequencing (WGS), comparing different methods of blood sample collection and storage for isolation of cfDNA and analysis of ctDNA, demonstrated that cfDNA remains stable for up to 24 h in the EDTA-treated blood plasma samples at room temperature, up to 48 h at 4°C, and for extended periods when plasma is appropriately processed and frozen [30]. However, the reported acceptable processing intervals for blood samples in EDTA tubes vary across studies, ranging from 1-2 h after blood collection [31, 32] to up to 24 h [30, 33, 34]. Therefore, in routine laboratory practice, blood samples collected in the EDTA tubes for the ctDNA analysis are recommended to be processed as soon as possible. The highest cfDNA stability is achieved using specialized blood collection tubes containing stabilizing reagents, whose performance is comparable across different manufacturers [30].

ctDNA has been detected in the patients with early-stage BC [35-37] and could serve as a prognostic biomarker [38-43]. It enables real-time monitoring of the treatment response, including detection of minimal residual disease [38-44], and could be applied to assess therapy efficacy in the patients with BC [45-53].

DETECTION OF ctDNA AS A VARIANT OF LIQUID BIOPSY

Analysis of ctDNA in the cancer patients – primarily focused on identifying driver mutations that determine tumor sensitivity to targeted therapy – represents a form of liquid biopsy. Although ctDNA is most commonly analyzed in the peripheral blood, other biological fluids such as urine, saliva, pleural or cerebrospinal fluid can also be used, depending on the tumor type and localization [54]. Liquid biopsy with ctDNA detection offers several advantages over conventional tissue biopsy. Classical biopsy is an invasive procedure that is not always feasible due to the tumor localization. Moreover, it often fails to capture the complete molecular and genetic profile of the malignancy because of tumor heterogeneity – that is, coexistence of multiple tumor cell clones. Presence of the so-called cancer stem cells contributes to the ongoing evolution of genetic and biological tumor properties during therapy and disease progression, which is nearly impossible to monitor using conventional tissue sampling. Performing repeated tissue biopsies to assess tumor evolution and response to therapy, is often complicated due to dispersion of tumor foci, size or localization, and overall condition of the patient. In particular, sampling from the metastatic nodes may be technically challenging or risky. In contrast, liquid biopsy sampling for ctDNA analysis is a simple, routine, and minimally invasive procedure. Therefore, biological material can be collected as frequently as needed to monitor disease dynamics and to obtain real-time information about the tumor [55, 56]. Thus, ctDNA analysis serves as a minimally invasive alternative for the molecular characterization of solid tumors.

METHODS FOR DETECTING CIRCULATING TUMOR DNA

In most solid tumors, including BC, analysis of ctDNA in blood samples is the most informative and technically accessible approach. For quantitative ctDNA analysis, blood plasma is preferred, as adherence to the rigorously standardized pre-analytical procedures involving double centrifugation minimizes contamination with the genomic DNA from blood cells, thereby increasing specificity and sensitivity of the method [57].

Since ctDNA constitutes only a very small fraction of the total cfDNA (Fig. 1), highly sensitive analytical techniques are required for its detection. The most common ctDNA detection approach relies on identifying tumor-specific mutations within the cfDNA [58]. Quantitative ratio of normal cfDNA to ctDNA in blood plasma of the cancer patients is expressed as the mutant allele fraction (MAF). MAF of 0.1% means that for every 999 molecules of cfDNA derived from normal tissue, there is 1 molecule of ctDNA. It is well established that the MAF values decline as tumor size decreases [59]. For accurate quantitative and qualitative analysis of ctDNA, various modifications

of dPCR and next-generation sequencing (NGS) are employed [60, 61].

The dPCR method allows detection of extremely small amounts of target DNA (in this case, ctDNA) even in the presence of a large amount of other DNA molecules [62], with sensitivity reaching MAF values as low as 0.001% [63]. Major limitation of the dPCR-based approaches is the requirement for prior knowledge of potential mutations and their genomic locations, which is necessary for primer and probe design. This method efficiently detects the known mutations, such as major driver mutations characteristic of the primary tumor or variants associated with therapeutic response in the specific tumor types [64]. It correlates well with histopathological findings, complements them, and provides additional information for more precise tumor classification, recurrence risk assessment, and therapy optimization.

Cancers lacking known mutations require a combined methodological approach for ctDNA analysis, involving prior identification of molecular–genetic alterations in the primary tumor [65]. This is particularly relevant for the triple-negative BC, in which no driver mutations have been identified. Whole-genome sequencing (WGS) [66] or whole-exome sequencing (WES) [67] can be used to identify somatic mutations in the tumor. Based on these findings, a panel of most representative somatic mutations is generated, including corresponding primer and probe systems for their detection by dPCR [39].

NGS-based approaches enable detection of a broad range of mutations – both previously described and novel ones [68]. ctDNA analysis using NGS can be performed in two formats: targeted sequencing of the selected genes or gene panels, and whole-genome approaches [69]. Whole-genome sequencing allows identification of genetic alterations without prior analysis of the primary tumor [58]. In particular, approaches based on the copy number aberration analysis using WGS data with different coverage depths have been proposed [70]. Tumor-specific mutations were identified based on the ratio of detected variants at different depths of WGS coverage, without the need for primary tumor analysis [70]. Some systems, such as the Guardant Reveal multi-cancer assay [71], evaluate not only genetic but also epigenetic alterations, namely, CpG methylation. Detection of ctDNA was performed not by comparing tumor and leukocyte DNA, but by comparing cfDNA sequencing results with the reference databases [72]. Despite numerous advantages and substantial technological progress, the NGS-based ctDNA analysis faces several challenges. Detection of rare mutations present at very low frequencies could be hampered by sequencing errors [73]. Therefore, specialized bioinformatic approaches and additional error-correction systems are required for analysis of the sequencing data aimed at studying ctDNA [1, 74-76].

An actively developing and increasingly clinically implemented approach involves creation of the personalized marker panels for ctDNA analysis. In this approach, both tumor biopsy material and blood samples are used for sequencing to identify somatic mutations specific to the individual patient. Whole-genome [66] or whole-exome [67] sequencing is typically applied, while targeted sequencing is used less frequently [77]. The phenomenon of clonal hematopoiesis [16, 78] should be considered when analyzing the paired blood sample [79]. The resulting mutation panel, reflecting the most abundant mutations in the tumor, is next used for ctDNA detection. An example of such approach is the commercially available Signatera™ assay (Natera, USA), developed for monitoring MRD, that is, molecular residual disease in BC and several other malignancies [80, 81]. Personalized systems can also evaluate nucleotide substitutions, particularly among the previously identified single-nucleotide variants (SNVs) [82], and are capable of detecting insertions and deletions [83]. The number of mutations assessed across different systems ranges from 1-2 up to 16 (in the Signatera™ assay). This approach enables tracking of changes in the clonal composition of the tumor, predicting therapeutic response. Furthermore, detection of ctDNA in the patient plasma may indicate the presence of micrometastases [83]. The CloneSight system, developed in Spain, enables monitoring of BC recurrence by detecting ctDNA at 6, 12, 18 months and beyond after completion of therapy [84].

Combined approaches to ctDNA analysis are also being actively developed [65]. These include the use of ELISA or conventional molecular biology techniques integrated with biosensor-based ctDNA detection platforms, such as electrochemical and fluorescent systems. In these methods, particular attention is paid to simplifying and accelerating sample preparation by reducing quality requirements for the biological material needed for analysis. Furthermore, electrochemical detection offers the potential for “point-of-care” ctDNA testing, since this approach does not require expensive equipment and specialized laboratory conditions. In the study by Wang et al. [85], several types of sensor elements are described, in which specific ctDNA-binding receptors – based on antibodies, aptamers, enzymatic systems, etc. – are immobilized on the sensor surface. Upon the ctDNA–receptor binding, a signal is transmitted to the electrode, followed by potentiometric or voltammetric analysis. Photometric sensors could be designed based on the same principle. Approaches for cfDNA detection involving probe-mediated reactions in solution are also under development. Such reactions are most often based on redox reactions, with a resulting product

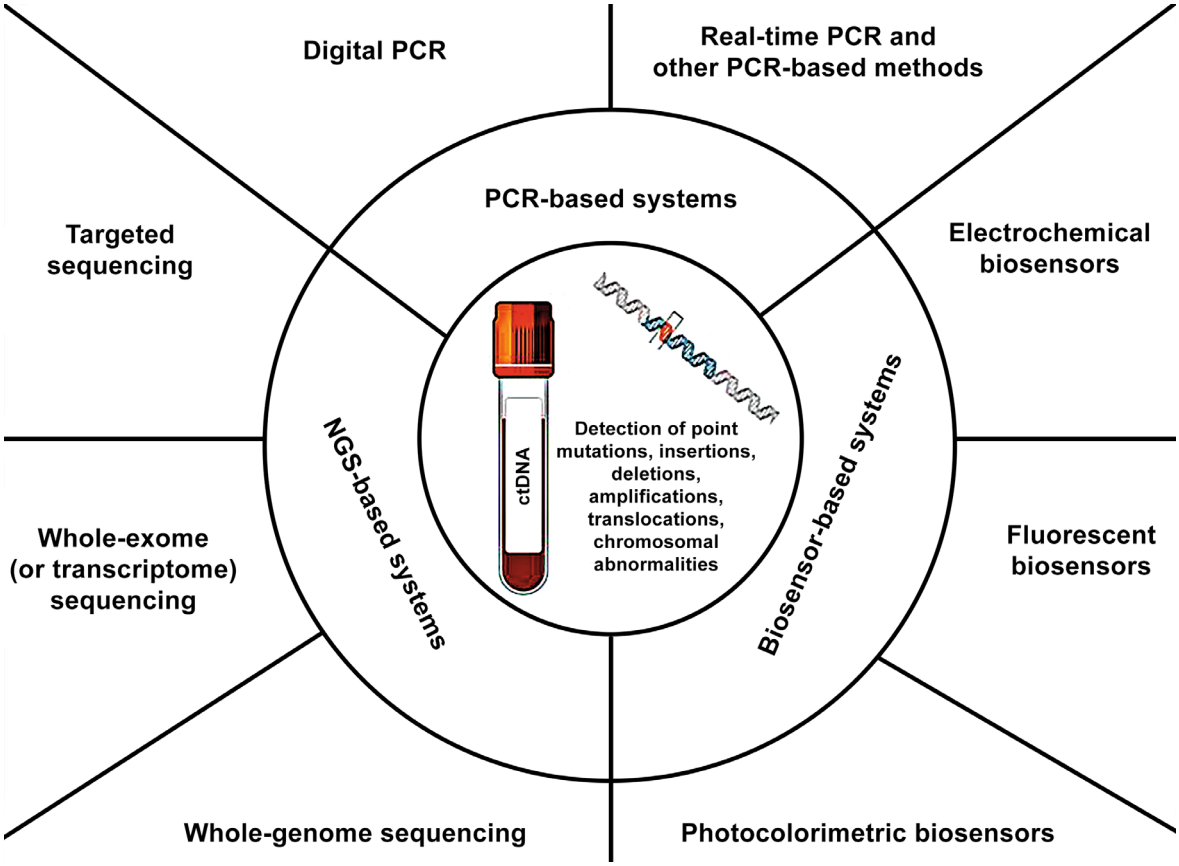

Fig. 2. Methods for detecting circulating tumor DNA (ctDNA).

that could be detected electrochemically, photometrically, or fluorimetrically [86]. The probes could include single-stranded DNA molecules complementary to the target ctDNA [87], RNA molecules [86], antibodies [88], the latter allows identification of ctDNA methylation. These studies focus on analyzing physical and chemical properties, as well as on quantitative assessment of the ctDNA content (Fig. 2) [89].

Due to the technical challenges in the development of such systems, they have so far been implemented only for the most frequently occurring driver mutations in the genes such as *PIK3CA* [90, 91], *KRAS* [92], *EGFR* [93], and *MGMT* [94].

At present, rapid progress in analytical technologies suggests that the diversity of approaches for ctDNA analysis will significantly increase in the near future, expanding the arsenal of available methods for ctDNA detection.

POTENTIAL OF ctDNA ANALYSIS FOR EVALUATING EFFICACY OF NEOADJUVANT DRUG THERAPY IN BREAST CANCER PATIENTS

Clinicopathological prerequisites for ctDNA analysis in neoadjuvant treatment of breast cancer patients. For the patients with BC, neoadjuvant drug therapy constitutes an integral part of the management of both early-stage and locally advanced disease. In these cases, achieving pathologic complete response (pCR) is a crucial prognostic factor and is associated with improved overall survival (OS) and disease-free survival (DFS) [95-102]. This relationship is most pronounced in the triple-negative breast cancer (TNBC) and HER2-positive BC. Typically, neoadjuvant therapy includes 6-8 cycles of chemotherapy, with addition of the targeted therapy when indicated (anti-HER2 therapy for HER2-positive tumors). Some oncologists believe that such treatment intensity may be excessive for the early-stage patients with high chemosensitivity. Monitoring ctDNA levels during therapy may facilitate treatment personalization, enabling optimization of the therapy duration for each patient.

The response to neoadjuvant chemotherapy (NACT) provides prognostic information that complements standard clinicopathological parameters of the primary tumor, such as molecular biological subtype, disease stage, and malignancy degree [98, 99]. Currently, efficacy of NACT is assessed using clinical parameters and pathomorphological examination of the resected tissue after surgery. Routine practice relies on conventional clinical and instrumental methods

(physical examination, palpation, ultrasound of breast and regional zones, mammography, MRI of mammary gland), which often fail to reflect the true therapeutic effect. The rate of complete response detection may be up to twofold lower when based on clinical and imaging assessment than when determined by pathomorphological examination of postoperative tissue [103].

A key parameter for assessing NACT efficacy is pCR, characterized by the absence of viable tumor cells in both breast tissue and lymph nodes upon pathomorphological examination. The standard method for evaluating the degree of treatment-induced pathomorphosis is routine pathological/histological examination, including determination of the ypT and ypN categories (post-NACT pathomorphological staging of tumor to T and N categories) or assessment of residual cancer burden (RCB). This method involves manual review of serial histological sections by a pathologist, which typically does not cover the entire volume of resected tissue, thereby contributing to potential errors due to heterogeneity of biological sample; furthermore, analytical precision varies widely across laboratories. Nevertheless, information about the degree of treatment-induced pathomorphosis is crucial for determining post-neoadjuvant therapeutic strategies in the TNBC and HER2-positive BC. A combined analysis has shown that the patients achieving pCR have a significant survival advantage compared to those with residual disease after NACT (residual tumor cells after treatment) [101, 102]. While pCR is a strong predictor of low recurrence risk, studies indicate that predicting early metastatic recurrence in the patients with residual tumors remains less reliable [102, 104, 105]. For example, survival analysis in the I-SPY2 Trial Consortium demonstrated that the 3-year DFS reached 95% among the patients achieving pCR [102], whereas metastases occurred in 22% of patients without pCR.

In the United States, several prognostic gene expression panels associated with BC progression have been developed, including MammaPrint (Agendia Inc.), Oncotype DX (Genomic Health), and Prosigna (Veracyte Inc.), which are designed to predict recurrence and/or metastasis. Based on analysis of these markers, a decision can be made regarding the necessity of adjuvant (postoperative) chemotherapy. These panels have proven their utility for the luminal ER-positive/HER2-negative tumors, which is the most common subtype of BC [104]. However, these assays do not reflect dynamic state of the tumor, its response to NACT (prediction of pCR), and therefore cannot be applied to guide decisions regarding the timing of chemotherapy completion.

Detection of ctDNA in blood may represent a more sensitive approach for evaluating the effect of NACT, showing a strong correlation with pathological methods used to assess the degree of pathological response. Development and implementation of a reliable marker reflecting tumor pCR could become an additional tool for therapy individualization and could also help determine optimal timing for the treatment completion. Moreover, a highly relevant emerging direction in the early-stage BC management is investigation of whether surgery could be safely omitted in the patients who achieve pCR. In such studies, pCR is typically confirmed by the tissue biopsy; however, this approach cannot fully capture the entire tumor burden. Analysis of ctDNA concentration and characteristics in these cases may provide a more informative, sensitive, and specific assessment, as it reflects molecular tumor dynamics in real time. For instance, in BC, ctDNA quantification enables a more accurate evaluation of tumor response to therapy and facilitates detection of minimal residual disease (MRD) and tumor resistance to therapy, i.e., molecular relapse [40, 41, 44, 105, 106]. ctDNA monitoring could be used for the early detection of the disease recurrence [65] and, in the treatment of metastatic disease, for selecting the most effective treatment regimens [46, 47, 81]. In this review, we focus on the potential of ctDNA analysis for assessing NACT efficacy in BC patients.

CtDNA analysis for detection of minimal residual disease during primary treatment. Analysis of ctDNA provides opportunities for detection of MRD, that is, presence of ctDNA in the absence of other clinical signs of recurrence in the patients who have completed therapy. Assessing treatment response based on MRD detection could become an important component of personalized management strategies, helping to guide further therapeutic decisions in subsequent observation of BC patients. Feasibility of using ctDNA to determine MRD following NACT and surgery has been demonstrated in several studies [1, 52, 105, 106]. Of particular importance is the dynamic change in ctDNA levels relative to baseline. As early as 2015, it was shown that ctDNA detection in the serial samples obtained from the patients after NACT and subsequent surgery was associated with early recurrence [39]. These findings were later confirmed by additional studies involving various subgroups of BC patients.

CtDNA assessment during NACT. Monitoring ctDNA during NACT enables prediction of treatment response, showing correlation with pCR, as well as with the risk of recurrence and metastasis. Moreover, the ctDNA positivity at mid-NACT (12 weeks after treatment initiation) was found to be strongly associated with the decreased OS and DFS (p = 0.0002 and p = 0.0034, respectively) [81].

Using a personalized whole-genome approach, the ctDNA analysis allows evaluation of the relationship

between the ctDNA presence, NACT response, recurrence risk, metastasis development, and 3-year survival. In this study, individualized mutation panels were designed for each patient based on the whole-exome sequencing of tumor tissue. The early treatment period of NACT (3 weeks after therapy initiation) was identified as a significant time point for pCR prediction (odds ratio (OR) = 4.33; $p = 0.012$). After completion of NACT, all patients who achieved pCR were ctDNA-negative. Among those who did not achieve pCR, the ctDNA-positive patients exhibited a higher risk of metastasis (OR = 10.4). Patients who failed to reach ctDNA negativity showed poor treatment response and increased metastatic risk (OR = 22.4; 95% CI; $p < 0.001$), whereas the ctDNA-negative state was associated with improved survival even in the absence of pCR [44]. These findings are consistent with the results of previous works [105, 107] and the study by Chen et al. [81], which demonstrated that the ctDNA absence at mid-NACT (12 weeks after treatment initiation) was associated with pCR achievement ($p = 0.02$).

Subsequent studies revealed relationship between the ctDNA-negative status and pCR achievement in the TNBC patients [108], as well as a positive correlation between the ctDNA levels and RCB [1, 109]. A correlation was also observed between the ctDNA positivity and reduced DFS regarding distant metastases, particularly at mid-NACT (12 weeks after treatment initiation) and after NACT but before surgery ($p < 0.0001$) [108].

Personalized ctDNA monitoring during NACT may provide a real-time assessment tool for treatment response and predict pCR as a surrogate survival marker [44, 104].

CtDNA assessment after NACT. CtDNA detection after completion of NACT but before surgery could be considered as a prognostic marker. The presence of ctDNA has been shown to be associated with poorer DFS [76, 110]. One of the earliest studies demonstrated an increased risk of early recurrence in the ctDNA-positive patients both after surgery and during subsequent observation of the patient, with hazard ratios (HR) of 25.0 and 12.0, respectively. 96% of the patients without disease recurrence had no detectable ctDNA ($p < 0.0038$) [39].

This correlation was most prominent in the study by Cailleux et al. (HR = 53; $p < 0.01$) [106]. In the ctDNA-positive patients after NACT who did not achieve pCR, a higher risk of distant metastasis was observed (HR = 5.89 for hormone receptor-positive BC and HR = 3.79 for TNBC) [108]. These findings were subsequently confirmed by additional studies involving various BC patient subgroups [41, 76, 109].

Using common mutations in the signaling pathways associated with BC progression is suggested to be a promising approach for ctDNA quantification and treatment response evaluation. It was found that in the BC patients carrying *PIK3CA* and/or *TP53* mutations in the tumor tissue, absence of these mutations in the cfDNA analysis after NACT corresponded to achievement of pCR in 93.33% of cases [111]. For the HER2-positive BC subtype, the analysis of *TP53* mutations in cfDNA has been shown to be a marker of resistance to anti-HER2 therapy with monoclonal antibodies [112], as well as the presence of *PIK3CA* and/or *TP53* mutations in cfDNA has been identified as a predictor of poor response to anti-HER2 treatment [64].

CtDNA assessment after NACT and surgery. For the ctDNA-positive patients after NACT and surgical treatment, an earlier disease recurrence (reduced RFS) was observed [113, 114]. CtDNA monitoring in the BC patients with residual tumors after NACT demonstrated that persistence of ctDNA positivity after NACT and surgery was associated with the decreased RFS. This approach could enable identification of the patient subgroups requiring more intensive therapy, improving their survival outcomes [115]. Association of ctDNA with the disease recurrence and progression was also observed during the post-NACT observation [72, 114].

Baseline ctDNA levels. It should be noted that ctDNA serves both as a predictive and as prognostic marker when analyzed during or after NACT, prior to surgery, and throughout subsequent treatment or observation. However, the pretreatment ctDNA levels are not significant in the TNBC or heterogeneous BC cohorts, as no association has been observed between the presence of ctDNA before NACT and RFS [44, 105, 106]. At the same time, correlation between the ctDNA levels and clinicopathological characteristics of BC – such as tumor size, lymph node metastasis, and tumor grade – has been reported [108]. However, in the HER2-positive subgroup, the ctDNA positivity (based on detection of *PIK3CA* and/or *TP53* point mutations) before initiation of anti-HER2 therapy was found to be associated with the lower rate of pCR achievement [64].

Potential of ctDNA analysis in TNBC patients. The triple-negative subtype represents the most aggressive form of BC, lacking known targets for hormone or targeted therapies, with chemotherapy continuing to be the standard therapeutic approach. In recent years, growing interest has been directed toward evaluating response to NACT in the TNBC patients through changes in the blood levels of ctDNA. For the TNBC patients, detection of the ctDNA-positive status has been associated with high incidence of metastatic disease (nearly 72%) [38]. The potential of ctDNA analysis for this most unfavorable BC subtype has been demonstrated in the long-term study with up to 12 years of observation, where the

Table 1. Key studies on ctDNA analysis for assessing the efficacy of NACT

Patients in the study	Main results	Reference
Early-stage BC	ctDNA positivity after NACT was associated with an increased risk of early recurrence, both after surgery and during subsequent observation, with hazard ratios (HR) of 25.0 and 12.0, respectively ($p < 0.0001$); ctDNA was undetectable in 96% of patients who remained recurrence-free, both at the postoperative time point ($p < 0.0038$) and during subsequent observation ($p < 0.0001$)	[39]
TNBC	absence of ctDNA in the middle of NACT (12 weeks after the initiation of therapy) was associated with achieving pCR ($p = 0.02$); ctDNA positivity at mid-NACT (12 weeks after the initiation of therapy) was strictly associated with the decreased OS and DFS ($p = 0.0002$ and $p = 0.0034$, respectively)	[81]
Stage I and II BC	presence of ctDNA after completion of NACT and prior to surgery was associated with shorter DFS	[111]
Early-stage BC	presence of ctDNA after completion of NACT and prior to surgery was associated with shorter DFS (HR = 53; $p < 0.01$)	[106]
High-risk stage II and III BC	before treatment, 73% of the patients were ctDNA-positive, and this proportion decreased during therapy; importantly, the early stage of NACT (3 weeks after treatment initiation) was identified as a critical time point for predicting pCR (odds ratio (OR) = 4.33; $p = 0.012$); a ctDNA-negative status at this early stage correlated with achieving pCR; after completion of NACT, all patients who achieved pCR were ctDNA-negative ($n = 17$; 100%); among those who did not achieve pCR ($n = 43$), the ctDNA-positive patients (14%) had an increased risk of metastasis (OR = 10.4); the patients who failed to reach ctDNA negativity demonstrated poor response to treatment and higher risk of metastasis (OR = 22.4; 95% CI, 2.5-201; $p < 0.001$), whereas ctDNA negativity was associated with improved survival even in the patients who did not achieve pCR	[44]
Localized BC	patients exhibited significantly higher levels of cfDNA from mammary gland compared to the healthy donors ($p = 5.3 \times 10^{-12}$; AUC ROC = 91.25% (83.79–98.71%)); BC patients could be identified with 80% sensitivity while maintaining 97% specificity; presence of mammary gland specific cfDNA at the end of chemotherapy reflected the presence of residual disease	[107]
Hormone receptor (HR)-positive/ HER2-negative BC and TNBC	ctDNA-negative status at 3 weeks after treatment initiation was associated with achieving pCR in the TNBC patients; correlation between the ctDNA levels during NACT and pCR achievement was observed in TNBC, but not in the HR-positive BC; ctDNA levels were also linked to clinicopathological features of BC such as tumor size, lymph node metastasis, and histological grade; among the patients who did not achieve pCR, the ctDNA positivity after NACT was associated with higher risk of distant metastasis (HR = 5.89 for HR-positive BC and HR = 3.79 for TNBC), whereas the ctDNA negativity indicated a favorable prognosis even in the patients with residual disease; tumor mRNA analysis before treatment revealed correlation between the ctDNA appearance in circulation, cell cycle, and immune-related signaling pathways	[108]

Table 1 (*cont.*)

Patients in the study	Main results	Reference
Stage II and III BC	patients who achieved complete response exhibited a more pronounced decrease in the ctDNA levels during postoperative therapy; early changes in the ctDNA levels during treatment had significant prognostic value for the BC patients; correlation was observed between the early ctDNA decline and longer DFS compared with the patients showing increase in the ctDNA levels (HR = 12.54; 95% CI 2.084-75.42; p = 0.0063)	[116]
Hormone receptor-positive/ HER2-negative BC and TNBC	a positive correlation between the ctDNA levels and RCB was established; in TNBC, a moderate negative correlation between the cfDNA concentration and RCB was detected at 3 weeks after treatment initiation; at the same time, ctDNA concentration showed a significant positive correlation with RCB at all time points (correlation coefficient R > 0.3; p < 0.05); in the HR-positive/HER2-negative BC, the cfDNA concentration was not associated with the response to NACT; however, the patients with high baseline cfDNA levels had significantly shorter distant DFS compared to those with low cfDNA levels (HR = 2.12; p = 0.037); in TNBC, survival differences between the patients with high and low cfDNA levels at all time points were not statistically significant; meanwhile, the ctDNA levels at all time points showed significant correlation with the distant DFS in both subtypes	[109]
Early-stage BC	positive correlation between the ctDNA levels and RCB was identified detection of ctDNA at mid-therapy was significantly associated with the higher RCB (OR = 0.062; 95% CI 0.01-0.48; p = 0.0077), allowing identification of the patients who would not achieve pCR and would be classified as RCB II/III	[1]
Early-stage TNBC with residual disease after NACT	presence of ctDNA after completion of NACT and prior to surgery was associated with the shorter distant DFS (median distant DFS, 32.5 months vs. not reached; HR = 2.99; 95% CI 1.38–6.48; p = 0.006); detection of ctDNA was also associated with worse DFS (HR = 2.67; 95% CI 1.28–5.57; p = 0.009) and OS (HR = 4.16; 95% CI 1.66–10.42; p = 0.002)	[76]
TNBC and HER2-positive BC	in the BC patients carrying *PIK3CA* and/or *TP53* mutations in the tumor tissue, absence of these mutations in the ctDNA after NACT corresponded to achievement of pCR in 93.33% of cases; moreover, presence of mutations in the ctDNA allowed exclusion of several patients from the pCR group with an accuracy of 89.47%	[111]
HER2-positive BC	presence of mutations in the *TP53* gene in the ctDNA was evaluated as a potential marker of resistance to anti-HER2 monoclonal antibody therapy in the HER2-positive subtype of BC; the patients harboring mutations in the *TP53* gene who received anti-HER2 therapy had significantly shorter DFS (p = 0.004); in the patients with mutations in the *TP53* gene, a trend toward poorer prognosis under the anti-HER2 antibody treatment was observed compared to those with wild-type *TP53* in the phase II study (p = 0.15), and this trend was confirmed in the combined analysis of the MutHER and SUMMIT cohorts (p = 0.01)	[112]

Table 1 (*cont.*)

Patients in the study	Main results	Reference
HER2-positive BC	significance of the *PIK3CA* and/or *TP53* mutations detected in the ctDNA as predictors of poor response to anti-HER2 therapy was demonstrated; ctDNA positivity, defined by the presence of point mutations in *PIK3CA* and/or *TP53*, was associated with the lower rate of pCR, particularly at the stage prior to initiation of anti-HER2 therapy; detection of ctDNA before NACT was associated with the reduced likelihood of achieving pCR (HR = 0.15; 95% CI 0.034-0.7; p = 0.0089), but not with DFS; notably, the patients with HER2-positive tumors and absence of ctDNA prior to treatment exhibited the highest pCR rates; whereas those with detectable ctDNA both before treatment and at week 2 had the lowest pCR rates	[64]
High-risk BC	in the patients, who remained ctDNA-positive after NACT and surgery, earlier recurrence was reported (reflected by decreased DFS); the Invitae Personalized Cancer Monitoring™ (PCM) panel, used for monitoring before and during neoadjuvant therapy, after surgery, and throughout observation, demonstrated 100% specificity and prognostic value; all patients (10 of 61; 16%) with detectable ctDNA during the monitoring period subsequently developed recurrence; detection of ctDNA during monitoring was associated with the markedly increased risk of future recurrence (HR = 37.2; 95% CI 10.5-131.9; p < 0.0001), with a median lead time from ctDNA detection to clinical recurrence of 11.7 months	[113]
Early-stage BC	the patients who remained ctDNA-positive after NACT and surgery experienced earlier recurrence (reflected by the reduced DFS); correlation between the ctDNA detection and disease recurrence or progression was observed, including during post-NACT observation; preoperative ctDNA detection was significantly associated with the decreased DFS (adjusted HR = 3.09; 95% CI 2.65-80.0; p = 0.001); after a median observation period consisted of 26.6 months among 11 patients, recurrence occurred in 5 cases, all of whom had detectable ctDNA at the 2-4-week postoperative time point; ctDNA clearance at this time point was associated with significantly longer DFS (p = 0.0009), whereas persistent ctDNA positivity after adjuvant therapy was observed in 36.4% (4 of 11) of the patients with stage III disease; during monitoring, ctDNA detection demonstrated sensitivity of 90.9% and specificity of 98.8% for predicting recurrence, with the median lead time of 9.7 months; the patients with detectable ctDNA exhibited significantly shorter progression-free survival than those without detectable ctDNA (adjusted HR = 207.05; 95% CI 41.38-1000; p = 0.001); therefore, the ctDNA status both before and after surgery could aid in risk stratification for recurrence in breast cancer patients	[114]
Non-metastatic BC with residual disease	monitoring of ctDNA in the BC patients with residual disease after NACT demonstrated that persistent ctDNA positivity following NACT and surgery was associated with the decreased DFS; the 4-year DFS rate was 100% in the patients who were ctDNA-negative before treatment, compared with only 67% in the ctDNA-positive patients (p = 0.032)	[115]

Table 1 (*cont.*)

Patients in the study	Main results	Reference
Early-stage BC	association between ctDNA and disease recurrence or progression was also observed during the long-term monitoring after NACT; in this study, only plasma samples were analyzed, without prior identification of tumor-specific mutations from the biopsy samples; to detect ctDNA, a bioinformatic classifier was developed to identify tumor-associated somatic variants and methylation profiles for the specific cancer types using the 5-Mb NGS panels; ctDNA was detected during or before distant recurrence in 11 of 14 (79%) patients, with sensitivity of 85% (11 of 13) among the samples collected within 2 years prior to recurrence; lead time was estimated in 4 of 6 (67%) of the ctDNA-positive samples collected before distant recurrence and ranged from 3.4 to 18.5 months; no ctDNA was detected in the samples from the patients without recurrence (n = 13)	[72]
Primary BC	prediction of recurrence with the lead time of up to 38 months (median, 10.5 months; range, 0-38 months) was demonstrated, with ctDNA positivity being associated with shorter distant DFS (p < 0.0001) and OS (p < 0.0001) during the long-term monitoring over 12 years; the most significant results were observed in the patients with TNBC; among all patients with recurrent TNBC (n = 7 of 23), the ctDNA positivity was detected within a median lead time of 8 months (range, 0-19 months), whereas in 16 TNBC patients without recurrence, ctDNA remained undetectable throughout a median observation period of 58 months (range, 8-99 months)	[65]
Early-stage TNBC	according to the results of the meta-analysis including 1202 patients with TNBC, ctDNA positivity detected after NACT, either before or after surgery, was associated with the risk of recurrence and OS (HR = 3.26; 95% CI 1.88-5.63) in this BC subtype	[117]
Stage II-III early TNBC	in the patients with early-stage TNBC, prognostic significance of ctDNA was demonstrated, allowing identification of the subgroups with high treatment sensitivity and increased risk of disease recurrence; a threshold value of the maximum allele frequency of 1.1% at baseline stratifies patients according to recurrence risk, as confirmed by both internal and external quality controls; the systemic tumor burden model integrating baseline and postoperative ctDNA represents an independent prognostic model (p = 0.022); combination of the systemic tumor burden with pathological response allows identification of a subgroup of the patients with high likelihood of cure and a subgroup of the patients with high-risk early-stage TNBC; long-term ctDNA monitoring enables detection of the patients with high risk of recurrence	[118]

most reliable results were obtained for TNBC [65]. Magbanua et al. [108] reported that correlation between the ctDNA levels during NACT and achievement of pCR was observed in TNBC, but not in the hormone receptor-positive BC. Several studies have also shown that the ctDNA-negative status correlates with improved RFS and distant metastasis-free survival in the patients with TNBC [108, 116]. Meta-analysis including 1,202 TNBC patients further confirmed correlation between the ctDNA positivity and increased risk of recurrence and decreased OS in this BC subgroup [117]. In another recent study involving

patients with early-stage TNBC, ctDNA demonstrated prognostic relevance, enabling identification of the patients highly sensitive to therapy as well as those at high risk of disease recurrence [118].

The key findings from the studies evaluating ctDNA analysis for NACT efficacy and disease prognosis are summarized in Table 1. The accumulated body of evidence has formed the basis for the development of the commercial Signatera assay for MRD detection, based on ctDNA analysis in blood plasma (Natera) of the BC patients. This assay employs whole-exome – and potentially whole-genome – sequencing of tumor tissue, followed by ultra-deep sequencing of plasma ctDNA and bioinformatic analysis. The method is already being used in clinical trials for ctDNA analysis [65, 81].

CONCLUSION

Thus, persistence of ctDNA during neoadjuvant therapy and after primary treatment is associated with an increased risk of recurrence and an unfavorable prognosis. In recent years, post-neoadjuvant therapy approaches have been supplemented with several highly effective agents. In particular, the CDK4/6 inhibitors are used in the hormone receptor-positive HER2-negative BC, TD-M1 in the HER2-positive BC, capecitabine and immunotherapy in TNBC, while the PARP inhibitor olaparib has proven effective in the BRCA-associated BC. However, despite their high efficacy, these agents do not achieve complete cure in all patients and are associated with considerable toxicity. Quantitative and qualitative assessment of ctDNA is important not only from the therapeutic and prognostic perspective for optimizing treatment strategies, but also from an economic standpoint, since personalized therapy can markedly reduce treatment costs. Detection of cfDNA after neoadjuvant therapy and surgical treatment could help identify patient subgroups, based on the ctDNA biological characteristics, for whom appropriate subsequent systemic therapy is indicated.

The ctDNA analysis also enables regular molecular monitoring throughout treatment and subsequent observation. Integration of ctDNA analysis, as a form of liquid biopsy, into routine clinical practice will make it possible to track real-time tumor response, predict disease progression, and guide decisions regarding treatment duration. Compared with the conventional tissue biopsy, the ctDNA testing is suggested to be a more convenient and minimally invasive biomarker, and could become an additional tool for personalized therapy optimization.

At present, investigation of ctDNA as a marker of response to neoadjuvant therapy in the BC patients is limited only to clinical trials. Nevertheless, the success of ctDNA-based approaches in colorectal cancer – for which the biomarker has already been incorporated into the ASCO (American Society of Clinical Oncology) recommendations – and the growing body of evidence from the ctDNA studies in BC suggest that clinical implementation of ctDNA as a sensitive predictive and prognostic BC biomarker is likely in the near future.

Abbreviations

95% CI	95% confidence interval
BC	breast cancer
cfDNA	cell-free DNA
ctDNA	circulating tumor DNA
DFS	disease-free survival
dPCR	digital PCR
HR	hazard ratio, risk ratio
MAF	mutant allele fraction
MRD	minimal residual disease
NACT	neoadjuvant chemotherapy
NGS	next-generation sequencing
OS	overall survival
RCB	residual cancer burden
pCR	pathologic complete response
TNBC	triple-negative breast cancer

Contributions

G.T.S. supervised the work and performed review of the final manuscript with subsequent approval; T.M.Z. came up with the concept of the work, carried out the literature search, prepared the manuscript including structuration and editing; I.V.P. provided the section “Methods for detecting circulating tumor DNA” and edited the manuscript; P.S.M. provided the section “Methods for detecting circulating tumor DNA” as well as technical preparation of the manuscript; S.V.Kh. provided the section “Potential of ctDNA analysis for evaluating the efficacy of neoadjuvant drug therapy in breast cancer patients” and participated in manuscript editing.

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Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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