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The Value of Cell-free DNA for Molecular Pathology

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Conflict of interest statement

DWYT is a former consultant of Inivata Ltd. DWYT is a contributor to patent on cell-free DNA detection methodologies and may receive royalties related to the licenses of those patents to Inivata Ltd, the terms of these royalties are managed by Cancer Research Technology and Cambridge Enterprise. DWYT has received travel sponsorship and honorarium from AstraZeneca. MFB receives research support from Illumina and is a consultant for Sequenom. All other authors have no conflict of interest to declare.

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Abstract

Over the past decade, advances in molecular biology and genomics techniques have revolutionized the diagnosis and treatment of cancer. The technological advances in tissue profiling have also been applied to the study of cell-free nucleic acids, an area of increasing interest for molecular pathology. Cell-free nucleic acids are released from tumour cells into the surrounding body fluids and can be assayed non-invasively. The repertoire of genomic alterations in circulating tumour DNA (ctDNA) is reflective of primary tumours as well as distant metastatic sites and can be sampled multiple times, thereby overcoming the limitations of the analysis of single biopsies. Furthermore, ctDNA can be sampled regularly to monitor response to treatment, to define the evolution of the tumour genome, and to assess the acquisition of resistance and minimal residual disease. Recently, clinical ctDNA assays have been approved for guiding therapy, an exciting first step translating cell-free nucleic acid research tests into clinical use for oncology.

In this review, we discuss the advantages of cell-free nucleic acids as analytes in different body fluids, including blood plasma, urine, cerebrospinal fluid, and their clinical applications in solid tumours and haematological malignancies. We will also discuss practical considerations for clinical deployment, such as pre-analytical factors and regulatory requirements.

Keywords: Liquid Biopsy, Cell-free nucleic acids, Circulating tumor DNA, Genomics, PCR, DNA sequencing, Molecular pathology, Cancer

Introduction

Molecular pathology is critical for cancer management due to the expanding application of targeted treatments that are prescribed based on tumour specific mutations. Although many methods exist to examine these mutations, next-generation sequencing (NGS) of tumour samples has demonstrated tremendous utility in the clinic. Ranging from small [1] or large gene panels [2] to whole exome sequencing (WES) [3], NGS analysis leads to not only new insights over the genomic landscape of cancer [4-7], but also the identification of clinically actionable genetic alterations and biomarkers that predict treatment outcome to specific therapies; for example, the detection of microsatellite instability [8-10] or overall tumour mutation burden [11,12] that predict responses to immunotherapy, or *EGFR* mutations that confer response to EGFR inhibitors [13].

Despite their many successes, tissue-based assays have limitations in the clinical setting [14]. Clinical samples such as formalin-fixed paraffin-embedded (FFPE) tissue may harbour chemically induced artefacts that require filtering with proper post-sequencing quality control metrics [14,15]. Additionally, biopsies can miss important drivers due to tumour heterogeneity or distant metastatic lesions. Biopsies are sometimes not available due to inaccessibility or widespread metastasis. In this regard, cell-free DNA (cfDNA)

has created new possibilities for non-invasive diagnosis and therapy monitoring [16,17].

Advantages of cfDNA as an analyte for molecular testing

cfDNA refers to DNA fragments present outside of cells in body fluids such as plasma, urine, or cerebrospinal fluid (CSF). In plasma, the majority of cfDNA originates from leukocytes and only a small fraction is tumour derived, known as circulating tumour DNA (ctDNA) [18,19]. ctDNA can contain mutations missed in biopsy studies due to tumour heterogeneity or lesions in distant sites, and is generally found in larger quantities in the bloodstream than circulating tumour cells [20]. The concentration of ctDNA varies amongst patients and differs depending on the type, location, and stage of cancer, with some producing extremely low concentrations [21]. The half-life of ctDNA is still unclear, although foetal cfDNA studies have highlighted its short duration (16 min – 2 h) [22,23]. This instability, which might be an issue on the pre-analytical level, can be used to our advantage by providing a very dynamic tool for tracking treatment response within hours [22,24].

The relationship between tumour biology and ctDNA release into the circulation is still unclear. Experimental data has shown that cfDNA is highly fragmented [25-28], in a largely chromatosomal pattern – indicating potential

association with nucleosomes, as well as transcription factors [29,30]. ctDNA fragments are slightly shorter, with the majority under 167 bp [19,26,27,31].

Clinical applications of cell-free DNA

Extensive research studies and trials have demonstrated the clinical applications of cfDNA profiling at multiple stages of treatment: prognosis, molecular stratification at diagnosis, detecting resistance mechanisms at relapse, and detecting minimal residual disease. Several key applications are summarized below and in Figure 1.

Prognostic value

cfDNA has been shown to predict prognosis and treatment response. The proportion of ctDNA correlates with both stage [21] and size [19,32-34] of the tumour. A study across multiple cancer types found that advanced tumours give 10X – 100X the amount of ctDNA in plasma compared with early stages [21] and additional increases can be observed in patients with metastatic disease [35]. As expected with high tumour load, patients with high proportions of ctDNA have worse survival outcomes [36-41]. This correlation suggests that ctDNA levels can be a measure of disease burden and prognosis. For instance, in a post-treatment study of stage I – III colorectal cancer (CRC) patients, those with detectable ctDNA levels had a 48% 2-year recurrence free survival rate compared to those without, who had 100%

survival [36], and in a separate CRC study, ctDNA analysis had 100% sensitivity and specificity for detecting post-surgery relapse [42]. Pre-treatment cfDNA levels are also prognostic, as shown in another CRC study where patients with low ctDNA (<25% quartile) had a disease control rate of 42%, while patients with high ctDNA (>75% quartile) had a 0% rate after 9 weeks [43]. High pre-treatment ctDNA values have also been associated with resistance in numerous cancer types [20,21,35,44-51]. Conversely, low or absent ctDNA levels have been shown to correlate with treatment response [20,22,24,41,52,53], a change that can be observed earlier than other clinical detection methods [20,24,54,55]. ctDNA analysis has great potential prognostic value in cancer management.

Molecular stratification

Detection of specific mutations can be used to stratify patients and guide therapy, including adjuvant therapy [44,56], endocrine therapy [57], and targeted therapies. For example, ctDNA testing using PCR for *EGFR* mutations is now used clinically in non-small cell lung cancer (NSCLC) [58-61]. Obtaining biopsy samples in NSCLC is difficult [62], and the high correlation between tumour and plasma mutations has accelerated the clinical implementation of ctDNA testing in this cancer type to detect *EGFR* hotspot mutations that confer sensitivity to EGFR-inhibitors. [63,64]. Two forms of the test have been approved by the European Medicines Agency and the US

Food and Drug Administration (FDA): Therascreen EGFR [65] and Cobas EGFR [60]. PCR based assays are ideal for situations where mutations are known, either as they are commonly occurring or identified during tumour sequencing, and where positive mutation identification will lead to enrolment in therapy, but are unable to identify additional informative mutations outside the regions and/or mutations covered in the assay.

More comprehensive assays interrogating multiple genes have been developed using NGS to guide treatment [66-69] or enrolment in clinical trials [58,70-72]. The size of the panels varies from a small number of genes relevant for a specific cancer type, to large panels encompassing hundreds of genes [73], or WES [74]. Smaller panels can be sequenced deeply to detect mutations with ~1% mutant allele fraction (MAF) [73,75,76], while larger panels are generally sequenced to a lower depth and therefore require >5% MAF for *de novo* mutation detection [77]. WES offers hypothesis-free comprehensive screening, but requires MAF in the ~10% range [74], which is rarely seen outside progression or metastatic settings. Confidently calling mutations that can be present at <1% of reads, near the level of sequencing noise, remains difficult and a limitation for implementing cfDNA NGS assays clinically. Methodological advances have led to improved sensitivity, for instance the incorporation of molecular barcodes, also known as unique molecular indexes (UMIs) or unique identifiers (UID), into sequencing libraries

allows *de novo* mutation detection down to 0.1% MAF [78-81]. Additional improvements in detection rates have been shown by applying size selection to enrich for ctDNA [27,82]. Further developments in technology, for instance though the use of long-read sequencers [83,84] or via CRISPR-based diagnostics [85] may continue to improve detection. Together, these advances expand the applicability of cfDNA testing in a wider patient population across diseases status.

Detecting resistance mechanisms

cfDNA can be used to monitor acquisition of resistance, through screening for known resistance mutations [86-94], or searching for novel mechanisms of resistance [95-97]. Serial sampling can be done to identify resistant clones prior to the onset of clinical progression [88,89]. For example, in a recent study of CRC patients receiving anti-EGFR therapy, *KRAS* resistant mutations emerged 5-6 months before radiographic disease became evident [90]. Timely identification of resistance mechanisms allows earlier clinical intervention.

Detecting minimal residual disease

The highly sensitive methods described above also have promise in detection of minimal residual disease (MRD). In a recent study of localized breast cancer patients, post-surgery ctDNA levels after apparently curative treatment

was predictive of metastatic relapse [98]. A similar study, done on early-stage CRC, found that the absence of ctDNA after resection was predictive of recurrence-free survival at 3 years, while those with ctDNA detection after serial sampling had 100% recurrence [99]. Highly sensitive approaches are required for MRD monitoring and typically these assays are patient and mutation-specific. This has been a challenge to implement clinically, however with increasing sensitivity broader panels could be used in the future.

Cell-free DNA profiling in haematological malignancies

The emerging clinical benefits of ctDNA analysis in solid tumours have led to an expansion of groups using ctDNA in haematological malignancies. ctDNA analysis was first demonstrated in haematological cancers in 1994 when tumour specific *NRAS* mutations were identified in the plasma of patients with myelodysplasia and acute myeloid leukaemia [100]. Over the last few years, research in this area has rapidly expanded with many studies now exploring the role of ctDNA for risk stratification, monitoring tumour burden, and response to treatment in both lymphoid and myeloid malignancies.

In the management of lymphoma, patient specific ctDNA analysis could be used to help monitor MRD and guide clinical decisions. In non-Hodgkin's lymphoma, patient specific IgH rearrangements are readily detectable through cfDNA analysis [101-104]. Tumour specific V(D)J recombination of the

immunoglobulin receptor genes can be used as a MRD monitoring strategy to identify individuals at increased risk of relapse, and detect relapse before clinical evidence of disease [105,106]. In addition, comprehensive analysis of the ctDNA of lymphoma patients has emerged as an important clinical tool to identify distinct biological subtypes and provide insights into the patterns of genomic evolution throughout treatment [107-110]. In parallel, several studies have shown ctDNA analysis to allow more comprehensive genomic characterisation of classical Hodgkin's lymphoma, a disease that has traditionally been difficult to study due to the rarity of Hodgkin and Reed-Sternberg cells in tumour biopsies [111,112].

Using ctDNA for disease monitoring has also recently been demonstrated in chronic lymphocytic leukaemia (CLL) [113]. In CLL, ctDNA analysis can reveal changes in the disease across tissue compartments, including the bone marrow and lymph nodes, providing additional information not assessed by monitoring the circulating disease alone [113]. Moreover, serial ctDNA analysis in CLL can allow monitoring of clonal dynamics and identify genomic changes associated with Richter’s syndrome [113]. ctDNA analysis is also a powerful tool in patients with myeloid malignancies, particularly myelodysplastic syndromes (MDS) [114-116], as well as plasma cell disorders such as multiple myeloma [117-120], as ctDNA can detect underlying genomic changes in the bone marrow and be used as a monitoring strategy to

limit invasive bone marrow biopsies [114,117]. The potential of these approaches is now coming to the forefront of research, with clinical applications likely to expand across the breadth of haematological malignancies.

Cell-free DNA profiling in other body fluids

Urine

Research findings suggest that cfDNA enters urine from blood after passing through the renal filtration system [31,121,122]. Additionally, for patients with disease affecting the renal or genitourinary tract, urine may serve as a more concentrated sample source for disease related cfDNA. The utility of urinary DNA analysis was initially evaluated through studies of urinary cells. In an early study, Sidransky *et. al.* showed that p53 mutations could be identified within the urine sediment of three patients with invasive bladder cancers [123]. Later studies in prenatal medicine have shown Y chromosome DNA can be detected in the supernatant of urine samples from pregnant women carrying male foetuses [122]. Studies have shown that urinary DNA has two size profiles <100 bp and 150-250kb, which is hypothesized to represent the cfDNA and genomic DNA arising from urinary tract cells, respectively [124,125].

In oncology, urine ctDNA has been studied in multiple cancer types, particularly cancers of the genitourinary tract. For example, urine ctDNA showed 88% concordance with primary tumour tissue for *EGFR* mutations in NSCLC [126], and in hepatocellular carcinoma (HCC), Hann *et. al.* showed that urinary biomarkers could be a promising tool to monitor for relapse [127]. More recently, longitudinal sampling of urine ctDNA in patients with muscle invasive bladder cancer before and after neoadjuvant chemotherapy was shown to help predict outcome [128]. Urine collection offers the advantages of being truly non-invasive and allows large sample volumes. It is particularly beneficial in the field of paediatric oncology, where obtaining tumour samples or large volumes of plasma is more challenging.

As noted above, a portion of the ctDNA found in urine originates from the circulatory system and is filtered through the kidneys [31,122,129]. Biological factors such as hydration status, kidney function, and primary tumour site may all contribute to the amount of ctDNA that can be detected in urine. The effects of these parameters on the performance of urine-based cfDNA tests would require further evaluation before clinical implementation.

Cerebrospinal fluid

Cerebrospinal fluid (CSF) circulates throughout the central nervous system (CNS) and is protected from the systemic circulation by the blood brain barrier

[130], as a consequence it possesses a low background level of normal cfDNA. Additionally, in cancers involving the CNS the concentration of plasma ctDNA is much lower than other solid tumours [21], thus CSF analysis has been proposed as an alternate potential reservoir for ctDNA.

Initial investigations identified mutations within tumours prior to evaluating the CSF for mutations [131-134]. Subsequent studies have adopted a more clinically feasible paradigm whereby mutations are *de novo* called through targeted mutation panels or WES [135,136]. The improvement in sensitivity associated with these techniques has enabled CNS tumour mutations to be identified in the majority of samples from patients with CNS cancers and at a greater concentration compared to plasma samples [133].

Many of these studies have relied upon mixed populations of primary brain tumour patients and those with metastatic disease within the CNS. The detection rate and the concentration of cfDNA vary between these pathological entities with primary brain tumours possessing lower levels than secondary metastatic lesions [135,136]. The largest study on primary brain tumours demonstrated a higher detection rate and an increase in MAF in high-grade tumours when compared with low-grade tumours [134]. Interestingly, in the same analysis tumour size was not a significant factor in ctDNA detectability, although positioning of the tumour influenced mutation

detectability: those entirely encapsulated within the CNS parenchyma had no detectable ctDNA in the CSF [134].

Whilst obtaining CSF is less invasive than a surgical tumour biopsy, it remains a procedure that possesses clinical risk and the potential for significant patient discomfort. The clinical utility of CSF analysis will ultimately be reliant upon the balance between the efficacy of the test and the importance of the clinical question. In this context, the molecular diagnosis of inoperable lesions or those with a non-surgical management paradigm may provide appropriate indications.

Other cell-free nucleic acids and strategies

cfDNA methylation studies

Analysis of DNA methylation can be applied to cfDNA to uncover methylation changes known to be important in cancer using methylation-specific PCR [137], microarray [138] or sequencing [139-143]. For instance, uncovering gene methylation status, such *BRCA1* in breast cancer [144]. Additionally, methylation deconvolution can be used to determine the originating tissues of cfDNA fragments [145-147], providing information about tissue dynamics in various disease states [146]. This technique has great potential for oncology in determining the tissue of origin in cancer of unknown primary [148] or identifying tumour subtypes. However, these techniques require a high tumour

fraction and a large proportion of the already limited starting material can be lost during bisulphite conversion.

Viral and bacterial cfDNA

Persistent infection with viruses such as HPV and Epstein-Barr virus (EBV) is associated with certain cancers, such as cervical cancer and nasopharyngeal carcinoma (NPC), respectively. Viral DNA is often profiled from tumour samples or used in screening for pre-cancerous states, but it can also be found within cfDNA samples, such as plasma [149], urine [150], and saliva [151]. For instance, HPV DNA has been detected in the plasma of patients with cervical cancer [152] and head and neck squamous cell carcinoma [151], and could potentially be used as a marker of the disease.

Similarly, short fragments of bacterial DNA have also been identified within plasma originating from commensal or infectious bacteria from throughout the body. Studies of bacterial cfDNA has led to the identification of hundreds of new species [153] and is of particular interest for identifying and monitoring infection post-transplantation or after therapy related immunosuppression [154]. It may also offer a tool to monitor the change in microbiome during cancer therapy.

Cell-free RNA

Previous work has shown that cell-free RNA (cfRNA) can be isolated from maternal plasma to non-invasively screen for foetal-specific transcripts [155]. In the oncology setting, several reports have demonstrated the isolation of cell-free miRNA from CSF of cancer patients [156-159] and cell-free mRNA from plasma [160,161]. One area of great potential for cfRNA is fusion gene detection. Genomic and transcriptomic sequencing has led to the identification of many gene fusions in solid and haematological cancers [162,163]. Gene fusions can have diagnostic and prognostic, but also therapeutic implications when involving targetable genes [164]. Unfortunately, DNA sequencing has limited ability to detect gene fusions or identify fusion partners due to potentially large intronic sequences. For instance, in a study of advanced stage NSCLC patients with ALK fusions, sensitivity for patients with localized disease was only 28.6% [165]. Additionally, expressed driver events may not be easily distinguished from passenger events [166].

RNA-based detection methods provide a direct readout of expressed gene fusions in tumour cells [167]. Furthermore, gene fusions in low purity tumours are more likely to be detected by RNA sequencing due to the higher abundance of fusion-derived RNA transcripts [168]. Therefore, RNA sequencing has been extensively used for the detection of gene fusions in tumour tissue [166,167]. The same strategy can be applied to cfRNA [169],

however as RNA is more unstable, systematic optimization of pre-analytical processing is required to ensure reproducibility and stability.

Early screening for cancer

Early screening of cancer has become increasingly effective though collecting information from multiple different biospecimens. A recent study, combining protein and cfDNA detection across 8 cancer types, achieved 73% sensitivity for stage II and 49% for stage I cancers with >99% specificity, although the study involved patients already diagnosed, it demonstrates the potential benefits of combining cfDNA with other markers for cancer screening [170].

Cell-based methods, such as the Papanicolaou test (Pap smear), have been tested for the diagnosis and screening of endometrial cancers [171,172], but have proved challenging. A recent study identified tumour-derived mutations in DNA extracted from Pap smears in 100% of endometrial cancer patients and 41% of ovarian cancer patients [173], offering potential for early screening. In ovarian cancer, where the sensitivity of Pap smears is low, perhaps combined Pap smear and mutation analyses may provide better performance for screening to compensate for the limitations of either approach alone [92,174,175].

Another strategy to improve the screening performance of cfDNA-based assays is to target biomarkers that are highly specific to the tumour and nearly absent in background cfDNA. For example, targeting EBV in NPC. EBV DNA was first identified in plasma of NPC patients in 1999 [149] and is cleared from plasma after treatment [150,176], making it an ideal biomarker [177]. In a longitudinal study, >20,000 asymptomatic participants were screened for EBV DNA in their plasma and 34 with NPC were identified [178]. These patients were diagnosed earlier and had improved 3-year progression free survival than historical cohorts, and only one participant with a negative EBV result developed NPC a year after testing [178]. As EBV DNA is highly specific for NPC, this test offers a sensitivity of 97.1% and specificity of 98.6%. This example demonstrates the potential of targeting highly specific tumour-derived information in cfDNA for early screening of cancer.

Practical considerations for clinical implementation

To date, the majority of clinical applications of cfDNA analysis have been demonstrated in prenatal testing and cancer diagnostics, but it has potential in many other physiological conditions such as trauma, stroke, sepsis, epilepsy, autoimmune diseases and post-transplantation monitoring, among others [45-49,179]. In prenatal testing, multiple molecular tests have been developed for foetal aneuploidy [180-183] or single-gene disorders [184-186], with some clinically implemented worldwide [187-190]. In oncology, there is tremendous

clinical potential for ctDNA to be incorporated in molecular diagnostics. However, before broad clinical implementation, practical considerations such as pre-analytical factors and regulatory requirements must be addressed in order to achieve consistent and reproducible results.

Pre-analytical considerations

Pre-analytic factors have different effects on cfDNA yield, quality, and downstream molecular applications. One factor is the storage time between blood draw and processing to isolate plasma. Delayed processing results in blood clotting and lysis, which releases large amounts of background genomic cfDNA, introducing additional challenges for identifying low-level somatic mutations [191]. Preservation tubes, such as EDTA tubes, that prevent blood clotting and minimize the release of genomic DNA from blood cells can address this issue [191], however they need to be processed within 2 – 24 hours [192-194], which is challenging to implement in a clinical setting. One potential solution is to use specialist blood collection tubes that can extend storage for multiple days before processing [195-197]. Other pre-analytical factors such as centrifugation protocols, number of freeze/thaw cycles, and extraction methods are also important [192,198]. Guidelines pertaining to pre-analytic factors must be put in place to ensure accurate and efficient genomic profiling.

Considerations for clinical test development

The two most important upfront decisions are the choice of platform and the scale of the analysis required. The choice of clinical sequencing platform and method depends on the sensitivity, type and complexity of the test, expected volume of testing, turnaround time, costs, laboratory infrastructure, and computational and human resources, to validate and perform the clinical testing.

The specific requirements for validation vary depending on intended use: single locus, low multiplex panel testing, targeted NGS, WES or genome wide analysis. Single locus or low multiplex assays, such as digital PCR, allow rapid detection and quantification of recurrent hotspot mutations and monitoring of well-established resistance mutations, with a rapid turnaround time for a relatively low cost. These assays are highly sensitive, although restricted in the number of loci assessed. Clinical validation encompasses the establishment of accuracy compared to a gold standard, inter- and intra-assay reproducibility, and establishment of sensitivity levels for each patient population.

For more comprehensive assays such as targeted NGS approaches, platform selection is pivotal to all further decisions of testing, validation, and capabilities for future expansion. Several important considerations include the

size of the panel, selection of genes, depth of sequencing, coverage, sensitivity of the assay, and complexity of analytical and clinical interpretation. All of these will factor into the cost of running the assay, which for large panels or WES, can be prohibitively expensive to implement clinically. Based on current guidelines for NGS testing, the lab should determine gene content based on available scientific evidence and clinical validity of the variants and utility of the NGS assay [199], documenting the scientific evidence in the validation protocol.

Considerations for test validation and clinical implementation

While specific guidelines for cfDNA testing are not yet established, regulatory requirements under Clinical Laboratory Improvement Amendments (CLIA) call for all non-FDA-approved tests to address accuracy, precision, reportable and reference ranges, analytical sensitivity, analytical specificity, and any other parameters that may be relevant to the assay performance. All clinical validations should be included in the outlined validation protocol, including the entire range of clinical samples expected for the patient population, and if appropriate, validation of the bioinformatics pipelines. Given the wide range of clinical platforms used for cfDNA testing, a comprehensive evaluation that would cover all is outside the scope of this review.

The wide variability of cfDNA content among samples introduces a high degree of complexity and more potential sources of error. Assessing all potential sources of error at every level of assay design, method validation, and quality control is critical to avoid potential harm to the patient by both false positive or false negative results. Special attention must be given to the qualification and quantitation of input cfDNA. Dedicated standard operating procedures that outline the pre-analytical steps for optimal collection, handling, extraction, isolation and storage of cfDNA samples must be validated with the same rigorous nature as the analytical platform.

Conclusions

Cell-free nucleic acids have tremendous potential for molecular pathology in cancer. Advances in the sensitivity and specificity of cfDNA methodologies open up possibilities for early detection of recurrence and cancer screening at asymptomatic stages. In the molecular diagnostic setting, we envision that tumour analysis will continue to play a critical role in diagnosis by revealing histological and genomic profiles. cfDNA will provide additional genomic information that a single biopsy may miss or when tumour is not available, as well as longitudinal monitoring of the evolving genome for timely treatment intervention. Further work needs will further define the specifications for broad clinical implementation, but undoubtedly, cell-free nucleic acid profiling is

creating a new paradigm of molecular pathology to improve cancer care through precision oncology.

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CMS and DWYT wrote the initial draft, with contributions from PK, FM, RM, SS, RB, AZ, BW, SJD, MEA and MFB. All authors approved the final version.

Figure Legend

Figure 1 Clinical applications of cell-free DNA during treatment journey

Serial monitoring of circulating tumour DNA in plasma allows non-invasive identification of clinically relevant genomic alterations at different stages: molecular stratifications at diagnosis, tracking tumour responses during treatment and identifying genetic mechanisms of resistance at progression. Colours represent tumour subclones with differing mutations, which release ctDNA at different frequencies as depicted in the graph below.

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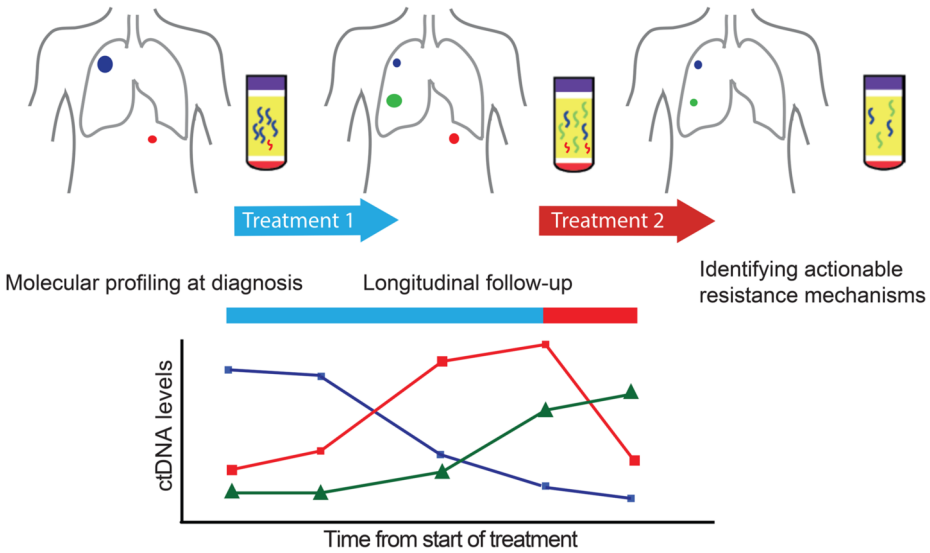

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