Liquid biopsies come of age: towards implementation of circulating tumour DNA

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Key points

- cfDNA is released predominantly by cell death into the bloodstream, though active secretion may play a role. Since the discovery of fetal cfDNA in the maternal circulation, cfDNA analysis has been rapidly implemented in clinical practice for non-invasive prenatal testing.
- Mutations were first detected in cfDNA over two decades ago, and interest in ctDNA as a non-invasive cancer diagnostic has increased dramatically with the development of molecular methods that permit the sensitive detection and monitoring of multiple classes of mutation.
- ctDNA may have utility at almost every stage of cancer patient management, including: diagnosis, minimally invasive molecular profiling, treatment monitoring, detection of residual disease, and identification of resistance mutations. ctDNA analysis may be broadly considered as a tool for both quantitative analysis of disease burden and for genomic analysis.
- The identification of ctDNA in individuals prior to a cancer diagnosis, and in pre-symptomatic individuals, suggests the possibility of ctDNA analysis as a

tool for earlier diagnosis or screening. Non-invasive cancer classification or sub-typing has also emerged as a possibility, though for early detection, both technical and biological factors introduce challenges to the detection of mutant DNA in plasma and its interpretation.

- Monitoring multiple mutations in parallel can enhance sensitivity for ctDNA detection, can be used to assess clonal evolution of patients' disease, and may identify resistance mutations before clinical progression is observed.
- ctDNA analysis is beginning to transition from the research setting into the clinic. The US Food and Drug Administration and the European Medicines Agency have approved ctDNA tests for specific indications in the absence of evaluable tumour tissue. Analysis of gene panels in plasma has now become available as a potential clinical tool. Larger studies are underway to establish the overall performance and clinical utility of such assays when a tumour biopsy is not available for analysis.
- Potential applications of ctDNA have been demonstrated by a number of proof-of-principle studies. Prospective clinical trials are beginning to assess the clinical utility of ctDNA analysis for molecular profiling and disease monitoring. Increasing acceptance of ctDNA is enabling the field to move from exploratory ctDNA studies towards clinical trials where ctDNA is guiding decision making.
- In order to fully exploit the potential utility of liquid biopsies, it is essential that the biology of cfDNA and ctDNA is explored further. Mechanisms of release and degradation, and the factors that affect the representation of ctDNA in plasma, are poorly understood. The nature of ctDNA will be clarified through both large, well-annotated clinical studies, and through *in vivo* studies, where variables may be controlled.

Preface

Improvements in genomic and molecular methods are expanding the range of potential applications for circulating tumour DNA (ctDNA), both in a research setting and as a 'liquid biopsy' for cancer management. Proof-of-principle studies have demonstrated the translational potential of ctDNA for prognostication, molecular profiling, and monitoring. The field is now at an exciting transitional period where ctDNA analysis is beginning to be applied clinically, although there is still much to learn about the biology of cell-free DNA. This is an opportune time to appraise potential approaches for ctDNA analysis, and to consider their applications in personalised oncology and in cancer research.

The presence of fragments of cell-free nucleic acids in human blood was first described in 1948 by Mandel and Métais[1]. The origins and characteristics of cell-free DNA (cfDNA) were studied intermittently in subsequent decades[2]. In healthy individuals, cfDNA concentration tends to range between 1-10ng/millilitre (ml) in plasma[3,4]. Raised cfDNA levels were first reported in the serum of cancer patients in 1977[5]; cfDNA concentration can also be raised by other physiological conditions or clinical scenarios, such as acute trauma[6], cerebral infarction[7], exercise[8], transplantation[9], and infection[10]. Furthermore, the identification of fetal DNA sequences in maternal plasma by Dennis Lo and colleagues in 1997[11] has led to multiple applications of cfDNA in prenatal medicine including sex determination[12], identification of monogenic disorders[13], and non-invasive prenatal testing (NIPT) for aneuploidies such as Down's Syndrome (trisomy 21). NIPT was first demonstrated in 2007 by Lo *et al*.[14] and has moved rapidly into widespread clinical use[15,16].

In 1989, Stroun, Anker *et al*. identified that at least some cfDNA in the plasma of cancer patients originates from cancer cells[2,17]. In 1991, Vogelstein, Sidransky and colleagues showed that DNA from urinary sediments (cell pellets) from patients with invasive bladder cancer carried mutations in *TP53*, setting the stage for the use of genomic analysis methods in liquid biopsy applications[18]. *KRAS* mutations were soon found in stool or sputum that matched mutations from colorectal[19], pancreatic[20] or lung[21,22] cancers. In 1994, mutated *KRAS* sequences were first reported to be

detected in plasma cfDNA of patients with pancreatic cancer using polymerase chain reaction (PCR) with allele-specific primers[23]. For each patient, the *KRAS* mutation found in the plasma was identical to that found in the patient's tumour, thereby confirming that the mutant DNA fragments in plasma were of tumour origin. Mutations in cfDNA are highly specific markers for cancer, which gave rise to the term circulating tumour DNA (ctDNA).

In the following decades, ctDNA was explored as a prognostic or predictive marker[24,25] and for cancer detection[26]. Such studies confirmed the potential of ctDNA, though the levels of ctDNA in different clinical contexts were not yet accurately defined. These studies nonetheless could demonstrate potential clinical applications, for example detection of KRAS mutations in plasma as a potential prognostic factor in colorectal cancer[27]. The introduction of a digital PCR (dPCR) method in 1999 by Vogelstein and Kinzler enabled the accurate identification and absolute quantification of rare mutant fragments[28]. A modification of this technique using beads in emulsions[29] and flow cytometry allowed the quantification of the mutant allele fraction of cancer mutations in the plasma of patients with different stages of colorectal cancer[30]. Diehl, Diaz et al. then showed in 2008 that ctDNA is a highly specific marker of tumour dynamics, and may be able to indicate residual disease[31]. In parallel, allele-specific PCR and other methods were devised and tested for their ability to identify epidermal growth factor receptor (*EGFR*) mutations in serum or plasma of lung cancer patients[32], following the elucidation of the role of such mutations in predicting response to treatment with molecularly targeted inhibitors[25,33,34].

The development of next generation sequencing-based technologies has facilitated the interrogation of the genome at a broader scale. In 2012, deep sequencing of multiple genes in cfDNA was demonstrated using panels of tagged amplicons, which allowed the identification of mutations directly in the plasma of cancer patients, and monitoring of multiple tumour-specific mutations in a single assay[35]. This method was subsequently applied to monitor ctDNA in a cohort of patients with metastatic breast cancer[36]. Shortly thereafter, whole-genome sequencing (WGS) of plasma cfDNA was

first shown to identify tumour-derived chromosomal aberrations[37], focal amplifications[38] and gene rearrangements[39], and hybrid-capture sequencing was introduced as a non-invasive method to analyse the evolving genomic profile of mutations in cancer across the entire exome[40].

There is a clear clinical need for novel diagnostic and molecular tools in oncology (Box 1). Conventional sampling methods such as needle biopsies are subject to procedural complications in up to one in six biopsies[41], difficulty in obtaining sufficient material of adequate quality for genomic profiling (reported failure rates range from <10% to >30% of cases)[42,43], and sampling biases arising from genetic heterogeneity[44–48]. Detection and monitoring of disease often relies on body fluid-based markers that often lack specificity[49], and imaging which exposes patients to ionising radiation[50] and has limited resolution (in both time and space). Recent advances in ctDNA research highlight the potential applications of liquid biopsies at each stage of patient management (Fig. 1a). These potential applications primarily arise from two types of information obtainable through ctDNA analysis: quantification of disease burden, and genomic analysis of cancer (Fig. 1b). These may be combined and/or leveraged through serial sampling in order to monitor disease burden and clonal evolution.

The increasing availability and reliability of techniques for PCR and high-throughput sequencing are facilitating novel high-sensitivity applications, the generation of large clinical datasets, and a better understanding of the origin of both cfDNA and ctDNA. This Review will highlight and explore recent advances in the field and the implications for oncology.

cfDNA and ctDNA biology

Characteristics of cfDNA and ctDNA

cfDNA is thought to be released from cells mostly through apoptosis and necrosis, and possibly also active secretion[2,51–54]. Outside of the blood circulation, cfDNA has been detected a variety of body fluids including urine[55–58,59], cerebrospinal fluid (CSF)[60–63], pleural fluid[64] and saliva[65]. Genetic and epigenetic modifications of cfDNA

molecules reflect the genome or epigenome of the cell of origin[66–68] (Fig. 2). Methylation analysis has revealed that the majority of cfDNA in plasma is released from haematopoietic cells in healthy individuals[67–69]. These have been suggested to be the source of cfDNA release following intense exercise[70]. Observational studies have determined the half-life of cfDNA in the circulation as between 16 minutes and 2.5 hours[31,71–73], which allows ctDNA analysis to be considered as a 'real-time' snapshot of disease burden. Other observational studies indicate that cfDNA is cleared from the circulation via nuclease action[72,74] and renal excretion into the urine[55,58,75]. cfDNA uptake in the liver and spleen, followed by degradation by macrophages, may also contribute[30,76]. The stability of individual fragments in the circulation may be increased through association with cell membranes, extracellular vesicles or proteins[2].

Nearly two decades ago, the modal size of cfDNA was determined using gel electrophoresis as ~180 base pairs (bp), indicating that cfDNA was likely to be nucleosome-associated[77]. Sequencing-based approaches have since refined this measurement, by identifying a prominent peak at 166bp[78,79], corresponding to the length of DNA wrapped around a nucleosome (~147bp), plus linker DNA associated with histone H1. Fragment size traces of cfDNA show a 10bp ladder pattern[54,75], ostensibly caused by nucleases cleaving the DNA strand at periodically exposed sites with each turn of the DNA double-helix. The fragmentation patterns of cfDNA differ between plasma and urine[75], potentially contributed to by a higher nuclease activity in urine[80].

ctDNA molecules are shorter than non-mutant cfDNA in plasma, demonstrated by PCR[4,81] and sequencing[68,82]. Animal xenograft experiments[79,81–83] provide an elegant means to interrogate ctDNA, since any human DNA sequences must have originated from the tumour xenograft. The modal length of ctDNA fragments has been measured in a rat xenograft model as between 134-144bp[82], though the cause of this shortening is not clear. Shortening of fragments is also observed in fetal cfDNA relative to maternal cfDNA[78], and between non-haematopoetically-derived vs. haematopoietically-derived cfDNA fragments in transplant patients[84,85]. Differences in

nucleosome wrapping or nuclease action between haematopoietic cells, which contribute most to the cfDNA pool, and other tissues may play a role. Long cfDNA fragments (>1000bp) have been observed in healthy individuals using long-read sequencing techniques[86], and may be released into the circulation in association with exosomes[51,52], or by tumour cells via necrosis[53]. Current extraction methods often poorly recover these long fragments[87,88] (Box 2). Commonly used library preparation methods introduce further biases: single-stranded DNA (ssDNA) library preparation[89] can recover DNA fragments with damaged ends, and when applied to cfDNA[66,90] uncovered a large proportion of fragments shorter than 100 bases. Diverse extraction and sequencing methods may therefore yield complementary data. Combining those with histological analysis of corresponding tissue samples could provide new insights into the biological determinants of cfDNA fragmentation, and the biological origins of cfDNA.

Physiological and pathological considerations

cfDNA has been proposed as a ligand for Toll-like receptor 9 (TLR9)[91,92], a sensor of exogenous DNA fragments, found primarily in tissues rich in immune cells. In mice, obesity-related adipocyte degeneration was shown to release cfDNA, which contributed to macrophage accumulation via TLR9 activation, leading to adipose tissue inflammation and insulin resistance[92]. Another study has suggested that cfDNA may inhibit pro-apoptotic caspases via TLR9-depedent signalling[91], which could imply a potential immunomodulatory role for cfDNA.

In vitro experiments suggest that cfDNA may be internalised by cells[2,93,94], raising the possibility that cfDNA molecules could mediate the horizontal transfer of genes or DNA. One report showed *in vitro* transformation of NIH-3T3 mouse cells that were in contact with samples of plasma from patients with *KRAS*-mutant colorectal cancers, despite being separated to avoid tumour cell contamination[95]. Another study demonstrated integration of ctDNA into the nuclear DNA of recipient cells, and suggested that this may occur through non-homologous end-joining[93]. A similar phenomenon has been observed with mitochondrial DNA[96]. Overall, it is clear that

there is a lot to learn about the biology of cfDNA and ctDNA, which could have an important impact on their potential applications in oncology.

Approaches for ctDNA analysis

Analysis of ctDNA ranges in scale from single mutations to whole-genome analyses (Fig. 3; See *Table 1* for a comparison of selected techniques). Appropriately designed assays for individual mutations can achieve high sensitivity using a simple workflow. Allele-specific PCR methods[97,98] have been applied since the mid-2000s for detection of hot-spot mutations in serum and plasma[25,33,34,99], and some assays are available as kits that are approved for clinical use[158,160], but have limited analytical sensitivity. dPCR assays on microfluidic platforms are quantitative and highly sensitive, and are used extensively for quantifying ctDNA levels[29,30,32,57,100–102]. Improved detection at selected loci has been demonstrated by methods such as single-base extension[103] or enrichment for mutant alleles by electrophoretic methods[131,132], nuclease activity[104] or modified PCR[105–107]. The multiplexing capacity of such assays, that rely on differential binding affinities of mutant and wild type alleles, and for the most part require primers or probes that are specific to a defined mutation or targeted locus, is limited. These are, therefore, generally suited to investigating a small number of mutations, and are often applied to analysis of cancer hot-spot mutations. If samples need to be split into multiple reactions, this increases sampling error, and may impair the overall performance of an assay for very low copy numbers of mutant DNA.

In order to interrogate a larger number of loci, targeted sequencing using PCR amplicons or hybrid-capture have been employed[35,40,108,109]. Regions for sequencing may range from individual exons of interest (kilobases), to the entire exome (~50 megabases). Current off-the-shelf panels for gene sequencing can detect mutations with an allele fraction greater than 1%[46,110]. By reducing background error rates of sequencing, for example by molecular barcoding (Fig. 3) or multiple replicates (Fig. 4), ctDNA can be detected at allele fractions below 0.1%[111–114] (*Table 1*). Amplicon-based assays that have been optimised for the purpose of ctDNA analysis can target

dozens to hundreds of amplicons across multiple kilobases[35,112,114]. Hybrid-capture-based approaches can increase the genomic region studied to dozens or hundreds of kilobases[108,109,111,115]. The sensitivity for ctDNA detection can be further enhanced, even with limited amounts of input material, by using multiplexed patient-specific panels in combination with targeted sequencing methods[35,111] (Fig. 4).

Amplifications and deletions may be identified through low-depth (~0.1x coverage) sequencing of the whole genome, with comparison of the relative number of sequencing reads between equally sized genomic regions across a sample or between samples and controls[39]. Such shallow WGS (sWGS) has been employed to detect fetal aneuploidies[38], and it can also be used to detect cancer-specific copy number alterations[37,39,116]. sWGS has a limit of detection of between 5%-10% mutant allele fraction (Table 1), and so has limited sensitivity for profiling earlier stage disease. If molecular profiling of a small number of recurrent copy number alterations is desired, higher sensitivity may be achieved through targeted sequencing of single nucleotide polymorphisms, which may detect copy number alterations as low as 0.5%[117].

The limit of detection for assays will vary based on whether the individual's disease status, and tumour mutations, are already characterised. Tumour burden in plasma has often been assessed by quantifying mutations (or other alterations) that were previously identified in the patient's tumour sample[30,31]. For mutation calling across a panel of genes or hotspots, the risk of false positives increases with the size of the panel due to multiple hypothesis testing, and filters need to be applied to increase specificity, which erodes sensitivity for rare variants. Prior knowledge of the mutation profile (e.g. from tumour sequencing data) enables the detection of known patient-specific mutations above the background error rate, as opposed to calling mutations *de novo*[35]. Thus, sequencing-based assays can be used as sensitive and quantitative tools for ctDNA measurement and monitoring, in addition to their use for mutation profiling[35,36,108] (Fig. 1b).

ctDNA can be quantified using different metrics, such as mutant allele concentration (i.e. copies per ml) or mutant allele fraction[118]. Each of these metrics would be affected in a different way by analytical, pre-analytical, and physiological characteristics. For example, metabolic changes to the rate of cfDNA turnover would affect the concentration of mutant alleles more than the mutant allele fraction, whereas pre-analytical factors affecting release of germline DNA from blood cells would reduce the mutant allele fraction to a greater extent. Analysis of ctDNA (both fraction and concentration)[118], as well as total cfDNA and cfDNA fragmentation[3,119], could therefore provide complementary information, and may have advantages in different applications or in combination.

ctDNA detection across cancer stages

ctDNA relates to stage and prognosis

The concentration of ctDNA in plasma has been shown to correlate with tumour size[79,120] and stage[121]. A study of 640 patients with various cancer types and stages[121] found a 100-fold increase in median ctDNA concentration between patients with Stage I and Stage IV disease. Measuring individual tumour mutations in each patient, patients with Stage I disease had fewer than 10 copies per 5 ml of plasma. In sharp contrast, patients with advanced prostate, ovarian and colorectal cancers had a median concentration of 100-1,000 copies per 5 ml of plasma. ctDNA levels vary greatly even within patients with the same type and stage of disease. This variability in ctDNA concentration is partially explained by differences in extent of metastatic spread or disease burden. In a recent report that compared ctDNA levels with tumour volume assessed by imaging in patients with relapsed high-grade serous ovarian cancer, ctDNA levels and disease volume were significantly correlated[118]. Mutant alleles in plasma increased in fraction by approximately 0.08%, and in concentration by 6 mutant copies per millilitre of plasma, for every cm^3 of disease[118]. Notwithstanding these correlations, substantial variation in ctDNA concentration may arise from inter-individual differences. For example, poor tumour vascularisation could hamper ctDNA release into the bloodstream, or conversely, could promote ctDNA release via producing hypoxia and cell death. Histological differences could forseeably influence both the rate and type of cell death. Patients with primary brain

tumours have very low levels of ctDNA, with a median concentration for individual mutations of less than 10 copies per 5 ml of plasma[121], while the fraction of tumour DNA in CSF was found to be significantly higher[61,62,121]. Although not directly proven, the blood-brain barrier has been suggested to impede the movement of cfDNA fragments into the circulation[61,62,121].

The relationship between ctDNA levels and cancer stage suggests prognostic utility for ctDNA. Patients with detectable ctDNA have been shown to have worse survival outcomes than those without[27,122–126]. In one of the earliest examples in the field, the 2-year overall survival rate for patients with colorectal cancer who had detectable ctDNA was 48%, as opposed to 100% for patients without[27]. In patients with detectable ctDNA, it has been found to be a more significant prognostic predictor than commonly used tumour markers[36,118], where an increasing concentration of ctDNA correlates with poorer clinical and radiological outcomes[36,118,121,127,128]. For example, in patients with metastatic breast cancer, a significant inverse correlation was shown between ctDNA concentration and overall survival up to 2000 copies/ml, with a uniformly poor prognosis above this level[36]. In addition to ctDNA levels, mutational patterns identified in ctDNA (Fig. 1b) can help group patients into molecular subtypes with different prognosis[129].

Earlier diagnosis of disease

Diagnosing cancer at an earlier stage, particularly before metastatic spread, may allow earlier intervention and could improve survival[130]. A number of studies have demonstrated the potential for non-invasive early diagnosis. Mutations have been detected in saliva and plasma from individuals up to two years prior to cancer diagnosis[21,131], and there have been reports of incidental pre-symptomatic detection of cancers in pregnant women who underwent NIPT[68,132,133], as WGS can identify copy number alterations of both fetal and tumour origin. Screening in asymptomatic populations introduces risks of over-diagnosis and false positives; implementation could therefore be explored in stages, and a first step could involve the use of ctDNA for earlier diagnosis of disease in symptomatic individuals, who at present may undergo lengthy investigation procedures.

In a survey across several cancer types, ctDNA was detected in 82% of patients with Stage IV disease, which fell to 47% for patients with Stage I disease[121]. The method they applied was benchmarked as being able to detect one copy of an individual cancer mutation per 5ml of plasma[121]. Using a sequencing gene panel that targeted a median of four mutations per patient, ctDNA was detected in 50% of patients with stage I non-small cell lung cancer (NSCLC)[108]. Targeting known tumour mutations in plasma using ddPCR assays in early-stage breast cancer showed a sensitivity of 93.3%[134]. A sWGS method adapted from an NIPT assay was recently shown to detect 6/16 (37.5%) cases of early ovarian cancer[135], though this approach may not perform as well in other cancer types with fewer copy number alterations. Together, these studies outline the possibility, and the challenge, of detection of ctDNA in early stage disease.

If analysis is performed on a few millilitres of plasma containing only a few thousand copies of the genome, increasing the analytical sensitivity beyond the range of ~1/1000 may not produce any sensitivity benefit since at low allele fractions, it becomes increasingly likely that the mutation of interest may not be found due to sampling noise. One approach may be to collect greater volumes of plasma (and more cfDNA) through methods such as plasmapheresis or implanted devices containing materials that bind cfDNA; similar approaches have been tested for enhancing the yield of circulating tumour cells (CTCs)[136]. Given these challenges, it is important that pre-analytical factors surrounding the collection, processing, and extraction of cfDNA are optimised (Box 2). Alternatively, for some cancer types, other minimally invasive samples may have a higher tumour DNA content, such as urine for bladder cancer[57] or stool for colorectal cancers[19]; or cytological specimens such as cervical smears[137], uterine lavage[138], or oesophageal brushings[139] for gynaecological or oesophageal cancers, respectively (Fig. 3c). For cancers with a viral aetiology, e.g. nasopharyngeal carcinoma or cervical cancer, detection of the cancer-associated viral DNA that may be present in body fluids in many more copies than tumour DNA can enhance the identification of individuals with early stage disease or pre-malignant lesions with a high risk for cancer[140,141].

Technical and biological advances facilitate enhanced mutation detection: the finding that ctDNA is shorter than cfDNA[4,81,82] suggests the utility of experimental or *in silico* size selection (Fig. 3d). Where the input amount of plasma or DNA is limiting, assays that interrogate multiple mutations in the same reaction (through broad genomic coverage or by patient-specific multiplexed panels) may produce a higher overall sensitivity for detecting the presence of *any* ctDNA[35,36,108,110,111], compared to focused analysis of individual loci (Fig. 4). Taken to a (currently impractical) extreme, ultra-deep sequencing of the entire genome could in the future allow sensitive detection of cancer even from small volumes of plasma[37] (or other fluids).

Although technical advances may improve sensitivity for ctDNA analysis further, biological and genomic factors may eventually become limiting. For confident *de novo* cancer detection using ctDNA, detected alterations should have a high positive predictive value for cancer. However, mutations known to be associated with cancer (e.g. in *TP53*, *KRAS*, and Notch pathway genes) have been found at low levels in skin biopsies of healthy individuals[142]. If non-tumourigenic clones were to increase to sufficient size and release mutated cfDNA, they could introduce biological noise. Clonal haematopoiesis with leukaemia-associated mutations has been observed in 10% of individuals older than 65 years of age, though the absolute risk of conversion to haematologic cancer is 1%[143]. Genomic alterations known to be associated with cancer have been found in plasma from healthy individuals[111,115,144,145]. Clinical outcomes for apparently healthy individuals in whom mutant DNA is detected in plasma should be characterised in order to understand the biological and clinical implications of such findings.

Cancer localisation

Tissue-of-origin information from liquid biopsies might be able to aid cancer localisation, for example, for cancers of unknown primary[146]. Methylation and nucleosome occupancy patterns in cfDNA have been found to encode tissue- and cell-specific information[66–68,147]: in one case, a pregnant woman had chromosomal abnormalities detectable by NIPT, and so tissue-specific methylation signals in

plasma were studied, enabling quantification of the relative contributions of each tissue to the total cfDNA pool[68]. This revealed an increased contribution of cfDNA from B-lymphocytes, consistent with a diagnosis of follicular lymphoma[68]. While it may be possible in the future to deduce the tissue type of origin of a cancer in this fashion, it remains to be seen whether the site or sites of metastatic spread can be determined through a measurable increase in tissue-specific cfDNA signal.

Non-invasive molecular profiling

Analysis of heterogeneity

The extent of genetic heterogeneity has been confirmed over recent years as multi-regional sequencing studies have demonstrated clear differences in mutation profiles between different tumour regions in the same patient[148,149] and between different specimens from primary and metastatic sites[150]. Although the potentially confounding effects of heterogeneity are recognised, it is often neither feasible nor desirable to perform multiple tumour biopsies on patients to try to account for this. Analysis of an individual biopsy might not accurately reflect the genomic architecture of a patient's cancer, introducing bias to the selection and efficacy of personalised medicines. Furthermore, in a recent study of patients with lung cancer treated with an EGFR inhibitor, the tumour *EGFR*^{T790M} allele fraction correlated with the degree of tumour shrinkage[151], suggesting that the current paradigm of treatment selection based on mutation presence or absence alone may be suboptimal.

Liquid biopsies sample ctDNA released from multiple tumour regions, and may thereby reflect both intratumour heterogeneity[45,47,62] and spatially separated disease foci[48,152–154]. While individual tumour biopsies from different tumour regions may differ in mutation profile due to intratumour heterogeneity[155,156], ctDNA analysis has detected mutations missed in corresponding tissue samples[45,48,157,158]. Multi-region tumour sequencing data show that stem mutations (shared by all tumour regions) show a higher allele fraction in plasma compared to private mutations[47,48]. Therefore, for tracking tumour burden in plasma, stem mutations would provide the most reliable detection. Alternatively, tracking a large set of mutations may compensate for potential biases of individual private mutations.

Hotspot mutations and gene panels

By comparing mutation detection in plasma against matched tumour samples, the sensitivity of ctDNA analysis has been estimated in retrospective studies as between 65%-98%[100,102,119,154,157,159–161]. For profiling specific loci, for example in order to stratify patients for matched molecular therapies, international studies have begun to demonstrate that large-scale testing is feasible and may be standardised, although the use of assays with limited analytical sensitivity resulted in low detection rates of ctDNA[162–164]. Using assays developed specifically to detect low levels of ctDNA[3], a blinded prospective study demonstrated sensitivity for KRAS and BRAF mutations in metastatic colorectal cancer of 92% and 100%, respectively, with concordance rates of 96% and 100% for each[119].

Considerable attention has been devoted to analysis of *EGFR* mutations in patients with NSCLC[34,99], as it is often challenging to obtain tissue biopsies to help inform treatment[41,42]. Meta-analysis of 27 selected studies published between 2007-2015, comprising altogether nearly 4000 patients, resulted in a pooled sensitivity of 60% and specificity of 94% for detection of *EGFR* mutations in plasma or serum, with a variety of methods[34]. In a phase IV study of the *EGFR* inhibitor gefitinib, mutation status was compared between tumour and plasma samples from 652 patients. The sensitivity and specificity for detecting mutations in plasma were determined as 65.7% and 99.8%, respectively[164,165], likely affected by the limited analytical sensitivity of the PCR-based method used[166], as the version of the kit used in that study had a limit of detection (at ≥95% analytical sensitivity) of 1.64% and 1.26% for *EGFR* deletions and L858R mutations, respectively[164]. Low rates of concordance of $EGFR^{T790M}$ status were also observed in a recent phase III trial of osimertinib[167], in which tissue testing was compared to plasma using an allele-specific PCR assay for ctDNA analysis that has a limit of detection (with ≥95% analytical sensitivity) of 100 copies of $EGFR^{T790M}$ per ml of plasma[168]. Using methods with greater analytical sensitivity, higher concordance rates were obtained[157,169]. The $EGFR^{T790M}$ mutation confers resistance to gefitinib and erlotinib, and frequently emerges following initial treatment with those EGFR tyrosine kinase inhibitors[33,170,171]. In plasma, the

sensitivity for detecting this mutation was lower than for *EGFR*^{L858R} and *EGFR*^{Ex19del} (70% vs. ≥90%), which occur earlier in the development of disease[157,169]. Initial data suggests that this may be due to heterogeneous presence of resistance mutations at disease relapse[157].

In retrospective analyses, despite limited concordance rates of *EGFR* mutation status between plasma and tumour samples observed in some studies, response rates for patients who were plasma positive for mutations in *EGFR* were similar to response rates of patients who were tissue positive[157,164,165,167,169]. Data showing the response of patients treated solely on the basis of ctDNA analysis are starting to emerge: patients who were treated with osimertinib based on detection of *EGFR*^{T790M} in plasma, and had response rates similar to the response rates of patients treated based on tissue analysis[172]. Interestingly, objective responses were also seen in patients with very low allele fractions of mutant *EGFR*^{T790M} in plasma (<0.5%)[172].

At present, the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) approve the use of information from ctDNA analysis to help select patients with *EGFR*-mutant NSCLC for gefitinib (EMA)[173], erlotinib (FDA)[168] or osimertinib (EMA and FDA)[168,174] therapy in the event that a tumour sample is not evaluable. This could offer a pragmatic solution to provide molecular profiling information for patients, while avoiding repeat biopsies for some individuals. Current recommendations[168,174] state that if liquid biopsies are carried out in advance of a tumour biopsy, ctDNA detection may abrogate the need for tissue biopsy, but if ctDNA analysis is negative, a tissue biopsy may still provide valuable genomic information.

Molecular profiling using ctDNA may have particular utility for stratifying patients in 'basket trials', which enrol patients independent of tumour histology, or 'umbrella trials', which assign patients to multiple investigational drugs or treatment options[175]. For example, a 54-gene panel detected ctDNA in 58% of patients across multiple cancer types[176]. Of the patients with alterations, 71.4% had at least one mutation actionable by an FDA-approved drug[176]. This panel is being used to test the

feasibility of matching patients with different metastatic cancer types to targeted therapies in a prospective clinical trial[177]. In another study presented at the 2016 *Molecular Analysis for Personalised Therapy* meeting, a 34-gene panel identified mutations in 79% of 174 patients with NSCLC, allowing 28 patients (17%) to receive personalised treatment based on ctDNA molecular profiling[178]. Personalised therapy selection presents challenges: even if mutations are successfully detected using ctDNA in patients, an efficacious molecularly targeted agent may not exist. However, data from a prospective clinical trial presented at the 2016 *Molecular Analysis for Personalised Therapy* meeting demonstrated that selecting therapies based on genomic analysis can improve outcomes for cancer patients, even when patients with well-established actionable targets (for which approved drugs are available) were excluded[179].

Improvements in the analytical sensitivity of molecular profiling tools could further increase detection and concordance rates or allow for sensitive multiplexed analysis (*Table 1*), though biological factors and heterogeneity may reduce sensitivity in some cancer types and stages[121,157,180]. The utility of ctDNA should, therefore, be assessed for different clinical indications. However, benchmarking ctDNA against individual tumour biopsies may be confounded by sampling error, as rare private mutations may be sampled in the biopsy, but release insufficient ctDNA into the bloodstream to be detectable.

Structural variants

Copy number alterations can be detected in cfDNA using WGS[38,181–183], amplicon-based[116,184,114], and hybrid-capture approaches[40,108,160,185]. In patients with hepatocellular carcinoma, WGS was able to identify amplifications and deletions in plasma matching those identified in tumour tissue[38,186]. Heterogeneous copy number changes were also identified in a patient with synchronous breast and ovarian cancers, as copy number changes unique to each cancer were detected in plasma[38]. In a study of 80 patients with prostate cancer, androgen receptor (*AR*) copy number gain prior to abiraterone therapy predicted a worse overall survival, thus identifying patients with primary resistance[187]. For patients with advanced disease, sWGS may

provide a relatively cost-effective measure of ctDNA level that is applicable across cancer types. This approach may have utility as a sample screening step in a ctDNA analysis workflow[116], where high-burden patient samples are triaged for exome sequencing[40].

Chromosomal rearrangements in plasma can be identified through both WGS[37] and hybrid-capture sequencing approaches[39,188], though the latter may be more economical due to the depth of coverage needed to confidently identify a rearrangement. In one study of patients with prostate cancer, sWGS was able to detect a deletion on chromosome 21 in 5 patients, though higher-depth capture sequencing was necessary to identify a rearrangement between exon 1 of *TMPRSS2* and exon 3 of *ERG*[39].

Longitudinal monitoring

Monitoring response

The short half-life of cfDNA,[31,71–73] as well as the ease and reduced risk of repeating liquid biopsies relative to imaging[50] or tissue biopsies[41], enables liquid biopsies to be used for real-time monitoring of cancer burden in response to therapy. Studies monitoring patients during treatment have shown that ctDNA dynamics correlate with treatment response[31,35,36,108,126], and may identify response earlier than clinical detection[36,189,190]. In patients with breast cancer, ctDNA showed the greatest range in concentration and provided the earliest measure of response to chemotherapies, as well as the earliest indication of impending relapse compared to imaging and other blood-based cancer markers, such as CTCs and Cancer Antigen 15-3 (CA 15-3, also known as MUC1)[36]. In relapsed ovarian cancer, pre-treatment ctDNA levels and the extent of ctDNA decrease after chemotherapy initiation were significantly associated with time to progression, and were more informative than levels of CA 125 (also known as MUC16) [118].

A recent study suggested that an early spike in ctDNA levels (allele fractions of BRAF mutations) in the first week following the initiation of immunotherapy in melanoma patients may predict response[191]. This may reflect a transient increase in

cell death. If these data are confirmed, sampling at early time points could be applied in the clinic as well as in drug development. However, the presence or timing of such spikes in cell death would likely vary based on the pharmacological properties and biological responses to treatments used. An early spike was not observed a few days after initiation of treatment with chemotherapy for patients with colorectal cancer[159] or with *EGFR* inhibitor for patients with NSCLC[190]. If analysis of plasma immediately after the start of therapy could reliably detect the destruction of sensitive cancer cells, this raises an exciting possibility that the existence of resistant sub-clones could be identified very rapidly through differential early dynamics of mutations. In the context of immunotherapy, liquid biopsies may provide the opportunity to monitor both ctDNA and the response of the immune system, for example through the analysis of cfDNA released from distinct T-cell clones[192].

Minimal residual disease and recurrence monitoring

Following surgery or treatment with curative intent, even in the absence of any other clinical evidence of disease, detection of ctDNA may signal the presence of minimal residual disease (MRD), which could identify patients who may be at a higher risk of relapse. Stratification of patients into high- and low-risk groups would enable adjuvant therapy to be given to patients who stand to benefit most, while sparing low-risk patients from unnecessary comorbidities and risks of adverse events. In a prospective study of 230 early-stage colorectal cancer patients, assessment of ctDNA at the first follow-up visit after surgical resection indicated that recurrence-free survival at 3 years was 0% for the ctDNA-positive and 90% for the ctDNA-negative groups[193]. In a separate study of 55 patients with early-stage breast cancer, assessment of ctDNA showed that detection of ctDNA at first follow-up could also indicate poor prognosis in early-stage breast cancer[194]. Furthermore, stratification based on mutation detection across serial samples improved prediction of relapse, and this and other studies have observed an interval of 7.9-11 months between ctDNA detection and clinical relapse[194–196], similar to that identified in the metastatic setting[36]. With more sensitive approaches (Fig. 4), earlier identification of clinical relapse should be possible. Combining monitoring for known mutations with molecular profiling assays could allow identification of potential targets for early

therapeutic intervention – ideally, such intervention would, in future, prevent or postpone overt relapse.

Patient-specific DNA rearrangements identified from sequencing tumour samples can be used to design assays to track tumour burden in plasma[196–199]. Curative surgery could provide an excellent opportunity to obtain tumour DNA that can be sequenced to guide the design of assays for post-operative monitoring. Patient-specific rearrangements may be more readily detected in ctDNA with high sensitivity[197,198], since rearrangements are less confounded by background noise. One of the challenges of individualised panel design is that sequencing an individual tumour biopsy may not sample every mutation in heterogeneous disease; therefore, sequencing matched body fluid and tumour samples may be desirable for comprehensive mutation profiling. In future, if tumour sequencing becomes routine, monitoring disease using patient-specific panels could become viable, although regulation of such assays will likely be more complex than fixed panels.

Clonal evolution and resistance

As discussed above, rising or falling ctDNA concentration may provide an indication of treatment effect on overall tumour burden. If multiple tumour mutations are interrogated, then the relative change between each may provide insight into molecular evolution of the patient's cancer[40,200,201]. Ratios between the levels of different mutations in plasma can indicate heterogeneity and may be informative to predict patient response to treatment targeting particular alterations[157,160]. Liquid biopsies have been shown to contain ctDNA from multiple tumour sites[45,47,48,62], and their analysis has a faster turnaround time than tissue biopsies[39,102,188], and may be less prone to biases resulting from the analysis of individual tumour biopsies[148,149,202].

Studies demonstrate that ctDNA can monitor clonal evolution and identify resistance mechanisms to treatment[110,158,160,200,203,204]. Serial ctDNA analysis in patients with colorectal cancer demonstrated the positive selection of mutant *KRAS* clones during *EGFR* blockade, which later decline upon the withdrawal of anti-*EGFR* therapy[158,205]. In patients with NSCLC undergoing treatment with *EGFR* inhibitors, resistance-

conferring mutations emerged in plasma ahead of clinical progression[170,171]. Exome sequencing of plasma DNA may identify resistance mechanisms in patients across cancer types[40], though the sensitivity of exome sequencing currently limits its application to advanced cancer patients where ctDNA levels are high (>5% mutant allele fraction). Design of patient-specific mutation panels[35,48,111,206] could be a more cost-effective alternative for high-sensitivity monitoring, though may miss subsequent *de novo* events unless appropriately designed. Serial sWGS analysis also demonstrates highly dynamic copy number adaptations in response to selection pressures, with a mean interval of 26.4 weeks between new amplifications[207].

Serial liquid biopsies may have particular utility for adaptive or reactive therapy, in which resistance mutations are prospectively identified, and therapy adapted in real-time (Fig. 5). In the clinical research setting, non-invasive monitoring could facilitate a clinical trials that prospectively identify efficacious treatment regimens or drug combinations, and identify resistance mechanisms to novel therapies. In addition, *in vitro* or *in vivo* experiments carried out in parallel may provide greater insight into cancer biology. For example, colorectal cancer cell line experiments carried out in parallel with ctDNA analysis showed that resistance mutations may arise from both the selection of pre-existing minor clones, and through ongoing mutagenesis[200]. Another study investigating resistance to a pan-tropomyosin-related kinase (TRK, also known as NTRK) inhibitor in colorectal cancer demonstrated that simultaneous analysis of patient-derived xenografts and liquid biopsies may characterise resistance more comprehensively than plasma alone[199].

Future directions

Proof-of-concept studies provide an excellent starting point for larger prospective studies into the clinical utility of ctDNA, and demonstrate that ctDNA may be a useful research tool for drug development, and for the study of intratumour heterogeneity and clonal evolution. Moving forwards, randomised trials comparing ctDNA-guided decision-making against the standard of care would be definitive, and the EMA have outlined good practice guidelines for the design of such trials[208]. Trials to test the clinical utility of ctDNA analysis for treatment monitoring are now being carried

out[209,210]. In one trial, patients with NSCLC receiving erlotinib are being prospectively monitored, and if resistance mutations emerge in plasma, then additional scans to search for signs of disease progression would be carried out[209]. Another clinical trial aims to demonstrate the efficacy of targeting mutations identified in plasma from patients with advanced breast cancer[211], which could support the future use of plasma-only mutation profiling and treatment stratification. Together, these studies highlight that the field is moving from exploratory ctDNA studies, towards clinical trials where ctDNA is guiding decision-making.

A better understanding of the origin and biology of cfDNA and ctDNA would aid the implementation of liquid biopsies[2]. The relative contributions of apoptosis, necrosis and active release, particularly at different time points during treatment, should be explored. Our limited understanding of the release and clearance mechanisms of cfDNA hampers interpretation of current studies. Studies of the dynamics and reproducibility of ctDNA measurement in the absence of intervention will become increasingly important as we aim to interpret ctDNA signal in response to treatment. It is also not clear whether all tumour subclones contribute proportionately to the total ctDNA pool, or whether their representation in the bloodstream is biased by other biological factors, such as tumour vascularity or metabolic activity. *In vivo* cellular barcoding experiments[212] and autopsy studies[48] could elucidate the contribution of individual subclones, and histological studies may clarify the factors that modulate ctDNA release. The differences in size between cfDNA and ctDNA fragments[4,68,81,82] suggest that optimising processing and extraction methods (as well as downstream assays) for recovery of selected fragment sizes may provide further improvement to overall performance.

While ctDNA can have greater sensitivity and specificity compared to other circulating biomarkers[36], taking a multi-marker approach may offer a more comprehensive insight into a patient's disease[3,119,213]. For example, total cfDNA concentration correlates with disease status[3,5] and is associated with prognosis[214]. Epigenetic analysis of cfDNA may identify cancer gene hypermethylation[27,153] or the cell-type giving rise to cfDNA fragments[66–68], and may provide a window into the

tumour microenvironment, which usually lacks somatic mutations. Other circulating nucleic acids such as mRNA and microRNA can provide additional layers of information[215]. Targeting multiple types of nucleic acid, with independent mechanisms of release, may increase sensitivity for detection of MRD, for example through the co-isolation of both exosomal RNA and cfDNA[216]. Actively released nucleic acids may be preferred for the detection of mutations in subclones resistant to therapy, whereas fragments arising from dying cells following the initiation of therapy may identify treatment-responsive subclones. Next, although it may be possible to infer gene expression patterns from cfDNA[217], sequencing RNA within exosomes[218], CTCs[219] or platelets[220] could provide more direct evidence. Analysis of cell-free DNA in plasma alongside other fluids such as urine[221] or CSF[45] can provide complementary information. We further echo the suggestion by Gormally, Hainaut and colleagues[145], made a decade ago, that characterisation of proteins associated with cfDNA may provide a rich source of information on an individual's disease, and the biology of cfDNA.

The clinical uptake of liquid biopsies will depend on the practical advantages for patients and clinicians, the infrastructure required, and its cost-effectiveness. Tissue biopsies will continue to play a key role in cancer management, particularly for the histological diagnosis and classification of cancers. At present, specialised laboratories handle CTC and ctDNA samples[222], though in future hospital laboratories may carry out analysis locally if appropriate processes can be established[223].

Point-of-care devices for the identification of individual hotspot mutations with clinically meaningful sensitivities are starting to be used for tissue and plasma samples[224,225]. The feasibility of single molecule (third generation) sequencing of maternal plasma DNA was first demonstrated in 2015[86], and subsequently it was shown that structural variants in cell line DNA can be detected[226]. The portability of such technologies was demonstrated by the real-time genomic surveillance in the field during the Ebola virus disease epidemic[227]. At present, such platforms are limited by a high error rate[226], making single nucleotide variant and indel detection challenging. Another challenge is that of sequencing short DNA fragments, which

requires optimised library preparation methods[228]. Sequencing capacity is also limited (currently to ~150 megabases)[229], though this is likely to increase in the near future, and specific amplicons may be targeted through real-time selective sequencing[230]. These studies support the possibility of molecular profiling at the point of care, especially if blood plasma can be interrogated without the relatively cumbersome and time-consuming step of DNA purification[87,231].

The initial approvals by the EMA and FDA for mutation detection in plasma as a companion diagnostic[168,173,174], and emerging ctDNA-guided clinical trials[209–211], represent key milestones towards the implementation of liquid biopsies in personalised oncology. Improving technologies are enabling an ever-wider scope for non-invasive molecular analysis of cancer, providing information that opens new avenues for genomic research and may aid in clinical decisions. In order to fully exploit the potential utility of liquid biopsies, it is essential that the biology of ctDNA be explored further. Thus far, liquid biopsies have demonstrated the potential for utility across a range of applications, and are beginning to be used for patient benefit.

Boxes

Box 1 - Clinical need for liquid biopsies in oncology

There are multiple areas of oncology where novel diagnostics may have utility and produce clinical benefit.

- **Cancer diagnosis** – earlier diagnosis of cancer would enable treatment to be initiated sooner, and curative surgery may be carried out if the tumour is diagnosed at an early stage. For symptomatic patients, sensitive and specific cancer detection may speed up the time to diagnosis and treatment. At a population level, pre-symptomatic individuals may be screened for markers of disease, enabling early intervention.
- **Prognosis, residual disease and risk of relapse** – assessment of risk of progression is essential to select the extent or aggressiveness of treatment. After treatment with curative intent, the identification of patients with residual disease who are at high risk of relapse may be used to stratify patients to adjuvant therapy. Effective stratification would also spare low-risk patients from overtreatment.
- **Treatment selection** – the introduction of a wide array of novel molecularly targeted and immunotherapy agents necessitates improved tools for molecular profiling of patients and treatment stratification. At present, tumour biopsies are the standard for obtaining tumour DNA; these cannot always be obtained and their interpretation may be confounded by intratumour heterogeneity[44–48], which could lead to false negative results and suboptimal therapy selection.
- **Monitoring disease burden** – treatment monitoring, presently performed through imaging or molecular methods, may identify response or progression, enabling clinicians and patients to adapt therapy accordingly. Current methods have limited accuracy, associated logistical burden or radiation exposure[50]. The ideal monitoring assay should be repeatable serially over time, with minimal risk to patients, and should provide an accurate read-out of tumour burden.

Box 2 – Pre-analytical considerations

In low-burden disease, or certain cancer types, the concentration of ctDNA molecules may be low and any loss of sampled material could reduce the sensitivity of molecular profiling. For quantitative applications, reproducibility of measurement is essential to achieving a robust result, and so the following pre-analytical factors should be considered:

- Samples should be collected in tubes containing an anticoagulant that is compatible with PCR, with EDTA being preferred. Plasma from heparinised blood leads to inhibition of PCR[232], although some studies have been able to utilise such samples[33].
- It is important that the first centrifugation of the blood is done within a few hours of the blood draw in order to remove blood cells that may lyse and release germline DNA which would dilute ctDNA[69,233–236]. Tubes containing fixative agents may stabilise cells and prevent lysis for several days at room temperature[234,236–239], including during shipping[239,240].
- Following centrifugation, buffy coat DNA from the same tubes can be used as a source of germline DNA, although this may contain small or trace amounts of ctDNA.
- From a blood draw, plasma is preferred over serum for ctDNA analysis[235]. Serum also contains ctDNA[241], but blood cell lysis during the preparation of serum samples could release DNA from non-cancerous cells, which would dilute any ctDNA signal. Other body fluids or cytological specimens may be used, and may contain a higher amount or concentration of tumour DNA depending on tumour proximity (Fig. 3c).
- cfDNA extraction may be carried out with affinity-column, magnetic bead, polymer, phenol-chloroform methods, or by filtration. Different methods show variation in their ability to recover particular fragment sizes[87,88], which could have implications for ctDNA detection, given the differences in size between cfDNA and ctDNA.

Figures

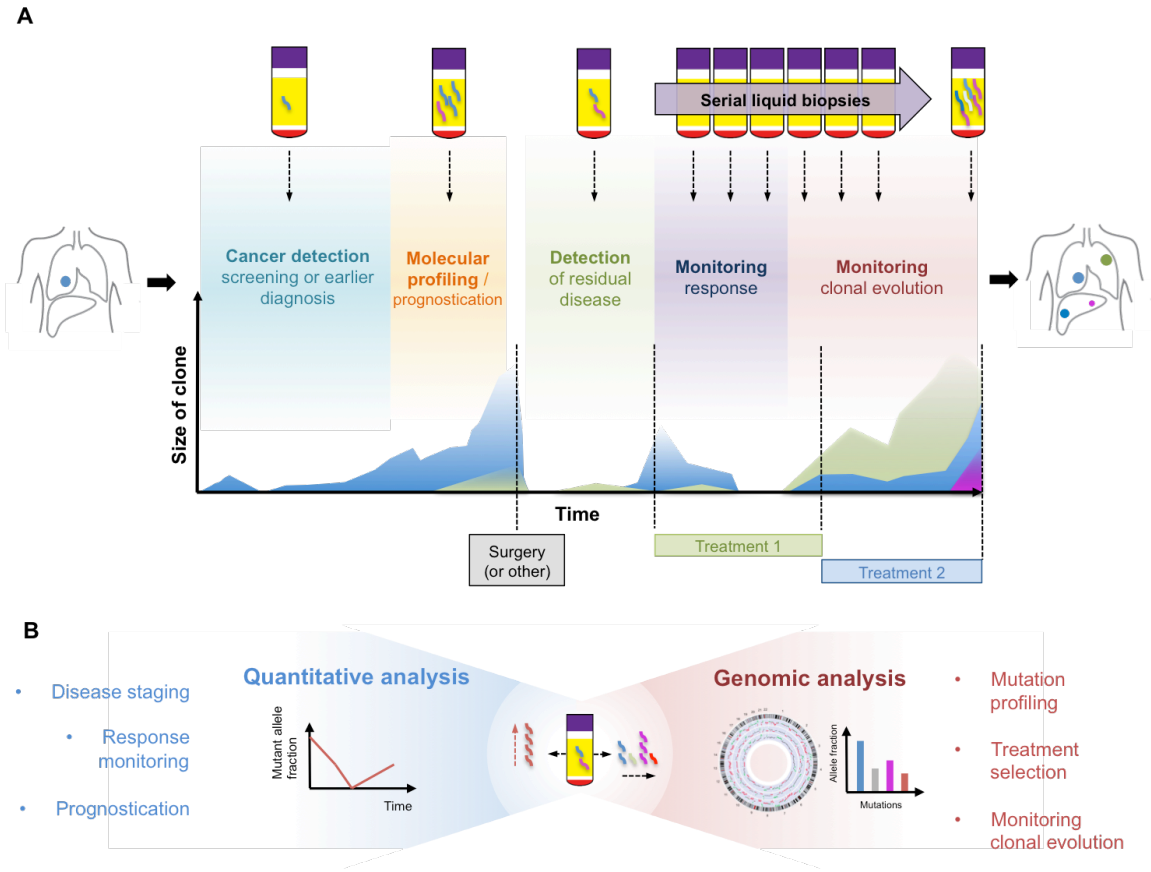

Figure 1 | **Applications of ctDNA analysis during the course of disease management**

(A) A schematic time course for a hypothetical patient who undergoes surgery (or other initial treatment) has a disease relapse, and then undergoes systemic therapy. The potential applications of liquid biopsies during this patient's care are indicated. The patient starts with one single disease focus, but multiple metastases and distinct clones emerge following treatment, depicted in different colours.

(B) The information extracted from ctDNA may be classified, broadly, into quantitative information (i.e. tumour burden) or genomic information. Quantification of ctDNA at a single time point may allow disease staging and prognostication, and genomic analysis can inform selection of targeted therapies. Therefore, longitudinal analysis allows the quantitative tracking of tumour burden, such as response monitoring; and by comparing genomic profiles over time, clonal evolution may be monitored. The whole genome sequencing image was kindly provided by Dennis Lo, based on data published by Chan et al.[38].

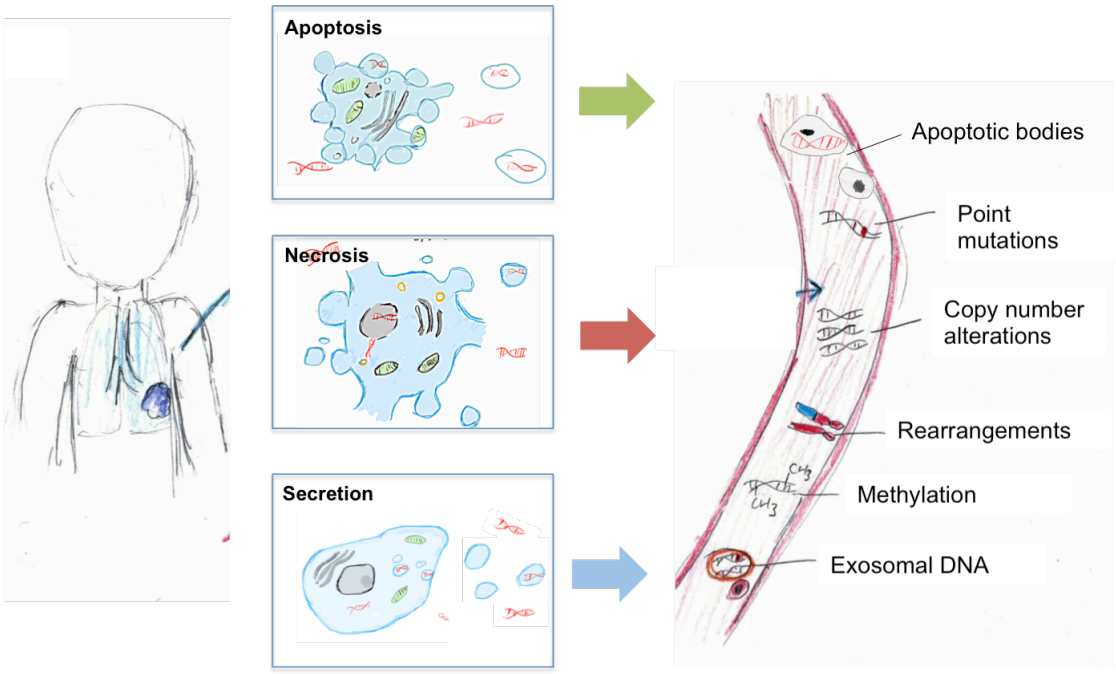

Figure 2 | **Origins and spectrum of alterations in cell-free DNA**

Cells release cfDNA through a combination of apoptosis, necrosis, and secretion. cfDNA can arise from cancerous cells but also from cells in the tumour microenvironment, immune cells, or other body organs. In the bloodstream cfDNA may exist as either free, or associated with extracellular vesicles such as exosomes[2]. Multiple classes of genetic and epigenetic alterations can be found in cell-free DNA. Adapted with permission from Schwarzenbach et al.[215].

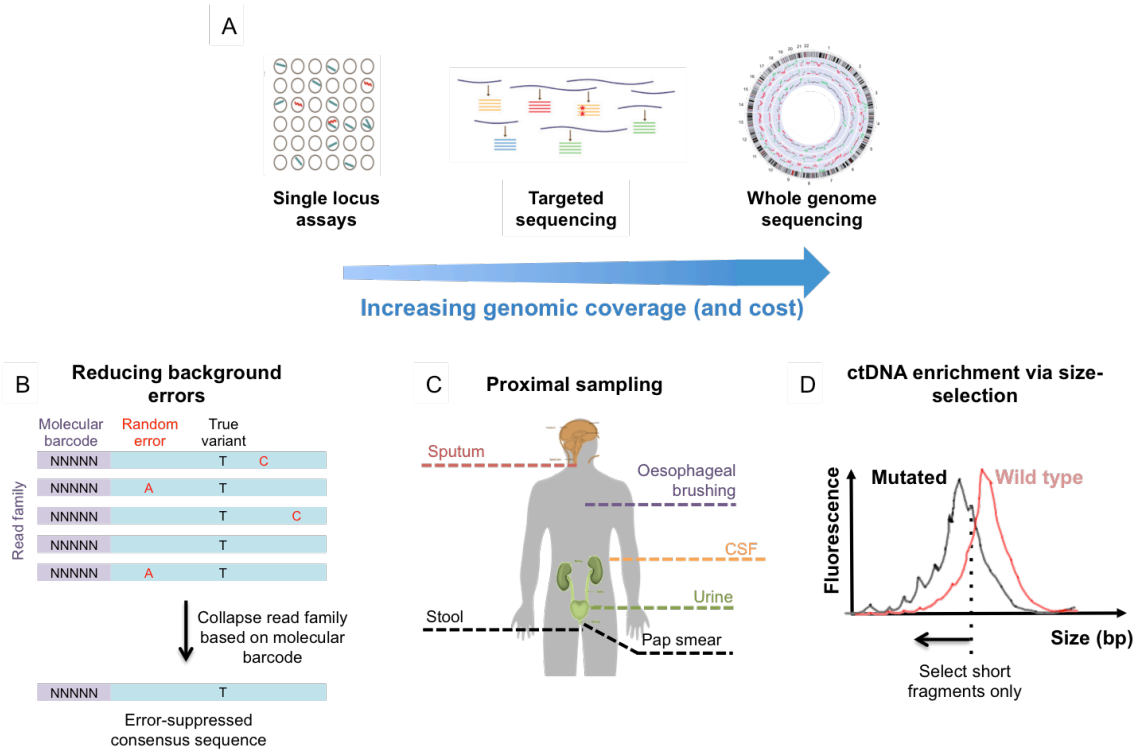

Figure 3 | **Current and future paradigms for sensitive detection of ctDNA**

(A) The analysis of cfDNA can range from the interrogation of individual loci, to analysing the whole genome (*Table 1*). Off-the-shelf dPCR assays can achieve high sensitivity with a simple workflow, but are limited by a low multiplexing capability. Targeted sequencing can allow the interrogation of multiple loci with high sensitivity, using methods that suppress background noise[242]. The targeted sequencing image is modified with permission from Forshew et al.[35] and the whole genome sequencing image was kindly provided by Dennis Lo, based on data published by Chan et al.[38].

(B) In molecular barcoding, unique molecular sequences are added to each molecule during library preparation so that sequencing reads originating from the same starting molecule can be identified. By comparing all reads from the same molecule, a single consensus sequence can be taken, which can suppress errors arising from PCR or sequencing.

(C) To improve sensitivity of analysis, for example for disease diagnosis or detection of MRD, other body fluids may be considered in combination with, or instead of, plasma. Sampling of body fluids or cytological specimens proximal to the tumour site may yield a higher concentration of DNA of tumour origin.

(D) ctDNA has been shown to be shorter than cfDNA[4,81,82,85]. Thus, selection of shorter fragments experimentally or *in silico* may enrich for sequences of cancer origin[82] and can improve sensitivity for samples with low fractions of ctDNA.

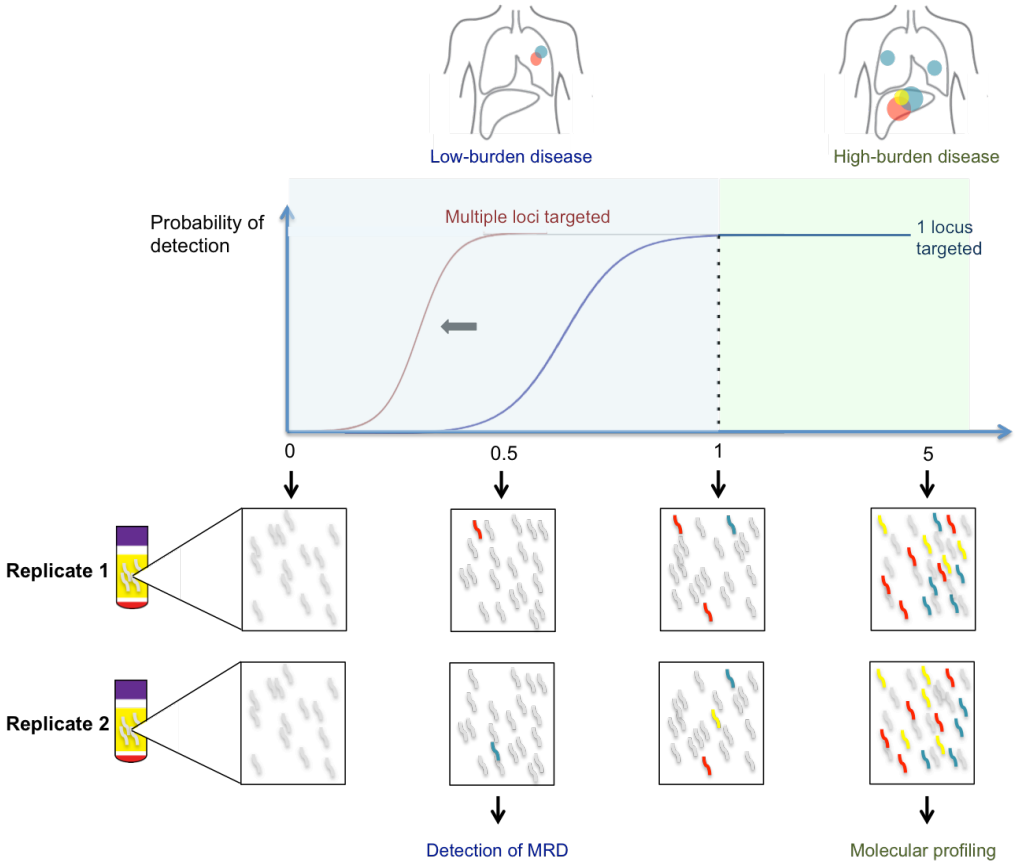

Figure 4 | **Leveraging multiple mutations to detect low-burden disease and overcome sampling noise**

Even with a perfectly sensitive assay, the probability of detection of ctDNA decreases as ctDNA concentration declines, as any single mutation of interest may not be present in a given volume of sample. At low ctDNA concentrations, due to sampling error, some mutations will be detected while others are missed. Sampling multiple pre-specified mutations in each reaction may improve detection of low levels of ctDNA, since every target provides an independent opportunity to test for the presence of a mutant molecule in the set of DNA molecules at that locus[35,111]. Sensitivity can be further improved by analysis of multiple replicates, with few molecules each, so that the mutant allele, where present in a reaction, will constitute a large fraction of the DNA template[113]. Boxes below the graph show hypothetical examples of sets of molecules that may be captured by each replicate in the analysis of a sample.

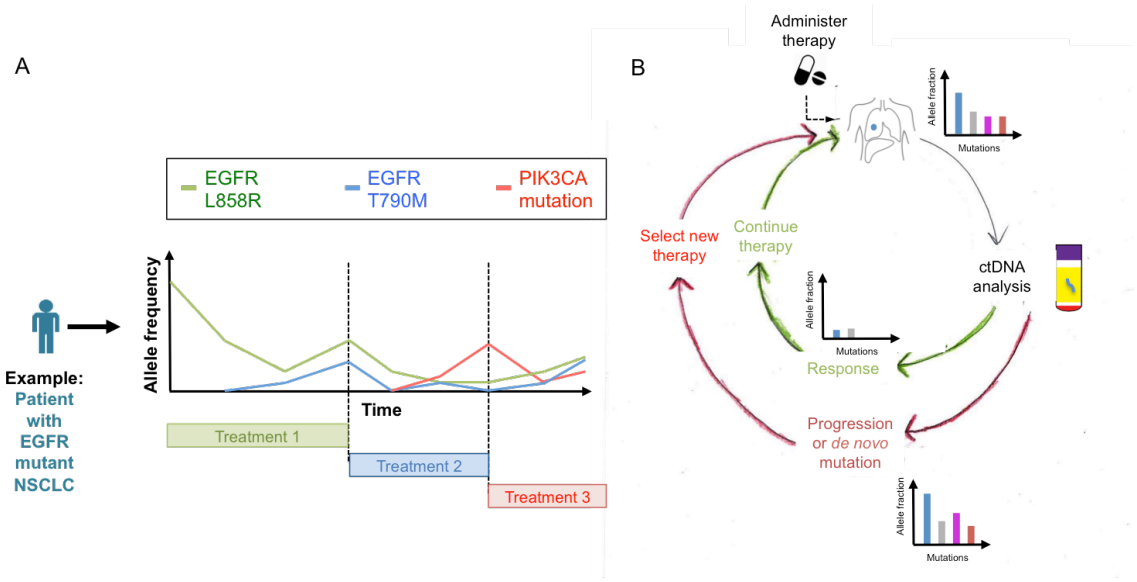

Figure 5 | **Adaptive or reactive treatment paradigms using liquid biopsies**

(A) During systemic anti-cancer therapy, serial liquid biopsies may identify biochemical response or progression. If progression is identified, the clinician may be able to switch therapy, or select a therapy to target arising clones carrying additional mutations that were identified by this analysis.

(B) This adaptive or reactive monitoring and treatment may continue as a loop, which would be facilitated by a fast turnaround time for ctDNA analyses, for example through the use of point-of-care diagnostics. The timeframes for this analysis can vary between hours and months; the former could allow analysis of early kinetics in response to therapy.

Tables

Table 1 | **Comparison and utility of technology platforms for ctDNA analysis**

Scale of analysis	Example technologies	Loci interrogated	Indicative limit of detection (mutant allele fraction or concentration)	Clinical utility
Single-locus or multiplexed assays	PCR-based: • Digital PCR[28,32,101,243] • BEAMing[29,30] • Intplex[3,119]	• 1 to 10 loci • Both ctDNA and cfDNA (IntPlex)	Varies by method, optimal implementations can reach sensitivity of 0.001%-0.01% or individual mutant copies/ml[30,119,243,246]	• Detection and quantification of recurrent hotspot mutations • Monitoring for recurrent resistance mutations • Rapid turnaround time
	Enrichment for mutant alleles: • COLD-PCR[105] • SCODA[244,245] • NaME-PrO[104]	• 10-100 loci		
	Allele-specific or ARMS-PCR kits for companion diagnostics (CDx):		Limit of detection (≥95% sensitivity):	Approved for in vitro diagnostic (IVD) use:
	• cobas EGFR[168]	7 mutation assays covering multiple variants	25-100 copies/ml[168]	• FDA-approved
	• therascreen EGFR[166]	3 mutation assays covering multiple variants	Median 1.42% (range 0.05%-12.47% for different variants)[166]	• CE-marked
Targeted sequencing approaches	Amplicon-based: • TAm-Seq[35] • Enhanced TAm-Seq[114] • Safe-SeqS[112] Hybrid capture-based: • Exome sequencing[40] • CAPP-Seq[108,111] • Digital Sequencing[109,115,185]	10 kilobases to 50 megabases	<0.01%-0.5% for purpose-built panels[35,109,111,112,176,114] 1% for off-the-shelf multiplexed panels[46,110]. 5% for exome sequencing[40]	• Profiling gene panels • Monitoring for *de novo* resistance mutations. • Monitoring clonal evolution in response to therapy. • Sensitivity for disease burden can be increased by testing multiple loci in parallel (Fig. 4)
Genome-wide	WGS: • Plasma-Seq[39] • PARE[197] Amplicon-based: • FAST-SeqS[184] • mFAST-SeqS[116]	3.2 gigabases (whole genome) 21.6 unique kilobases of LINE-1[184]	5%-10%[39]	• Identification of structural variants • Stratification of patient samples based on disease burden • Detecting the presence of chromosomal aberrations

Table 1 | Abbreviations used: BEAMing, beads, emulsion, amplification, and magnetics; CAPP-Seq, cancer personalized profiling by deep sequencing**;** COLD-PCR, co-amplification at lower denaturation temperature PCR; FAST-SeqS, fast aneuploidy screening test-sequencing system; LINE-1, long interspersed nucleotide element-1; mFAST-SeqS, modified fast aneuploidy screening test-sequencing system; NaME-PrO, nuclease-assisted minor-allele enrichment with probe-overlap; PARE, personalized analysis of rearranged ends; SCODA, synchronous coefficient of drag alteration; TAm-Seq, tagged amplicon deep sequencing

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Glossary

Liquid biopsy – analysis of tumour material (e.g. cells or nucleic acids) obtained in a minimally or non-invasive manner, through sampling of blood or other body fluids.

Hybrid-capture sequencing – DNA sequencing of kilobases to megabases of the genome, in which DNA to be sequenced is selected using complementary oligonucleotide baits that hybridise to the target DNA. The DNA is then captured in solution, commonly through binding to magnetic beads.

Limit of detection – The threshold below which mutations cannot be confidently discriminated from background noise; for sequencing-based approaches, this is often determined by technical artefacts such as PCR or sequencing errors.

Stem mutations – Mutations that occur early in a cancer's development and are present in all cells.

Private mutations – Mutations that are present only in a specific region of a tumour, or in a subset of cells, due to intratumour heterogeneity.

Molecular barcoding – Unique molecular sequences that are added to each molecule when creating a sequencing library, so that reads originating from the same molecule may be identified and the consensus taken, correcting for some PCR or sequencing errors.

Mutant allele fraction – the proportion of mutant DNA fragments at a given locus.

Mutant allele concentration – the number of mutant DNA fragments at a given locus, per unit volume.

Digital PCR - Many micro-litre or nano-litre scale PCR reactions are run in parallel within physically separated reaction chambers, or droplets in an emulsion (droplet dPCR, ddPCR). By partitioning molecules into hundreds or up to millions of reactions, rare mutant molecules may be accurately identified and quantified.

Targeted Sequencing – massively parallel (next generation) sequencing that uses methods such as PCR amplification or hybrid capture to focus on a subset of the genome, which can range from few genes or mutation loci, to large fractions of the genome such as the entire exome. Smaller panels yield higher sequencing depth at lower costs.

Acknowledgements

The authors would like to thank all members of the Rosenfeld Lab, in particular Christopher G Smith, Davina Gale, Keval M Patel and Wendy N Cooper for expert advice and proofreading of this manuscript. We would like to thank Dennis Lo for providing the image used in Figure 3a. We would like to acknowledge the support of The University of Cambridge, Cancer Research UK (grant numbers A11906, A20240, A15601) (to N.R., J.D.B.), the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013) / ERC Grant Agreement n. 337905 (to N.R.), and Hutchison Whampoa Limited (to N.R.), AstraZeneca (to R.B., S.P.), the Cambridge Experimental Cancer Medicine Centre (ECMC) (to R.B., S.P.), and NIHR Biomedical Research Centre (BRC) (to R.B., S.P.). J.G.C. acknowledges clinical fellowship support from SEOM.

Competing interests statement

NR is the CSO of Inivata. NR and JDB are cofounders and shareholders of Inivata. NR and FM are co-inventors of patent applications describing methods for analysis of DNA fragments and applications of ctDNA.