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

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Abstract | 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 in an exciting transitional period in which 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 to ctDNA analysis, and to consider their applications in personalized oncology and in cancer research.

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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 concentrations tend to range between 1 and 10 ng ml^{-1} in plasma[3,4]. Raised cfDNA levels were first reported in the serum of patients with cancer in 1977 (REF. 5); cfDNA concentrations can also be raised in other physiological conditions or clinical scenarios, such as acute trauma[6], cerebral infarction[7], exercise[8], transplantation[9] and infection[10]. The identification of fetal DNA sequences in maternal plasma by Lo *et al.*[11] in 1997 has led to multiple applications of cfDNA in prenatal medicine, including sex determination[12], the identification of monogenic disorders[13] and noninvasive prenatal testing (NIPT) for aneuploidies such as Down 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 and colleagues[2,17] reported that at least some cfDNA in the plasma of patients with cancer originates from cancer cells. In 1991, Sidransky *et al.*[18] showed that DNA from urinary sediments (cell pellets) from patients with invasive bladder cancer carried mutations in *TP53* (which encodes p53), and this set the stage for the use of genomic analysis methods in liquid biopsy applications. *KRAS* mutations that matched the mutations in colorectal[19], pancreatic[20] or lung[21,22] cancers were soon found in stool or sputum samples. In 1994, mutated *KRAS* sequences were first reported to be detected in the plasma cfDNA of patients with pancreatic cancer by 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 the plasma were of tumour origin. Mutations in cfDNA are highly specific markers for cancer, and this 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, although the levels of ctDNA in different clinical contexts were not yet accurately defined. These studies nonetheless could demonstrate potential clinical applications, for example, the 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[28] enabled the accurate identification and absolute quantification of rare mutant fragments. A modification of this technique using beads in emulsions[29] and flow cytometry enabled the quantification of the mutant allele fraction of cancer mutations in the plasma of patients with various stages of colorectal cancer[30]. Diehl, Diaz *et al.*[31] then showed in 2008 that ctDNA is a highly specific marker of tumour dynamics and may be able to indicate residual disease. 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 patients

Box 1 | **The clinical need for liquid biopsies in oncology**

There are multiple areas of oncology in which novel diagnostics may have utility and produce clinical benefit, including:

- 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, screening for markers of disease could enable early intervention in presymptomatic individuals.
- Prognosis, residual disease and risk of relapse: the 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 for treatment stratification. At present, tumour biopsies are the standard for obtaining tumour DNA; however, these cannot always be obtained and their interpretation may be confounded by intratumour heterogeneity[43–47], which could lead to false-negative results and suboptimal therapy selection.
- Monitoring disease burden: treatment monitoring, which is currently performed using imaging or molecular methods, may identify response or progression, enabling clinicians and patients to adapt therapy accordingly. Current methods have limited accuracy, or are associated with a logistical burden or radiation exposure[49]. 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.

with lung cancer, following the elucidation of the role of such mutations in predicting response to treatment with molecularly targeted inhibitors[25,32,33].

The development of next-generation sequencing-based technologies has facilitated the interrogation of the genome at a broader scale than previously possible. 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 patients with cancer and enabled the monitoring of multiple tumour-specific mutations in a single assay[34]. This method was subsequently applied to monitor ctDNA in a cohort of patients with metastatic breast cancer[35]. Shortly thereafter, whole-genome sequencing (WGS) of plasma cfDNA was shown to identify tumour-derived chromosomal aberrations[36], focal amplifications[37] and gene rearrangements[38], and hybrid-capture sequencing was introduced as a noninvasive method of analysing the evolving genomic profile of mutations across the entire exome in cancer[39].

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[40], difficulty in obtaining sufficient material of adequate quality for genomic profiling (reported failure rates range from <10% to >30% of cases)[41,42] and sampling biases that arise from genetic heterogeneity[43–47]. The detection and monitoring of disease often relies on body fluid-based markers that often lack specificity[48], and on imaging, which exposes patients to ionizing radiation[49] and has limited resolution (in terms of 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 arise primarily from two types of information that are 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 for ctDNA, the generation of large clinical data sets, and a better understanding of the origins of both cfDNA and ctDNA. This Review highlights and explores recent advances in the field, and discusses 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[2,50–53]. Outside the blood circulation, cfDNA has been detected in various body fluids, including urine[54–58], cerebrospinal fluid[59–62], pleural fluid[63] and saliva[64]. Genetic and epigenetic modifications of cfDNA molecules reflect the genome or epigenome of the cell of origin[65–67] (FIG. 2). Methylation analysis has revealed that the majority of cfDNA in plasma is released from haematopoietic cells in healthy individuals[66–68]. These cells have been suggested to be the source of cfDNA release following intense exercise[69]. Observational studies have determined the half-life of cfDNA in the circulation as between 16 minutes and 2.5 hours[31,70–72], which enables 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[71,73] and renal excretion into the urine[54,58,74]. cfDNA uptake by the liver and spleen, followed by degradation by macrophages, may also contribute[30,75]. 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 bp, indicating that cfDNA was likely to be associated with nucleosomes[76]. Sequencing-based approaches have since refined this measurement by identifying a prominent peak at 166 bp[77,78], which corresponds to the length of DNA wrapped around a nucleosome (~147 bp) plus linker DNA associated with histone H1. Fragment size traces of cfDNA show a 10 bp ladder pattern[53,74], which is 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[74], and this is potentially related to relatively higher nuclease activity in urine[79].

ctDNA molecules are shorter than non-mutant cfDNA molecules in plasma, as demonstrated by PCR[4,80] and sequencing[67,81]. Animal xenograft experiments[78,80–82] provide an elegant means by which to interrogate ctDNA, as any human DNA sequences must have originated from the tumour xenograft. The modal length of ctDNA

Liquid biopsy
Analysis of tumour material (for example, cells or nucleic acids) obtained in a minimally invasive or noninvasive manner through the sampling of blood or other body fluids.

Digital PCR
(dPCR). An assay in which many microlitre- or nanolitre-scale PCR reactions are run in parallel within physically separated reaction chambers or as droplets in an emulsion (droplet dPCR (ddPCR)). By partitioning molecules into hundreds or up to millions of reactions, rare mutant molecules can be accurately identified and quantified.

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

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

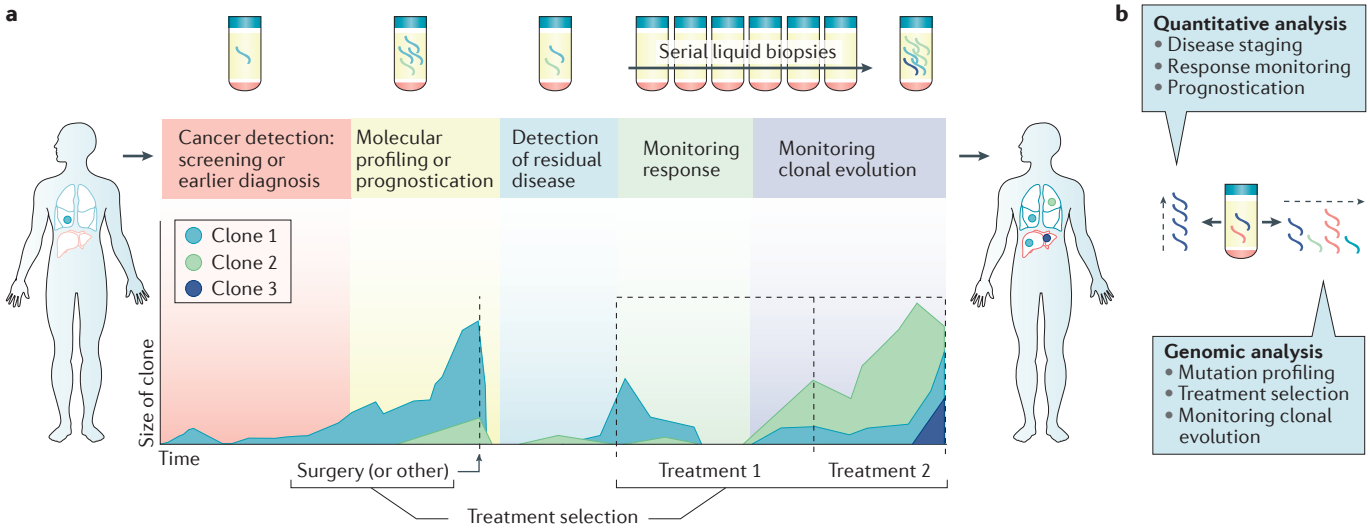

Figure 1 | **Applications of circulating tumour DNA analysis during the course of disease management. a** | A schematic time course for a hypothetical patient who undergoes surgery (or other initial treatment), has a disease relapse and then receives systemic therapy. The potential applications of liquid biopsies during this patient's care are indicated. The patient starts with one single disease focus, but multiple metastases and distinct clones (depicted in different colours) emerge following treatment. **b** | The information extracted from circulating tumour DNA (ctDNA) may be classified, broadly, as quantitative information (that is, relating to tumour burden) or genomic information. Quantification of ctDNA at a single time point may allow disease staging and prognostication, and genomic analysis can inform the selection of targeted therapies. Therefore, longitudinal analysis allows the quantitative tracking of tumour burden to monitor treatment response, for example, and by comparing genomic profiles over time, clonal evolution may be monitored.

fragments has been measured in a rat xenograft model as between 134 and 144 bp[81], although the cause of this shortening is not clear. Shortening of fragments is also observed in fetal cfDNA relative to maternal cfDNA[77] and in non-haematopoetically derived relative to haematopoietically derived cfDNA fragments in patients who have received transplants[83,84]. Differences in nucleosome wrapping or nuclease action between haematopoietic cells, which contribute most to the cfDNA pool, and other tissues may have a role. Long cfDNA fragments (>1,000 bp) have been identified in healthy individuals using long-read sequencing techniques[85] and may be released into the circulation in association with exosomes[50,51] or by tumour cells via necrosis[52]. Current extraction methods often poorly recover these long fragments[86,87] (BOX 2). Commonly used library preparation methods introduce further biases: single-stranded DNA library preparation[88] can recover DNA fragments with damaged ends, and when applied to cfDNA[65,89] this approach identified a large proportion of fragments shorter than 100 bases. Diverse extraction and sequencing methods may therefore yield complementary data. Combining those data 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)[90,91], which is a sensor of exogenous DNA fragments that is 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 and led to adipose tissue inflammation and insulin resistance[91]. Another study has suggested that cfDNA may inhibit pro-apoptotic caspases via TLR9-dependent signalling[90], which could imply a potential immunomodulatory role for cfDNA.

In vitro experiments suggest that cfDNA may be internalized by cells[2,92,93], which raises the possibility that cfDNA molecules could mediate the horizontal transfer of genes or DNA. One report showed that NIH-3T3 mouse cells that were in contact with samples of plasma from patients with *KRAS*-mutant colorectal cancers underwent *in vitro* transformation, despite being separated to avoid tumour cell contamination[94]. Another study demonstrated the integration of ctDNA into the nuclear DNA of recipient cells and suggested that this may occur through non-homologous end-joining[92]. A similar phenomenon has been observed for mitochondrial DNA[95]. Overall, it is clear that there is a lot to learn about the biology of cfDNA and ctDNA, and improved knowledge could have an important impact on their potential applications in oncology.

Approaches to ctDNA analysis

The 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[96,97] have been applied since the mid-2000s for detection of hot-spot mutations in serum and plasma[25], and some assays are available as kits that are approved for clinical use[98,99], but have limited analytical sensitivity.

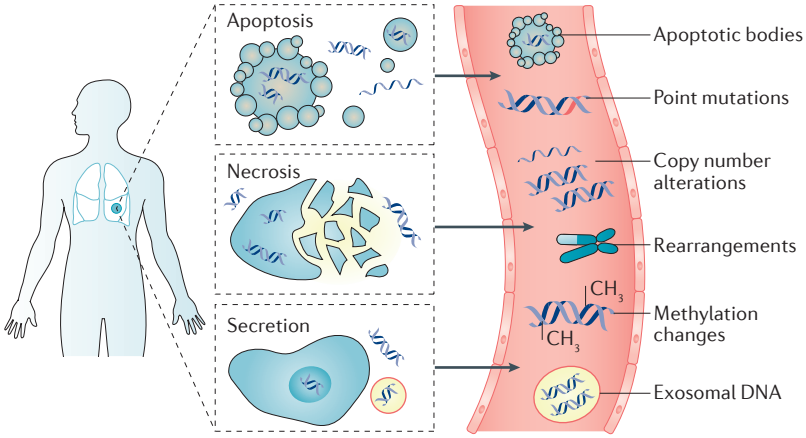

Figure 2 | **Origins and range of alterations in cell-free DNA.** Cells release cell-free DNA (cfDNA) through a combination of apoptosis, necrosis and secretion. cfDNA can arise from cancerous cells but also from cells in the tumour microenvironment, immune cells or other body organs. In the bloodstream, 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 cfDNA. Adapted with permission from REF. 215, Macmillan Publishers Ltd.

dPCR assays on microfluidic platforms are quantitative and highly sensitive, and are used extensively to quantify ctDNA levels[29,30,56,100–103]. Improved detection at selected loci has been demonstrated by methods such as single-base extension[104] or enrichment for mutant alleles by electrophoretic methods[105,106], nuclease activity[107] or modified PCR[58,108,109]. The multiplexing capacity of such assays, which 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 used[34,39,110,111]. Regions for sequencing may range from individual exons of interest (kilobases) to the entire exome (~50 Mb). Current off-the-shelf panels for gene sequencing can detect mutations with an allele fraction greater than 1%[45,112]. By reducing the background error rates of sequencing[34,113]— for example, by molecular barcoding (FIG. 3b) or running multiple replicates (FIG. 4) — ctDNA can be detected at allele fractions below 0.1%[114–117] (TABLE 1). Amplicon-based assays that have been optimized for the purpose of ctDNA analysis can target dozens to hundreds of amplicons across multiple kilobases with high sensitivity[34,115,117]. Hybrid capture-based approaches can increase the genomic region studied to dozens or hundreds of kilobases[110,111,114,118]. The sensitivity of 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[34,114] (FIG. 4).

Amplifications and deletions may be identified through low-depth (~0.1× coverage) sequencing of the whole genome, and subsequent comparison of the relative number of sequencing reads in equally sized genomic regions across a sample, or in samples versus controls[38]. Such shallow WGS (sWGS) has been used to detect fetal aneuploidies[14], and it can also be used to detect cancer-specific copy number alterations[36,38,119]. sWGS has a limit of detection of a mutant allele fraction of between 5% and 10% (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%[120].

The limit of detection for assays will vary on the basis of whether the individual's disease status and tumour mutations have already been characterized. 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 hot spots, the risk of false positives increases with the size of the panel owing to multiple hypothesis testing, and filters need to be applied to increase specificity; however, their application erodes sensitivity for rare variants. Prior knowledge of the mutation profile (for example, from tumour sequencing data) enables the detection of known patient-specific mutations above the background error rate, as opposed to calling mutations *de novo*[34]. 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[34,35,110] (FIG. 1b).

ctDNA can be quantified using various metrics, such as mutant allele concentration (that is, copies per millilitre) or mutant allele fraction[121]. Each of these metrics would be affected in a different way by analytical, pre-analytical and physiological characteristics. For example, metabolic changes in the rate of cfDNA turnover would affect the concentration of mutant alleles more than the mutant allele fraction, whereas pre-analytical factors affecting the release of germline DNA from blood cells would reduce the mutant allele fraction to a relatively greater extent. The analysis of ctDNA (both fraction and concentration)[121], as well as total cfDNA and cfDNA fragmentation[3,122], 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[78,123] and stage[124]. A study of 640 patients with various types and stages of cancer found a 100-fold increase in the median ctDNA concentration in patients with stage IV disease compared with those who had stage I disease[124]. Quantification of individual tumour mutations in each patient revealed that patients with stage I disease had fewer than 10 copies per 5 ml of plasma. In sharp contrast, patients with advanced prostate,

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 proportions of the genome, such as the entire exome. Smaller panels yield a higher sequencing depth at lower costs than do larger panels.

Molecular barcoding
The addition of unique molecular sequences to each molecule when creating a sequencing library, so that reads originating from the same molecule can be identified and the consensus taken, correcting for some PCR or sequencing errors.

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.

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

ovarian or colorectal cancer had a median concentration of 100–1,000 copies per 5 ml of plasma. ctDNA levels vary greatly even between patients with the same type and stage of disease. This variability in ctDNA concentration is partially explained by differences in the 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[121]. The mutant alleles in plasma increased in fraction by approximately 0.08%, and in concentration by 6 mutant copies per millilitre of plasma, for every cubic centimetre of disease[121]. Notwithstanding these correlations, substantial variation in ctDNA concentration may arise from interindividual differences. For example, poor tumour vascularization could hinder ctDNA release into the bloodstream or, conversely, could promote ctDNA release by producing hypoxia and cell death. Histological differences could foreseeably influence both the rate and type of cell death. Patients with primary brain tumours have very low levels of plasma ctDNA, with a median concentration for individual mutations of less than 10 copies per 5 ml of plasma[124], whereas the proportion of tumour DNA in cerebrospinal fluid was found to be significantly higher[60,61,124]. Although not directly proved, the blood–brain barrier has been suggested to impede the movement of cfDNA fragments into the circulation[60,61,124].

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[27,125–129]. 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 significantly better prognostic predictor than are commonly used tumour markers[35,121]; specifically, an increasing concentration of ctDNA correlates with poorer clinical and radiological outcomes[35,121,124,130,131]. For example, in patients with metastatic breast cancer, a significant inverse correlation was shown between ctDNA concentration and overall survival up to 2,000 copies per millilitre, with a uniformly poor prognosis above this level[35]. In addition to ctDNA levels, mutational patterns identified in ctDNA (FIG. 1b) can help to group patients into molecular subtypes with different prognoses[132].

Earlier diagnosis of disease. Diagnosing cancer at an earlier stage, particularly before metastatic spread, may allow earlier intervention and could improve survival[133]. A number of studies have demonstrated the potential for noninvasive early diagnosis. Mutations have been detected in saliva and plasma from individuals up to 2 years before cancer diagnosis[21,134], and there have been reports of incidental presymptomatic detection of cancers in pregnant women who underwent NIPT[67,135,136], as WGS can identify copy number alterations of both fetal and tumour origin. Screening in asymptomatic populations introduces the 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 investigative procedures.

In a survey across several cancer types, ctDNA was detected in 82% of patients with stage IV disease, and this fell to 47% for patients with stage I disease[124]. The method applied by the investigators was benchmarked as being able to detect one copy of an individual cancer mutation per 5 ml of plasma[124]. 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)[110]. Targeting known tumour mutations in plasma using droplet dPCR (ddPCR) assays in early-stage breast cancer showed a sensitivity of 93.3%[137]. A sWGS method adapted from an NIPT assay was recently shown to detect 6 of 16 (37.5%) cases of early ovarian cancer[138], although this approach may not perform as well in other cancer types that have fewer copy number alterations. Together, these studies outline the possibility, and the challenge, of ctDNA detection in early-stage disease.

If analysis is performed on a few millilitres of plasma that contain only a few thousand copies of the genome, increasing the analytical sensitivity beyond the range of ~1 in 1,000 may not produce any sensitivity benefit, as it becomes increasingly likely at low allele fractions that the mutation of interest may not be found owing to sampling noise. One approach may be to collect greater volumes of plasma (and more cfDNA) through methods such as plasmapheresis or implanted

Box 2 | **Pre-analytical considerations**

In low-burden disease, or certain cancer types, the concentration of circulating tumour DNA (ctDNA) molecules may be low, and any loss of sampled material could reduce the sensitivity of molecular profiling. For quantitative applications, the reproducibility of measurement is essential to achieve 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; ethylenediaminetetraacetic acid (EDTA) is preferred. PCR is inhibited by plasma from heparinized blood[232], although some studies have been able to utilize such samples[32].
- 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[68,233–236]. Tubes containing fixative agents may stabilize 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 than that in plasma, depending on tumour proximity (FIG. 3c).
- Cell-free DNA (cfDNA) extraction may be carried out using affinity column-based, magnetic bead-based, polymer-based and phenol–chloroform-based methods, or by filtration. Different methods show variation in their ability to recover particular fragment sizes[86,87], and this could have implications for ctDNA detection, given the differences in size between cfDNA and ctDNA.

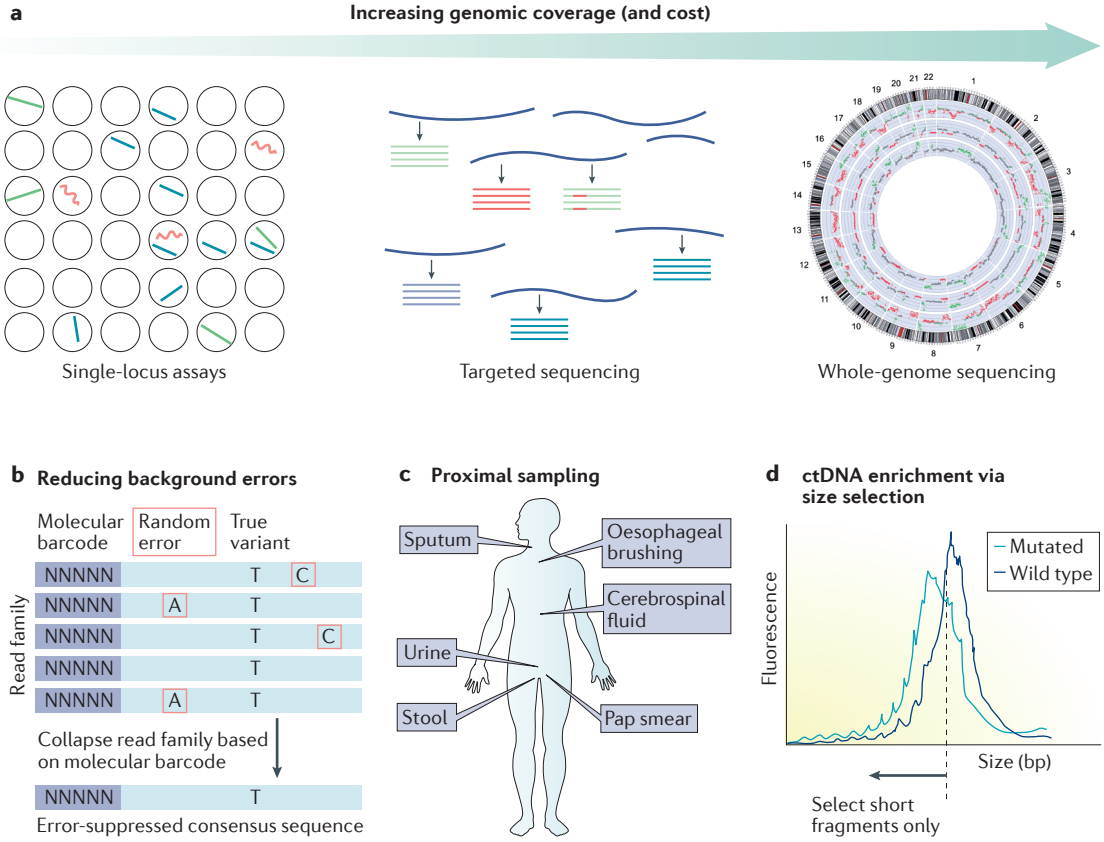

Figure 3 | **Current and future paradigms for the sensitive detection of circulating tumour DNA. a** | The analysis of cell-free (cfDNA) can range from the interrogation of individual loci to analysis of the whole genome (TABLE 1). Off-the-shelf digital PCR (dPCR) assays can achieve high sensitivity with a simple workflow but are limited by a low multiplexing capability. Targeted sequencing can allow the interrogation of multiple loci with high sensitivity through the use of methods that suppress background noise[242]. **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, thus suppressing errors arising from PCR or sequencing. **c** | To improve the sensitivity of the analysis — for example, for disease diagnosis or detection of minimal residual disease — 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 than is found in plasma. **d** | Circulating tumour DNA (ctDNA) has been shown to be shorter than cfDNA[4,80,81,84]. Thus, experimental or *in silico* selection of shorter fragments may enrich for sequences of cancer origin[81], and can improve sensitivity for samples with low proportions of ctDNA. In part **a**, the targeted sequencing image is adapted from REF. 34 (Forshew, T. *et al.* Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci. Transl Med.* **4**, 136ra68 (2012)). Reproduced with permission from the AAAS. The whole-genome sequencing image is courtesy of Dennis Lo (The Chinese University of Hong Kong) and is based on data published in REF. 37.

devices containing materials that bind cfDNA; similar approaches have been tested for enhancing the yield of circulating tumour cells (CTCs)[139]. Given these challenges, it is important that pre-analytical factors relating to the collection, processing and extraction of cfDNA are optimized (BOX 2). Alternatively, for some cancer types, other minimally invasive samples may have a higher tumour DNA content than plasma: for example, urine for bladder cancers[56]; stool for colorectal cancers[19]; cytological specimens such as cervical smears[140] or uterine lavage[141] for gynaecological cancers, or oesophageal brushings[142] for oesophageal cancers (FIG. 3c). For cancers with a viral aetiology — for example, 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 those with premalignant lesions and a high risk of cancer[143,144].

Technical and biological advances facilitate enhanced mutation detection: the finding that ctDNA is shorter than cfDNA[4,80,81] suggests the utility of experimental or *in silico* size selection (FIG. 3d). If the input amount of plasma or DNA is limiting, assays that interrogate multiple mutations in the same reaction (through broad genomic coverage or the use of patient-specific multiplexed panels) may produce a higher overall sensitivity for detecting the presence of any ctDNA[34,35,110,112,114] than could be achieved by a focused analysis of individual loci (FIG. 4). Taken to a (currently impractical) extreme,

ultra-deep sequencing of the entire genome could enable sensitive detection of cancer even from small volumes of plasma (or other fluids) in the future[36].

Although technical advances may improve the sensitivity of 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 (for example, those in *TP53*, *KRAS* and Notch pathway genes) have been found at low levels in skin biopsies from healthy individuals[145]. If non-tumorigenic clones were to increase to a 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, although the absolute risk of conversion into haematological cancer is 1%[146]. In addition, genomic alterations known to be associated with cancer have been found in plasma from healthy individuals[114,118,134,147]. Clinical outcomes for apparently healthy individuals in whom mutant DNA is detected in plasma should be characterized in order to understand the biological and clinical implications of such findings.

Cancer localization. Tissue-of-origin information from liquid biopsies might be able to aid cancer localization, for example, for cancers of unknown primary[148]. Methylation and nucleosome occupancy patterns in cfDNA have been found to encode tissue-specific and cell-specific information[65–67,149]: in one case, a pregnant woman had chromosomal abnormalities that were detectable by NIPT, and so tissue-specific methylation signals in the plasma were studied, enabling the quantification of the relative contributions of each tissue to the total cfDNA pool[67]. This revealed an increased contribution of cfDNA from B lymphocytes, which was consistent with a diagnosis

Table 1 | **Comparison and utility of technology platforms for circulating tumour DNA analysis**

Scale of analysis	Example technologies	Loci interrogated	Indicative limit of detection (mutant allele fraction or concentration)	Clinical utility
Single-locus or multiplexed assays	Microfluidic or allele-specific PCR: • Digital PCR[28,101,103,194] • BEAMing[29,30] • Intplex[3,122]	Microfluidic or allele-specific PCR: • 1–10 loci • Both ctDNA and cfDNA (Intplex)	Varies by method, optimal implementations can reach sensitivity of 0.001%–0.01% or individual mutant copies per millilitre[30,122,243,244]	• Detecting and quantifying recurrent hot-spot mutations • Monitoring for recurrent resistance mutations • Rapid turnaround time
	Enrichment for mutant alleles: • COLD-PCR[108] • SCODA[105,106] • NaME-PrO[107]	Enrichment for mutant alleles: 10–100 loci		
	Allele-specific or ARMS-PCR kits for companion diagnostics: • Cobas EGFR[99] • Therascreen EGFR[98]	• Cobas EGFR: 7 mutation assays covering multiple variants • Therascreen EGFR: 3 mutation assays covering multiple variants	Stated limit of detection (≥95% sensitivity): • Cobas EGFR: 25–100 copies per millilitre[99] • Therascreen EGFR: median 1.42% (range 0.05%–12.47% for different variants)[98]	Approved for *in vitro* diagnostic use: • Cobas EGFR: FDA approved • Therascreen EGFR: CE marked
Targeted sequencing approaches	Amplicon-based: • TAm-Seq[34] • Enhanced TAm-Seq[117] • Safe-SeqS[115]	10 kb to 50 Mb	• <0.01%–0.50% for purpose-built panels[34,111,114,115,117] • 1% for off-the-shelf multiplexed panels[45,112] • 5% for exome sequencing[39]	• 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)
	Hybrid capture: • Exome sequencing[39] • CAPP-Seq[110,114] • Digital sequencing[111,118,185]			
Genome-wide	WGS: • Plasma-Seq[38] • PARE[197]	• 3.2 Gb (whole genome) • 21.6 kb unique to LINE-1 (REF. 184)	5%–10%[38]	• Identifying structural variants • Stratifying patient samples on the basis of disease burden • Detecting the presence of chromosomal aberrations
	Amplicon-based: • FAST-SeqS[184] • mFAST-SeqS[119]			

ARMS, amplification-refractory mutation system; BEAMing, beads, emulsion, amplification and magnetics; CAPP-Seq, cancer personalized profiling by deep sequencing; CE, *Conformité Européene* (European Conformity); cfDNA, cell-free DNA; ctDNA, circulating tumour DNA; COLD-PCR, co-amplification at lower denaturation temperature PCR; EGFR, epidermal growth factor receptor; FAST-SeqS, fast aneuploidy screening test-sequencing system; FDA, US Food and Drug Administration; LINE-1, long interspersed nucleotide element 1; mFAST-SeqS, modified FAST-SeqS; NaME-PrO, nuclease-assisted minor-allele enrichment with probe-overlap; PARE, personalized analysis of rearranged ends; Plasma-Seq, plasma sequencing; Safe-SeqS, safe sequencing system; SCODA, synchronous coefficient of drag alteration; TAm-Seq, tagged amplicon deep sequencing; WGS, whole-genome sequencing.

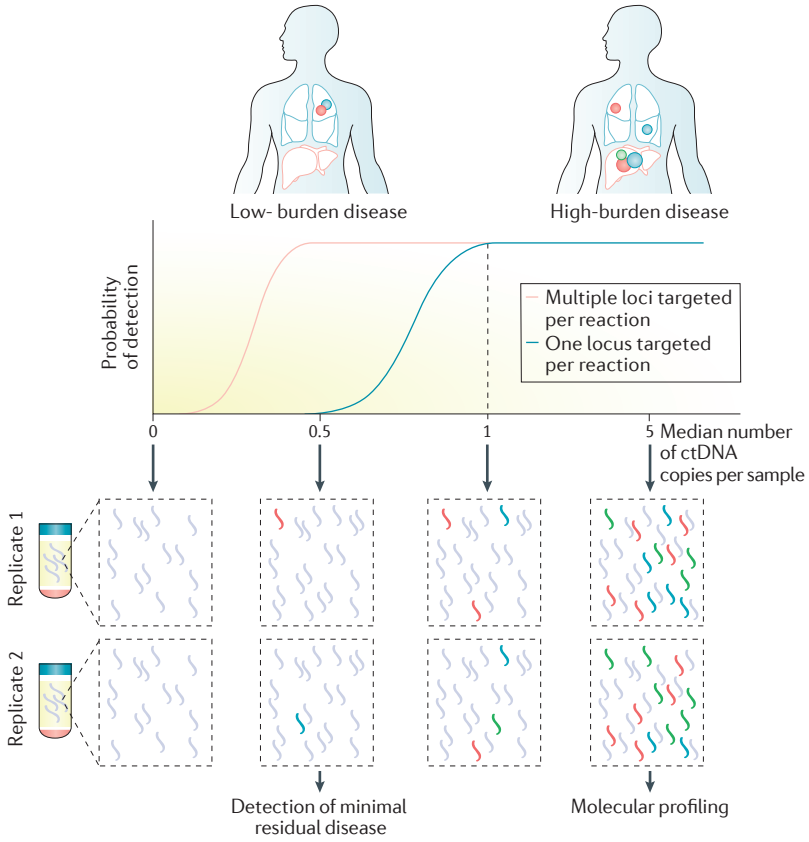

Figure 4 | **Leveraging multiple mutations to detect low-burden disease and overcome sampling noise.** Even with a perfectly sensitive assay, the probability of detecting circulating tumour DNA (ctDNA) decreases as ctDNA concentration declines, as any single mutation of interest may not be present in a given volume of sample. At low ctDNA concentrations, some mutations will be detected while others are missed owing to sampling error. Sampling multiple pre-specified mutations in each reaction may improve the detection of low levels of ctDNA, as every target provides an independent opportunity to test for the presence of a mutant molecule in the set of DNA molecules at that locus[34,114]. Sensitivity can be further improved by analysing multiple replicates that each contain few molecules, so that the mutant allele — where present in a reaction — will constitute a large proportion of the DNA template[116]. The dashed boxes below the graph show hypothetical examples of sets of molecules that may be captured by each replicate in the analysis of a sample.

of follicular lymphoma[67]. Although it may be possible in the future to deduce the tissue type of origin of a cancer in this manner, it remains to be seen whether the site or sites of metastatic spread can be determined through a measurable increase in the tissue-specific cfDNA signal.

Noninvasive molecular profiling

Analysis of heterogeneity. The extent of genetic heterogeneity has been confirmed in recent years, as multiregional sequencing studies have demonstrated clear differences between the mutation profiles of different tumour regions in the same patient[150,151], and those of different specimens from primary and metastatic sites[152]. Although the potentially confounding effects of heterogeneity are recognized, 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, and this could introduce bias to the selection and efficacy of personalized medicines. Furthermore, in a recent study of patients with lung cancer who were treated with an EGFR inhibitor, the tumour *EGFR*^{T790M} allele fraction correlated with the degree of tumour shrinkage[153], which suggests 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[44,46,61] and spatially separated disease foci[47,154–156]. Although individual tumour biopsies from different tumour regions may differ in their mutation profiles owing to intratumour heterogeneity[157,158], ctDNA analysis has detected mutations that have been missed in corresponding tissue samples[44,47,159,160]. Multiregion tumour sequencing data show that stem mutations (which are shared by all tumour regions) have a higher allele fraction in plasma than do private mutations[46,47]. Therefore, stem mutations would provide the most reliable method of tracking tumour burden in plasma. Alternatively, tracking a large set of mutations may compensate for potential biases that may be introduced by heterogeneous or private mutations.

Hot-spot mutations and gene panels. By comparing mutation detection in plasma against that in matched tumour samples, the sensitivity of ctDNA analysis has been estimated in retrospective studies as between 65% and 98%[100,102,122,156,159,161–163]. For profiling specific loci — for example, in order to stratify patients to receive matched molecular therapies — international studies have begun to demonstrate that large-scale testing is feasible and can be standardized, although the use of assays with limited analytical sensitivity resulted in low detection rates of ctDNA[164–166]. Using assays developed specifically to detect low levels of ctDNA[3], a blinded prospective study demonstrated sensitivities of 92% and 100% (and concordance rates of 96% and 100%) for *KRAS* and *BRAF* mutations, respectively, in metastatic colorectal cancer[122].

Considerable attention has been devoted to the analysis of *EGFR* mutations in patients with NSCLC[33,167], as it is often challenging to obtain tissue biopsies from these patients to help to inform treatment[40,41]. A meta-analysis of 27 selected studies published between 2007 and 2015 that used a variety of methods and comprised nearly 4,000 patients, found a pooled sensitivity of 60% and specificity of 94% for detection of *EGFR* mutations in plasma or serum[33]. 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[166,168]; these measurements were probably affected by the limited analytical sensitivity of the allele-specific PCR method used[98], as the version of the kit that was used in that study had limits of detection (at ≥95% analytical sensitivity) of 1.64% and 1.26% for *EGFR* deletions and L858R mutations, respectively[166]. Low rates of concordance of *EGFR*^{T790M} status were also observed in

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, owing to intratumour heterogeneity.

a recent phase III trial of osimertinib[169], in which tissue testing was compared with 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 millilitre of plasma[99]. Using methods with greater analytical sensitivity, higher concordance rates can be obtained[159,172]. The *EGFR*^{T790M} mutation confers resistance to gefitinib and erlotinib and frequently emerges after initial treatment with EGFR tyrosine kinase inhibitors[32,170,171]. In plasma, the sensitivity for detecting this mutation was lower than for *EGFR*^{L858R} and *EGFR*^{Ex19del} mutations (70% versus ≥90%), which occur earlier in the development of disease[159,172]. Initial data suggest that this may be due to the heterogeneous presence of resistance mutations at disease relapse[159].

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 the response rates of patients who were tissue positive[159,166,168,169,172]. Data showing the response of patients treated solely on the basis of ctDNA analysis are starting to emerge: patients who were treated with osimertinib on the basis of the detection of *EGFR*^{T790M} in plasma showed response rates that were similar to those of patients treated on the basis of tissue analysis[173]. Interestingly, objective responses were also seen in patients with very low allele fractions of mutant *EGFR*^{T790M} in plasma (<0.5%)[173].

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 to select patients with *EGFR*-mutant NSCLC for gefitinib (EMA)[174], erlotinib (FDA)[99] or osimertinib (EMA and FDA)[99,175] therapy in the event that a tumour sample is not evaluable. This could offer a pragmatic solution that provides molecular profiling information for patients while avoiding repeat biopsies for some individuals. Current recommendations[99,175] 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 independently of tumour histology, or 'umbrella trials', which assign patients to multiple investigational drugs or treatment options[176]. For example, a 54-gene panel detected ctDNA in 58% of patients across multiple cancer types[177]. Of the patients with alterations, 71.4% had at least one mutation that was actionable by an FDA-approved drug[177]. This panel is being used to test the feasibility of matching patients who have different metastatic cancer types to targeted therapies in a prospective clinical trial[178]. 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, and this allowed 28 patients (17%) to receive personalized treatment on the basis of ctDNA molecular profiling[179]. Personalized 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 on the basis of genomic analysis can improve outcomes for patients with cancer, even when patients with well-established actionable targets (for which approved drugs are available) were excluded[180].

Improvements in the analytical sensitivity of molecular profiling tools could further increase detection and concordance rates, or allow for sensitive multiplexed analysis (TABLE 1), although biological factors and heterogeneity may reduce sensitivity in some cancer types and stages[124,159,181]. 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 ctDNA carrying such mutations may be present at levels that are too low to be detectable.

Structural variants. Copy number alterations can be detected in cfDNA using WGS[36,37,182,183], amplicon-based [117,119,184] and hybrid-capture approaches[39,110,162,185]. In patients with hepatocellular carcinoma, WGS was able to identify amplifications and deletions in plasma that matched those identified in tumour tissue[37,186]. 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[37]. In a study of 80 patients with prostate cancer, androgen receptor (*AR*) copy number gain before 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 levels that is applicable across cancer types. This approach may have utility as a sample screening step in a ctDNA analysis workflow[119], such that high-burden patient samples are triaged for exome sequencing[39].

Chromosomal rearrangements in plasma can be identified through both WGS[36] and hybrid-capture sequencing approaches[38,188], although the latter may be more economical owing 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 five patients, although higher-depth hybrid-capture sequencing was necessary to identify a rearrangement between exon 1 of *TMPRSS2* (which encodes transmembrane protease serine 2) and exon 3 of *ERG*[38].

Longitudinal monitoring

Monitoring response. The short half-life of cfDNA[31,70–72], as well as the ease and reduced risk of repeating liquid biopsies relative to imaging[49] or tissue biopsies[40], 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[31,34,35,110,129]

and may identify response earlier than does clinical detection[35,189,190]. 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 when compared with imaging and other blood-based cancer markers, such as CTCs and cancer antigen 15-3 (CA15-3; also known as MUC1)[35]. In relapsed ovarian cancer, pretreatment 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 CA125 (also known as MUC16)[121].

A recent study suggested that an early spike in ctDNA levels (specifically, an increase in the allele fractions of *BRAF* mutations) in the first week following the initiation of immunotherapy in patients with melanoma 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 probably vary on the basis of the pharmacological properties of the treatments used and the biological responses to them. An early spike was not observed a few days after the initiation of treatment with chemotherapy in patients with colorectal cancer[161] or treatment with an EGFR inhibitor in patients with NSCLC[190]. If the 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 subclones could be identified very rapidly by detecting the 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 through, for example, the analysis of cfDNA released from distinct T cell clones[192].

Minimal residual disease and recurrence monitoring. Following surgery or treatment with curative intent, detection of ctDNA may signal the presence of minimal residual disease (MRD) even in the absence of any other clinical evidence of disease, and the presence of ctDNA could identify patients who may be at higher risk of relapse. Stratification of patients into high-risk and low-risk groups would enable adjuvant therapy to be given to patients who are likely to benefit most, while sparing low-risk patients from unnecessary comorbidities and the risk of adverse events. In a prospective study of 230 patients with early-stage colorectal cancer, the 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 group and 90% for the ctDNA-negative group[193]. In a separate study of 55 patients with early-stage breast cancer, assessment of ctDNA showed that the detection of ctDNA at first follow-up could also indicate poor prognosis[194]. Furthermore, stratification based on mutation detection across serial samples improved the prediction of relapse, and this and other studies have observed an interval of 7.9–11.0 months between ctDNA detection and clinical relapse[194–196], which is similar to that identified in the metastatic setting[35]. With more sensitive approaches (FIG. 4), earlier identification of clinical relapse should be possible. Combining monitoring for known mutations with molecular profiling assays could enable the 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 postoperative monitoring. Patient-specific rearrangements may be more readily detected in ctDNA at high sensitivity[197,198], as rearrangements are less confounded by background noise than mutations are. One of the challenges of individualized panel design is that sequencing an individual tumour biopsy may not sample every mutation in heterogeneous disease; therefore, sequencing of 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 the routine implementation of such assays may face challenges related to validation, regulation and quality assurance of individual assays.

Clonal evolution and resistance

As discussed above, a rising or falling ctDNA concentration may provide an indication of the effect of treatment on overall tumour burden. If multiple tumour mutations are interrogated, then the relative change between each may provide insight into the molecular evolution of a patient's cancer[39,200,201]. Ratios between the levels of different mutations in plasma can indicate heterogeneity and may be informative for the prediction of patient response to treatments that target particular alterations[159,162]. Liquid biopsies have been shown to contain ctDNA from multiple tumour sites[44,46,47,61], and their analysis can have a faster turnaround time[38,102,188] and might be less prone to biases[150,151,202] resulting from the analysis of individual tumour biopsies.

Studies demonstrate that ctDNA can be used to monitor clonal evolution and identify mechanisms of resistance to treatment[112,160,162,200,203,204]. Serial ctDNA analysis in patients with colorectal cancer demonstrated the positive selection of mutant *KRAS* clones during EGFR blockade, and a later decline in the mutant *KRAS* clones upon the withdrawal of anti-EGFR therapy[160,205]. In patients with NSCLC undergoing treatment with EGFR inhibitors, resistance-conferring mutations emerged in plasma ctDNA ahead of clinical progression[170,171]. Exome sequencing of plasma DNA may identify resistance mechanisms in patients with various cancer types[39], although the sensitivity of exome sequencing currently limits its application to patients with advanced cancer in whom ctDNA levels are high (>5% mutant allele fraction). The design of patient-specific mutation panels[34,47,114,206] could be a more cost-effective alternative

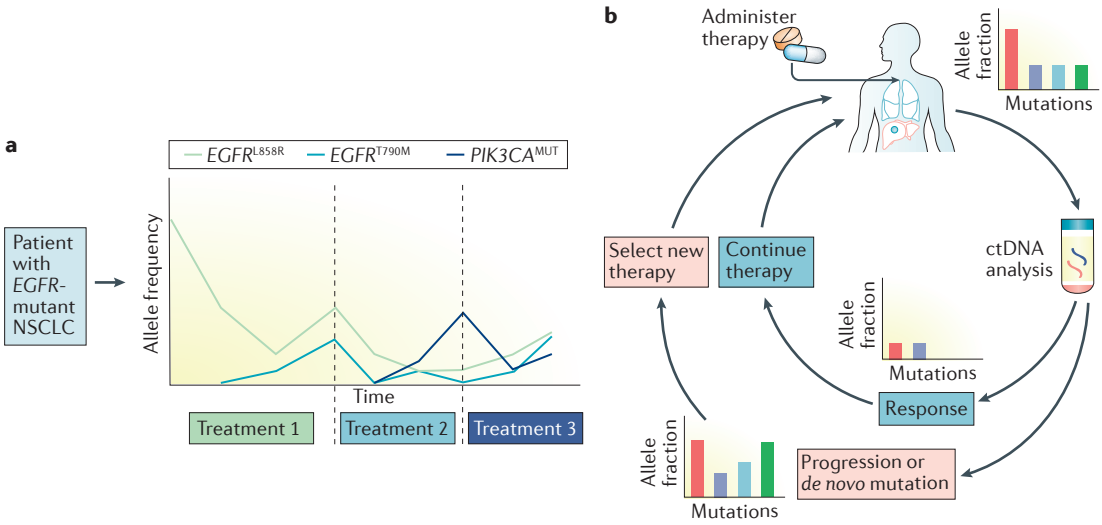

Figure 5 | **Adaptive or reactive treatment paradigms using liquid biopsies. a** | During systemic anticancer therapy, serial liquid biopsies may identify biochemical response or progression. If progression is identified, the clinician may be able to switch therapy or select a therapy to target arising clones that carry additional mutations that were identified by the analysis. **b** | This adaptive or reactive monitoring and treatment may continue as a loop, which would be facilitated by a fast turnaround time for circulating tumour DNA (ctDNA) analysis, for example through the use of point-of-care diagnostics. The time frames for this analysis can vary between hours and months; a time frame of hours could allow the analysis of early kinetics in response to therapy. *EGFR*, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer; *PIK3CA*^{MUT}, mutant PI3K catalytic subunit-α.

for high-sensitivity monitoring, although these panels 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 is adapted in real time (FIG. 5). In the clinical research setting, noninvasive monitoring could facilitate 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 further insight into cancer biology. For example, experiments in a colorectal cancer cell line that were carried out in parallel with ctDNA analysis showed that resistance mutations may arise both from 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 the simultaneous analysis of patient-derived xenografts and liquid biopsies may characterize resistance more comprehensively than the analysis of plasma alone[199].

Future directions

Proof-of-concept studies provide an excellent starting point for larger prospective studies of 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. In the future, randomized 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 will 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], and the findings of this study 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 in which 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 the 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[47]

could elucidate the contribution of individual subclones, and histological studies may clarify the factors that modulate ctDNA release. The difference in size between cfDNA and ctDNA fragments[4,67,80,81] suggests that optimizing processing and extraction methods (as well as downstream assays) for the recovery of selected fragment sizes may provide further improvement to overall performance.

Although ctDNA analysis can have increased sensitivity and specificity compared with the analysis of other circulating biomarkers[35], taking a multi-marker approach may offer a more comprehensive insight into a patient's disease[3,122,213]. For example, the total cfDNA concentration correlates with disease status[3,5] and is associated with prognosis[214]. Epigenetic analysis of cfDNA may identify cancer gene hypermethylation[27,155] or the cell type that gives rise to cfDNA fragments[65–67], and may provide information about 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 that have independent mechanisms of release (for example, through the co-isolation of exosomal RNA and cfDNA[216]) may increase the sensitivity of detecting MRD. Actively released nucleic acids may be preferred for the detection of mutations in subclones that are resistant to therapy, whereas fragments that arise 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 cfDNA in plasma alongside other fluids such as urine or cerebrospinal fluid can provide complementary information. We further echo the suggestion made by Gormally *et al.*[221] a decade ago that the characterization of proteins associated with cfDNA may provide a rich source of information about an individual's disease and about 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 have a key role in cancer management, particularly for the histological diagnosis and classification of cancers. At present, specialized laboratories handle CTC and ctDNA samples[222], although in future, hospital laboratories may carry out analysis locally if appropriate processes can be established[223].

Point-of-care devices that have clinically meaningful sensitivities for the identification of individual hotspot mutations 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 (REF. 85), 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], which makes single-nucleotide variant and indel detection challenging. Another challenge is that of sequencing short DNA fragments, which requires optimized library preparation methods[228]. Sequencing capacity is also limited (currently to ~150 Mb)[229], although this is likely to increase in the near future, and specific amplicons can 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 analysed without the relatively cumbersome and time-consuming step of DNA purification[86,231].

The initial approvals by the EMA and FDA for mutation detection in plasma as a companion diagnostic[99,174,175], and emerging ctDNA-guided clinical trials[209–211], represent key milestones towards the implementation of liquid biopsies in personalized oncology. Improving technologies are enabling an ever-wider scope for noninvasive 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 is explored further. Thus far, liquid biopsies have demonstrated potential utility across a range of applications, and they are beginning to be used for patient benefit.

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Competing interests statement

The authors declare competing interests: see Web version for details.