Integrating liquid biopsies into the management of cancer

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Abstract | During cancer progression and treatment, multiple subclonal populations of tumour cells compete with one another, with selective pressures leading to the emergence of predominant subclones that replicate and spread most proficiently, and are least susceptible to treatment. At present, the molecular landscapes of solid tumours are established using surgical or biopsy tissue samples. Tissue-based tumour profiles are, however, subject to sampling bias, provide only a snapshot of tumour heterogeneity, and cannot be obtained repeatedly. Genomic profiles of circulating cell-free tumour DNA (ctDNA) have been shown to closely match those of the corresponding tumours, with important implications for both molecular pathology and clinical oncology. Analyses of circulating nucleic acids, commonly referred to as ‘liquid biopsies’, can be used to monitor response to treatment, assess the emergence of drug resistance, and quantify minimal residual disease. In addition to blood, several other body fluids, such as urine, saliva, pleural effusions, and cerebrospinal fluid, can contain tumour-derived genetic information. The molecular profiles gathered from ctDNA can be further complemented with those obtained through analysis of circulating tumour cells (CTCs), as well as RNA, proteins, and lipids contained within vesicles, such as exosomes. In this Review, we examine how different forms of liquid biopsies can be exploited to guide patient care and should ultimately be integrated into clinical practice, focusing on liquid biopsy of ctDNA — arguably the most clinically advanced approach.

Cancers arise owing to the accumulation of molecular alterations in genes that control cell survival, growth, proliferation, and differentiation within the nascent tumour. Currently, the molecular profile of cancers is typically assessed using DNA and/or RNA obtained from a fragment of the primary tumour or a single metastatic lesion; therapeutic strategies are subsequently defined according to the molecular profile of the tissue. Importantly, however, the molecular profile of tumours evolves dynamically over time. The ability of tumours to evolve in response to a wide variety of endogenous and exogenous selective pressures has several implications: firstly, the genetic make-up of individual cancers is highly heterogeneous; secondly, within a single patient, distinct metastatic lesions can be molecularly divergent; thirdly, therapeutic stress exerted on tumour cells, particularly by targeted drugs, can dynamically modify the genomic landscape of tumours1–3. Of note, human blood samples contain materials — including cell-free DNA (cfDNA) and RNA (cfRNA); proteins; cells; and vesicles (such as exosomes) — that can originate from different tissues, including cancers. Indeed, the rapid turnover of cancer cells is postulated to result in the constant release of tumour-derived nucleic acids and vesicles into the circulation, and viable tumour cells can also separate from the tumour to enter the bloodstream. Thus, the ability to detect and characterize circulating cell-free tumour DNA (ctDNA) and/or tumour-derived RNA (predominantly microRNAs (miRNAs)), and circulating tumour cells (CTCs), has enabled clinicians to repeatedly and non-invasively interrogate the dynamic evolution of human cancers (Fig. 1). The possibility of probing the molecular landscape of solid tumours via a blood draw, with major implications for research and patient care, has attracted remarkable interest among the oncology community; the term ‘liquid biopsy’ is often used to describe this approach4.

Many studies have illustrated the potential of liquid biopsy approaches to determine the genomic profile of patients with cancer, monitor treatment responses and quantify minimal residual disease, and assess the emergence of therapy resistance. In addition to blood, several other body fluids such as urine5, saliva6, pleural effusions6, and cerebrospinal fluid (CSF)6, as well as stool6, have been shown to contain tumour-derived genetic material, and our ability to exploit liquid biopsies for diagnostic purposes will further expand in the future. The analysis of liquid biopsy specimens is, however, challenging because
REVIEWS

Key points

- Patient selection is central to the success of targeted therapy; identification of tumour-specific molecular landscapes is pivotal to guiding treatment choices
- The genomic landscape of each individual tumour is heterogeneous and changes over time as a result of the Darwinian clonal evolution imposed on cancer cells by selective pressures, including targeted therapy
- Longitudinal surveillance of clonal evolution is essential for precision medicine, but cannot be effectively achieved using tissue biopsy specimens, owing to sampling issues
- The blood of patients with cancer contains diverse tumour-derived materials, including circulating cell-free tumour DNA (ctDNA), circulating tumour cells, and exosomes
- The sampling and analysis of ctDNA or other circulating tumour components present in biological fluids, termed ‘liquid biopsy’, enables minimally invasive monitoring of tumour evolution over time in the clinic
- Two different liquid biopsy companion diagnostic tests for EGFR mutations in plasma ctDNA have been approved by the regulatory agencies in Europe and the USA for the selection of patients with non-small-cell lung cancer for anti-EGFR treatment in clinical practice

ctDNA is fragmented and highly under-represented compared with germ line cfDNA, and only a limited number of CTCs can be isolated from a blood sample. Thus, analysis of tumour material obtained by liquid biopsies requires highly sensitive assays, which became available only within the past 5 years. Herein, we provide a brief overview of the various types of tumour-derived material that can be sampled using liquid biopsies. Subsequently, we discuss how different forms of liquid biopsy can be exploited in patient care and should ultimately be integrated into clinical practice, focusing primarily on those related to ctDNA that, arguably, have the greatest clinical utility at present.

Blood-based liquid biopsies

A range of tumour components can be isolated from the blood. Molecular analysis of these different components can provide distinct and complementary information (Table 1).

CTCs: content analysis and experimental models. CTCs are tumour cells that have, presumably, intravasated or been passively shed from the primary tumour and/or metastatic lesions into the bloodstream. The existence of CTCs was first reported in 1869 by the Australian physician Thomas Ashworth, but their clinical utility was not appreciated until the late 1990s. CTCs can be isolated from the blood of patients with cancer, either as single cells or in cell clusters, and the number of CTCs detected has been associated with treatment outcomes and overall survival. The abundance of CTCs in the blood is low (approximately 1 cell per 1 × 10⁶ blood cells in patients with metastatic cancer), however, and varies between tumour types.

Multiple technologies have been implemented to isolate CTCs, and have been extensively reviewed elsewhere. Briefly, CTCs can be isolated through negative-enrichment based on their size and other biophysical properties, or by positive enrichment using markers commonly expressed on the surface of these cells, such as epithelial cell adhesion molecule (EpCAM). Of note, we still lack markers that enable CTCs to be distinguished from nonmalignant epithelial cells, although direct imaging has been used in combination with functional assays to identify CTCs. Size-based selection methods exploit the fact that CTCs are usually larger than normal blood cells. Importantly, however, this approach probably results in considerable loss of CTCs that could be overcome by using cocktails of antibodies for positive selection of these cells. Moreover, various approaches, including protein, DNA, and RNA analyses, can be applied to explore the content and facilitate the identification of CTCs.

Interestingly, once isolated ex vivo, CTCs can be molecularly characterized and used in functional assays in vitro and in vivo, in order to provide insights into the cancer biology, as reported by several groups. Notably, Yu and colleagues, isolated CTCs from six patients with metastatic luminal breast cancer and cultured them in vitro for >6 months. Establishing cell lines of CTCs derived from other cancer types has, however, proved challenging. For instance, creation of permanent cell lines using CTCs isolated from patients with colorectal cancer is ineffective, with only one successful example reported to date. By contrast, efforts to expand CTCs in immunocompromised mice have met with considerable success. Xenograft models have been established using CTCs obtained from patients with metastatic luminal breast cancer. Moreover, Hodgkinson and colleagues have demonstrated that CTCs from patients with either chemoresistant or chemoresistant small-cell lung cancer (SCLC) form tumours in immunocompromised mice, and that the response to chemotherapy in these CTC-derived xenografts (CDXs) models seem to mirror that of the donor patient. Importantly, whole-genome sequencing (WGS) analysis revealed that the isolated CTCs and corresponding CDXs had comparable genomic profiles. Using this approach, blood samples collected from a patient before and after drug-resistant disease relapse could potentially be used to generate CDX models that could subsequently be characterized and compared in order to search for new druggable targets; routine in vivo testing of the corresponding targeted therapies would also be possible, thus facilitating the development of personalized medicine strategies. The timescale required to produce such models will almost certainly preclude application of the findings to tailor therapy for the original CTC donor, but the information gleaned could be used to generate hypotheses for future research. In addition to CDX models, CTCs isolated from patients with prostate cancer have been successfully cultured as 3D organoids, which might recapitulate the molecular complexity of different prostate cancer subtypes.

Remarkably, Khoo and colleagues demonstrated an efficient approach for evaluating treatment sensitivity using patient-derived CTCs cultured in microfabricated tapered microwells coupled to a microfluidics platform. Drug screening was performed without pre-enrichment of CTC clusters, enabling rapid feedback (after 2 weeks) and, therefore, immediate intervention upon detection of drug resistance or tolerance. The CTC-culturing procedure was clinically validated using 73
Exosomes. Several types of microvesicles can be released from non-neoplastic and tumour cells. Extracellular vesicles (EVs) can be classified into two groups: the first comprises microvesicles shed directly from the cell membrane via budding; the second consists of exosomes, which are exuded via exocytosis when multivesicular bodies (MVBs) fuse with the plasma membrane. Exosomes were first described in 1983 by Pan and Johnstone, and are EVs of 40–100 nm in size that are released by several cell types into the extracellular space and a variety of body fluids. Blood cells, endothelial cells, immunocytes, platelets, and smooth-muscle cells are known to release exosomes. Exosomes can be extracted from body fluids by normal density-gradient centrifugation. Alternatively, exosomes can be isolated through ultracentrifugation, visualized by transmission microscopy, or selected based on the presence of specific protein markers, such as the tetraspanin proteins CD63, CD9, and CD81. Given their content, exosomes have important roles in exchanging molecular information between cells: they have been shown to contain proteins as well as a range of nucleic acids, including DNA, mRNAs, and miRNAs, suggesting that they can modulate the activity of the recipient cells. Exosomal miRNAs seem to modulate the activity of the recipient cells. Given their content, exosomes and other EVs could potentially be exploited as cancer biomarkers. Moreover, exosomal miRNAs seem to be involved in disease progression; for example, they can stimulate angiogenesis and promote metastasis. Harvesting of exosomes from biological fluids enables the isolation and subsequent analysis of mRNA, and thus the detection of mutations, splice variants, and gene fusions in tumours. Liquid biopsies of tumour components in the blood, including circulating cell-free tumour DNA (ctDNA) and RNAs (ctRNA), exosomes, and circulating tumour cells (CTCs), can be leveraged to capture the molecular heterogeneity of distinct tumour lesions harbouring different genetic alterations. The tables illustrate the detection of point mutations in oncogenes (G12D, G12C, and G13D mutations in KRAS; V600E mutation of BRAF; and K57N mutation of MAP2K1 (MEK1)) through candidate-gene or next-generation sequencing analysis of ctDNA in plasma. Identification of a LMNA–NTRK gene fusion is depicted in the sequence alignment. Gene copy-number variations can also be detected using fluorescence in situ hybridization analysis of CTCs isolated from the blood, as shown in the fluorescence micrograph, and via whole-exome analysis of blood-derived tumour DNA by next-generation sequencing (as demonstrated for FLT3 and ERBB2 (HER2) amplification).
gene fusions, as well as gene-expression profiling. In comparison with ctDNA fragments, of which only two copies are essentially present in the tumour cell of origin, mRNA originating from a highly expressed gene could occur in thousands of copies per cell and might be shed into the circulation (within EVs or as cfRNA) at higher concentrations; therefore, analysis of exosomal mRNA might be advantageous, especially in patients with limited amounts of detectable ctDNA.

Circulating RNAs. In 1996, the detection of circulating tumour-associated mRNA was first described in the blood of patients with melanoma, and soon after, other forms of RNAs — mostly miRNAs and long non-coding RNAs (lncRNAs) — were identified in the circulation of patients with solid cancers. The presence of tumour-derived mRNA in blood is of clinical relevance for a number of reasons, including the identification of tumour-specific gene-expression profiles. Somatic mutations in DNA only represent a subset of the molecular alterations associated with cancers, and do not fully recapitulate changes in gene-expression profiles that might result from epigenetic alterations, the effects of miRNAs, or other mechanisms. If feasible, therefore, blood-based RNA profiling of cancers could provide highly valuable information.

miRNAs are the most abundant cfRNA molecules in the blood, and can be carried in exosomes, apoptotic bodies, protein–miRNA complexes, and tumour-educated platelets (TEP). The landscape of miRNAs in blood seems to correlate with that of the solid tumours from which they originate. The amount and composition of exosomal miRNAs differs between patients with cancer and individuals without this disease, implicating miRNAs as potential non-invasive diagnostic biomarkers. Whether cfRNA originates preferentially from tumour cells, or whether cells of the haematopoietic system make a strong contribution to cfRNA levels (possibly owing to response of immune cells to the disease), remains controversial. Most of the analyses of mRNA and miRNA in blood remain exploratory, and validation in clinical studies with standardized protocols is required to substantiate the value of cfRNAs in the clinical setting. The clinical implications of cfRNA are currently unclear; an in-depth discussion of this topic is beyond the scope of this Review, and this subject has been comprehensively reviewed in this journal and in others.

An example of clinical utility is, however, provided by the NETest, which can provide insight into the activity of neuroendocrine tumours via targeted expression profiling of 51 genes using mRNA isolated from peripheral blood samples.

| Table 1 | Comparison between the applications of ctDNA, CTCs, and exosomes |
|-----------------|-----------------|-----------------|-----------------|
| Potential to fully recapitulate spatial and temporal tumour heterogeneity | Yes |
| Yes²⁴,³⁰⁴ No No | CTCs |
| Yes²⁴,³⁰⁴ | | Exosomes |
| Yes²⁴,³⁰⁴ | | |
| Assessment of pre/post-analytical variability | Yes²⁴,³⁰⁴ No No |
| Yes²⁴,³⁰⁴ | Yes²⁴,³⁰⁴ Yes³⁵ |
| Detection of somatic mutations, InDels, copy-number alterations and gene-fusions | Yes²⁴,³⁰⁴ Yes³⁵ |
| | Yes²⁴,³⁰⁴ |
| Evaluation of methylation patterns | Yes²⁴,³⁰⁴ |
| Yes²⁴,³⁰⁴ | Yes²⁴,³⁰⁴ |
| Analysis of mRNA/miRNA/lncRNA/RNA splice variants | Yes³⁵ |
| Yes³⁵ |
| Analysis of RNA expression | No Yes³⁵ |
| Yes³⁵ |
| Cell morphology and functional studies ex vivo | No Yes³⁵ |
| No Yes³⁵ |
| Demonstration of signal colocalization | No Yes³⁵ |
| No Yes³⁵ |
| Proteomics analysis | No Yes³⁵ |
| No Yes³⁵ |

“Yes” indicates that the approach is feasible, possible, and/or published studies are available; “No” indicates that the application is not feasible and/or no studies are available. CTCs, circulating tumour cells; ctDNA, circulating tumour DNA; InDels, DNA insertions and/or deletions; lncRNA, long noncoding RNA; mRNA, messenger RNA; miRNA, microRNA.
clearance of cfDNA from the blood

**Liquid biopsy of other body fluids**

Several studies have revealed the presence of tumour-derived nucleic acids in other body fluids, such as urine, saliva, and CSF (Fig. 2). In patients with lung cancer, ctDNA has also been detected in bronchial washings and pleural fluids. Indeed, the localization of the primary tumour and of any metastatic lesions seems to have a major effect on the abundance of ctDNA in different body fluids.

**Urine.** More than 20 years ago, Zhang and co-workers reported that DNA fragments corresponding to the Y-chromosome gene SRY could be detected in cell-free urine supernatant from female recipients of renal transplants from a male donor; however, why genomic DNA can be detected in the urine after renal filtration was not demonstrated until 10 years later, when analyses demonstrated that ctDNA in urine, also known as transrenal DNA (tr-DNA), is present as a result of renal clearance of ctDNA from the blood (Fig. 2). In principle, therefore, urine could be a useful source of tumour-derived DNA, particularly considering that urine collection is simple and non-invasive, and the fact that we know very little about the properties of tr-DNA in patients with cancer is somewhat surprising. Glomerular filtration of plasma is highly selective; only molecular complexes <6.4 nm in diameter and with a molecular weight of <70 kDa, equating to ~100 bp of DNA, can transit into the lumen of the nephron. The clinical condition of individual patients probably also affects the passage of nucleic acids from the blood into the urine. For example, smoking has been shown to influence the amount of tr-DNA found in urine.

Limited data are available on the correlation between tumour-derived nucleic acids in urine and in matched blood samples from patients with urogenital cancers, although the final concentrations of tumour-derived tr-DNA in the urine after glomerular filtration do not seem to reflect transport of ctDNA from the blood.

Instead, the vast majority of urinary tumour tr-DNA fragments detected in patients with urogenital cancers are thought to result from shedding of tumour cells or their breakdown products directly into the urinary tract.

According to their size, tr-DNA fragments in urine can be divided in two main groups: high-molecular-weight DNA (>1 kbp) and low-molecular-weight DNA (<100 bp). Next-generation sequencing (NGS) approaches have been applied to assess the size of tr-DNA fragments in the urine of pregnant women, revealing that most maternal and fetal tr-DNA fragments contain fewer than 100 bp, with a peak at 29 bp. As in blood, DNase I is the major DNA-hydrolysing enzyme present in urine, but the activity of this enzyme in urine is more than 100-fold higher than that in the blood, potentially explaining the greater fragmentation of tr-DNA versus cfDNA.

Owing to a high concentration of RNA-hydrolysing enzymes, mRNA is not preserved in urine; however, miRNAs are more resistant to nucleases owing to their small size (20–25 nucleotides) and can be detected in urine. Moreover, miRNAs are more stable than mrNAs, as they are often present within EVs, such as exosomes, or bound to the protein argonaute-2 (Ref. 87).

Importantly, with regard to clinical relevance, tumour-specific genetic alterations commonly detected in plasma ctDNA, such as point mutations and methylation profiles, can also be detected in urinary tr-DNA from patients with cancer. For instance, in one study, KRAS mutations in tr-DNA were detected in five of eight patients with stage IV pancreatic cancer, and in four of five patients with stage III/IV colorectal adenocarcinoma. Reckamp and co-workers reported the ability to detect mutations in exons 19, 20, and 21 of EGFR in both plasma and matched urine samples from patients enrolled on the phase I/II TIGER-X trial (NCT01526928) of rociletinib in previously treated patients with EGFR-mutated advanced-stage non-small-cell lung cancer (NSCLC). Using the mutation status of 60 evaluable tissue samples as a reference, the sensitivity of EGFR-mutation detection in urine was 72% (34 of 47 patients) for T790M, 75% (12 of 16 patients) for L858R, and 67% (28 of 42 patients) for exon 19 deletions, with higher sensitivities — comparable to those achieved with plasma — reported for samples that met the recommended volume. Remarkably, combined urine and plasma testing resulted in the identification of 12 additional T790M-positive patients, in whom T790M mutations were missed using tumour tissue analysis, indicating that urine ctDNA analysis might provide complementary information about a patient’s mutational status that is not captured by plasma or tissue tests.
At present, quantification of tumour-derived tr-DNA in urine is technically challenging, owing mainly to the low amounts present, although the continuous development of DNA-amplification and sequencing technologies will probably facilitate this approach. Moreover, we should be mindful of the possibility that liquid biopsy of urine could be preferable to the use of other body fluids, as this approach is a truly non-invasive alternative to biopsy sampling and can be performed at home by the patients themselves. Liquid biopsy of tr-DNA offers the fascinating possibility of monitoring minimal residual disease (MRD) after surgery with curative intent.

**Cerebrospinal fluid.** CSF is secreted by the choroid plexuses of the ventricles of the brain. Given that CSF is in direct contact with all the cells of the central nervous system (CNS), including any cancer cells present, this biological fluid has been exploited to profile ctDNA in patients with brain tumours (FIG. 2). Of note, although ctDNA can be detected in plasma from patients with brain tumours, individuals with brain tumours, including those with high-grade gliomas and medulloblastomas, have only low levels of ctDNA in their blood, probably owing to the presence of the blood–brain barrier. Lumbar puncture to obtain CSF is an invasive procedure, but is routinely performed in patients with brain tumours (including meningiomas, glioblastomas and medulloblastomas) as well as those with leptomeningeal carcinomatosis and lymphomas. Evidence suggests that CSF is also suitable for analysis of tumour-derived DNA in patients with tumours that have metastasized to the brain. Importantly, De Mattos-Arruda and colleagues have observed a strong association of the tumour type (grade III or IV glioma, medulloblastoma, or ependymoma) and tumour location (proximity to a CSF reservoir or the cortical surface) with the presence of tumour-derived DNA in the CSF. Considering, however, that CSF quickly circulates throughout the ventricles and spinal reservoirs, liquid biopsies of CSF obtained through lumbar puncture could potentially be exploited for ctDNA analyses.

The potential of CSF-ctDNA for characterizing and monitoring brain tumours has been investigated, in comparison with the use of plasma ctDNA. Matched samples of CSF, plasma, and tumour tissue DNA from patients with glioblastoma, medulloblastoma, or brain metastases from lung or breast cancer were analysed. Notably, ctDNA derived from tumours located in the CNS was found to be more abundant in CSF than in plasma, and CSF-ctDNA could be used to detect somatic mutations as well as to longitudinally monitor tumour burden. Additional studies are needed to compare ctDNA and cytology analysis of CSF from patients with cancer, but the two approaches will probably complement the use of other biomarkers, radiological imaging, and clinical parameters.

miRNAs can also be identified in CSF samples; in a few studies, analysis of circulating free miRNA has been exploited to diagnose primary CNS lymphoma, and exosome-derived miRNA has been used as a biomarker for monitoring therapeutic responses in patients with glioblastoma. Interestingly, CSF exosomes seem to be enriched for miRNAs relative to other types of CSF EVs (specifically, microvesicles), whereas the distribution of miRNA between different plasma EV types is less predictable.

**Saliva.** Few studies have examined the presence of tumour-derived DNA in saliva (FIG. 2). Notably, however, Wang and colleagues analysed DNA in saliva for potential tumour biomarkers in 93 patients with head and neck squamous-cell carcinoma (HNSCC). The authors hypothesized that tumour DNA might be released from the basal side of HNSCC cells. Thus, saliva and plasma samples were screened for human papillomavirus (HPV) genes or somatic mutations in genes or genomic regions commonly altered in HNSCC (such as TP53, PIK3CA, CDKN2A, HRAS, and NRAS) using multiplex PCR and massively parallel sequencing, and when both plasma and saliva were tested in combination, ctDNA was identified in 96% of patients independent of tumour site. In saliva, however, tumour DNA was found in 100% of patients with oral cancers, compared...
with 47–70% of patients with cancers of the other sites (oropharynx, larynx, oropharynx), while ctDNA was found in plasma from 80% and 100% of these patient groups, respectively. The authors, therefore, concluded that, whereas plasma is enriched for tumour DNA from other sites, saliva is enriched for tumour DNA originating from the oral cavity, making it a valuable biomarker for the detection of oral HNSCC. Importantly, in this proof-of-principle study, even early stage oral cancers were associated with highly detectable levels of tumour-derived DNA in saliva. This finding demonstrates the importance of examining specific bodily fluids according to the anatomical location of the tumour in order to achieve optimal sensitivity of ctDNA detection. If confirmed in other studies, saliva-based tests could be incorporated into diagnostic practice, to complement routine examinations, as well as disease monitoring and clinical decision-making. Saliva also contains EVs (including exosomes) that can carry distinct miRNAs implicated in oral cancers, such as oral squamous-cell carcinoma, and their identification holds promise for both diagnostic and prognostic biomarker assessments.

**Pleural effusion fluids and bronchial washings.** Pleural effusion fluid and bronchial washing samples collected with physiological saline solutions are currently used in diagnosing cancers of the respiratory system. In this setting, detection of EGFR mutations through cytology approaches is feasible, although often difficult owing to the limited number of cancer cells that are usually available for analysis. In an alternative approach, Kimura et al. assessed the feasibility of detection of activating EGFR exon 18–21 mutations in cfDNA present in pleural effusion fluid from patients with NSCLC. Their findings indicate that the EGFR mutational status can be accurately ascertained using tumour-derived cfDNA from pleural effusion fluid, and is correlated with responsiveness to EGFR tyrosine-kinase inhibitors (TKIs).

In another study, the feasibility of identifying EGFR mutations in tumour-derived DNA collected through bronchial washings, termed cytology cell-free DNA (ccfDNA), was examined. The results demonstrated the high sensitivity and specificity (88% and 100%, respectively) of this approach compared with the analysis of DNA from tumour tissue, suggesting that activating EGFR mutations can be accurately detected in ccfDNA. Thus, ccfDNA might be a valuable alternative to cytological samples, although larger investigations are needed to validate this diagnostic approach.

A limited number of studies have investigated the diagnostic, prognostic, or predictive value of miRNAs in pleural effusion fluid from patients with NSCLC. In one study, the authors found that a signature comprising five miRNAs in the effusion samples was predictive of the overall survival of patients with NSCLC and malignant pleural effusion.

**Technologies to analyse ctDNA**

As discussed, circulating ctDNA is primarily composed of germ line DNA that originates from normal cells, with a relatively small and highly variable fraction of ctDNA present in patients with cancer. As such, the sensitivity of traditional approaches to DNA analysis (such as Sanger sequencing) is insufficient for detection of somatic mutations in plasma ctDNA from patients with cancer. To overcome these limitations, digital-PCR-based technologies with a high level of analytical sensitivity and specificity have been developed, enabling high-throughput, targeted amplification of the mutant gene of interest on the background of abundant wild-type alleles, reaching limits of detection below 0.0001% (TABLE 2) — which is mandatory for the detection of rare aberrations in ctDNA.

In addition, nontargeted genome-wide analyses enable the identification of tumour-specific alterations without prior knowledge of the aberrations likely to be present in the tumour (TABLE 2); therefore, such approaches can be exploited for de novo discovery of genetic changes underlying therapy resistance and the identification of new actionable targets in patients with cancer. NGS is a technique that involves immobilization of DNA fragments on a solid support and reading of the sequence as a part of a DNA-synthesis process. Using NGS, millions of ctDNA sequences can be produced in a single reaction, and are subsequently aligned and compared with a reference genome or to the germ line DNA obtained from the same patient (that is, from nonmalignant tissue, typically peripheral blood mononuclear cells), making it possible to identify nucleotide changes (variants or mutations) relative to the reference sequence. Several NGS-based methods have now been devised that enable the detection of not only point mutations and insertions, deletions or rearrangements, but also copy-number alterations and gene fusions.

In the future, digital PCR and NGS will probably both be used complementarily in liquid biopsy analyses. The former approach enables dynamic profiling of individual mutations, but requires a priori knowledge of the mutant allele, whereas the latter technique enables the discovery of novel mutated variants, but has higher costs and cannot be readily applied to monitor patients longitudinally. Further discussions of the technical aspects of liquid biopsy are beyond the scope of this Review.

**Clinical applications of liquid biopsies**

The potential of liquid biopsy assays is far reaching, and their wide-ranging clinical applications are only starting to emerge. Liquid biopsies can be exploited for diagnostic purposes, to identify and track tumour-specific alterations during the course of the disease, and to guide therapeutic decisions. Clinical implementation will only be achievable, however, if standardized procedures are defined and large validation studies are performed (BOX 1).

**CTCs as biomarkers and their clinical utility.** At present, the clinical value of CTC analysis remains controversial, although evidence indicates that the abundance of tumour cells in the blood of patients with cancer has prognostic value, and that CTC numbers after treatment can be predictive of response to therapy and, thus, treatment outcomes. These findings must be considered...
with caution, however, because CTC numbers are highly variable between different tumour types, and are subject to biases relating to the variety of CTC-detection methods used. Moreover, the correlation between the number of CTCs detected and patient survival is far from being defined\textsuperscript{108}, and this limitation is likely to be overcome only by combining different technologies to improve assay performance.

The only FDA-approved platform for the isolation and enumeration of CTCs in patients with metastatic breast, colorectal, or prostate cancer is CellSearch\textsuperscript{110–112}, a semi-automated system that enables positive selection of CTCs based on the expression of the epithelial marker EpCAM and the lack of expression of the leukocyte-specific molecule CD45. Using CellSearch, CTCs have been shown to be present in the peripheral blood of patients with most types of carcinomas (including those of the prostate, breast, ovary, colorectum, or lung), but not in the blood of those without cancer\textsuperscript{111}. In a separate study, the numbers of CTCs detected at baseline and in subsequent blood draws were found to be good predictors of the progression-free survival (PFS) and overall survival of patients with metastatic breast cancer\textsuperscript{114}. Data suggest that CellSearch can also be applied in the nonmetastatic setting, as long as expert training and central image review is performed, with the detection of CTCs in blood from patients with stage I–III breast cancer independently predicting worse disease-free, metastasis-free, breast-cancer-specific, and overall survival, compared with the outcomes of patients without detectable CTCs\textsuperscript{115}.

The biology and clinical implications of CTCs will hopefully become better understood with the availability of more-comprehensive molecular characterization and functional analyses. RNA in situ hybridization enables differentiation of epithelial from mesenchymal cancer cells according to the expression levels of markers specific for these cell types\textsuperscript{116}; importantly, RNA can also be extracted from CTCs and sequenced, and in patients with prostate cancer, this approach has enabled the identification of specific gene fusions (such as TMPRSS2–ERG fusions)\textsuperscript{117}. In another study\textsuperscript{118}, microfluidic-based single-cell mRNA-expression analysis enabled transcriptional profiling of 87 cancer-associated and reference genes in individual CTCs from patients with breast cancer, revealing elevated expression of genes associated with metastasis and with epithelial-to-mesenchymal transition, as well as providing insights into tumour heterogeneity.

Moreover, CTCs can be exploited to investigate the presence of drug targets, as a surrogate for tumour biopsy specimens\textsuperscript{119}. In this regard, intratumoural expression of programmed cell death 1 ligand 1 (PD-L1) has been highlighted as a key factor that prevents the

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<td><strong>Approach</strong></td>
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<td>Deep-sequencing targeted</td>
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ARMS, amplification refractory mutation system; BEAMing, beads, emulsion, amplification, magnetic; CAPP-Seq, cancer personalized profiling by deep sequencing; COLD-PCR, complete enrichment amplification at lower denaturation temperature PCR; ctDNA, circulating cell-free tumour DNA; ddPCR, droplet digital PCR; EMA, European Medicines Agency; FDA, US Food and Drug Administration; iDES, integrated digital error suppression; LNA/DNA-PCR, locked nucleic acids/DNA chimera PCR; LoD, limit of detection; NA, not applicable; PARE, parallel analysis of RNA ends; PNA clamp-PCR, peptide nucleic acids clamp PCR; qPCR, quantitative PCR; SAFE-SeqS, safe-sequencing system; TAm-Seq, tagged-amplion deep sequencing; WES, whole-exome sequencing; WGS, whole-genome sequencing. *The Therascreen EGF RQ PCR kit and cobas EGR Mutation Test v2 are qPCR assays approved by the EMA and FDA, respectively, for the analysis of plasma ctDNA for EGFR mutations that determine eligibility of patients with non-small-cell lung cancer for treatment with EGFR tyrosine-kinase inhibitors. 1Semiquantitative technologies. 2qPCR allele-specific blocker qPCR involves the construction of original and specific PCR primers. 3Endpoint PCR incorporates an increased number of cycles, which enables an amplification plateau to be reached.
immune system from destroying cancer cells\textsuperscript{120}; therefore, characterization of tumour cells for PD-L1 expression will probably be fundamental to the success of this form of immunotherapy. Mazel \textit{et al.}\textsuperscript{121} have provided evidence that PD-L1 is frequently expressed on CTCs in the blood of patients with hormone-receptor-positive, HER2-negative breast cancer. PD-L1-expressing CTCs were present in 11 of 16 (68.8\%) patients with tumour cells detectable in the blood, with the fraction of PD-L1-positive CTCs varying from 0.2–100\%. This CTC-based PD-L1 assay might be used in future clinical trials for stratification of patients and monitoring of response to immune-checkpoint blockade.

\textbf{ctDNA in cancer diagnosis}. Quantitative analysis of ctDNA can be used to assess tumour burden — with diagnostic implications. For example, the amount of cfDNA present in plasma is substantially higher in patients with cancer than in healthy individuals or in patients with benign diseases, and seems to increase with tumour stage (and presumably, therefore, tumour volume)\textsuperscript{122,123}. Moreover, ctDNA measurements could potentially be used to determine if a patient is disease free after curative surgery\textsuperscript{122–124} (and ctDNA analysis will be more useful in this regard: see ‘MRD monitoring and early diagnosis of relapse — ctDNA as prognostic biomarker’). In the diagnosis of cancer, the absolute levels of cfDNA in the circulation provide limited information\textsuperscript{122–124}; however, when levels of cfDNA are coupled with identification of somatic mutations (that is, focusing on ctDNA), they provide valuable diagnostic information\textsuperscript{124,125}. Somatic mutations are tumour specific and, as a result, evaluation of these aberrations in ctDNA offers the potential for better diagnostic accuracy than can be achieved with standard protein biomarkers, such as carcinoembryonic antigen (CEA)\textsuperscript{122–124}. A number of studies have demonstrated the potential of liquid biopsy assays in the early diagnosis of cancer. For example, ctDNA analysis of Epstein–Barr virus (EBV) has been used for the early detection of nasopharyngeal carcinoma\textsuperscript{126}. Specifically, Chan \textit{et al.}\textsuperscript{126} screened 1,318 asymptomatic volunteers and detected viral DNA in 69, with further investigation uncovering the presence of nasopharyngeal cancers in three of the EBV-positive patients, demonstrating the utility of ctDNA-based screening. In a separate study, KRAS mutations were detected in 13 out of 1,098 healthy volunteers, and within 25 months, six of the 13 individuals were found to have cancer (of the bladder or respiratory apparatus)\textsuperscript{127}. An open question is how liquid-biopsy-based approaches to early detection of cancer might affect patient outcomes. Improvements would be expected through detection of tumours at an earlier stage, which are generally more amenable to curative treatment. Importantly, however, many precancerous benign conditions have been shown to carry common mutations shared with malignant tumours; therefore, highly sensitive ctDNA analyses could lead to high rates of false positives and, consequently, to overdiagnosis and overtreatment, similar to the results obtained by highly sensitive radiological screening\textsuperscript{128}.

Importantly, the results of several studies have demonstrated a high concordance between mutational profiles of candidate genes in matched tumour and plasma DNA samples from patients with breast cancer\textsuperscript{92,129,130}, colorectal cancer\textsuperscript{132,131–133}, or NSCLCs\textsuperscript{134,135}. For example, Bettegowda \textit{et al.}\textsuperscript{132} characterized a large population of patients with different tumour types using both digital PCR and NGS approaches; mutant ctDNA was identified in 75\% of 640 patients with advanced-stage pancreatic, ovarian, bladder, gastroesophageal, breast, hepatic, colorectal, or head and neck cancers, or melanoma, and in >50\% of patients with early stage cancers\textsuperscript{62}. In a study focused on pancreatic ductal adenocarcinoma, actionable somatic mutations and gene amplifications were identified in nearly 30\% of patients, with allele frequencies of mutant KRAS in plasma cfDNA varying from undetectable to 87.7\%\textsuperscript{136}. In an ongoing Canadian study (NCT02251314; Supplementary information S1 (table)), BRAF mutations in pre-mortem and postmortem plasma cfDNA, and in matched tumour DNA samples from patients with melanoma are being evaluated in order to understand the quantitative relationship between ctDNA and total tumour burden. In addition to the potential to non-invasively estimate tumour burden, the correlation between mutations present in ctDNA and tumour tissue samples is increasingly important in diagnosing specific molecular tumour subtypes, with implications for precision medicine, as discussed extensively in the following sections.

\textbf{Methylation profiles in ctDNA — predicting response to chemotherapy}. Promoter hypermethylation at specific CpG sites associated with tumour-suppressor genes occurs in many cancers; therefore, methylated ctDNA is a promising biomarker. Several studies have compared aberrant methylation in tumour tissues and matched ctDNA from blood samples, in settings such as lung, gastrointestinal\textsuperscript{137}, breast\textsuperscript{138}, ovarian, prostate\textsuperscript{139}, testicular\textsuperscript{140}, and head and neck cancer, and in most cases a good correlation was reported\textsuperscript{141}. Detection of promoter hypermethylation in ctDNA might have higher sensitivity than analyses of instability in microsatellite DNA (at unique GT/CA repeats, either mononucleotide microsatellite sites, such as BAT25 and BAT26, or dinucleotide sites, such as D2S123, D5S346, and D17S250), which could potentially be further improved if combined with mutational analysis\textsuperscript{142}. A seminal study that included both methylation and copy-number analysis at a genome-wide level was carried out by Lo’s group\textsuperscript{143}. The results indicate that copy-number alterations could be inferred from bisulfite DNA-sequencing data in patients with nonmetastatic cancer, with a diagnostic sensitivity and specificity of 74\% and 94\%, respectively.

Methylation of the \textit{MGMT} gene (encoding 6-O-methylguanine-DNA methyltransferase, an enzyme involved in repairing alkylated guanine in DNA) in ctDNA was first investigated in patients with glioblastoma multiforme (GBM): Balaña \textit{et al.}\textsuperscript{144} reported a high level of concordance between \textit{MGMT} promoter
methylation in matched tissue and serum DNA, and a potential correlation between MGMT promoter methylation and clinical response to treatment. In particular, increased serum MGMT promoter methylation was shown to predict a better response and time to progression after treatment with cytotoxic alkylating agents\textsuperscript{144}. Of note, MGMT silencing by promoter methylation has been used to identify patients with GBM or metastatic colorectal cancer who are most likely to respond to the alkylating agents dacarbazine or temozolomide\textsuperscript{145}. Assessment of the prognostic and predictive value of MGMT promoter methylation testing in plasma ctDNA and tumour tissue samples using a technology named ‘methyl-BEAMing’ (beads, emulsion, amplification, magnetics) revealed that this approach is more specific than other techniques, such as methylation-specific PCR and bisulfite pyrosequencing\textsuperscript{146}. Of note, the quantitative assays methyl-BEAMing and bisulfite pyrosequencing outperformed methylation-specific PCR in patients with metastatic colorectal cancer, thus enabling better prediction of treatment response and PFS.

**MRD monitoring and early diagnosis of relapse — ctDNA as prognostic biomarker.** In principle, liquid biopsy approaches might be well-suited to measuring MRD, as residual tumour components can be detected with high sensitivity, and data from proof-of-concept studies have shown that ctDNA levels can be used to monitor MRD following surgery or other curative treatments\textsuperscript{147,148}. Beaver et al.\textsuperscript{148} were able to detect PIK3CA mutations in DNA from plasma samples obtained before surgery in 93% of patients with localized breast cancer and a limited tumour burden; matched blood samples were also collected after surgery from 10 patients (within 14 days for five patients, per protocol, and between 15 and 72 days after surgery in the others), and ctDNA remained detectable in five of these patients, suggesting incomplete eradication of disease after surgery alone, and predicting the development of recurrent disease in one patient. Similarly, in a prospective cohort of 55 patients with early stage breast cancer receiving neo-adjuvant chemotherapy\textsuperscript{149}, detection of ctDNA in plasma after treatment predicted metastatic relapse with a high level of accuracy; further longitudinal mutation tracking increased the sensitivity for prediction of relapse, anticipating clinical relapse by a median of 7.9 months.

Indeed, ctDNA surveillance, aimed at identifying recurrence in patients with no evidence of disease after primary treatment with curative intent, is a key use of liquid biopsy in clinical trials (Supplementary information S1 (table)). The power of NGS analysis of ctDNA in disease surveillance has been evaluated in a retrospective analysis\textsuperscript{150} in 107 patients with diffuse large-B-cell lymphoma (DLBCL) who had achieved complete remission with the EPOCH regimen (comprising etoposide, prednisone, vincristine cyclophosphamide and doxorubicin) in any of three different trials that used EPOCH as a control treatment. All three trial protocols included the longitudinal collection of plasma samples at pivotal therapeutic timepoints. Clonotypes were retrospectively defined in tumour tissues for 86% of the patients. In the same patients, the clonotype was assessed by liquid-biopsy-based assays, as a measure of disease recurrence, and the findings were compared with those of CT and/or PET imaging (the gold standards for disease assessment) at matched timepoints. After a median follow-up duration of 11 years, patients who developed detectable clonotypic ctDNA had a hazard ratio for clinical progression 228 times greater than that of patients with undetectable ctDNA\textsuperscript{150}. The positive and negative predictive values (PPV and NPV) of the liquid biopsy approach were 88% and 98%, respectively, and recurrence was identified a median of 3.5 months before clinical evidence of disease, suggesting that interim liquid biopsy is a promising biomarker to identify patients at high risk of treatment failure\textsuperscript{150}.

In a landmark study, Diehl and co-workers\textsuperscript{151} used a BEAMing approach to detect mutational frequencies as low as 0.01% in ctDNA from patients with colorectal cancer, and those with MRD detected generally had disease relapse within 1 year of localized surgery. More recently, Tie and colleagues\textsuperscript{152} reported the results of a prospective trial evaluating the relationship of postoperative ctDNA levels with tumour recurrence in patients with stage I1 colorectal cancer; the recurrence rate was >10-fold higher in patients with detectable postoperative ctDNA than that of patients in whom ctDNA was undetectable, suggesting that the presence of ctDNA could be a predictive biomarker. As for the early diagnosis of cancer, whether identification of MRD-positive patients at increased risk of relapse can improve patient outcomes through early diagnosis of relapse and proactive ‘consolidative’ or ‘rescue’ treatment remains to be clarified. Monitoring of MRD also raises the possibility of de-escalating treatment in MRD-negative patients.

**Evaluating response and predicting resistance to therapy.** Similarly to monitoring MRD and thus relapse risk after definitive treatments, liquid biopsies can be applied to the monitoring of response and/or resistance to systemic therapy. For example, Dawson and colleagues\textsuperscript{153} exploited WGS and candidate-gene analysis of ctDNA from patients with metastatic breast cancer to detect mutations in PIK3CA and TP53, which had previously been identified in tumour tissue. Furthermore, the amount of mutant ctDNA was better correlated with responses to standard treatment compared with serum CA15-3 levels or CTC enumeration\textsuperscript{150}. High ctDNA levels were also correlated with unfavourable overall survival, and, importantly, ctDNA assessment was able to provide the earliest measure of treatment response in 53% of the patients analysed using all the monitoring modalities tested (increased ctDNA levels were detectable on average 5 months before the detection of progressive disease using imaging)\textsuperscript{150}. In addition, Schiavon and colleagues\textsuperscript{154} analysed ESR1 mutations in ctDNA to demonstrate the evolution of resistance during therapy in 171 patients with advanced-stage breast cancer. ESR1 mutations were detected only in patients with oestrogen receptor (ER)-positive disease previously treated with aromatase inhibitors, and were associated with a substantially shorter PFS on subsequent therapy\textsuperscript{152}. 

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In a proof-of-concept study in patients with metastatic breast or ovarian cancer, Murtaza and co-workers\textsuperscript{46} performed whole-exome sequencing of plasma ctDNA collected from patients before initiating treatment and at disease recurrence, enabling the identification of mutations that were associated with the emergence of drug resistance.

The utility of ctDNA analysis in monitoring therapeutic response has also been reported in patients with NSCLC\textsuperscript{154}, a reduction in the levels of ctDNA harbouring EGFR mutations that reflect sensitivity to EGFR TKIs was observed in 96% of the patients after the first treatment cycle, which provided an early indication of treatment response; however, the EGFR T790M mutation, which is a ‘gatekeeper’ mutation associated with resistance to first-generation EGFR TKIs, was detected in ctDNA before clinical disease progression. Moreover, ctDNA sequencing led to the discovery of novel mutations responsible for resistance to third-generation EGFR TKIs, which also target mutant EGFRs harbouring the T790M mutation\textsuperscript{154}.

Similarly, liquid biopsies have been successfully applied to identify mechanisms of resistance to antibody-mediated EGFR blockade in patients with metastatic colorectal cancer. KRAS mutations could be detected in plasma at high levels in almost 40% of patients after 6 months of treatment with cetuximab or panitumumab, whereas tissue biopsy samples obtained before the initiation of treatment did not reveal any mutations in this gene\textsuperscript{155}. Interestingly, the emergence of resistant KRAS-mutant clones could be detected up to 10 months before radiographic confirmation of disease progression\textsuperscript{155}. Indeed, Diaz and co-workers\textsuperscript{47} uncovered the presence of multiple KRAS mutations in the circulation of single patients with metastatic colorectal cancer receiving panitumumab treatment. Importantly, mathematical modelling applied to their ctDNA-analysis data indicated that colorectal cancers probably contain resistant KRAS-mutant cells before therapy\textsuperscript{155}, with subsequent selection of these resistant cells under therapeutic pressure, in a Darwinian manner. MET amplification has also been detected in the plasma ctDNA of patients with metastatic colorectal cancer and acquired resistance to EGFR blockade\textsuperscript{156}.

Mutations in the EGFR extracellular domain (ECD) that negate binding of cetuximab and panitumumab have been detected in patients with colorectal cancer who achieved a partial response or stable disease after treatment with these anti-EGFR antibodies\textsuperscript{48}. Interestingly, the same patients then received treatment with MM-151, a novel mixture of oligoclonal anti-EGFR antibodies with affinity that is not affected by ECD mutations, which led to reductions in the levels of ctDNA containing EGFR ECD mutations\textsuperscript{49}. Notably, the reduction in the allelic frequency of the EGFR p.G465E mutation observed in one patient anticipated a marked reduction in tumour volume that was measured about 5 weeks later using CT\textsuperscript{47}, thus confirming the sensitivity of the EGFR-ECD-mutant clones to MM-151. These findings illustrate the potential of ctDNA analyses for the monitoring of disease evolution to guide therapeutic decisions.

The detection of oncogenic gene rearrangements in ctDNA is another exciting area of research. For example, Russo et al.\textsuperscript{138} longitudinally monitored plasma ctDNA to assess LMNA–NTRK1 gene-fusion status in a patient with metastatic colorectal cancer during treatment with the TRK tyrosine-kinase receptor inhibitor entrectinib. In addition to monitoring response and resistance, this liquid biopsy approach enabled characterization of previously unknown mutations that confer resistance to entrectinib: two novel NTRK1 kinase domain mutations (G595R and G667C) that were undetectable in the ctDNA obtained before treatment became detectable as resistance developed\textsuperscript{138}.

Indeed, liquid biopsies can be used to monitor dynamic clonal evolution in response to selective pressures exerted by therapy (FIG. 3). For example, ctDNA has been analysed in order to genotype colorectal cancers and track clonal evolution at multiple points before, during, and after treatment with anti-EGFR antibodies\textsuperscript{6}. This study revealed that the abundance of KRAS-mutant clones that often emerge during EGFR blockade declines upon withdrawal of anti-EGFR antibody therapy\textsuperscript{7}, indicating that clonal evolution continues beyond clinical progression. Of note, ctDNA profiles of individuals who benefit from multiple challenges with anti-EGFR antibodies exhibit pulsatile levels of mutant KRAS, providing a molecular explanation for the efficacy of rechallenge therapies based on EGFR blockade\textsuperscript{1}. Beyond colorectal cancer, evidence of successful rechallenge strategies with targeted therapies can be found in patients with other tumour types, particularly melanoma or NSCLC\textsuperscript{159–163}, but the molecular evolution has not been documented using liquid biopsy approaches.

Importantly, ctDNA analyses have been instrumental in demonstrating that responses to targeted therapies can be driven by distinct resistance mechanisms arising within separate tumour lesions in the same patient\textsuperscript{1,164}. This finding suggests that evaluations of tissue and liquid biopsy samples should be integrated with radiological imaging to monitor the effect of individual oncogenic alterations on lesion-specific treatment responses. Murtaza and colleagues\textsuperscript{4} reported an extensive analysis of matched tumour biopsy specimens and plasma samples collected from a patient with ER+/HER2+ metastatic breast cancer first treated with tamoxifen and trastuzumab followed by lapatinib; tumour evolution was followed for more than 3 years. Remarkably, the findings of plasma ctDNA mutational analysis reflected the clonal hierarchy determined via sequencing of multiregional tumour tissues, and enabled tracking of varying treatment responses across different lesions\textsuperscript{6}. The results of these studies further suggest that mutations that occurred early during tumour development (clonal or ‘truncal’ mutations) are ideal candidates for monitoring tumour burden using ctDNA, as they are present in essentially all cancer cells in a single patient. Subclonal or ‘branch’ mutations that arose later in the tumour’s phylogeny might, however, have utility in informing patient stratification and, consequently, in guiding the choice of targeted treatment.
Liquid biopsy brings to the clinic the precious asset of convenience. Patient selection lies at the core of successful targeted therapy and precision medicine. At present, elucidation of molecular landscapes of tumour tissue is pivotal to guide treatment choices in clinical practice and in the context of clinical trials. Tissue biopsies are, however, fraught with issues. For example, accessibility is a problem when tumour lesions are in difficult anatomical locations, such as the mid-lung or retroperitoneum. Moreover, safety can be questionable, for instance, regarding sampling of tumours surrounding major vessels or in eloquent regions of the brain, or in patients with major comorbidities. In addition, assessments of tumour specimens can be restricted by time-related hindrances, including the reliability of molecular tests using years-old archival samples, and the unfeasibility of performing multiple longitudinal tests to follow tumour evolution and thereby expose mechanisms of secondary resistance to treatment. Evaluation of tumour-derived cfDNA in plasma, or other body fluids, clearly offers the potential to overcome these barriers. On the other hand, a limitation of liquid biopsy approaches is that transcriptome profiling and gene-expression analysis are not possible, as cfRNA is present in insufficient amounts in plasma and other body fluids. CTCs could, however, be amenable to transcriptomic studies, especially in those tumour types for which gene-expression profiles have an established role in molecular diagnosis and/or treatment choices for advanced-stage or early stage disease settings, such as breast cancer. CTCs can also be exploited to understand the plasticity of tumour biology and guide treatment decisions based on the phenotypic status of the tumour cells, rather than their genotype, as indicated by the findings of Jordan and colleagues. This group showed that CTCs from patients with originally ER+/HER– breast cancer can interconvert to a HER2+ phenotype under cytotoxic treatment, with activation of different signalling pathways in each population, without acquisition of additional genetic aberrations. Of note, HER– CTCs had activation of Notch and DNA-damage-repair pathways, therefore, displaying insensitivity to cytotoxic chemotherapy, but sensitivity to Notch-targeted drugs. By contrast, HER+ CTCs had reduced activation of these pathways, but were more proliferative. Thus, coexistence or interconversion between these states might enable tumour cells to overcome different stressors.

### Figure 3

**Monitoring clonal evolution using liquid biopsies.** Treatment with targeted therapies places tumour cells under selective pressure, thereby triggering clonal evolution that can be captured using liquid biopsy approaches. The data obtained can provide important insights into mechanisms of resistance, and can guide therapeutic decision-making. This schematic depicts the dynamic changes in the abundance of tumour-cell subclones harbouring different mutations in a patient with metastatic colorectal cancer treated with anti-EGFR antibodies. Monitoring of a clonal mutation in APC that is present in essentially all tumour cells (clone 1, blue line) tracks tumour burden, while subclonal mutations (KRAS<sup>G12D</sup>, KRAS<sup>Q61H</sup>, and EGFR<sup>G465R</sup> in clones 2, 3 and 4, respectively) provide a measure of clonal evolution during therapy. Subclonal mutations can be lesion-specific, leading to variations in responses at different disease sites. In this hypothetical patient, primary treatment with the anti-EGFR antibody leads to a substantial decline in tumour burden through targeting of the majority of tumour cells, but outgrowth of the resistant KRAS<sup>G12D</sup>-mutant subclone eventually causes tumour regrowth, necessitating a therapeutic switch. The change in therapy decreases the size of the KRAS<sup>G12D</sup>-cell population, but the resistant subclones (clones 3 and 4) with other mutations expand and drive tumour growth. A third-line of treatment restricts the growth of clone 3, but clone 4 continues to proliferate.
The integration of liquid biopsy assays into the management of patients with cancer is dependent on the accuracy of ctDNA sequencing in blood samples, defined as the positive predictive value (PPV) of plasma ctDNA levels versus ‘gold standard’ sequencing of matched tissue DNA. The clinical utility of liquid-biopsy-based techniques for treatment decision-making is well-exemplified by the determination of EGFR mutations in plasma ctDNA to guide the use of EGFR TKIs in patients with advanced-stage NSCLC — a clinical setting in which invasive diagnostic procedures are often deemed impossible. The first attempt at tissue–plasma pairwise comparison of EGFR mutations was reported a decade ago. Since then, reports from numerous studies, including IGNYTE and ASSESS (two large, multinational, diagnostic, noncomparative intervention trials), and two meta-analyses, have confirmed that EGFR-sensitizing mutations are detectable in ctDNA with high specificity (>93%), but improvable sensitivity (<70%), making plasma genotyping of NSCLCs a viable alternative to tissue-based genotyping when the latter is not feasible.

Two randomized trials of first-generation EGFR TKIs have addressed the clinical utility of liquid biopsy and ctDNA analysis for EGFR genotyping. EURTAC was the first trial to investigate ctDNA as a surrogate for EGFR testing of tissue biopsy samples and the effect of the liquid biopsy approach on outcomes. The trial randomly assigned 173 patients with activating EGFR mutations to receive erlotinib or a platinum–based chemotherapy doublet, with analysis of EGFR mutations in serum or plasma DNA included as a secondary end point. The authors developed a multiplex real-time PCR assay for EGFR exon 19 deletions and L858R mutation, and were able to show that blood and tissue biomarkers had comparable predictive power. Specifically, they found that L858R mutation, whether detected in tissue DNA or ctDNA, negatively affected median PFS and overall survival in erlotinib-treated patients compared with the outcomes of patients with tumours harbouring exon 19 deletions, as expected (L858R mutation is an established predictor of unfavourable outcome). Mok and colleagues used the cobas EGFR Blood Test to evaluate predictive biomarkers in the FASTACT-2 trial of induction chemotherapy plus either erlotinib or placebo, followed by erlotinib or placebo maintenance treatment. This test, at the time still in development, enabled the detection of G719A/C/S mutations in exons 18; 29 deletions in exon 19; S768I and T790M mutations and five insertions in exon 20; and L858R and L861Q mutations in exon 21. In the planned retrospective analysis by Mok et al., testing of samples from approximately 50% of the randomized patients revealed that baseline EGFR-mutation-positive patients who became EGFR-negative in plasma ctDNA at the end of the induction period had better outcomes, in terms of PFS and overall survival, than those who remained EGFR-mutation-positive.

Perhaps the most-valuable asset of a blood-based EGFR-mutation test is the capacity to monitor for resistance-associated mutations, such as the T790M gatekeeper mutation, because of their impact on ongoing treatment decision-making and patient survival. Importantly, the standard of care in this clinical space is rapidly changing owing to the availability of third-generation EGFR TKIs designed to have low activity against wild-type EGFR while retaining activity against EGFR isoforms with activating mutations as well as those harbouring some resistant mutations (including the T790M mutation). In a seminal study by Oxnard et al., the investigators retrospectively established that patients with T790M–positive tumours treated with osimertinib, one such third-generation EGFR TKI that binds irreversibly to EGFRs harbouring common activating mutations and the T790M resistance mutation, had equivalent outcomes whether the mutation was detected in DNA from the tumour tissue or the blood. This finding is important because repeated tissue biopsy sampling is seldom feasible in patients with NSCLC. More importantly, the Oxnard group prospectively confirmed and expanded these findings in patients with newly diagnosed NSCLC and those with acquired resistance to EGFR-TKI therapy. All patients underwent concomitant tissue and blood DNA genotyping using the cobas Blood Test, followed by longitudinal blood-based monitoring using the same test for EGFR exon 19 deletions, L858R and T790M mutations, and/or all KRAS codon 12 (G12X) alterations. The PPV of plasma ctDNA analysis was 100% for EGFR 19 deletions, EGFR L858R, and KRAS G12X, and 79% for T790M; sensitivity for the EGFR mutations was in the range of 74–88%, and was 64% for the KRAS mutations. Interestingly, sensitivity for both EGFR and KRAS was higher among patients with multiple metastatic sites and in those with hepatic or bone metastases than in patients with a single metastatic site outside of these organs. The assay also enabled detection of EGFR T790M mutations missed using tissue genotyping owing to tumour heterogeneity in patients with resistant disease. The high specificity and a short median turnaround time of testing (12 days; range 1–54 days) make this assay a promising tool for guiding precision medicine.

Early primary tumour detection (diagnosis) might well be the holy grail of liquid biopsy, coupled with assays such as those used to detect EGFR mutations in NSCLC; however, this topic can only be addressed by very large trials in (apparently) healthy individuals, and is being pursued by several screening trials worldwide. In the USA and Korea, the umbrella LUNAR trial is expected to enrol thousands of individuals to demonstrate, firstly, the feasibility and, secondly, the efficacy of early detection of breast, ovarian, lung, colorectal and pancreatic cancers. The LUNAR trial will integrate the use of ctDNA analyses, imaging, and germ line risk assessment, and has already collected samples from multiple trial sites, with reporting of pilot data expected late in 2016. In China, a cohort of 1,000 people with hepatitis B will undergo longitudinal liquid biopsy sampling to prove that aberrant shortening of ctDNA molecules can be used to indicate the presence of liver tumours before an ultrasonography-based clinical diagnosis.

On the other hand, the US FDA is investigating the manufacturers of the multiple-gene CancerIntercept Detect test, because the manufacturer was marketing the
test directly to consumers (at a cost of US$699); the FDA declared that the test constitutes a medical device that is not approved for direct marketing nor has it been adequately clinically validated within a clinical trial\textsuperscript{186}. Indeed, liquid biopsy technologies are considered by regulatory agencies, such as the FDA and the European Medicine Agency (EMA), as diagnostic \textit{in vitro} devices and are, therefore, subject to approval based on safety and effectiveness criteria. To accelerate development, advancement, and reimbursement of liquid biopsy assays of blood (and other bodily fluids), these generic rules must be translated into standards for clinical concordance, validation, and utility studies (BOX 1). The FDA have published a draft guidance document to facilitate this process\textsuperscript{187}. The position of the FDA regarding liquid biopsy technologies is crucial in view of the fact that Illumina, a prominent manufacturer of NGS apparatus, and other large companies, such as Johnson & Johnson, Qiagen, and Roche, as well as a plethora of at least 30 other new companies, are steadfastly developing diagnostic blood tests for multiple cancer types. In February 2016, the business consulting firm RNCOS estimated that the global liquid biopsy industry “could cross the US$1 billion mark by 2020” [REF. 188].

To date, however, liquid biopsy has entered clinical practice only for the management of NSCLC. In January 2015, the EMA granted \textit{In Vitro Diagnostic Medical Device (IVD) marketing approval in Europe to the therascreen EGFR RQPCR Kit}, and the following year, the FDA approved the cobas EGFR Mutation Test v2 (an updated version of the prior cobas EGFR Mutation Test) for use in the USA. Both of these IVDs can detect \textit{EGFR} mutations in plasma ctDNA with comparable accuracy to that of bidirectional Sanger sequencing of DNA from tumour tissue specimens, as established within the framework of large clinical trials of small-molecule EGFR TKIs in patients with \textit{EGFR}-mutated NSCLC.

The therascreen assay enables the detection of 19 different exon 19 deletions and three distinct exon 20 insertions (although it cannot be used to distinguish which specific deletion or insertion is present), as well as L858R, L861Q, G719X, S768I, and T790M mutations in \textit{EGFR}. In the Lung-LUX trial\textsuperscript{189}, in which the efficacy of frontline chemotherapy was compared with that of afatinib (an irreversible second-generation EGFR TKI), the overall percentage agreement (OPA) between the therascreen test and the tissue-based test results was 92.2% (95% CI 89.0–94.8%); for 27 of 28 discordant sample pairs (96%), the presence of an \textit{EGFR} mutation was detected in plasma ctDNA, but not in DNA from tumour tissue samples\textsuperscript{190}. The clinical utility of the therascreen test was confirmed in a phase IV trial of gefitinib\textsuperscript{191}, in which 12 of 201 patients with no tumour tissue available for genotyping were found to harbour \textit{EGFR} mutations using this liquid biopsy assay. On the other hand, the test had a 34% false-negative rate (36 out of 105 patients tested positive for \textit{EGFR}-mutations in tissue DNA, but had no \textit{EGFR} mutations detected in plasma ctDNA)\textsuperscript{192}. Understandably, therefore, the EMA has amended the drug label for gefitinib to state that the detection of \textit{EGFR} mutations in ctDNA should only be attempted for patients without an evaluable tumour sample\textsuperscript{192}.

The cobas EGFR Mutation Test v2 enables identification of 42 \textit{EGFR} mutations, including exon 19 deletions, exon 20 insertions, and S768I, L861Q, L858R and T790M mutations, in both tissue and plasma\textsuperscript{193,194}. The plasma test, however, is FDA-approved as a companion diagnostic for determining the eligibility of patients with NSCLC for erlotinib treatment based only on the presence of \textit{EGFR} exon 19 deletions and the L858R substitution mutation; patients who test negative for these alterations must undergo routine biopsy sampling and testing for \textit{EGFR} mutations in formalin-fixed paraffin-embedded tissue samples\textsuperscript{195}. The approval of this test was based on the results of the phase III ENSURE trial (NCT01342965) conducted in China and Southern Asia to evaluate the efficacy and safety of erlotinib versus that of chemotherapy as first-line treatment in 217 patients with stage IIIB/IV NSCLC. Plasma samples were available for 517 of 610 (86.0%) of the patients screened for inclusion, and 214 of the 217 patients (98.6%) ultimately enrolled in this study\textsuperscript{196}. Plasma samples were negative for \textit{EGFR} exon 19 deletions and L858R mutation in 98.2% (95% CI 95.4–99.3%) of tissue-negative patients; however, only 76.7% (95% CI 70.5–81.9%) of the patients who tested positive for \textit{EGFR} exon 19 deletions and the L858R mutation in tissue also had these alterations detected in plasma samples\textsuperscript{195,194}. This relatively low positive percentage agreement (76.7%) between the tissue and plasma test results is what prompted the FDA to specify that patients without detectable mutations in plasma should undergo retesting using tumour tissue (if available)\textsuperscript{196,194}. The efficacy of erlotinib based on the detection of \textit{EGFR} mutations in plasma using the cobas EGFR Mutation Test v2 was also evaluated by ‘bridging’ to the drug efficacy results of the same trial based on tissue assessment using the cobas EGFR Mutation Test v1 [REF. 194]; among the patients who tested positive for exon 19 deletion and/or an L858R mutation in plasma, those treated with erlotinib had improved progression-free survival (PFS) compared with those who received chemotherapy\textsuperscript{196}. The cobas EGFR Mutation Test v2 has also been approved by the FDA for the detection of T790M mutation in tissue samples\textsuperscript{194}, but not yet in plasma samples. Interestingly, the performance of the cobas EGFR Mutation Test v2 in detecting the T790M resistance mutation in ctDNA has been evaluated in a pooled retrospective analysis of two single-arm phase II registration studies of osimertinib (AURA extension, NCT01802632; AURA2, NCT02094261)\textsuperscript{195}; the positive percentage agreement and negative percentage agreement between the findings of tissue and plasma testings was 61.4% and 78.6%, respectively\textsuperscript{195}, which is not dissimilar to the results obtained for detection of exon 19 deletions and L858R mutations in ctDNA using the same test\textsuperscript{193,194}.

Notably, in a study conducted at the Dana-Farber Cancer Institute (Boston, Massachusetts, USA), the clinical utility of a similar approach to liquid biopsy analysis of \textit{EGFR} mutations (as well as \textit{KRAS} mutation), at diagnosis or relapse, was prospectively validated in 180 patients with advanced-stage NSCLC. Mutations were detected using a digital PCR platform with high specificity (>79%) and with a median turnaround time of only 3 days, compared...
with 12 days and 27 days for tissue genotyping of newly diagnosed and relapsed tumours, respectively. Outside of NSCLC, eligibility for treatment with targeted therapies is increasingly based on molecular inclusion and/or exclusion criteria for many cancer types, including melanoma, and colorectal and breast cancers. At present, most decisions are informed by data from tumour tissue specimens, but in the future, such assessments could potentially be replaced with liquid biopsy approaches, which might be of particular importance when surgery is not indicated and tissue biopsy samples are difficult to obtain, or when genetic heterogeneity could compound decision-making based on limited tissue specimens. Extensive research is needed, however, to prove the utility and reliability of ctDNA analysis in such diverse settings.

Of note, the preliminary results from a large validation trial of Guardant360 (REF 197), a digital DNA-sequencing technology test encompassing a broad panel of 70 genes, including all of the current clinically actionable genes with approved targeted drugs, have been presented at the 2016 ASCO Annual Meeting198. The study included 15,191 patients with advanced-stage lung, breast, colorectal, or other cancers198. Accuracy was assessed indirectly by comparing the frequencies of specific changes in ctDNA with tumour-tissue-based data from patients included in The Cancer Genome Atlas (TCGA), and directly for a subset of almost 400 patients — mostly with NSCLC or colorectal cancer — using matched plasma and tissue samples. Correlations between the TCGA and ctDNA data ranged from 92–99% across multiple cancer genes and different classes of alterations. The EGF\(\text{R}\) T790M resistance mutation, however, was only detected in plasma DNA from patients treated with EGF\(\text{R}\) TKIs — that is, it is not detectable in pretreatment samples. The matched plasma-tissue comparison showed a PPV of 87% that, importantly, increased to 98% when the plasma and tissue samples were collected <6 months apart. The Guardant360 test is quite sensitive (mutated ctDNA accounting for 0.4% of the total cfDNA in the blood could be detected), and enabled the detection of resistance mutations in EGF\(\text{R}\), ALK, and KRAS that were not detectable in the matched tumour biopsy samples in almost one-third of the patients. Overall, the test identified a molecular alteration in 64% of the patients, including 362 patients with NSCLC with no available tumour tissue, which could potentially be targeted using an FDA-approved drug or an experimental drug currently being tested in a clinical trial. In several instances, mutations were identified at progression after an initial response to a targeted agent that can be successfully inhibited with additional lines of therapy, or lead to therapeutic switching, emphasizing the potential clinical significance of repeated assessment of ctDNA for targetable mutations throughout the disease course. For example, EGF\(\text{R}\) mutations that emerge during the treatment of patients with NSCLC with erlotinib or gefitinib can be successfully targeted with rociletinib; similarly, second-generation inhibitors are available for ALK mutations that emerge after initial ALK blockade. The same principles apply to other mutations associated with a range of solid and haematological cancers.

Conclusions

Applications of liquid biopsies in oncology have emerged and developed at an incredible rate over the past 5 years. Many clinical trials performed in patients with solid tumours now incorporate longitudinal blood collections; however, most of the studies have included only small cohorts of patients. Implementation of liquid biopsy approaches in clinical practice will occur only after extensive controlled studies are performed. Currently, more than 60 trials (Supplementary information S1 (table)), with a projected accrual of more than 20,000 patients across 11 cancer types, are addressing the challenges posed by liquid biopsy. Limitations to be tackled include: the standardization of the blood collection procedure to improve the stability of samples at room temperature, thus reducing preanalytical variability; defining ctDNA quantification methods; standardization of ctDNA isolation to improve yield; and improving the sensitivity of ctDNA detection for rare molecular alterations in order to anticipate drug resistance (BOX 1).

Exploiting liquid biopsy approaches in patient screening could provide a more comprehensive view of tumour characteristics, including aggressiveness and the overall molecular landscape. Comprehensive implementation in the clinical setting will probably include the initial analysis of a subset of candidate genes for known hotspot mutations using PCR-based approaches, as a benchmark test and to confirm the presence of sufficient amounts and quality of ctDNA, followed by broader ctDNA sequencing to uncover actionable therapeutic targets.

In conclusion, the next generation of ‘liquid biopsy’ studies will be key to definitively establishing the clinical applicability of blood-based genomic profiling. Liquid biopsy approaches will probably provide improved diagnostic power, but a key question remains: will liquid-biopsy-driven treatments translate into improved outcomes?

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