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► To cite this version:

Javier Pascual, Gerhardt Attard, François Clément Bidard, Giuseppe Curigliano, Leticia de Mattos-Arruda, et al.. ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group. *Annals of Oncology*, 2022, 33 (8), pp.750-768. 10.1016/j.annonc.2022.05.520 . hal-03775791

HAL Id: hal-03775791

<https://hal.science/hal-03775791>

Submitted on 31 Oct 2022

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SPECIAL ARTICLE

ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group

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Available online 6 July 2022

Circulating tumour DNA (ctDNA) assays conducted on plasma are rapidly developing a strong evidence base for use in patients with cancer. The European Society for Medical Oncology convened an expert working group to review the analytical and clinical validity and utility of ctDNA assays. For patients with advanced cancer, validated and adequately sensitive ctDNA assays have utility in identifying actionable mutations to direct targeted therapy, and may be used in routine clinical practice, provided the limitations of the assays are taken into account. Tissue-based testing remains the preferred test for many cancer patients, due to limitations of ctDNA assays detecting fusion events and copy number changes, although ctDNA assays may be routinely used when faster results will be clinically important, or when tissue biopsies are not possible or inappropriate. Reflex tumour testing should be considered following a non-informative ctDNA result, due to false-negative results with ctDNA testing. In patients treated for early-stage cancers, detection of molecular residual disease or molecular relapse, has high evidence of clinical validity in anticipating future relapse in many cancers. Molecular residual disease/molecular relapse detection cannot be recommended in routine clinical practice, as currently there is no evidence for clinical utility in directing treatment. Additional potential applications of ctDNA assays, under research development and not recommended for routine practice, include identifying patients not responding to therapy with early dynamic changes in ctDNA levels, monitoring therapy for the development of resistance mutations before clinical progression, and in screening asymptomatic people for cancer. Recommendations for reporting of results, future development of ctDNA assays and future clinical research are made.

Key words: circulating tumour DNA (ctDNA), liquid biopsy, precision medicine

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INTRODUCTION

Liquid biopsies (LBs) are a broad concept that encompasses the analysis of circulating nucleic acids, tumour cells or exosomes as a tool to molecularly profile tumours to guide clinical decision making.¹ LB technologies are rapidly

advancing with increasing evidence of clinical utility. To be routinely implemented in the clinic, analytical and clinical validity must be shown, clinical utility demonstrated, and quality requirements must be met with reporting standards clearly defined and supported by evidence.² In this recommendation manuscript, we address these critical considerations focusing specifically on assays for circulating tumour DNA (ctDNA) detected in plasma as LB analyte, and highlight the evidence that ctDNA assays have sufficient evidence to be used routinely in clinical practice to genotype advanced cancers to direct molecularly targeted therapies. We acknowledge the value of other LBs such as assays of circulating tumour cells (CTCs) and different species of circulating RNAs or extracellular vesicles as well as the use of ctDNA detected in other biological fluids like urine, saliva or cerebrospinal fluid, although these will not be covered by this recommendation. We suggest the reader refer to the glossary at the end of the document for detailed definitions of commonly used terms in the field of LBs.

METHODS

To provide some consensus over the many unstandardised aspects of genomic testing using ctDNA and its potential use in clinics, the European Society for Medical Oncology (ESMO) Precision Medicine Working Group convened a group of experts to provide recommendations. The group reviewed the many different technical aspects of ctDNA assays that need to be taken into account when interpreting a positive or negative result, aiming to provide some quality standards required for decision making when using laboratory developed or commercial ctDNA assays. The group of experts then reviewed the evidence of ctDNA as a tool in the many different phases of cancer care, giving some general and tumour-specific recommendations. We also give some insight about what to expect on ctDNA testing in future scenarios like clinical trial design or cancer screening.

TECHNICAL CONSIDERATIONS OF CTDNA ANALYSIS

Introduction and challenges of ctDNA analysis

Plasma DNA constitutes DNA fragments bound to proteins that protect the fragments from degradation in blood. In healthy people, plasma DNA arises primarily from cells of the haematopoietic lineage,³⁻⁶ with concentrations ranging from negligible amounts to up to 100 ng of plasma DNA/ml of plasma.⁷ The release of plasma DNA preferentially occurs as a result of cell death, apoptosis and necrosis, however other biological processes may contribute.⁸ In cancer patients, a variable fraction of plasma DNA derives from tumour (i.e. ctDNA fraction).⁶ Theoretically, ctDNA represents a mix of DNA released by the many different tumour subclones, capturing the heterogeneity of a given cancer, and therefore giving a better description of the genomic landscape that characterise the tumour.

The DNA fragments released by normal and tumour cells appear to differ modestly in size; whilst normal cell-derived plasma DNA fragments display a peak around 166bp, consistent with the size of DNA wound on a nucleosome

and linker, ctDNA fragments are enriched for smaller fragments, around 143-145bp, perhaps consistent with the size of a mononucleosome without the linker.^{9,10} The size, genomic location and epigenetic marks of ctDNA can provide information to distinguish normal from cancer samples and to define the site of origin or location of the tumour.

Plasma DNA has a relatively short half-life. Studies of foetal DNA in maternal plasma demonstrate clearance in two phases (i.e. a rapid phase happening in the first 10 min to 1 h and a slow phase with a half-life of 13 h).¹¹ In cancer patients, the ctDNA half-life appears to be <2 h.^{12,13} Clearance of plasma DNA takes place primarily in the liver, although kidney may clear smaller fragments.¹¹

ctDNA has multiple potential clinical applications, including its use for screening, characterisation of early disease, detection of molecular residual disease (MRD) after definitive local treatment, prediction of relapses, genotyping advanced cancer, early assessment of treatment efficacy, monitoring of response and identification of mechanisms of resistance to therapy (Figure 1).

In cancer patients, the ctDNA fraction varies according to tumour features, including tumour site, disease burden, rates of proliferation and apoptosis, extent of necrosis, inflammation, tumour microenvironment as well as host-related phenomena. Germane to the success of ctDNA analyses is the appropriate selection of the type of ctDNA assays to address specific scientific and clinical questions. At present, there is no single ctDNA assay that would be fit for all purposes (e.g. early detection, MRD analysis, genetic alteration identification and assessing tumour genetic heterogeneity, identifying molecular mechanisms of resistance and subsequent decay). For example, ultrasensitive assays (e.g. methylation pattern-based or patient-specific sequencing assays) requiring limited amounts of ctDNA may be needed in the context of early disease detection, monitoring of patients with early-stage disease or MRD analysis, whereas a different set of assays may be required in the context of the identification of therapeutic resistance-related mutations and tumour genetic heterogeneity in the metastatic setting. Different assays have distinct limits of detection (LoD; the lowest analyte concentration likely to be reliably distinguished from the noise) and limits of quantitation (LoQ; the lowest level of analyte where the concentration can be accurately determined), which ultimately determine the amount of plasma DNA and ctDNA fraction needed for an informative result to be provided.¹⁴

Pre-analytical variables

The analysis of ctDNA requires the comprehension of pre-analytical and analytical parameters that may impact the accuracy and reproducibility of the results in a given clinical context. The main pre-analytical variables are patient-specific factors affecting ctDNA release, sample volume, the collection tube, storage conditions and processing. Patient-specific factors include physiological conditions (e.g. strenuous exercise), inflammation as well as acute and chronic medical conditions. Levels of ctDNA and ctDNA fraction may be

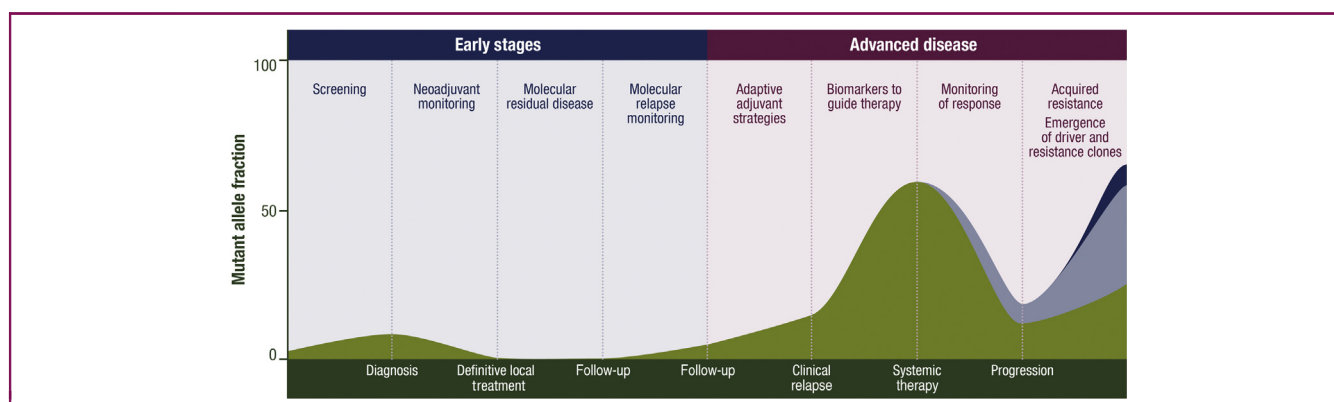


Figure 1. Clinical applications of ctDNA assays for patients with cancer and expected DNA levels in different phases of the disease. ctDNA, circulating tumour DNA.

affected by treatments including chemotherapy, targeted therapy, immunotherapy and radiation therapy and can be divided into two phases: acute changes (days to weeks) that arise from the direct impact of treatment on tumour and normal cells, and longer-term dynamics (weeks to months) that relate to tumour shrinkage by treatment. Hence, the timing of plasma collection should be carefully planned depending on the scientific question and clinical context. The quantity of plasma DNA available to test is directly proportional to the volume of plasma extracted from and therefore volume sampling must be carefully planned in advance, to ensure that sufficient analyte is available to address the clinical question. Cell preservation tubes that prevent the *ex vivo* rupture of leukocytes and other cells can allow extension of the time required to plasma extraction from 4 to 6 h (in EDTA K2 tubes¹⁵) to several days. Care should be taken not only to avoid the lysis of leukocytes during sample collection and processing, but also in the selection of collection tubes and standard operating procedures (SOPs) that would be compatible with the ctDNA assays to be deployed. Variation in blood storage duration in ambient temperature should be minimised as much as possible. The methods for plasma DNA extraction and quantification should also be taken into account and selected on the basis of their compatibility with the assays to be used. Processing of the samples should always follow validated SOPs and be preferentially carried out in dedicated areas of laboratories minimising the risk of contamination.

Different modalities of ctDNA assays, based on the type of alterations detected [e.g. copy number alterations (CNAs), somatic point mutations, epigenetic features and fragmentomics] or detection technology [e.g. next-generation sequencing (NGS), droplet digital PCR, mass spectrometry-based technology and others] may be differently influenced by pre-analytical variables. Studies by individual investigators^{16,17} and consortia^{18,19} have sought to provide guidance as to the pre-analytical parameters and SOPs.

Analytical variables and considerations

False-negative results. Many patients, even with relatively extensive advanced cancer, may have low levels of ctDNA in

their plasma. The inability to detect a variant in ctDNA testing might be due to absence of the tested variant(s) in the patient's cancer (a true negative) or low ctDNA levels or fraction that prevent detection of the variant (a false-negative).

Multiple technical factors also need to be considered in the possibility of a false-negative test result, such as the amount of plasma DNA analysed, and the assay sensitivity which could vary between different types of variants. For example, the ability of a test to detect a single nucleotide variant (SNV or mutation), may be different from a structural variant (e.g. gene fusion), or CNA (e.g. amplification). A ctDNA assay that detects SNVs, and small insertions or deletions, with high sensitivity, may have reduced sensitivity for detection of copy number variations or gene fusions. In tissue-based testing, RNA-based assays are being increasingly used for detection of gene fusions and splicing variants, and such variants may be detected less sensitively with ctDNA assays, even when sufficiently high purity ctDNA is present. Variants such as loss of heterozygosity, as well as low level copy number gains or losses, are technically more difficult to detect in ctDNA assays, with low sensitivity in many current assays.

The ctDNA concentration in plasma correlates with tumour stage and volume.^{20,21} Several clinical and pathological factors are also associated with ctDNA levels. For example, in non-small-cell lung cancer (NSCLC) higher plasma ctDNA concentrations were found in patients with squamous cell carcinomas versus adenocarcinoma, and in cases with higher proliferation index and necrosis.²¹ The anatomical site of the tumour also correlates with ctDNA levels in plasma [intrathoracic versus extrathoracic for NSCLC; liver versus peritoneum versus lung for colorectal cancer (CRC)], with brain tumours associated with the lowest levels.^{20,22-25}

Interpretation of ctDNA test results must therefore take into account the possibility that the plasma sample does not contain sufficient levels of ctDNA to detect different types of variants. New approaches based on tumour fraction estimation using, for example, CNAs, DNA methylation patterns or plasma DNA fragment patterns, may increase the possibility of giving confident negative results,²⁶⁻²⁸ by ensuring that sufficient tumour-derived DNA is present.

Some examples of non-informative and false-negative results are given in [Supplementary Figure S1](#), available at <https://doi.org/10.1016/j.annonc.2022.05.520>.

Biological false positives—clonal haematopoiesis and germline variants. Throughout life, somatic alterations accumulate in normal tissues due to multiple causes such as environmental and chemical exposures, or replication errors during cell division.²⁹ If these somatic alterations confer a selective growth advantage, it may cause clonal expansions, resulting in somatic mosaicism, and increasing the probability for sequential driver mutations and potentially the development of cancer. Plasma DNA in blood originates mainly from apoptotic haematopoietic cells,³⁰ and therefore clonal expansions in these cells risk false-positive ctDNA results, and this risk is prone to change over time. The frequency of this clonal haematopoiesis of indeterminate potential (CHIP) increases with age, previous systemic cancer treatment and smoking.³¹⁻³⁴ The presence of CHIP, and certain associated findings (number of variants, allele fraction and genes involved), is associated with higher risk of a subsequent haematologic malignancy and cardiovascular disease, and possibly adverse outcome in advanced cancers.^{33,35}

Genes mutated in CHIP partially overlap with solid tumour drivers. It has been conceptually demonstrated that CHIP variants are detectable in plasma DNA and could lead to false-positive calls in CHIP-related genes (e.g. *TP53*, *ATM*) in genotyping assays of a large fraction of advanced prostate, lung and breast cancer patients.^{6,36-38} These false positives can be largely excluded with sequencing of white blood cell (WBC) DNA, or minimised by paired sequencing of a tumour tissue sample,^{6,36,37} although the vast majority of currently commercially available assays approved for clinical use in Europe and the USA only analyse plasma DNA. Recent papers are developing lists of genes that are altered in CHIP.³⁹ Many gene alterations are very unlikely to be false-positive findings due to CHIP even without analysing WBC DNA. Examples are hotspot mutations in *VHL* or *SPOP* mainly occurring in kidney and prostate cancer, respectively, *EGFR* mutations in lung cancer patients and *PIK3CA* and *ESR1* ligand-binding domain mutations in breast cancer. Others, such as *KRAS* mutations can occur in CHIP, but at low incidence, so that *KRAS* mutations in colorectal and lung cancer patients are substantially more likely to be true positives than false.

In contrast, plasma DNA-only profiling is challenging for many tumour suppressors (such as *TP53*) or genes associated with DNA repair (*ATM* and *CHEK2*), and essentially uninterpretable for genes commonly mutated in CHIP such as *DNMT3A* and *TET2*. This is a non-negligible problem, as recently demonstrated in a small cohort of 69 men with metastatic prostate cancer in which almost half of the detected DNA repair deficiency variants originated from CHIP.⁴⁰ In addition, another clinically approved plasma DNA-only assay was applied on 3334 men with metastatic prostate cancer in which variants in *ATM* and *CHEK2* occurred with double frequency in ctDNA relatively to the expected frequency from tumour tissue.⁴¹ For clinical

genotyping ctDNA assays interrogating genes commonly harbouring CHIP variants, or for clinically actionable tumour suppressor genes such as DNA repair genes, synchronous profiling of plasma DNA and WBC DNA is therefore recommended. If this is not possible, we encourage conservative reporting to encompass only those variants estimated to occur in the same variant allele frequency range as a confident disease-specific alteration (e.g. a truncating *ATM* mutation with a similar variant allele frequency as a *SPOP* mutation in metastatic prostate cancer), especially in the lower ctDNA fraction ranges (0.1%–5%) in which CHIP variants will accumulate and the sensitivity to detect disease-specific alterations will decrease.^{6,37} It is not possible to use allele fraction alone to differentiate CHIP from ctDNA, as true-positive tumour-derived variants are often of low allele fraction. This was highlighted by the median true-positive variant allele frequency of 0.41% in a study on 21 807 patients using a clinically approved plasma DNA-only assay for *de novo* genotyping of advanced cancer,⁴² and low VAF at this time cannot be interpreted as inferring a variant is less likely to be somatic and clinically relevant.

Consideration should also be given for variants being biological true positives in patients being evaluated with a ctDNA test (e.g. a patient with advanced carcinoma who has a *JAK2* V617F mutation in ctDNA may have an undiagnosed myeloproliferative disorder). Indeed, ctDNA profiling has the potential to identify specific alterations of occult second malignancy in patients managed for another cancer.

Reporting standards

Reporting recommendations are inherently dependent on the ctDNA assay type and intended use of the test. In addition to general clinical molecular laboratory reporting recommendations,^{43,44} recommended reporting elements and approaches with particular relevance to ctDNA assays are included in [Table 1](#) and [Supplementary Table S1](#), available at <https://doi.org/10.1016/j.annonc.2022.05.520>. Reporting of pre-analytical parameters, like sample acquisition date and treatment exposure at the time of acquisition, is recommended. Reporting language should convey the potential for discordance with tumour testing, especially in cases where a variant is not detected in plasma DNA, by using language such as ‘non-informative or not detected’, instead of ‘negative’. Assays that are able to measure ctDNA fraction/purity in plasma DNA should communicate the ctDNA fraction and assist clinicians in estimating the likelihood of whether failure to detect a somatic variant is due to the variant not being present in the tumour or from insufficient ctDNA in the specimen.

It is likewise important to alert clinicians to variants detected in ctDNA assays which may be germline or contributed by non-tumour origins (e.g. CHIP). Analyses, confirmatory testing, patient counselling and reporting of potential germline variants should generally follow ESMO recommendations for germline-focused analysis of tumour-only sequencing⁴⁵ ([Table 1](#)). Likewise, reporting of ctDNA

Table 1. Recommended reporting elements and approaches for ctDNA assays	
Reporting element	Examples and considerations
Pre-analytical variables	Date of sample acquisition and treatment exposure (on/off treatment) at time of acquisition should be reflected.
Result	Cases where a variant is not detected are reported as 'non-informative' or 'not detected', instead of 'negative'.
Potential germline variants	Follow recommendations from ESMO Precision Medicine Working Group on germline-focused analysis of tumour-only sequencing. ⁴⁵ This includes: <ul style="list-style-type: none"> Flagging deleterious and/or pathogenic variants in genes associated with heritable cancer predisposition that are identified at an allele frequency consistent with germline origin. Providing patient informed consent before follow-up clinical testing of germline DNA to determine whether the variant is germline or somatic.
Variants potentially associated with CHIP	Variants in genes commonly implicated in CHIP should be flagged to caution the clinician about the potential non-tumour origin of these variants.
Variant allele fractions for quantitative assays	Variant allele fractions should be reported as they may provide information suggestive of possible germline origin, clonal relatedness of variants in the same panel and the potential for a false-positive result.
Targeted variant or regions examined by assay	This could range from a single variant for digital PCR assays (e.g. <i>EGFR</i> , c.2369C>T, p.T790M) to hundreds of genes for an expanded NGS-based panel.
Variant type and/or genomic features detected by assay	SNVs, small insertions/deletions, amplifications, copy number losses, gene fusions, MSI, TMB and LOH.
Limit of detection for different variant types	The limit of detection for each variant type should be determined and reported, ideally with an associated confidence interval. In cases where input plasma DNA is limiting, the reported sensitivity is adjusted or a warning is inserted in the report.
Assay limitations	Currently, many ctDNA assays have a substantial amount of discordance with tumour testing, so reporting language should communicate this potential discordance, especially in cases where a variant is not detected.

CHIP, clonal haematopoiesis of indeterminate potential; ctDNA, circulating tumour DNA; LOH, loss of heterozygosity; MSI, microsatellite instability; NGS, next-generation sequencing; SNVs, single nucleotide variants; TMB, tumour mutation burden.

variants in genes commonly implicated in CHIP should alert and caution the clinician about the potential non-tumour origin of these variants.^{6,31} If follow-up testing of leukocyte DNA is available to determine whether the variant is associated with CHIP, this should be noted in the report.

Technical considerations of ctDNA analysis recommendations

- The timing of blood sampling intended for ctDNA analysis should be carefully selected depending on the clinical question, as different factors can affect release of ctDNA (e.g. treatments being received, concurrent inflammatory processes, surgery). For the purpose of detecting MRD after surgery this should ideally happen at least 1 week after surgery, and likely 2 weeks or longer for major surgeries

that have longer healing times. For the purpose of advanced cancer genotyping, blood sampling should be avoided during active therapy of responding or non-progressing tumours to minimise false-negative results.

- Care should be taken in choosing collection tubes for blood samples, which will depend on time-to-processing and the assay used.
- If plasma is stored before DNA extraction, this should be at -80°C for long-term storage, with minimal temperature variation and successive freeze-thaw processes should be minimised as much as possible.
- False negatives (non-identification of a variant of interest when actually present in the tumour) is an important issue for ctDNA assays, and can be a result of low levels of plasma DNA analysed, insufficient assay sensitivity or 'non-shedding' from the tumour.
- CHIP is a common cause of false positives in ctDNA testing, when interrogating genes that commonly harbour CHIP variants. For clinically actionable testing of tumour suppressor genes such as DNA repair genes, synchronous profiling of plasma DNA and WBC DNA is recommended. For such testing, routine collection of buffy coat (enriched for WBC) from patients undergoing plasma ctDNA testing is recommended, to have available material to rule out CHIP if necessary.
- Pathogenic germline variants in cancer susceptibility genes may be detected in ctDNA (such as *BRCA1*, *BRCA2*, *PALB2*), and detection of such variants requires reflex germline testing with a validated assay to confirm somatic versus germline nature.
- Clinical genotyping assays should in the future be adapted to assess tumour purity to allow confident predictions of undetected results, and allow confident true-negative predictions. This could occur through informatic analysis, for example detection of an alternative tumour, and not CHIP, derived mutation at sufficiently high allele fraction, or orthogonal purity analysis.

POTENTIAL CLINICAL INDICATIONS—EVIDENCE FOR CLINICAL UTILITY

Advanced cancer genotyping

Precision medicine relies on identification of genomic aberrations in the tumour to refine prognosis and therapeutically target tumour-driver biomolecular traits. This is traditionally accomplished via tumour tissue biopsies that have several limitations. In addition to patient discomfort, risk and morbidity, tumour biopsies may not be feasible. In patients requiring urgent treatment, delays in obtaining tissue biopsies and their molecular results may restrict treatment options, with LBs providing faster results.⁴⁶ Tissue biopsy-based genotyping may be limited by low tumour cellularity and fixation problems, such as bone biopsies and NSCLC biopsies, and in some situations ctDNA testing may allow a greater informative yield through consistent coverage and fewer assay failures.^{46,47} In addition, ctDNA testing is appropriate when tissue is not available, or it is

foreseen to be technically challenging, or prone to delay, in sample acquisition. The molecular portrait depicted by a tumour biopsy is limited to single site (space) and 'frozen' in time. Spatial and temporal heterogeneity are established features of malignancies.⁴⁸ ctDNA has the advantage of being easier to access serially, and provide access to a 'genomic pool' originating from several metastatic sites in the patient. LBs may provide more accurate genotyping in patients with metachronous metastases, when tissue testing is only implementable in the primary tumour.

ctDNA release is believed to be proportional to tumour growth, which is linked to cell death and turnover, with the fastest-growing tumour clones shedding the most ctDNA, which are theoretically the most clinically relevant.⁴⁹ There can be exceptions, however, and there is still confounding evidence⁵⁰ and more research is needed to understand the molecular basis of ctDNA shedding into blood. Repeat LBs may allow detection of acquired resistance variants, for example to selective pressures from kinase inhibitors, to allow best selection of the next line of therapy on the basis of acquired resistance genotype and relationship with parental driver.

ctDNA testing also has some limitations compared with tissue testing, mainly higher rates of false negatives and positives as discussed in prior sections, but also low tumour fraction present in the sample, which limits reliable assessment of variant allele fraction (VAF) and limits copy number alteration analysis.

Evidence for ctDNA in advanced cancer genotyping. Technologies used for liquid genotyping can be broadly classified to sensitive PCR-based technologies whether real time PCR (RT-PCR) or digital PCR or NGS platforms. Digital PCR can only identify a restricted set of genomic alterations at lower cost. In contrast, error-corrected NGS approaches enable high throughput detection of a variably broad set of mutations, though at higher cost. Multiple prospective and retrospective studies have reported excellent specificity and positive predictive values between ctDNA LB and tissue-based PCR and NGS testing in advanced malignancies (95%-99%).⁵¹⁻⁵⁴ Recent large prospective studies of LBs have confirmed high accuracy of LBs in breast,⁴⁷ gastrointestinal (GI)⁴⁶ and lung cancer^{51,53} for SNVs, and when used in the patient populations recruited into the studies. Although most targeted therapies were licensed on the basis of tissue testing, the high positive predictive value of ctDNA testing provides confidence in assumed evidence of clinical utility for ctDNA testing. Of note, the 'clinical' accuracy parameters of LB platforms essentially refer to result comparisons with tissue-based assays, and should not be confused with analytical accuracy parameters. Interpretation of clinical accuracy parameters of LBs is challenged by tumour heterogeneity/evolution, and in these comparisons tissue-based sequencing of a single biopsy may not always reflect the gold standard, due to limited sampling.

The level of evidence for the clinical validity of ctDNA assays is such that validated and adequately sensitive ctDNA testing can be used in routine practice for advanced disease genotyping, provided that limitations are understood and taken into

account (see prior section on technical considerations of ctDNA analysis). This recommendation applies to testing for single nucleotide and small insertion and deletion variants. This recommendation is supported by prospective studies from lung,⁵¹ cholangiocarcinoma,^{55,56} breast,⁴⁷ GI cancer⁵⁷ and others,⁵⁸⁻⁶⁰ demonstrating similar efficacy using LB only to guide therapy. The evidence base is now sufficiently strong that assays with evidence of clinical validity can be assumed to have clinical utility in guiding therapy for tier I actionable variants, an approach endorsed by some specialist societies.⁶¹ Tumour-specific recommendations for LBs are listed in [Table 2](#).

The VAF may provide information on the likely subclonal nature of the variant, and in theory subclonal variants may be less likely to benefit from a therapy targeting that variant. At this time, however, VAF should not be used to make decisions in clinical practice because (i) it is unclear whether LBs can assess subclonality accurately and (ii) there is limited evidence to suggest that true subclonal variants predict for lack of response.

Limitations of LBs for advanced cancer genotyping. As mentioned before, a major potential clinical limitation of LB is incomplete sensitivity, with risk of false-negative results. If an assay is used when interrogating for an actionable genetic aberration and shows an undetected result, this should be considered a 'non-informative' result and confirmation with tissue testing (reflex tissue testing) is advised.

Reflex testing may not be required in certain circumstances, which assists in differentiation of a true 'negative' result from a 'non-informative assay', although evidence that these can robustly reduce false-negative results is limited cautioning use in routine clinical practice.

- Molecular confirmation of sufficient levels of ctDNA (ctDNA fraction) in the assay to detect the variant if it is present in the tumour. For example, detection of a somatic mutation at high levels of VAF, when such a mutation is confidently not arising from CHIP or germline. When such an SNV with moderate/high VAF is identified there can be reasonable assumption that other SNVs would also have been identified, and variants not identified represent a true 'negative' result. This should only be carried out by expert users, who are confident in their understanding of characteristics of the assay used, such as the limit of detection, and consideration of confounding variables such as the potential impact of copy number variation on VAF. Interpretation of a sample as 'truly negative' for fusion variants, or copy number variations, using ctDNA remains difficult.
- Molecular confirmation of the presence of high levels of ctDNA using an orthogonal approach, discussed in the technical considerations section. Although assays for detection of tumour fraction are in development, they are still experimental, and not available for routine clinical practice.

LBs may have limitations for the detection of specific aberrations. Somatic copy number variations in ctDNA samples can only be robustly identified with high ctDNA

Table 2. Tumour-specific table for advanced cancer genotyping

Tumour type	Indications	ESCAT tier and level of evidence	Recommendation
Non-small-cell lung cancer	<i>EGFR</i> (for common, uncommon, exon 20 insertions, T790M and other resistance mutations e.g. C797X). <i>ALK</i> (for fusions and acquired resistance kinase domain mutations). <i>MET</i> (for exon 14 splice site mutations, and acquired resistance mutations) <i>KRAS</i> (for G12C and non-tier 1 other <i>KRAS</i> mutations) <i>BRAF</i> (for V600E) <i>RET</i> (for fusions and acquired resistance kinase domain mutations) <i>ROS1</i> (for fusions and acquired resistance kinase domain mutations) <i>NTRK 1/2/3</i> (for fusions and acquired resistance mutations) <i>MET</i> (for high-level copy number gain/amplification) <i>ERBB2</i> (for exon 20 insertions and transmembrane mutations, and amplification) <i>BRAF</i> (for non-V600E class I-III mutations)	IA ¹²⁰ IA ¹²¹⁻¹²⁵ IB ^{126,127} IB ¹²⁸ IB ^{129,130} IB ¹³¹ IB ^{132,133} IC ¹³⁴ IIA ¹³⁵ IIB ¹³⁶⁻¹³⁸ IIB ¹³⁹	ctDNA genotyping recommended in treatment-naive cancer patients and resistance upon prior TKIs. Caution should be kept as ctDNA assays will miss histological trans-differentiation. ctDNA testing may not have adequate sensitivity to detect <i>MET</i> true high copy number gain as resistance mechanism to osimertinib or lorlatinib. Amplification and fusion detection is suboptimal with ctDNA assays, and should be repeated in tissue where possible.
Breast cancer	<i>PIK3CA</i> mutations <i>ERBB2</i> amplification <i>BRCA1/2</i> mutations <i>ESR1</i> mutations MSI-H <i>NTRK 1/2/3</i> fusions	IA ¹⁴⁰ IA ^{141,142} IA ^{143,144} IB ^{145,146} IC ¹⁴⁷ IC ¹³⁴	<i>ESR1</i> mutations should preferentially be tested in ctDNA. <i>ERBB2</i> amplification and <i>NTRK</i> fusions only when advanced tissue biopsy not available.
Gastric cancer	<i>ERBB2</i> amplification MSI-H <i>NTRK 1/2/3</i> fusions	IA ¹⁴⁸ IC ¹⁴⁷ IC ¹³⁴	ctDNA testing if tissue not available or when fast turnaround time is needed for urgent therapeutic decision making.
Pancreatic cancer	<i>NTRK 1/2/3</i> fusions MSI-H	IC ¹³⁴ IC ¹⁴⁷	ctDNA testing if tissue not available.
Hepatocellular cancer	MSI-H <i>NTRK 1/2/3</i> fusions	IC ¹⁴⁷ IC ¹³⁴	ctDNA testing if tissue not available.
Cholangiocarcinoma	<i>IDH1</i> mutations <i>FGFR2</i> fusions MSI-H <i>NTRK 1/2/3</i> fusions	IA ¹⁴⁹ IA ¹⁵⁰ IC ¹⁴⁷ IC ¹³⁴	ctDNA testing if tissue not available or when fast turnaround time is needed for urgent therapeutic decision making.
Colorectal cancer	<i>BRAF</i> (for V600E mutation) MSI-H <i>NTRK 1/2/3</i> fusions <i>KRAS/NRAS</i> mutations (exon 2,3,4) <i>ERBB2</i> amplification <i>EGFR-ECD</i> (for mutations in the extracellular domain S492, G465, S464, V441)	IA ¹⁵¹ IA ^{147,152} IC ¹³⁴ N/A (resistance biomarker) IB ^{57,153,154} IB ⁷³	<i>KRAS/NRAS/BRAF</i> ^{V600E} /MSI for chemotherapy-naive metastatic colorectal cancer is recommended when tissue testing is not feasible or urgent therapeutic decision making. <i>KRAS/NRAS/BRAF/EGFR-ECD</i> for pretreated patients if <i>EGFR</i> rechallenge is planned.
Ovarian cancer	<i>BRCA1/2</i> mutations MSI-H	IA ¹⁵⁵ IC ¹⁴⁷	In women with no germline pathogenic <i>BRCA1/2</i> variant found, testing for <i>BRCA1/2</i> pathogenic or likely pathogenic somatic variants may be carried out if tissue not available.
Endometrial cancer	MSI-H	IC ¹⁴⁷	ctDNA testing if tissue not available.
Prostate cancer	<i>BRCA1/2</i> mutations MSI-H <i>ATM</i> mutations <i>PTEN</i> mutations/deletions <i>PALB2</i> mutations	IA ¹⁵⁶ IC ¹⁴⁷ IIA ¹⁵⁶ IIA ¹⁵⁷ IIB ^{156,158}	<i>BRCA1/BRCA2/ATM</i> for potential PARPi therapy. Caution is needed when interpreting results of ctDNA assays due to false-positive CHIP mutations in DNA repair genes.
Urothelial cancers	<i>FGFR</i> mutations <i>FGFR3 (FGFR3-TACC3)</i> fusions <i>NTRK 1/2/3</i> fusions	IB ¹⁵⁹ IB ¹⁵⁹ IC ¹³⁴	ctDNA testing if tissue not available.
Thyroid cancer	<i>BRAF</i> mutations <i>RET</i> mutations <i>NTRK 1/2/3</i> fusions	IB ^{160,161} IB ^{162,163} IC ¹³⁴	ctDNA testing if tissue not available.
Soft tissue sarcoma	<i>NTRK 1/2/3</i> fusions	IC ¹³⁴	ctDNA testing if tissue not available.

ESCAT tier I refers to evidence for tissue target-drug match resulting in improvement of meaningful clinical outcomes (synonymous to clinical utility). ESCAT tier II refers to investigational targets that likely define a patient population that benefits from a targeted drug, but additional data are needed. Readers are directed to individual ESMO practice guidelines for detailed discussion of individual tumour types.

CHIP, clonal haematopoiesis of indeterminate potential; ctDNA, circulating tumour DNA; *EGFR*, epidermal growth factor receptor; ESCAT, ESMO Scale for Clinical Actionability of molecular Targets; MSI, microsatellite instability; PARPi, poly(ADP-ribose) polymerase inhibitor; TKI, tyrosine kinase inhibitor.

fraction,^{62,63} and copy number assessment should only replace tissue assessment when tissue assessment is not possible. Given their potential roles as predictive biomarkers for immunotherapy, microsatellite instability (MSI)

status and tumour mutation burden (TMB) have been studied in ctDNA by tailored methodologies. MSI status analysis with plasma samples has shown high clinical validity, and therefore the potential to be used to guide

therapy.⁶⁴ Several targeted panel-based sequencing assays of ctDNA offer to measure blood TMB (bTMB) as a surrogate of tissue-based TMB. At this time, evidence suggests that patients should not be selected for immunotherapy on the basis of bTMB alone. bTMB is highly correlated with the amount of ctDNA and thus a minimum amount of ctDNA is required for valid scoring (this problem similarly applies to tissue-based testing when there is low tumour cell content). CHIP and subclonal mutations may elevate bTMB compared with tissue TMB. Moreover, the clinically relevant cut-off for prediction of immunotherapy response may vary according to the type of assay used and the tumour type. Nonetheless, initial evaluation of tumour burden with ctDNA assays is an active area of research and hopefully could help better select patients for immunotherapies, in particular in cases with difficult or questionable imaging findings.

Tumour-specific aspects. A summary of tumour-specific recommendations for tier I and II variants can be found in Table 2.

Lung cancer. Current ESMO Clinical Practice Guidelines for metastatic NSCLC recommend genotyping in all patients with non-squamous NSCLC and squamous subtype NSCLC with special clinical characteristics (e.g. never smokers). ctDNA assays can be undertaken in treatment-naïve patients and is especially recommended when a significant delay is expected in obtaining tumour tissue for genotyping, when invasive procedures may be risky or contraindicated, or bone is the only site that could be biopsied.⁶⁵ In treatment-naïve NSCLC, ctDNA can be considered complementary or alternative to tissue NGS for biomarker evaluation. Small-volume predominantly intrathoracic tumours, or predominantly intracranial disease, are associated with high false-negative results. ctDNA assay tends to have reduced sensitivity for gene fusions. In addition, it must be noted that tissue RNA sequence may identify a greater breadth of splice site variants such as *MET* exon 14 'skipping' mutations and fusion variants (e.g. *ALK*, *RET*, *ROS1* or *NTRK 1/2/3*⁶⁶). If available, tissue testing with such assays remains the gold standard compared with ctDNA, although any tissue assay can be limited by low tumour cellularity or quality.

For patients with pretreated disease, resistance *EGFR* T790M mutation enabling osimertinib treatment⁶⁷ established a clinical paradigm of ctDNA assay first for T790M mutation detection with tissue sampling and tissue genotyping if T790M is not detected in blood, thereby avoiding an invasive tissue sampling procedure. Histological change, such as neuroendocrine or squamous differentiation, cannot currently be identified by this approach and ctDNA should not be relied on if clinical suspicion arises. ctDNA NGS is now well established as a tool to identify acquired resistance mechanisms in patients with oncogene-addicted NSCLC following on from this T790M strategy, especially in *EGFR*-mutant, *ALK*- or *ROS1*-positive disease. Therefore, for oncogene addicted NSCLC, LBs can be an acceptable initial approach for identification of mechanisms of resistance to targeted therapies. Whilst *MET* amplification is recognised

as a resistance mechanism for a number of kinase inhibitors, including osimertinib and lorlatinib, robust identification in ctDNA remains an area of research.

Breast cancer. Testing may be indicated for *PIK3CA* and *ESR1* mutations in oestrogen receptor (ER)-positive, human epidermal growth factor receptor 2 (HER2)-negative disease, and for MSI detection. Testing may be indicated for detection of *HER2* amplification only in situations where HER2 testing cannot be carried out on an advanced disease biopsy, as *HER2* amplification detection is suboptimal in ctDNA (varies by assay). *ESR1* mutations are acquired subclonally in the cancer, and robust identification of the presence of an *ESR1* mutation is more likely when done through an LB, with tissue biopsies often reporting false-negative results. Testing for *BRCA1/2* variants may be carried out with ctDNA assays, but as currently the indication of poly(ADP-ribose) polymerase (PARP) inhibitors in breast cancer is restricted to germline variants, reflex germline testing is indicated to confirm if the variant is present in germline. Irrespectively, given the actionability of *BRCA1/2* germline variants in breast cancer, germline testing is recommended even in the case of a negative LB finding. Details of other variants recommended as options for testing are given in recent guidance.⁶⁸

Upper GI cancers. In view of their incidence and availability of approved therapies, liquid testing for *ERBB2* in gastric cancer, *IDH1* and *FGFR2* in cholangiocarcinoma are recommended when tissue testing is not feasible or when urgent decision making for fast therapeutic intervention is required.

Colorectal cancer. An initial liquid test including at least *KRAS/NRAS/BRAF*^{V600E}/*MSI* for chemotherapy-naïve metastatic colorectal cancer is recommended when tissue testing is not feasible, or when quick therapeutic decisions are required, and longitudinal *KRAS/NRAS* and epidermal growth factor receptor (EGFR) extracellular domain (*EGFR*-ECD, mutations in S492, G465, S464 and V441) tests are recommended when a rechallenge is considered with an anti-EGFR monoclonal antibody.⁶⁹⁻⁷³ Although *KRAS* mutations may occur as a result of CHIP, the true rate of *KRAS* mutation detection is substantially higher than the contribution from CHIP, such that the detection of *KRAS* mutation in LB is considered sufficient to not give anti-EGFR monoclonal antibody treatment.

Prostate cancer. Aberrations of DNA damage repair genes are important to identify in patients with advanced prostate cancer, as ~20% of patients with castration-resistant prostate cancer have been shown to harbour germline and/or somatic alterations in DNA damage repair genes, such as *BRCA1*, *BRCA2* or *ATM*.⁷⁴ CHIP may cause false-positive results, however, and additionally ctDNA assays cannot robustly detect homozygous deletions (a common feature in *BRCA1/2* and other tumour-suppressor genes). Tissue testing is still recommended for decision making to direct PARP inhibitor therapy.

General tumour site recommendations. Sensitivity of ctDNA assays is reduced in central nervous system only metastatic disease, and in primary brain tumours, and LBs are generally unsuited to genotyping such patients, although can be attempted if this is the only source of sample for genotyping. Data suggest that genotyping may be obtained from cerebrospinal fluid analysis (not reviewed in this recommendation²⁵). In oligometastatic, and nodal only disease, the burden of disease implies potential low shedding of ctDNA, and high false-negative results. Tissue testing should be especially considered, or interval ctDNA testing when higher tumour bulk is present.

Advanced cancer genotyping recommendations

- LB assays with very high analytical and clinical specificity, and therefore positive predictive values, may be used in routine practice when the results will affect standard treatment options. The limitations of ctDNA assays, however, must be taken into account.
- Given the important practical advantages of LBs—faster results, the ability to test most patients regardless of access to interventional radiology—an LB first strategy is recommended as an alternative option to tissue genotyping, in particular for aggressive tumour types where time to result is clinically important, such as advanced NSCLC. The same applies when tissue biopsy is unavailable or inappropriate.
- LB assays for genotyping should be collected when cancer is progressing, either treatment naive or after prior lines of therapy. Samples collected when a tumour is responding to therapy will have decreased sensitivity.
- For genotyping of advanced cancer, the choice between RT-PCR, digital PCR and NGS assays in a clinical practice setting should be defined by availability, reimbursement status and the number of tier I actionable genetic aberrations in a tumour-specific context.
- Caution should be carried out in interpretation of pathogenic variants in high penetrance cancer susceptibility genes (such as *BRCA1*, *BRCA2*, *PALB2*); validated germline testing should be carried out to confirm germline or somatic nature.
- Given the modest clinical sensitivity and negative predictive value, a not-detected LB result for an actionable variant should prompt reflex tissue testing. For expert users, reflex testing may not be required if the presence of sufficient ctDNA purity in the sample can be confirmed.
- ctDNA assays have lower sensitivity for detection fusions and copy number events, and these variants should be tested in tissue when this is available.
- All oncology physicians should have access to a molecular tumour board, for education early in use to ensure correct interpretation of results, and for discussion of difficult cases to ensure appropriate decisions are made.

Advanced cancer ctDNA dynamics for cancer monitoring

ctDNA dynamics for early assessment of treatment efficacy. Due to the short half-life of ctDNA¹² and the possibility of non-invasive repeated sampling, blood ctDNA

allows real-time monitoring of disease during therapy. Studies monitoring cancer patients through therapy have shown that ctDNA dynamics correlate with treatment response, and may identify responses earlier than clinical/radiological detection.^{12,75-77} Across multiple different tumour types, and type of treatment (chemotherapy, targeted therapy and immunotherapy), patients who respond to treatment drop ctDNA levels within weeks of starting therapy. The initial early drop may reflect reduced release of ctDNA as a result of cell cycle arrest, and later reflects reduced tumour bulk. It should be noted that in addition, a few days after starting cytotoxic therapy, there may be a short-time rise in ctDNA levels, possibly reflecting a brief period of increased release.⁷⁸

In metastatic breast cancer, ctDNA provided more accuracy than standard serum markers such as cancer antigen 15-3 (CA 15-3),⁷⁶ with ctDNA dynamics associated with progression-free survival (PFS) in chemotherapy,⁷⁸ endocrine-based⁷⁹ and targeted combination therapies.⁸⁰ In 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 CA 125 levels.⁸¹ In advanced NSCLC, ctDNA dynamics can stratify patients with radiologically stable disease who are responding versus not responding to immunotherapy,^{77,82} and in *EGFR* mutation-positive NSCLC early ctDNA dynamic changes associated with outcomes.⁸³ In metastatic colorectal cancer, a prospective trial showed that a 10-fold decrease in ctDNA after cycle 2 of first-line chemotherapy was associated with PFS, and another study showed that evolution in ctDNA concentration after one or two cycles of chemotherapy predicted response and PFS.^{84,85} In GI malignancies, decreased ctDNA after 4 weeks of chemotherapy predicted partial response and clinical benefit more effectively than carcinoembryonic antigen (CEA) tumour marker, with a sensitivity of 60% versus 24%, respectively.⁸⁶

There is increasing evidence that tracking changes in ctDNA levels in serial plasma samples of patients receiving immune checkpoint inhibitors for metastatic cancer can enable assessment of prognosis and therapeutic benefit.⁸⁷⁻⁸⁹ In a pan-cancer analysis of immune checkpoint inhibition that evaluated almost 1000 patients with locally advanced/metastatic tumours treated with immune checkpoint blockade, on-treatment ctDNA dynamics appear to be predictive of long-term benefit from immunotherapy across tumour types.⁸⁹ Analysis of serial ctDNA allowed early identification of patients with molecular response, which was associated with RECIST response and improved survival among patients with initially radiologically stable disease. A clinical utility of monitoring ctDNA levels could be to differentiate between true clinical radiologic progression and pseudoprogression to anti-programmed cell death protein 1 and anti-programmed death-ligand 1 antibodies, which are observed in 5%-10% of patients receiving immunotherapy.^{90,91}

Longitudinal monitoring for emergent resistance mutations. Sequential ctDNA analysis can also be used to assess for the emergence of genomic mechanisms of drug resistance before clinical progression. Several studies in cohorts of patients with different tumour types treated with targeted therapies have shown the ability of longitudinal ctDNA analysis to detect the early emergence of mutations of resistance before clinical progression. For example, in colorectal cancer patients treated with an anti-EGFR monoclonal antibody, *RAS* and *EGFR*-ECD mutations are detected in ctDNA up to 10 months before radiological progression, and diminish after anti-EGFR drug withdrawal.^{71,92-94} In this setting, several clinical trials are including ctDNA to guide anti-EGFR rechallenge decision, and the CHRONOS trial has shown clinical benefit of anti-EGFR rechallenge in patients with no detection of *RAS*, *EGFR*-ECD and *BRAF* in ctDNA before rechallenge. Other studies have shown similar results.^{73,95,96} For patients with advanced breast cancer on aromatase inhibitor (AI) and cyclin-dependent kinase 4/6 (CDK4/6) inhibitor therapy, monitoring for *ESR1* mutation development has potential clinical utility. In the PADA-1 trial, at the time of *ESR1* mutations detection, patients randomised to fulvestrant and continued on CDK4/6 inhibitor had improved PFS compared with continued AI and CDK4/6 inhibitor.⁹⁷ The PFS gain observed with the early use of fulvestrant, following *ESR1* mutation detection, might not be caught up by a later use of fulvestrant given after disease progression. These data suggest resistance-associated mutations might be more actionable when detected sooner by ctDNA rather than later, at radiological progression, maybe due to a lower burden of resistant cells. Additional confirmatory trials are recruiting.

The optimal assay for monitoring advanced cancer patients has not been established, with both tumour-informed versus tumour-agnostic strategies investigated. In the former, sequencing of tumour tissue biopsies identifies key mutations that will be used in LBs for serial molecular profiling, while in the latter, ctDNA genotyping assays are used without prior knowledge of the tumour mutational profile. A tumour-informed strategy may be most accurate in tracking molecular response, but incurs the additional cost/time of baseline tumour tissue sequencing, and if too restrictive, may not identify emerging resistance mutations. A tumour-agnostic strategy can identify the emergent molecular heterogeneity of the tumour if a broad gene panel is used, but detection of CHIP mutations may complicate ctDNA dynamic assessment.

Areas for future research. Further studies are needed to define the optimal timing of ctDNA dynamic assessment and the most accurate threshold for response prediction. For monitoring for emergence of resistance mutations before clinical progression, further studies on the frequency of monitoring would be required.

Randomised interventional studies are required to assess whether changes of treatment on the basis of ctDNA dynamics assessment can improve outcome, or improve

quality of life through avoidance of unnecessary side-effects, or minimising economical costs.⁹⁸ These could include studies randomising patients with an insufficient fall in ctDNA to a switch in therapy, or to augmentation of therapy.

Advanced cancer monitoring recommendations

- There is insufficient evidence to use regular monitoring of ctDNA during therapy. Although early ctDNA dynamics associate strongly with outcome, and resistance mutations may be identified many months before clinical progression, there is insufficient evidence that acting on such findings improves outcome. We note one trial assessing clinical utility of *ESR1* mutation monitoring during AI and CDK4/6 inhibitor therapy for breast cancer, and ctDNA-guided anti-EGFR rechallenge in CRC. Randomised interventional clinical trials are required to assess the utility of ctDNA monitoring.

Early-stage cancer MRD and molecular relapse monitoring

Substantial evidence suggests that detection of ctDNA following potentially curative treatment is associated with a high risk of future relapse, with two terms commonly used. MRD is a term used in solid tumours to describe the molecular evidence of residual cancer cells soon after curative-intent treatment of the primary tumour with surgery and/or chemo-radiation.⁹⁹ MRD may be detected only by molecular techniques such as PCR or sequencing or CTC analysis, but is not detectable by conventional tests such as current blood-based protein tumour markers or imaging. Molecular relapse (MR) refers to the molecular detection of occult disease at a later timepoint during adjuvant treatment or surveillance.

Clinical evidence for MRD and MR monitoring in early-stage cancer. A rapidly growing body of evidence supports the clinical validity of ctDNA analysis for MRD detection and MR monitoring. Data stemming mainly from case/control and longitudinal cohort studies have demonstrated that detection of ctDNA immediately after completion of therapy or during surveillance predicts a high risk of recurrence in early-stage breast,¹⁰⁰⁻¹⁰² colorectal,¹⁰³⁻¹⁰⁶ lung¹⁰⁷ and bladder cancers,¹⁰⁸ among many other studies.¹⁰⁹ Detection of MRD/MR requires assays specifically optimised for this setting—assays developed for advanced cancer genotyping do not offer sufficient sensitivity, along with the risk of false-positive CHIP mutations.

Across both ctDNA assay and cancer types, clinical specificity of ctDNA detection for predicting relapse in the absence of further treatment is high, often $\geq 90\%$ if no further treatment is given after the positive test result.^{106-108,110} In most studies, however, clinical sensitivity of MRD detection, with current assays, shortly after completion of therapy is suboptimal and often $\leq 50\%$.^{103,104,111} Furthermore, even tumour-informed (i.e. detection of molecular alterations in ctDNA with a highly sensitive assay that specifically targets alterations found previously on tissue), bespoke ctDNA

assays generally display lead-times from ctDNA detection to clinical relapse of <6 months. Existing ctDNA MRD assays, therefore, mainly detect patients destined to experience early relapses (i.e. relapses occurring in the first year after the primary treatment) and may not detect disease that will result in late recurrences. To maximise lead-time and limit false-negative cases, it is critical that the most sensitive ctDNA detection techniques are used and the development of assays that can detect ultra-low ctDNA concentrations (i.e. <0.01%) remains an important area of research.¹¹²

Unlike clinical validity, the clinical utility of ctDNA MRD and MR monitoring remains to be established, and prospective randomised trials are needed. The most obvious potential utility of ctDNA MRD detection is that it could allow personalisation of adjuvant or consolidation systemic therapies. Specifically, MRD-positive patients might benefit most from additional treatment. Indeed, data from early-stage lung and bladder cancer suggest that the benefit of adjuvant/consolidation immunotherapy is potentially restricted to ctDNA-positive patients.^{111,113} Definitive proof of clinical utility will require randomised trials, as it is unknown to this point whether treating on the basis of an MRD-positive result can affect the natural history of such patients, and prospective studies testing personalisation of adjuvant therapy based on ctDNA MRD are underway. Separately, adjuvant therapies might theoretically be de-intensified in patients without MRD. The suboptimal sensitivity and high rate of false-negative results with currently available ctDNA MRD assays, however, suggests caution with using assays to guide de-intensification. Longitudinal post-treatment MR monitoring, which consists of serial ctDNA testing during surveillance, could potentially address the issue of false negatives shortly after completion of curative therapy by detecting MRD at later time points but still before clinical recurrence. MRD-based de-intensification strategies, whether based on ctDNA testing immediately after curative therapy or during surveillance, will need to be carefully compared with the current standard of care in randomised trials with non-inferiority designs in order to demonstrate clinical utility in a definitive manner.

Considerations for future clinical trials

Level of evidence. Evidence to prove clinical utility (whether ctDNA testing can improve clinical outcomes and add value to clinical decision making compared with not using the test) in the adjuvant setting should be of the highest level (Level 1) generated with one of the following two approaches as suggested by Hayes¹¹⁴:

- A prospective randomised clinical trial, where the primary objective is to assess ctDNA testing and the test result is used to direct adjuvant treatment or surveillance strategy.
- A prospective-retrospective study, using archival blood samples collected within a previously conducted prospective trial which is not designed primarily to assess ctDNA-directed management (often as an exploratory

endpoint). Two or more independent studies producing similar results are required to establish clinical utility.

Design and endpoints. It is noteworthy that the clinical utility of ctDNA is very much context-dependent, contingent on disease types and stages, available treatment that could effectively eradicate MRD and intended use (e.g. guiding adjuvant therapy after surgery or following standard adjuvant treatment, monitoring). Multiple ctDNA-based randomised clinical trials are currently underway to establish the clinical utility of ctDNA in early-stage solid tumours. Broadly, ctDNA-guided trials can be conducted in the following clinical settings (Table 3):

- A few weeks after definitive treatment: to examine if ctDNA testing can be used to de-intensify adjuvant treatment in ctDNA-negative patients and/or to escalate/intensify treatment in ctDNA-positive patients. Both non-inferiority (for de-intensification strategy) and superiority (for escalation strategy) designs can be used.
- Soon after completing standard adjuvant therapy: the primary objective is to investigate if additional 'second-line' or 'post-adjuvant' novel therapy can improve cure rate in patients with detectable ctDNA but no evidence of disease on imaging.
- During surveillance: to determine whether ctDNA-guided surveillance compared with standard surveillance protocol will result in earlier detection of recurrence, more patients undergoing curative intent resection of metastases or improvement in survival.

The value of a diagnostic ctDNA analysis in addition to post-surgery analysis remains uncertain. Hypothetically, excluding patients with a negative ctDNA before any definitive treatment (low-shedding tumour) may reduce the rate of false-negative results after definitive treatment. The turnaround time of the ctDNA results may also affect the study design particularly in the immediate post-surgery setting where oncologists are often eager to commence adjuvant treatment 4-6 weeks after surgery.

Early cancer MRD/MR recommendations

- Detection of ctDNA following curative therapy for early-stage cancers, with validated assays optimised for the setting, is associated with a high risk of future relapse, with clinical validity shown in multiple studies.
- There is insufficient evidence for adoption in routine practice, in the absence of prospective clinical trial evidence of clinical utility such as improved outcome, or safe de-intensification, with MRD/MR-guided therapy

Early or advanced stage—assisting in initial diagnosis of cancer

ctDNA assays may be integrated as part of the diagnostic workflow for patients suspected as having cancer on imaging. In individuals with tumours that are difficult to

Table 3. Potential randomised clinical trial designs to establish the clinical utility of ctDNA as a marker of MRD and MR

Timing of ctDNA testing/intended use	Clinical context	Potential trial designs	Objectives/potential endpoints	Examples of ongoing trials
A few weeks after definitive treatment of primary tumour/guide adjuvant therapy	Disease type/stage with sufficiently high recurrence risk (for example >10%) but no or modest benefit with standard adjuvant treatment (e.g. stage II colon cancer, stage I NSCLC)	<p>ctDNA-guided strategy design</p> <p>All patients randomised to:</p> <ul style="list-style-type: none"> Control (blinded to ctDNA result)—observation or standard chemotherapy based on standard pathologic features ctDNA-guided—ctDNA-positive cases receive standard treatment; ctDNA-negative cases are observed 	<p>Potential dual objectives to demonstrate non-inferiority for relapse-free survival and reduction in treatment use with ctDNA-guided approach</p> <p>Endpoints</p> <ul style="list-style-type: none"> Number of patients treated with adjuvant treatment Time-to-recurrence Recurrence-free survival Overall survival QoL/QALY 	<p>Stage II colorectal</p> <ul style="list-style-type: none"> DYNAMIC (ACTRN-12615000381583) MEDOCC-CrEATE (NL6281/NTR6455) Rectal cancer DYNAMIC-Rectal (ACTRN-12617001560381)
		<p>ctDNA-by-treatment interaction design</p> <p>ctDNA-positive patients randomised to:</p> <ul style="list-style-type: none"> Control: observation Experimental: standard adjuvant treatment (if available) or novel therapy <p>ctDNA-negative patients are:</p> <ul style="list-style-type: none"> Managed off trial OR Observed on trial 	<p>Superiority of treatment compared with observation in ctDNA-positive patients</p> <p>Endpoints</p> <ul style="list-style-type: none"> ctDNA clearance rate Time-to-recurrence Recurrence-free survival Overall survival 	<p>Stage II colorectal</p> <ul style="list-style-type: none"> CIRCULATE (NCT04089631) CIRCULATE- PRODIGE 70 (NCT04120701) COBRA (NCT04068103) CIRCULATE Spain (EudraCT 2021-000507-20)
	Disease type/stage with established benefit with standard adjuvant treatment (e.g. stage III colon cancer, stage II/III NSCLC, breast cancer)	<p>ctDNA-guided strategy design</p> <p>All patients randomised to:</p> <ul style="list-style-type: none"> Control (blinded to ctDNA result)—standard adjuvant treatment ctDNA-guided—ctDNA-positive cases receive escalated treatment or standard treatment; ctDNA-negative cases receive a de-intensified treatment regimen (e.g. single agent rather than combination chemotherapy, shorter duration, or no treatment) 	<p>Potential dual objectives to demonstrate non-inferiority for RFS and reduction in treatment use with ctDNA-guided approach</p> <p>Endpoints</p> <ul style="list-style-type: none"> Number of patients treated with adjuvant treatment Time-to-recurrence Recurrence-free survival Overall survival QoL/QALY 	<p>Bladder cancer</p> <ul style="list-style-type: none"> IMvigor011 (NCT04660344) <p>Stage III +/- high-risk stage II colorectal</p> <ul style="list-style-type: none"> DYNAMIC-III (ACTRN-12617001566325) TRACC (Part C) (NCT04050345)
After completing standard adjuvant therapy or with molecular relapse monitoring	Disease type/stage with established adjuvant therapy	<p>ctDNA-by-treatment interaction design</p> <p>ctDNA-positive patients randomised to:</p> <ul style="list-style-type: none"> Control: standard treatment Experimental: standard treatment + novel therapy <p>ctDNA-negative patients randomised to:</p> <ul style="list-style-type: none"> Control: standard treatment Experimental: observation 	<p>ctDNA-positive: superiority of novel strategy compared with standard treatment</p> <p>ctDNA-negative: non-inferiority of observation compared with standard treatment</p> <p>Endpoints</p> <ul style="list-style-type: none"> ctDNA clearance rate Time-to-recurrence Recurrence-free survival Overall survival 	<p>Stage II/III NSCLC</p> <ul style="list-style-type: none"> MERMAID-1 (NCT04385368) ER+/HER2-BC LEADER (NCT03285412) <p>High-risk stage II/low-risk stage III colorectal</p> <ul style="list-style-type: none"> CIRCULATE JAPAN — VEGA (jRCT1031200006)
		<p>ctDNA-positive patients are randomised to:</p> <ul style="list-style-type: none"> Control: placebo/standard therapy Experimental: novel therapy with emerging or established efficacy in the metastatic setting for that tumour type 	<p>Superiority of novel strategy compared with observation</p> <p>Endpoints</p> <ul style="list-style-type: none"> ctDNA clearance rate Time-to-recurrence Recurrence-free survival Overall survival 	<p>HR+ HER2-BC</p> <ul style="list-style-type: none"> DARE (NCT04567420) ZEST (NCT04915755) TRAK-ER (NCT04985266) <p>Triple-negative BC</p> <ul style="list-style-type: none"> c-TRAK-TN (NCT03145961) ZEST (NCT04915755) <p>Stage II/III colorectal</p> <ul style="list-style-type: none"> CIRCULATE JAPAN — ALTAIR (NCT04457297) ACT-3 (NCT04259944) NCT04486378

Continued

Table 3. Continued				
Timing of ctDNA testing/intended use	Clinical context	Potential trial designs	Objectives/potential endpoints	Examples of ongoing trials
During surveillance/ monitor for molecular relapse	Disease type/stage with established surveillance strategy	<p>Patients randomised to:</p> <ul style="list-style-type: none"> Control: standard surveillance protocol with tumour markers and/or imaging Experimental: ctDNA-guided surveillance—imaging only for patients with positive ctDNA during surveillance 	<p>Dual objectives to demonstrate non-inferiority of overall survival (or alternatively weaker survival endpoints) but reduction in imaging with ctDNA-guided surveillance and superiority in overall survival with ctDNA-guided surveillance</p> <p>Endpoints</p> <ul style="list-style-type: none"> Time to detection of recurrence Percentage of patients undergoing curative intent resection of metastases (if applicable for the tumour type) Overall survival 	<p>NSCLC</p> <ul style="list-style-type: none"> MERMAID-2 (NCT0464246) Stage III/high-risk stage II colorectal IMPROVE-IT2 (NCT04084249)

BC, breast cancer; ctDNA, circulating tumour DNA; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; MR, molecular relapse; MRD, molecular residual disease; NSCLC, non-small-cell lung cancer; QoL, quality-adjusted life-year; QoL, quality of life; RFS, recurrence-free survival.

biopsy, identification of a pathogenomic mutation using advanced cancer ctDNA genotyping assay may confirm the presence of cancer.¹¹⁵ CHIP mutations must not be confused with pathogenomic mutations in this context, and expertise in using assays for this purpose is essential. In patients with aggressive tumours, ctDNA assays may result in faster start of targeted therapy, compared with tissue biopsies. If ctDNA assays are used to augment tissue biopsies for diagnosis, the false-negative rate must be taken into consideration.

Screening asymptomatic populations for cancer

The ultimate application of ctDNA for cancer care is the potential for identifying early-stage cancers and precancerous conditions in asymptomatic individuals with a view to take actions to increase cure rates or even prevent invasive cancer development. Large population studies are required to provide sufficient level of evidence for this concept to become a reality. A requisite for a standardised and reliable screening tool is to achieve high levels of specificity while maintaining clinically useful levels of sensitivity. This remains technically challenging using ctDNA, particularly as early-stage cancers shed low amounts of ctDNA. Ideally, ctDNA-based screening should also be informative of the cancer tissue of origin, which is far from optimal at this stage.

Sensitivity mainly depends on the ability to detect ctDNA confidently at very low purity, with no prior knowledge of mutations present in the cancer, and with an ability to discriminate population level single nucleotide polymorphisms and CHIP mutations.³² Large efforts have been conducted in this field so far, with studies demonstrating high specificity and encouraging sensitivity with error-corrected sequencing¹¹⁶ that may be combined with protein biomarkers,¹¹⁷ genome-wide fragmentation patterns¹¹⁸ and methylation-based ctDNA assays.¹¹⁹ Data are awaited from large studies conducted in true screening populations, to assess ctDNA assays as a multi-cancer screening tool, but at this point screening cannot be considered as a validated use for ctDNA assays.

CONCLUSION

LBs, and in particular ctDNA assays, are increasingly used in clinical practice and there is already sufficient evidence of clinical utility for genotyping advanced cancer to guide therapies as an alternative to a tumour-guided strategy, especially in situations where tissue biopsies are suboptimal or time is crucial. Incomplete sensitivity must be factored into clinical use, in particular lower sensitivity for gene fusions and copy number events. Further assay development to robustly differentiate 'true' from possibly 'false' positive or negative results, is a major priority for future advanced genotyping assays. The potential for 'false' positive results arising from CHIP mutations remains a significant weakness of ctDNA genotyping assays. ctDNA testing has the theoretical advantage of capturing intra-patient spatial and temporal tumour heterogeneity more accurately than tissue

sequencing, and major clinical trials are now required to assess how detection of such heterogeneity can provide clinically useful information to improve treatment.

Lack of evidence of utility prevents a recommendation to incorporate ctDNA assays for other possible purposes like screening, MRD assessment, MR monitoring and early assessment of treatment response. New technologies currently in development, such as methylation pattern-based sequencing, fragmentation pattern-based sequencing or novel ultra-sensitive mutation detection methods, have the potential to optimise utility in these new settings. Multiple clinical trials are underway that may provide the evidence base to adopt ctDNA assays for decision making in multiple clinical scenarios.

GLOSSARY

CHIP: clonal haematopoiesis of indeterminate potential—somatic mutations that accumulate in cells of haematopoietic lineage as a result of age and environmental processes, easily picked up by plasma sequencing and can be wrongly attributed to tumour origin.

CTCs: circulating tumour cells—tumour cells that can be detected in the bloodstream.

ctDNA: circulating tumour DNA—small fragments of DNA released by a tumour cell into circulation.

ctDNA fraction: the fraction of DNA found in circulation coming from tumour cells relative to the total amount of DNA from multiple origins; can also be termed purity.

Fragmentation pattern-based sequencing: a DNA sequencing approach that utilises DNA fragment patterns commonly associated with tumour origin to refine sensitivity in mutation finding.

Germline: present in all cells from an individual as a result of inheritance.

LB: liquid biopsy.

LoD: limit of detection—the quantitative limit of an assay to reliably distinguish the target information from noise.

LoQ: limit of quantitation—the quantitative limit of an assay to consistently determine concentration and therefore allow for dynamic assessments.

Methylation-based ctDNA assays: a DNA sequencing approach that utilises DNA methylation patterns associated with cancer and tumour origin to refine sensitivity in mutation finding.

Molecular response: a drop in ctDNA levels suggesting clearance or shrinkage of tumour.

MR: molecular relapse—molecular detection of occult disease at a later timepoint during adjuvant treatment or surveillance (not yet identified by standard radiological assessments).

MRD: molecular residual disease—the presence of minimal amounts of detectable ctDNA suggesting some degree of tumour persistence even when not detectable with radiographic methods.

Plasma DNA: the fraction of DNA found in plasma.

Purity: the fraction of DNA found in circulation coming from tumour cells relative to the total amount of DNA from multiple origins; can also be termed ctDNA fraction.

Reflex tissue testing: testing again the molecular alteration of interest in a tissue sample.

Somatic: present exclusively in tumour cells from an individual.

Tumour-informed assay: detection of molecular alterations in ctDNA with a highly sensitive assay that specifically targets alterations found previously on tissue.

VAF: variant allele frequency—the fraction of counts of an assay attributed to the mutation of interest relative to the reference counts of an assay, often wild-type counts.

ACKNOWLEDGEMENTS

This is a project initiated by the ESMO Translational Research and Precision Medicine Working Group. We would also like to thank ESMO leadership for their support in this manuscript.

FUNDING

This project was funded by the European Society for Medical Oncology (no grant number).

DISCLOSURE

GA reports receipt of honoraria for participation in Advisory Board from Astellas, AstraZeneca, Bayer, Janssen, Novartis, Orion, Pfizer, Sanofi, Sapience, receipt of honoraria as invited speaker from Astellas, AstraZeneca, Janssen, receipt of royalties for licensing fees from Janssen, receipt of institutional research grants from Astellas and Janssen, non-remunerated activities as a PI in Astellas, Janssen, and non-remunerated advisory role in AstraZeneca and Janssen; FCB reports receipt of honoraria for participation in Advisory Board from Archer, BioNTech, Lilly, Novartis, Pfizer, receipt of honoraria to institution for participation in Advisory Board from AstraZeneca, receipt of honoraria as invited speaker from AstraZeneca, Novartis, Pfizer, Roche, Seagen, receipt of honoraria to institution as invited speaker from Pfizer, Sanofi, receipt of honoraria for expert testimony from Hikma, institutional non-financial interest for research as a coordinating PI from AstraZeneca, Pfizer, ProLynx, Saga and Seagen; GC reports receipt of honoraria for participation in Advisory Board from AstraZeneca, Bristol Myers Squibb (BMS), Daiichi Sankyo, Ellipsis, Exact Sciences, Lilly, Merck, Pfizer, Roche, Veracyte, receipt of honoraria as invited speaker from AstraZeneca, Daiichi Sankyo, Novartis, Pfizer, Roche, receipt of honoraria for writing engagement from Pfizer, institutional research grant for investigator initiated clinical trial from Merck, institutional funding for conduct of phase I studies from Astellas, AstraZeneca, Blueprint Medicine, BMS, Daiichi Sankyo, Kymab, Novartis, Philogen, Roche, Sanofi, non-remunerated activities as an officer of the Italian National Health Council—Advisor for Ministry of Health, non-remunerated advisory role as a member of the Scientific Council of Europa Donna, non-remunerated advisory role in Fondazione Beretta, and non-remunerated member of Board of Directors of the Lega Italiana Lotta ai

Tumori; LDMA reports receipt of honoraria as invited speaker, participation in speaker bureau from Roche, institutional research collaboration grant from NanoString and receipt of education grant from BMS; MD reports receipt of honoraria for participation in Advisory Board from AstraZeneca, Boehringer Ingelheim, Genentech, Gritstone Oncology, Illumina, receipt of honoraria for consultancy from BioNTech, Novartis, RefleXion, Roche, has ownership interest with CiberMed, Foresight Diagnostics, receipt of royalties for licensing fees from Foresight Diagnostics, Roche Diagnostics, receipt of institutional financial interest as a coordinating PI from AstraZeneca, Varian, receipt of funding from Genentech, non-financial interest for receipt of reagents from Illumina, non-remunerated activities as a member of Board of Directors of Foresight Diagnostics; AI reports receipt of honoraria for participation in Advisory Board from Bayer, Chugai Pharmaceutical Co, GlaxoSmithKline, Philips, Roche, institutional non-financial interest as a coordinating PI from AstraZeneca, Bayer, Ipsen, Merck, Merck Sharp & Dohme (MSD) and Roche; JL reports receipt of honoraria to institution as invited speaker from Roche; JDM reports receipt of honoraria for participation in Advisory Board from Illumina, BMS, receipt of honoraria for consultancy from PierianDx, receipt of royalties for licensing fees administered by Stanford University from the United States Patent Office, non-remunerated activities as a member of Board of Directors of the Association for Molecular Pathology, non-remunerated leadership role as a Chair of the Informatics Subdivision of the Association for Molecular Pathology, non-remunerated leadership role as a Vice Chair of the CLSI MM23—Molecular Diagnostic Methods for Solid Tumors Committee of the Clinical and Laboratory Standards Institute (CLSI), non-remunerated activities in the US NIH/NCI as a PI for the NIH 1-UG1-CA233333-01, UNITS: The UNC/UT National Clinical Trials Network Group Integrated Translational Science Production and Consultation Center; CM reports receipt of honoraria for participation in Advisory Board from Biocartis, Merck Serono, receipt of honoraria as invited speaker from Amgen, Guardant Health, Merck Serono, and Pierre Fabre, Roche, receipt of royalties for licensing fees administered by Institut Investigació Hospital del Mar; NN reports receipt of honoraria for participation in Advisory Board from AstraZeneca, Bayer, Biocartis, Incyte, Novartis, Qiagen, Roche, receipt of honoraria as invited speaker from BMS, Eli Lilly, Illumina, Merck, MSD, Sanofi, Thermo Fisher, receipt of institutional research grants from AstraZeneca, Biocartis, Blueprint, Illumina, Incyte, Merck, Qiagen, Roche, Thermo Fisher, leadership non-remunerated role as a President of the International Quality Network for Pathology (IQN Path) and President of the Italian Cancer Society (SIC); KP reports receipt of honoraria for participation in Advisory Board from MSD, Menarini, Hello Healthcare, Sanofi, receipt of honoraria as invited speaker from Abcam, Ipsen Pharma, Medac, Agena, institutional financial interest from Angle plc, European Liquid Biopsy Society, Böhringer Ingelheim and for participation in IMI JT ID Cancer from EU/IMI Cancer-ID

consortium; SP reports receipt of honoraria for participation in Advisory Board from Amgen, AstraZeneca, Bayer, BeiGene, Blueprint, BMS, Boehringer Ingelheim, Daiichi Sankyo, Guardant Health, Janssen, Eli Lilly, Merck KGaA, Novartis, Roche, Takeda, institutional financial interest for research as a coordinating PI from Ariad, Boehringer Ingelheim, Celgene, Daiichi Sankyo, Takeda, Turning Point Therapeutics, as a local PI from AstraZeneca, GlaxoSmithKline, Roche, Trizell, as a sub-investigator from Amgen, MSD, non-remunerated advisory role in ALK Positive UK, International Association for the Study of Lung Cancer, Lung Cancer Europe, Ruth Strauss Foundation, non-remunerated leadership role in the British Thoracic Oncology Group as a Chair of Steering Committee, European Thoracic Oncology Platform as a Foundation Council Member, non-remunerated member of Thoracic malignancy Faculty in the European Society for Medical Oncology, non-remunerated member of Board of Directors in the Mesothelioma Applied Research Foundation; JSRF reports consultancy fees from Goldman Sachs, Repare Therapeutics, Paige.AI and Eli-Lilly, membership of the Board of Directors Group Oncoclinicas, stock ownership of Repare Therapeutics, and honoraria for *ad hoc* participation in the Scientific Advisory Board of Repare Therapeutics, Paige.AI, Roche Tissue Diagnostics, Novartis, Roche/Genentech, Invicro and Personalis; JT reports consultancy fee from Haystack Oncology, receipt of honoraria for participation in Advisory Board from AstraZeneca, BMS, MSD, Inivata, Pierre Fabre, receipt of honoraria as invited speaker from Merck Serono, Amgen and Servier; JS reports ownership interest as a co-founder of Mosaic Biomedicals SL, member of Board of Directors of Northern Biologics Inc., receipt of institutional research grants from Roche Glycart AG, Mosaic Biomedicals SL, Hoffmann-La Roche Ltd, Northern Biologics Inc., RidgeLine Therapeutics, non-remunerated activities as a member of Board of Directors of Asociación Española Contra el Cáncer and a member of Board of Directors, Secretary General of the European Association for Cancer Research (AACR); TY reports receipt of honoraria as invited speaker from Bayer, Chugai, Eli Lilly, Merck Biopharma, Ono, Taiho, receipt of institutional research grants from Amgen, MSD, Ono, Taiho, receipt of institutional financial interest as a local PI from Chugai, Daiichi Sankyo, MSD, Ono, Parexel International, Sanofi and Sumitomo Dainippon; NT reports receipt of honoraria for participation in Advisory Board from Arvinas, AstraZeneca, BMS, GlaxoSmithKline, Lilly, MSD, Novartis, Pfizer, Repare Therapeutics, Roche/Genentech, Zentalis Pharmaceuticals, institutional funding for research from AstraZeneca, MSD, Pfizer, Roche/Genentech, institutional non-financial interest for provision of material for research from BioRad and for provision of assays from Guardant Health. JS is co-founder of Mosaic Biomedicals and has ownership interests from Mosaic Biomedicals and Northern Biologics and reports receipt of grant/research support from Mosaic Biomedicals, Northern Biologics, Roche/Glycart and Hoffmann la Roche. All other authors have declared no conflicts of interest.

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