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# Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review

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## ABSTRACT

### Purpose
Clinical use of analytical tests to assess genomic variants in circulating tumor DNA (ctDNA) is increasing. This joint review from ASCO and the College of American Pathologists summarizes current information about clinical ctDNA assays and provides a framework for future research.

### Methods
An Expert Panel conducted a literature review on the use of ctDNA assays for solid tumors, including pre-analytical variables, analytical validity, interpretation and reporting, and clinical validity and utility.

### Results
The literature search identified 1,338 references. Of those, 390, plus 31 references supplied by the Expert Panel, were selected for full-text review. There were 77 articles selected for inclusion.

### Conclusion
The evidence indicates that testing for ctDNA is optimally performed on plasma collected in cell stabilization or EDTA tubes, with EDTA tubes processed within 6 hours of collection. Some ctDNA assays have demonstrated clinical validity and utility with certain types of advanced cancer; however, there is insufficient evidence of clinical validity and utility for the majority of ctDNA assays in advanced cancer. Evidence shows discordance between the results of ctDNA assays and genotyping tumor specimens and supports tumor tissue genotyping to confirm undetected results from ctDNA tests. There is no evidence of clinical utility and little evidence of clinical validity of ctDNA assays in early-stage cancer, treatment monitoring, or residual disease detection. There is no evidence of clinical validity and clinical utility to suggest that ctDNA assays are useful for cancer screening, outside of a clinical trial. Given the rapid pace of research, re-evaluation of the literature will shortly be required, along with the development of tools and guidance for clinical practice.

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## INTRODUCTION

The use of assays that assess genomic variants in circulating tumor DNA (ctDNA) is increasing in the oncology clinical setting, despite uncertainties around pre-analytical considerations, analytical validity, and clinical validity and utility. ASCO and the College of American Pathologists convened a joint panel of oncology and pathology experts (Appendix Table A1, online only) to review available evidence and develop this review about ctDNA assays as a cancer biomarker in various clinical scenarios. This joint review is intended to provide an assessment of the evidence on ctDNA assays in oncology and a framework for future research and clinical practice guidelines to help better inform clinical practice.

The review is limited to analysis of variants in ctDNA for solid tumors and the analysis of sequence or copy number variants in DNA. Topic areas addressed include pre-analytical variables, analytical validity, interpretation and reporting, and clinical validity and utility.

### *Methodology*

*Literature review.* A literature search was completed on March 20, 2017. The search strategies were developed in collaboration with a medical

ASSOCIATED CONTENT

Appendix
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librarian for the concepts of liquid biopsies; blood; cancer abnormalities; and pre-analytical, analytical, interpretation, reporting, utility, and validity variables (Appendix, online only). The Expert Panel supplemented the search with additional articles, in particular to cover areas not targeted by the literature search. As noted in the QUOROM (Quality of Reporting of Meta-Analyses) diagram in Appendix Figure A1 (online only), a total of 1,338 unique publications were identified in the search, and 390 articles were selected for full-text review. The Expert Panel supplied an additional 31 references. Ultimately, 77 articles were selected for inclusion in the review.

*Writing and review.* The Expert Panel was divided into four writing groups to review the evidence relevant to the four topic areas. The entire Expert Panel was involved in the evidence review and development of the article. External reviewers provided comments on the draft manuscript. Table 1 lists the terms and definitions that were applied. More detailed explanations, definitions, and examples are provided in the respective sections. The article was reviewed and approved by ASCO and College of American Pathologists leadership. A listing of findings regarding the current status of ctDNA testing in patients with solid tumors is provided in Table 2.

## BACKGROUND

The term liquid biopsy was coined nearly a decade ago by Pantel and Alix-Panabières[1] to imply the use of a blood test to provide the same diagnostic information included in a tissue biopsy. Compared with a classic biopsy, liquid biopsies are more convenient and present minimal procedural risk to the patient (Table 3). Furthermore, their collection is less expensive. Therefore, they can be performed on a serial basis. In theory, a liquid biopsy may also deliver more complete information regarding the patient's entire tumor burden, because the sample theoretically represents all tumor DNA present in the circulation, as opposed to the spatial limitations of a biopsy sampling of a single lesion within a single anatomic site.

The term liquid biopsy can include measurement of soluble factors, such as proteins, tumor markers (eg, carcinoembryonic antigen), circulating tumor cells, and circulating cell-free nucleic acids. This review focuses on the recent advances in molecular technology that have facilitated detection and quantification of cancer-related genomic variants in the cell-free DNA, which are believed to reflect ctDNA.[2] The literature regarding ctDNA assays is rapidly growing, but the synthesis of this information is cumbersome because of broad variability in definitions, analytical approaches, and assessment of clinical significance.

Three semantic terms critical to the assessment of clinical significance were first proposed by the Evaluation of Genomic Applications in Practice and Prevention initiative of the Centers for Disease Control with regard to genetic testing[3] and later adopted and refined by a panel of the Institute of Medicine.[4] The three terms are analytical validity, clinical validity, and clinical utility. Analytical validity refers to the ability of a test to accurately and reliably detect the variant(s) of interest and includes measures of accuracy, sensitivity, specificity, and robustness. Clinical validity implies that the test may accurately detect the presence or absence of a pathologic state or predict outcomes for groups of patients whose test results differ. Clinical utility is documented when high levels of evidence exist to demonstrate that the use of the test improves patient outcomes compared with not using it.

To determine clinical validity or utility, one must define the intended use of the marker. In broad terms, but specific to cancer, possible uses may include categorization for risk of disease, screening unaffected patients for the disease, differential diagnosis of a proven malignancy, prognosis in the absence of further treatment, prediction that a specific treatment is likely to be effective, and monitoring disease activity—either to detect impending recurrence in a patient presumed free of disease or to determine whether a patient with known cancer has evidence of progressive disease. In solid tumors, the latter few uses may differ in implications, depending on the stage of the disease (ie, early *v* advanced and/or metastatic).

**Table 1.** Terms and Definitions

| Term | Definition |
|---|---|
| Cell-free DNA | Total amount of cell-free DNA in plasma or serum, which can be derived from multiple sources, including tumor cells. |
| ctDNA | Circulating tumor DNA: the fraction of cell-free DNA that originates from tumor cells. The presence of ctDNA in cell-free DNA is generally inferred by the detection of somatic variants; consequently, the presence of ctDNA in cell-free DNA is usually not confirmed until after a ctDNA assay is performed. |
| ctDNA assay | A clinical test designed to detect somatic variants in ctDNA. This encompasses targeted assays that may interrogate a single variant in one gene to broad assays that may interrogate numerous variants in many genes. Other terms for ctDNA assays include circulating cell-free plasma DNA assays and plasma genotyping assays. |
| Liquid biopsy | A broad category for a minimally invasive test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for fragments of tumor-derived DNA that are in the blood. Tumor genetics or genomics from ctDNA assays are one example. |
| Variant allele fraction | The fraction of alleles in a specimen that contain the variant, or mutation. As an example, a pure population of tumor cells in which one allele contained a *BRAF* V600E variant and the other *BRAF* allele was wild type (ie, not variant/mutated) would have a *BRAF* V600E variant allele fraction of 50%. |
| Pre-analytical | Issues regarding collection, handling, transport, processing, and storage of a specimen that may affect the subsequent analysis. |
| Analytical validity | Ability of an assay to detect and measure, with statistical significance, the presence of a biomarker of interest accurately, reproducibly, and reliably. |
| Clinical validity | Ability of an assay to divide, with statistical significance, one population into two or more groups on the basis of outcomes, such as presence of cancer or treatment response. |
| Clinical utility | Ability to demonstrate, with statistical significance, improvement in the diagnosis, treatment, management, or prevention of cancer, with the use of the assay compared with not using the assay. |

  

**Table 2.** Summary of Key Findings on the Use of ctDNA Analysis in Patients with Cancer

| Topic | Key Findings |
|---|---|
| Pre-analytical variables for ctDNA specimens | • Evidence suggests that plasma is the optimal specimen type for ctDNA analysis.<br>• Evidence supports the use of either cell-stabilizing tubes or EDTA anticoagulant tubes. However, EDTA tubes need to be processed as expediently as possible within 6 hours of collection. Leukocyte stabilization tubes allow up to 48 hours from collection to processing, and longer with some tubes.<br>• Further studies are required to address other pre-analytical variables that may affect ctDNA testing, including specimen collection, handling variables, storage condition and time, and patient-related biologic factors. |
| Analytical validity | • Analytical validity needs to be clearly established for any clinical ctDNA assay, with particular attention paid to detection of variants near the reported lower limit of detection of the assay. Ideally, validation will include evaluation of standardized samples that facilitate cross-assay comparisons.<br>• Evidence has not established optimal lower limits of detection for various types of somatic variants. Optimal lower limits of detection may vary depending on the intended use of the ctDNA assay, but are lower than for tumor genotyping assays.<br>• Different ctDNA assays may not give the same results because of different assay performance characteristics, such as differing limits of detection.<br>• Future studies should focus on cross-assay comparisons, assay robustness, and the development of proficiency testing mechanisms. |
| Interpretation and reporting | • Evidence demonstrates the importance of integrating clinical information, and available information from tumor analysis, with the identification of an actionable somatic variant in a ctDNA assay, to inform the appropriate selection of therapy.<br>• The proportion of ctDNA as a fraction of total cell-free DNA in plasma varies substantially between different patients, and the potential prognostic and therapeutic implications of variant allele fractions from ctDNA assays need further study.<br>• Caution is important when interpreting ctDNA variants found in genes that are mutated in clonal hematopoiesis of indeterminate potential. Additional research is necessary to determine how to interpret and report variants in these genes.<br>• ctDNA assays in which a somatic variant is or is not identified should be reported in a way that conveys the potential for discordance with tumor tissue testing. |
| Clinical validity and utility | • Aside from assays that have received regulatory approval, most assays have insufficient evidence to demonstrate clinical validity, and most have no evidence of clinical utility. Well-designed clinical trials or equivalence studies are needed to demonstrate clinical utility for most assays.<br>• Evidence shows discordance in results between ctDNA assays and tumor tissue genotyping and supports value of tumor tissue genotyping to confirm undetected ctDNA findings.<br>• For advanced cancer, the evidence indicates that more reliable test results occur when the ctDNA assay is performed at the time of disease progression and not when responding to prior therapy.<br>• There is evidence that positive findings from well-validated ctDNA assays may support initiation of a targeted therapy option where an assay for the relevant genomic marker has demonstrated clinical utility when performed in tissue.<br>• For monitoring therapy effectiveness, evidence of clinical validity is still emerging, and there is currently no evidence of clinical utility to suggest that ctDNA assays are useful in this context, outside of a clinical trial.<br>• For early-stage cancer, evidence of clinical validity is still emerging, and there is currently no evidence of clinical utility to suggest that ctDNA assays are useful at diagnosis or in the adjuvant setting after completing treatment, outside of a clinical trial.<br>• For cancer screening, there is no evidence of clinical validity and clinical utility to suggest that ctDNA assays are useful in this context, outside of a clinical trial. |

Abbreviation: ctDNA, circulating tumor DNA.

## PRE-ANALYTICAL VARIABLES FOR ctDNA SPECIMENS

Pre-analytical variables for ctDNA include all steps preceding analysis of the specimen. The variables inherent in these steps may affect the quality of the specimen and its fitness for cell-free DNA extraction and ctDNA testing.[5,6] Pre-analytical variables that increase degradation of cell-free DNA in the specimen, or increase contamination of the plasma with normal DNA from leukocytes, are the most likely to compromise analytical success.

**Table 3.** Comparison of ctDNA Versus Tumor Tissue Testing

| Consideration | ctDNA Assay | Tissue Assay |
|---|---|---|
| Logistics | Easy to draw | Invasive, more challenging to obtain |
| | Variable venipuncture risks | Variable biopsy risks |
| | Easy serial testing | Serial testing more difficult |
| Biology | Cannot directly correlate ctDNA results with histology or cellular phenotype | Can correlate with histology and cellular phenotype |
| | More likely to represent whole tumor, but differential tumor cell turnover may bias representation | Represents one small tumor region |
| Pre-analytical | Easier to standardize across sites | More difficult to standardize across sites |
| | Requires special processing and handling unless using cell-stabilization tubes | Uses existing, validated tissue processing and handling approaches |
| | Limited data on confounding patient-related factors | |
| Clinical utility | Limited evidence for treatment selection in advanced cancer | Substantial evidence for treatment selection in multiple malignancies for early and advanced cancers |
| | No evidence for other potential indications | |

Abbreviation: ctDNA, circulating tumor DNA.

### Optimal Specimen Type

Current evidence suggests that the optimal specimen type for analysis of ctDNA in blood is plasma. Both serum and plasma consist of the liquid, cell-free fraction of whole blood. The major difference between them is that serum is devoid of clotting factors.

The concentration of total cell-free DNA (normal and ctDNA) from identical blood samples is higher in serum than in plasma. Most cell-free DNA in blood results from leukocyte lysis occurring during clotting.[7] The amount of normal DNA derived from leukocyte lysis, which dilutes the ctDNA, is much lower in plasma, especially if it is separated from the leukocyte fraction soon after the blood draw or if the blood is drawn into collection tubes containing a leukocyte stabilizer.[8,9]

### Specimen Collection

*Blood draw.* The majority of published studies include little detail on the blood draw procedure. Blood is typically acquired from peripheral veins, but no data currently exist on the comparative effects on ctDNA analysis of specimen acquisition from other sites (eg, central veins, either directly or from an intravascular port, or arteries) or other blood draw variables (eg, use of a discard tube, tube fill level, tube inversions, and draw order). In the absence of these data, the phlebotomist should follow the tube manufacturer's instructions for use.

*Tube type and specimen handling.* The type of blood collection tube is the most commonly studied pre-analytical variable. Standard lavender top tubes containing the anticoagulant $K_2$EDTA are suitable for cell-free DNA specimen collection. A critical consideration with the use of $K_2$EDTA tubes is that time to processing should be as expedient as possible, within 6 hours from collection, to avoid lysis of white blood cells, which can dilute the ctDNA with normal leukocyte DNA.[10-13] The use of leukocyte stabilization tubes allows greater flexibility in the time to processing of up to 48 hours, or longer with some tubes, without compromise of ctDNA detection or quantification.[7,10,12,14-16] However, a head-to-head performance comparison of all tube types used for blood collection for ctDNA analysis has not been reported.

Once peripheral blood is collected, it is typically processed through filtration or a sequential pair of centrifugations at low speed and high speed.[9-12] The significant excess of white cells compared with ctDNA in peripheral blood underscores the importance that either filtration or the first, low-speed centrifugation step occurs within hours of collection in EDTA tubes to minimize leukocyte lysis.

The influence of storage temperature and time on unprocessed whole blood has been variable, and this issue remains unresolved. Studies have shown up to a 10-fold increase in levels of DNA, reflecting leukocyte lysis, from tubes with stabilizing agents stored for 3 to 5 days refrigerated or warmed to 40°C.[10,14,17] There has also been at least one report that plasma volume decreases by $>$ 1 mL when unprocessed tubes with stabilizing agents are stored refrigerated or warmed.[15]

There is consensus among studies that storage of frozen plasma before DNA extraction has no effect on subsequent ctDNA analysis. However, studies indicate that plasma must be isolated before freezing, and freezing unspun whole blood should not be performed. Although exposure of plasma to a single freeze-thaw cycle does not affect downstream ctDNA analysis, multiple freeze-thaw cycles may result in nucleic acid degradation and decreased ability to detect ctDNA.[5,12] Therefore, current evidence suggests that processed plasma be aliquoted into single-use fractions for future ctDNA extraction and analysis.

*Transport.* Shipping exposes samples to unfavorable handling and temperature conditions, such as agitation and extreme cold or hot temperatures. If plasma is separated and frozen before shipping, studies generally kept the samples frozen to avoid freeze-thaw cycles.[5,12] Unprocessed samples requiring overnight shipping necessitate collection in tubes with stabilizing agents and packaging to maintain room temperature and minimize temperature fluctuations.[14] Although a recent study of agitation of samples in tubes with stabilizing agents did not detect altered ctDNA yield or genomic DNA release,[15] sample protection in secure foam boxes to reduce sample agitation is common practice.

*DNA purification.* There are several different cell-free DNA purification methods, numerous different kits based on these methods, and various protocol modifications.[6] These varying methods and modifications lead to a wide range of cell-free DNA purification approaches that may affect cell-free DNA yield and purity. Therefore, consideration of the tube type and other pre-analytical variables, as well as downstream analytical methods, may contribute to the optimal DNA purification approach.

*Knowledge gaps.* Insufficient evidence exists to resolve major remaining questions regarding retrospective studies, and whether using archived serum or plasma not collected into leukocyte stabilization tubes or processed rapidly accurately reflects clinical validity or utility, especially in terms of sensitivity of the assay for ctDNA. Little is known about the effects of different storage temperatures or duration on ctDNA assays. Therefore, although the presence of ctDNA suggests that the performance of the assay in such specimens might be feasible, it is unknown whether patients who are considered negative are truly negative and whether serial values truly reflect increase or decrease of the biomarker.

Furthermore, limited data are available regarding the effect of blood draw procedures and potentially confounding patient-related factors that may contribute to the release of cell-free DNA. These factors include diurnal or other biologic influences, smoking, pregnancy, exercise, and numerous nonmalignant disorders such as inflammatory conditions, anemia, heart disease, metabolic syndrome, and autoimmune disorders. Future studies would require banked specimens with well-documented pre-analytical variables and patient factors to address these limitations.

## ANALYTICAL VALIDITY

Multiple assays and methods are available for ctDNA analysis, which can be categorized into two general classes—those targeted for a single or small number of variants, and those aiming for broader coverage.[18] Targeted assays detect known recurring somatic variants and generally use one of several polymerase chain reaction (PCR)–based strategies, such as real-time or digital PCR.[19] Targeted assays are useful for detection of specific known variants,

  

often at very low levels, in a single gene or small number of genes. These targeted assays are generally used for select applications, such as identification of variants that are associated with response to drugs in individual tumor types (eg, *EGFR* variants in patients with non–small-cell lung cancer [NSCLC]). In contrast to targeted assays, broad-coverage assays generally use next-generation sequencing (NGS)–based approaches and have the capability of detecting a larger number of variants in multiple genes, often examining parts of > 50 genes. Broader panels are usually designed to be applied to multiple different tumor types. Two different ctDNA assays may or may not provide the same results because of different assay performance characteristics. For example, the assays may have different lower limits of detection, or they may interrogate different genomic regions. It is therefore not possible to assume that the assays are interchangeable, and to do so would require rigorous cross-assay comparisons.

The most commonly used approach for assessing analytical validity in published studies of ctDNA assays has been to compare concordance between variants detected in tumors and plasma. There are many biologic factors that may affect concordance independent of analytical factors (eg, tumor type, stage, tumor heterogeneity, time between tumor tissue and blood sampling, and whether the variant is clonal *v* subclonal).[20-23] Consequently, analytical validity studies designed in this way may confound issues of analytical validity with issues of clinical validity. In a situation where a somatic variant is identified in a tumor tissue specimen, but not by the ctDNA assay, or vice versa, it may be unclear whether this discordance is caused by analytical or biologic factors. For applications such as detection of *EGFR* variants in NSCLC, concordance between tissue and plasma variant detection for leading platforms ranges from 70% to 90%, with the positive predictive value of ctDNA assays being higher than the negative predictive value.[23-25]

To overcome the issues discussed above, future studies of analytical validity need to include evaluation of standardized samples, reference materials with known variants at specified variant allele fractions and variant copies per assay (eg, *EGFR* T790M variant at 1%, with 10 variants per assay). These reference materials could include the use of cell lines, engineered cell lines, or artificial DNA constructs diluted in an appropriate matrix. Analytical validity is best determined within groups of specimens ranging through low, intermediate, and high levels of the analyte, and examination of analytical validity must include evaluation of both the wet laboratory and bioinformatics portion of an assay. Such reference materials allow assessment of the analytical performance of the assay independent of the potential biologic factors that confound comparisons between tumor and plasma specimens. Use of such reference materials has allowed documentation of the lower limit of detection for single variants ranging from < 0.1% to > 1%, depending on the assay. Given the low limits of detection required for ctDNA assays, it is critical that laboratories ensure validation studies clearly demonstrate their routine ability to detect variants near the reported lower limit of detection of their assay. However, optimal lower limits of detection for various types of somatic variants remain to be established. These lower limits of detection will vary depending on the intended use of the ctDNA assay, but they are likely at least two orders of magnitude lower than for tumor genotyping assays for some applications.

Analytical specificity for assays has generally been shown to be > 95%.[21] Cross-platform comparisons have been undertaken in a few small studies, with high concordance between assays for specific variants with discrepancies largely explained by differences in analytical sensitivity among assays.[24,25] Few studies have examined cross-platform comparisons of broad NGS ctDNA assays.

Future research in the area of analytical validity needs to focus on more and larger cross-platform comparisons to clearly define the performance of various assays. In addition, more studies are urgently needed on assay robustness to changes in pre-analytical and analytical variables. To ensure quality control and to allow unbiased comparison between assays, proficiency testing using standardized samples and administered by independent groups would be highly desirable, and several such efforts are in development. Finally, studies are needed to define the minimal levels of analytical sensitivity and specificity that will maximize clinical utility across the spectrum of envisioned clinical applications for ctDNA assays.

## INTERPRETATION AND REPORTING

A comprehensive discussion of the interpretation and reporting content for ctDNA assay results is beyond the scope of this review. This section focuses on areas that present particular challenges to ctDNA assays. Previously published general guidance about interpretation and reporting of clinical molecular assays should be reviewed.[26-28]

Selection of therapy is a nuanced process guided by numerous factors, including tumor type, grade, stage, patient performance status, prior therapies, and genetic findings. The same variant may have different therapeutic consequences, depending on the primary tumor site. Caution is needed when reporting actionability of a particular genomic variant on a ctDNA report. It is advisable to limit discussion of potential actionability to general associations between a variant and therapy options that have established clinical utility in the same primary tumor site, to avoid specific therapeutic recommendations for the patient, and to emphasize that variant data must be integrated with other clinical information for appropriate selection of therapy.

As is discussed in the analytical validity section, targeted PCR-based ctDNA assays focus on the detection of known somatic variants. Broad NGS-based approaches detect these somatic (acquired) variants, but they also may identify germline (heritable) variants. Evidence suggests that a variant is somatic if it meets certain criteria, including a variant allele fraction that is substantially less than 50%, which is the expected allele fraction for heterozygous germline variants; the variant is a known, commonly recurring somatic variant with clinical significance in cancer; and the variant is not commonly observed in population databases. The presence of all three criteria strongly suggests that a variant is somatic, but there are ambiguous cases on rare occasions. High allele fraction alone does not strongly discriminate between somatic and germline, as some somatic variants in cell-free DNA may be found with high allele fraction (eg, a variant allele from a locus that is genetically amplified). In cases where the variant could be germline in nature, follow-up testing of germline DNA with a clinical germline sequencing assay could aid clinical decision

making (eg, determination if a *BRCA1* pathogenic or likely pathogenic variant is germline or somatic).

The proportion of ctDNA as a fraction of total free DNA in plasma (may be referred to as purity) varies substantially between different patients, and allele fractions of variants in ctDNA need to be interpreted with great caution. The relative abundance of leukocyte DNA may vary in different specimens on the basis of pre-analytical issues (as noted above). Comparison of relative allele fractions between different variants identified in the same assay might identify variants that are not present in all cancer cells, identifying intrapatient tumor heterogeneity.[29] Such subclonal variants may have a lower response to therapies targeting the mutation,[30] although there is no evidence of validity for this approach, and further research is required. Furthermore, it can be difficult to calculate the actual fraction of cell-free DNA composed of ctDNA, especially with targeted assays. Although targeted assays can provide accurate quantitation of variant allele fraction, a single measurement may not be representative of the actual fraction of cell-free DNA composed of ctDNA. For example, the variant could be subclonal or the variant could be present in a region of copy number variation (eg, on an amplified allele).

Not all somatic variants identified in circulating cell-free DNA originate from the cancer. Somatic variants may be found in apparently healthy people,[31,32] arising in part from clonal hematopoiesis. Age-related clonal hematopoiesis, also referred to as clonal hematopoiesis of indeterminate potential, is characterized by the detection of recurring somatic variants most commonly associated with hematologic cancers in the peripheral blood.[33-36] These variants are observed with increasing frequency from approximately the fifth decade of life, detected in approximately 5% of persons 60 to 69 years of age and 10% of persons $\geq$ 70 years of age.[33] The substantial majority of individuals with clonal hematopoiesis do not have hematologic cancer, but it does confer an increased risk.[31,32] The most commonly involved genes include *DNMT3A*, *TET2*, and *ASXL1*; however, other frequently mutated genes include *TP53*, *JAK2*, *SF3B1*, *GNB1*, *PPM1D*, *GNAS*, and *BCORL1*.[33-35] Although most studies examining clonal hematopoiesis of indeterminate potential have been performed with peripheral blood, these mutations also appear in plasma,[31,32] because hematopoietic cells are the origin of the majority of cell-free DNA in healthy individuals.[37] Given the limited evidence, caution is needed when interpreting ctDNA variants in these genes, and further work is needed to determine how to interpret and report ctDNA variants in these genes.

All ctDNA assays have an appreciable rate of discordance with tumor testing, and the ctDNA assay may not detect the variant observed in the tumor specimen in some patients. In part, this reflects very low release of tumor DNA into plasma in some patients with cancer. Such discordant results are particularly frequent in cancers of the central nervous system,[20] potentially because the blood-brain barrier blocks release of tumor DNA into the systemic circulation. Failure to detect a somatic variant in a ctDNA assay, consequently, may result from the variant being absent in the tumor or from an insufficient amount of ctDNA being present in the specimen. In contrast, with standard tissue-based molecular testing in which histologic assessment of the specimen is used to evaluate for sufficient neoplastic cell content, similar confirmation of the presence of sufficient ctDNA is not generally available in ctDNA assays. For these reasons, reporting of ctDNA assays necessitates clear communication of this limitation when a somatic variant is not detected by including a prominent note or comment in the report. Terms such as not detected, undetected, or uninformative are generally more precise than reporting the lack of detection of somatic variants as negative.

## CLINICAL VALIDITY AND UTILITY

Once a tumor biomarker test has demonstrated adequate analytical validity, the next step is to demonstrate clinical validity and, most importantly, clinical utility. These elements are essential for clinicians and patients to use these tests to inform treatment decisions. Although it is highly unlikely that a ctDNA test would have clinical utility if it has not previously been shown to have clinical validity, the reverse is not true. An assay may have clinical validity but not have clinical utility. Demonstration of clinical validity does not confer or imply clinical utility. Several methods of establishing clinical utility have been proposed, either as prospective clinical trials[38,39] or as retrospective characterization of archived specimens from previous prospective clinical trials.[40] For ctDNA assays, the pre-analytical issues discussed above render the latter particularly problematic, unless care has been taken to collect, process, and store the specimens appropriately.

As noted, there are several contexts in which a ctDNA assay might be applied. We principally focus on the use of ctDNA assays in metastatic cancer, because there is generally substantially less evidence regarding ctDNA assays in other settings.

### *Evidence on the Use of ctDNA Assays for Treatment Selection in Advanced Cancer*

The clinical validity of ctDNA assays has been the subject of multiple studies in select cancer types. In general, PCR-based assays for detection of oncogenic driver variants have very high diagnostic specificity, but more modest diagnostic sensitivity. For example, in lung cancer, in a review of five studies that used tissue genotype as the reference standard, specificities for canonical driver variants averaged 96% (95% CI, 83% to 99%), and sensitivities averaged 66% (95% CI, 63% to 69%).[41-45] For variants selected before treatment, such as the *EGFR* T790M variant in the setting of acquired resistance, sensitivities remained moderate, whereas specificities showed more variability (range, 40% to 78%), a difference believed to be a result of the genomic heterogeneity of treatment resistance.[30,46-49] PCR-based ctDNA assays for *KRAS* genotyping in colorectal cancer have also been systematically analyzed and demonstrate high specificity and moderate sensitivity.[50]

Fundamentally, there are two paradigms to demonstrate clinical utility and the adoption of ctDNA as a clinically useful test. The most reliable are prospective clinical trials to test the clinical utility of ctDNA as a stand-alone diagnostic test. No such trial has been reported to date. A second strategy is to assess whether ctDNA provides the same information as tissue genomic evaluation. If tissue genomic evaluation has proven clinical utility with high levels of evidence, demonstrating that a ctDNA assay has high agreement with tumor tissue genotyping may provide sufficient

  

evidence of utility for ctDNA assays in driving patient treatment decisions.

Definitively establishing the clinical utility of ctDNA assays, as compared with a standard biopsy for tumor genotyping, is challenging, because prospective trial data are lacking. At present, one PCR-based ctDNA assay for the detection of *EGFR* variants in patients with NSCLC has received regulatory approval in the United States and Europe, and PCR-based ctDNA assays for *EGFR* in NSCLC and *KRAS* in colorectal cancer are available for commercial use in Europe. These assays have demonstrated clinical validity,[51-53] but the clinical utility in this setting is based on retrospective analyses. Evidence demonstrated that, although positive *EGFR* testing results may effectively be used to guide therapy, undetected results should be confirmed with analysis of a tissue sample, if possible. Cases in which the variant is not detected in the ctDNA but is detected in the tissue sample are relatively common, so undetected ctDNA assay results should be confirmed in tumor tissue testing (Fig 1).[54] As a general point, the literature demonstrates that treatment selection in advanced cancer is optimized when ctDNA assays are performed in the context of disease progression rather than while a patient is still demonstrating response to prior therapy. ctDNA levels may decrease when a tumor is responding to treatment, and sensitivity of ctDNA assays may be reduced if the samples are taken while a tumor is responding to therapy.

The challenges of demonstrating clinical utility are illustrated in NSCLC. A major potential issue is that the patient population selected for study inclusion may not be representative of those targeted for the intended clinical use of the ctDNA assay. In NSCLC, this can occur for at least two reasons. First, although recent prospective data are lacking, older trials have estimated that approximately 20% of patients with NSCLC with resistance to *EGFR* tyrosine kinase inhibitors either cannot (or were not willing to) undergo biopsy or biopsy tissue was inadequate.[55] Although several trials have demonstrated that patients with NSCLC with an *EGFR* variant in plasma do just as well on *EGFR* tyrosine kinase inhibitors as those with an *EGFR* variant in the tumor,[51,54,56] these studies did not include patients who could not obtain tumor tissue genotyping. Second, most trials preselected patients with positive tumor tissue genotyping for treatment; therefore, plasma-positive cases often were double positives, both in tumor tissue and plasma, which was not representative of the intended clinical use of ctDNA assays. One post hoc analysis of an osimertinib trial in NSCLC included 18 patients with *EGFR* T790M detected in plasma, but not in tumor tissue, and this small cohort of patients did less well than patients with T790M detected on tumor tissue genotyping.[30] To date, few trials have prospectively tested the outcomes of treatment when a targeted therapy was selected solely on the basis of a ctDNA assay result.[57]

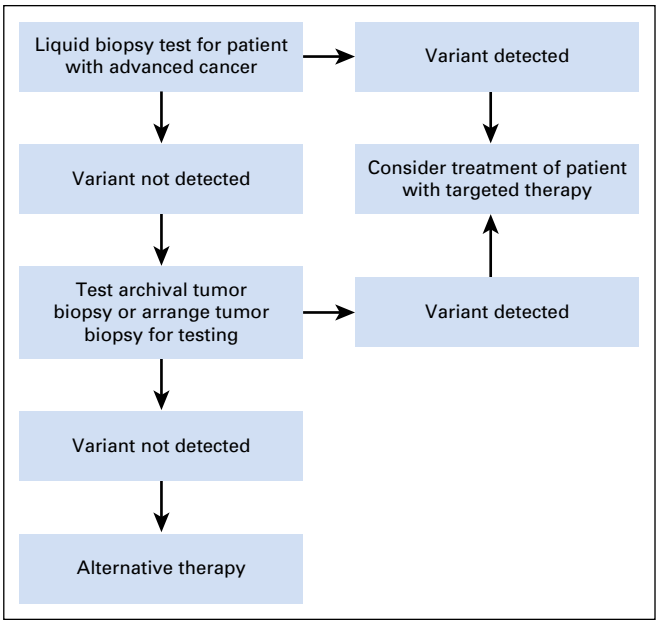


**Fig 1.** Reflex tumor biopsy testing in patients with negative liquid biopsy results. The evidence indicates that current liquid biopsy assays have suboptimal sensitivity and have an appreciable rate of discordance with tumor tissue genotyping. There are two potential reasons for a not-detected circulating tumor DNA (ctDNA) result: no variant in the tumor, or the variant is present in the tumor tissue but not detected by the ctDNA assay. A diagnostic approach relying only on ctDNA analysis could fail to identify relevant information from tumor tissue.

There is limited evidence of clinical validity of ctDNA analysis in other tumor types and for variants that were not analyzed as part of the ctDNA studies for *EGFR* in lung cancer and *KRAS* in colorectal cancer. A wide range of ctDNA assays have been developed and clinically studied for detection of potentially targetable variants, such as *BRAF* variants in melanoma[58] and *PIK3CA* and *ESR1* variants in breast cancer,[29,59] and the diagnostic performance characteristics are in line with the assays described previously. Nevertheless, the clinical utility of these assays has not been established.

The large number of potential genetic driver events in advanced cancers has raised interest in NGS-based panel ctDNA assays, with the potential to detect a wide range of simple and complex genomic events, including targetable gene rearrangements (eg, *ALK* and *ROS1*).[60,61] Determination of clinical validity for these broad NGS-based approaches is challenging, given that they generally target multiple tumor types. Initial studies seem to demonstrate similar overall concordance with tissue-based genotyping as PCR-based assays, although concordance may be reduced for variants found in ctDNA at a low variant allele fractions ($< 1\%$).[62]

Advanced cancers may be genetically heterogeneous, and this presents a potential challenge to ctDNA testing, in particular because the ctDNA assays may sample tumor DNA arising from all sites of metastasis, whereas tissue genotyping is conducted on a biopsy of a single metastatic site or on the archival primary (Fig 2).[63] High-sensitivity ctDNA assays may detect subclonal variants, and such subclonal variants may theoretically not predict for durable responses to therapies that target the variant. The extent of the subclonality likely differs depending on the variant, whether the variant can be selected by prior therapy, and the patient population. For example, genetic heterogeneity likely does not appreciably affect the utility of ctDNA testing for *EGFR*-activating variants in therapy-naïve advanced lung cancer, because the variants are rarely subclonal. The extent to which genetic heterogeneity affects the utility of ctDNA testing for treatment-selected *EGFR* T790M variants, where variants may be subclonal, has not been robustly established in the literature. Limited current data suggest that the incidence of subclonal *EGFR* T790M variants is

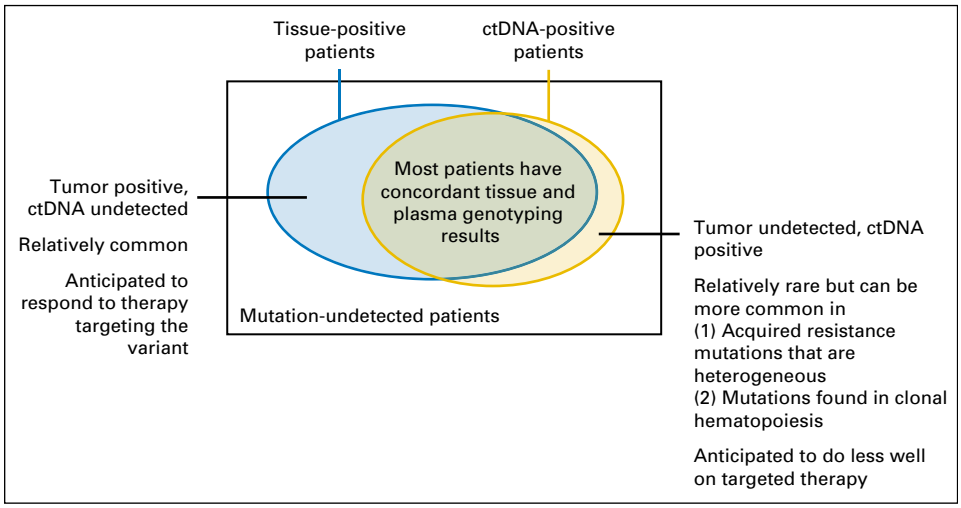


**Fig 2.** Overlap between tissue and circulating tumor DNA (ctDNA) genotyping in advanced cancer. The box represents a cohort of patients genotyped with tissue tumor and plasma ctDNA assays, many of whom will be undetected with both assays. For patients with mutation detected in tissue (blue circle) or ctDNA (gold circle), most are expected to have concordant tissue and ctDNA results. Discordant tissue and ctDNA genotyping results are most commonly a result of the low sensitivity of ctDNA assays in tumors with low shedding of DNA into the blood. These patients with tissue-positive, ctDNA-undetected disease would be expected to potentially respond to matched targeted therapy. Discordance with tissue-undetected, ctDNA-positive results is most likely to result from either temporal heterogeneity (an archival tumor specimen), spatial heterogeneity (a subclonal mutation), or assay error (false-negative tissue genotyping or false-positive ctDNA genotyping). Clonal hematopoiesis of indeterminate potential may cause discordance from some mutations. Adapted from G.R. Oxnard, American Association for Cancer Research, 2016.[63]

sufficiently low, with the studied PCR assay, to not affect utility.[30] Further research is required to assess for which variants, and in which contexts of testing, subclonality may undermine the clinical utility of ctDNA assays.

### *Establishing Clinical Validity and Utility of ctDNA Assays*

Future research studies to establish clinical validity and utility of ctDNA assays should include a patient cohort that matches the intended-use population as closely as possible and samples collected from a prospective study with defined entry criteria. Data will most frequently come from a phase II or phase III study in the patient population where it is anticipated the assay would be used in subsequent clinical practice, with the frequency of the variant under study approximately equal to that in an unselected clinical population. In prospective studies of targeted therapies, the entry criteria should allow inclusion of patients in which the variant under study is observed in the plasma, but not in the tissue analysis, to evaluate the treatment response of this population with discordant genotyping results.

### *Evidence on the Use of ctDNA Assays for Noninvasive Monitoring of Advanced Cancer*

Another potential use for ctDNA assays is monitoring treatment effect, involving quantitative measurement of ctDNA over time, in response to cancer treatments. Blood-based monitoring of treatment response and progression via ctDNA analysis is attractive because it is minimally invasive, does not involve ionizing radiation, and could ultimately be less expensive than current approaches to response assessment. Indeed, assays for tumor-associated proteins, such as carcinoembryonic antigen, prostate-specific antigen, CA-125, MUC1, and CA19-9, are well established in routine clinical care for patients with documented metastatic colorectal, prostate, ovarian, breast, and pancreatic cancers, respectively.

However, validation of an assay quantitation of tumor burden is more technically challenging than an assay that merely dichotomizes patients into ctDNA variant detected or not detected. First, the efficiency and reproducibility of pre-analytical and analytical steps are critical to allow reliable quantitation of variant ctDNA. Compatibility and interoperability of results, in terms of the measured variant ctDNA load from different laboratories, will also be necessary. Quantitation needs to be uniform and reproducible between laboratories for results to be comparable within and between patients and to allow for results from different laboratories and trials to be comparable. Furthermore, the best unit for quantifying DNA burden is not established; most current approaches measure either the somatic variant allele fraction or detected somatic variant events per unit of plasma.[64,65] Because the former method is a ratio of somatic variant to nonvariant ctDNA, it controls for the amount of plasma DNA input. However, this ratio could be affected by the levels of non–cancer-origin cell-free DNA, which can fluctuate over time, and may also conceivably be affected by certain therapies. The best approach to quantitation is currently unclear and will likely evolve in concert with what is needed for clinical utility and patient management.

Correlations between changes in ctDNA levels and tumor responses or outcomes have been demonstrated in small proof-of-principle studies in a variety of cancer types, such as lung cancer,[44,49,66] colorectal cancer,[67,68] breast cancer,[2,69] lymphoma,[70,71] and melanoma.[72] In addition, studies of multiple cancer types indicate that ctDNA analysis can identify the emergence of resistant mutations months earlier than standard radiologic studies,[68,73,74] creating an opportunity to test whether changing therapy before clinical progression could improve outcomes.[75]

However, currently there is a lack of rigorous evidence on clinical validity, let alone clinical utility, because few large, prospective validation studies have been performed on ctDNA-based monitoring. Published studies are mostly retrospective, and few

  

rigorous comparisons to established response metrics have been performed. In addition, no studies convincingly demonstrate improved patient outcomes or any cost savings when compared with standard-of-care monitoring approaches. There is no evidence supporting changing treatment at the time of ctDNA progression before clinical progression. Finally, some data suggest that ctDNA responses do not always parallel imaging-based responses.[76] This could complicate validation of ctDNA-based monitoring and suggests that studies will ultimately need to assess clinical outcome in addition to correlation with radiographic responses.

### *Evidence on the Use of ctDNA Assays to Detect Residual Disease in Early-Stage Cancer*

There is hope that ctDNA assays can be used for detection and monitoring of residual tumor after curative therapy for solid tumors, in the way that detection of leukemic cells in blood after completion of initial therapy (or minimal residual disease) has entered routine clinical practice in the management of leukemia.[77] ctDNA can be detected before treatment in patients with early-stage primary cancer; however, ctDNA is generally detected at a lower rate than in advanced cancer.[20,60,78] Persistent detection of ctDNA after local therapy (surgery or radical radiotherapy) predicts for a high risk of relapse in proof-of-principle studies in colon cancer,[64,65,79] breast cancer,[80,81] pancreatic cancer,[82] and lung cancer.[83] In these studies, the primary tumor is often sequenced to identify somatic genetic events that can then be tracked in plasma as evidence of residual disease. Evidence is lacking to demonstrate the ability of ctDNA assays to detect a similarly low level of residual disease that would correctly be referred to as minimal residual disease detection similar to the use of the term in leukemia management.

Importantly, current studies are retrospective and findings have not been validated in prospective studies, providing limited evidence of clinical validity. No studies have systematically conducted imaging at the point of ctDNA detection to confirm that overt metastatic disease has not already developed at the point of ctDNA detection. The false-negative rate of ctDNA analysis in this setting (patients who relapse without ctDNA being detected) and the false-positive rate (patients who do not relapse despite the ctDNA assay being positive),[84] have not been established sufficiently for any assay. Large, prospective studies are needed to establish clinical validity for this purpose.

The theoretical potential of detection of residual disease in this fashion is that early treatment, triggered by changes in ctDNA, could eradicate residual disease and prevent or delay relapse. The clinical utility of such an approach has not been established; there is no evidence that treatment on the basis of the detection of ctDNA improves outcome. Indeed, prospective randomized trials of circulating protein markers have failed to demonstrate survival benefits from screening for occult recurrences in breast and ovary cancers, although there are data to suggest they are helpful in colorectal and prostate cancers.[85-89] Evidence of clinical utility can only be obtained from future prospective randomized studies.

### *Evidence on the Use of ctDNA Assays in Screening for Cancer in Asymptomatic Individuals*

Given that ctDNA can be detected in some patients diagnosed with early-stage cancer, there is substantial interest in the potential of using ctDNA in early detection of cancer in asymptomatic individuals and populations. Case reports of detection of cancer during maternal cell-free DNA testing, to detect fetal DNA aneuploidy, raise the potential of this approach.[90] However, at this time there are no data on clinical validity in this setting and no evidence of clinical utility. The extent to which assays may have false-positive test results (both technical and biologic), diagnosing the presence of cancer in a patient without cancer, and determining tissue of origin, have not been established. It is also possible that circulating genomic variants could arise in cells that have taken the first step toward transformation but were never destined to become clinically important. This form of biologic false positive, commonly termed overdiagnosis, has been well documented in breast cancer with mammographic screening[91] and prostate cancer with prostate-specific antigen screening.[92]

Although assays detecting viral DNA inserted into cancer DNA were not reviewed in this statement, an important prospective study has demonstrated the potential of screening for nasopharyngeal carcinoma in China; a ctDNA assay for Epstein-Barr virus DNA detected early-stage cancers with a positive predictive value of 11%.[93] Although this study highlights the potential of ctDNA analysis for cancer screening, the analytical challenges of detecting a nonhuman genome like Epstein-Barr virus are substantially different from common solid tumor early detection. At present, there is no evidence of clinical validity and utility for ctDNA assays in patients without a cancer diagnosis.

## CONCLUSION

ctDNA assays could play a future role in the treatment of patients with cancer. Despite the extremely high level of current enthusiasm, deployment of ctDNA assays in routine clinical practice requires evidence of clinical utility. There is little evidence of clinical validity and clinical utility to support widespread use of ctDNA assays in most patients with advanced cancer, with the exception of those with demonstrated clinical utility or those with regulatory approval. The increasing uptake of ctDNA assays in clinical care highlights the clear demand to inform clinical decision making. Robust research is needed in several areas, as discussed in this article, to enable development of clinical practice recommendations. Tumor genotyping is a rapidly evolving area of research in many areas of cancer care. Over time, it is highly likely that evidence will emerge to enable better assessment of the clinical validity and utility of ctDNA assays.

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

## AUTHOR CONTRIBUTIONS

**Manuscript writing:** All authors
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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

**Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review**

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## Appendix

**Table A1.** Expert Panel Members for the ASCO and College of American Pathologists Joint Review on Circulating Tumor DNA Analysis in Patients With Cancer

| Panel Member Name | Affiliation |
|---|---|
| ASCO representatives | |
| Nicholas C. Turner, MD, PhD, co-chair | Royal Marsden Hospital and Institute of Cancer Research |
| Maximilian Diehn, MD, PhD | Stanford University School of Medicine |
| Daniel F. Hayes, MD, FACP, FASCO | University of Michigan Comprehensive Cancer Center |
| Geoffrey R. Oxnard, MD | Dana Farber Cancer Institute and Harvard Medical School |
| Richard L. Schilsky, MD, FACP, FSCT, FASCO | American Society of Clinical Oncology |
| Apostolia M. Tsimberidou, MD, PhD | The University of Texas MD Anderson Cancer Center |
| College of American Pathologists representatives | |
| Jason D. Merker, MD, PhD, co-chair | Stanford University School of Medicine |
| Carolyn Compton, MD, PhD | Arizona State University |
| Alexander J. Lazar, MD, PhD | The University of Texas MD Anderson Cancer Center |
| Neal Lindeman, MD | Brigham and Women's Hospital and Harvard Medical School |
| Christina M. Lockwood, PhD, DABCC, DABMGG | University of Washington<br>Representative, Association for Molecular Pathology |
| Alex J. Rai, PhD | Columbia University Medical Center<br>American Association for Clinical Chemistry Liaison to the College of American Pathologists Molecular Oncology Committee |
| ASCO staff | |
| Patricia Hurley, MSc | Director, Research and Analysis, Staff Lead |
| Thomas K. Oliver | Director, Guidelines Development |
| Suanna S. Bruinooge, MPH | Division Director, Research and Analysis |
| Courtney Davis | Program Coordinator, Research and Analysis |
| College of American Pathologists staff | |
| Patricia Vasalos | Technical Manager, Proficiency Department, Staff Lead |
| Brooke L. Billman | Medical Librarian |

### Literature Search Methodology

Literature search strategies were developed in collaboration with a medical librarian for the concepts of liquid biopsies, blood, cancer abnormalities, and pre-analytical, analytical, interpretation, reporting, utility, and validity variables. The search strategies were created using standardized database terms and text words. PubMed searches were completed on March 20, 2017, and were limited to articles published in English from January 2007 to March 2017, inclusive. Comments, editorials, and letters were excluded. The Cochrane search filter for humans was applied. Additional searches for gray literature were run April 3, 2017, in the Cochrane Library, National Guideline Clearinghouse, ClinicalTrials.gov, and applicable US and international organizational websites.

### Literature Review Results

As noted in the Quality of Reporting of Meta-Analyses diagram in Figure A1, a total of 1,338 unique publications were identified through database searching. Title and abstract screening resulted in 390 articles for full-text review. The Expert Panel supplied an additional 31 references for use in the manuscript. Ultimately, 77 articles were selected for inclusion in the review. Note: In the few instances where citations were published before 2007, there were no more recent publications found.

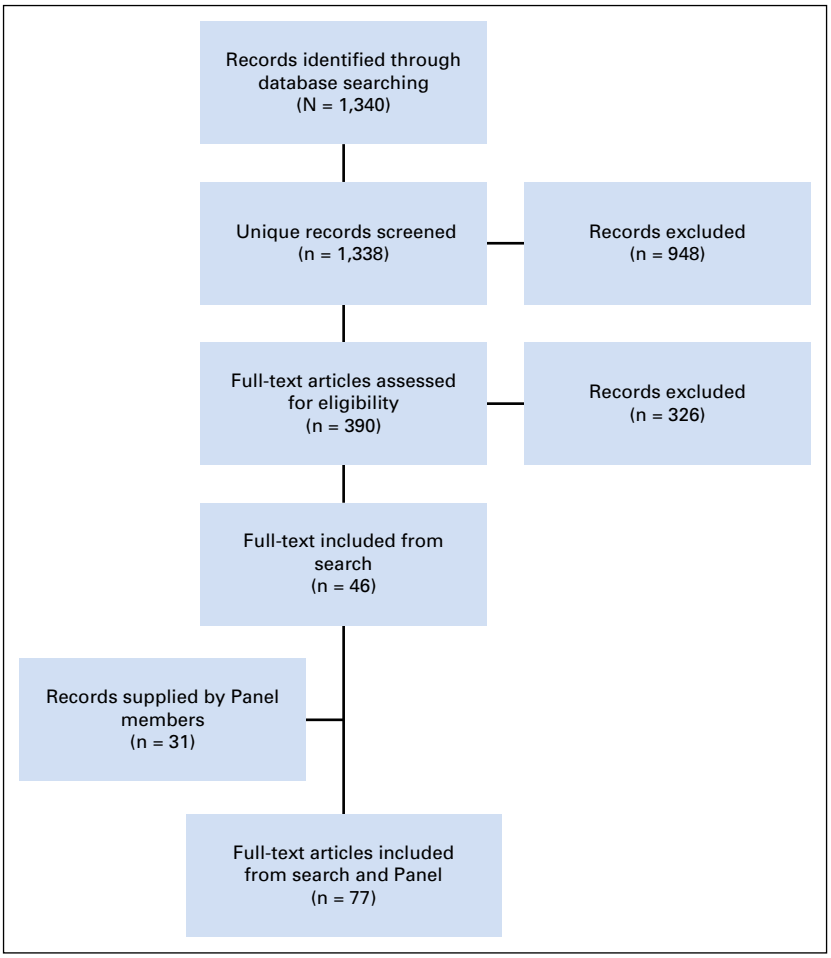


**Fig A1.** QUOROM (Quality of Reporting of Meta-Analyses) diagram.