Sentara Health Plans

Liquid Biopsy

Policy Number: AHS – G2054 – Liquid Biopsy	Prior Policy Name and Number, as applicable: • AHS-G2054 Detection of Circulating Tumor Cells and Cell Free DNA in Cancer Management • Consolidated: AHS-M2140 – Liquid Biopsy
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I. Policy Description

The National Cancer Institute (NCI) defines "liquid biopsy" as a test done on a sample of blood, urine, or other bodily fluid to look for cancer cells from a tumor or small pieces of DNA, RNA, or other molecules released by tumor cells into a person's body fluids. Liquid biopsies are non-invasive blood tests since circulating tumor cells (CTCs) and cell-free tumor DNA (cfDNA) fragments are shed into the bloodstream from existing tumors and can be detected in blood (Curigliano, 2014; Haber & Velculescu, 2014). The presence of CTCs can be indicative of metastatic disease (Alix-Panabieres & Pantel, 2013).

For guidance concerning Tumor Mutational Burden Testing (TMB) and/or Microsatellite instability (MSI) analysis please refer to AHS-M2178-Microsatellite Instability and Tumor Mutational Burden Testing policy.

II. Indications and/or Limitations of Coverage

Application of coverage criteria is dependent upon an individual's benefit coverage at the time of the request. Specifications pertaining to Medicare and Medicaid can be found in Applicable State and Federal Regulations of this policy document.

1) For individuals diagnosed with non-small cell lung cancer (NSCLC), cell-free DNA/circulating tumor DNA (cfDNA/ctDNA) testing **MEETS COVERAGE CRITERIA** in **any** of the following situations:
   a) When tissue-based testing is infeasible (i.e., quantity not sufficient for tissue-based test or invasive biopsy is medically contraindicated).
   b) In the initial diagnostic setting when there is insufficient tissue to allow testing for broad molecular analysis following pathological confirmation of NSCLC (if an oncogenic driver is not identified, follow-up tissue-based analysis should be considered).

c) In the initial diagnostic setting when tissue-based molecular analysis does not completely assess all recommended biomarkers due to tissue quantity or testing methodologies available. Recommended biomarkers include:

i) *ALK* rearrangements.

ii) *BRAF* mutations.

iii) *EGFR* mutations.

iv) *ERBB2* (*HER2*) mutations.

v) *KRAS* mutations.

vi) *MET*ex14 skipping mutations.

vii) *NTRK1/2/3* fusions.

viii) *RET* rearrangements.

ix) *ROS1* rearrangements.

x) PD-L1 expression levels.

d) To aid in biomarker evaluation for treatment selection in the initial diagnostic setting (when the feasibility of timely tissue-based testing is uncertain).

2) For individuals diagnosed with HR-positive/HER2-negative breast cancer and who are being considered for targeted therapy, cfDNA/ctDNA testing for *PIK3CA* **MEETS COVERAGE CRITERIA**.

3) For individuals diagnosed with castration-resistant prostate cancer, cfDNA/ctDNA testing of the following biomarkers **MEETS COVERAGE CRITERIA**:

a) Androgen receptor variant 7 (AR-V2) to guide therapy selection in the post-abiraterone/enzalutamide metastatic CRPC setting.

b) Somatic analysis of *BRCA1* and *BRCA2* to select patients for rucaparib treatment.

4) For individuals meeting the above criteria, cfDNA/ctDNA testing (annually) **MEETS COVERAGE CRITERIA**.

The following does not meet coverage criteria due to a lack of available published scientific literature confirming that the test(s) is/are required and beneficial for the diagnosis and treatment of a patient's illness.

5) For all other situations not described above, liquid biopsy testing for screening, detecting, **and/or** monitoring any other malignancy or tumor **DOES NOT MEET COVERAGE CRITERIA**.

6) For all situations not addressed above, analysis of PD-L1 by liquid biopsy **DOES NOT MEET COVERAGE CRITERIA**.

7) For the screening, detection, and/or diagnosis of cancer, urinary liquid biopsy (i.e., use of cell-free DNA ["UcfDNA"] or circulating tumor DNA obtained in a urine sample) (e.g.,

SelectMDX) **DOES NOT MEET COVERAGE CRITERIA**.

8) Liquid biopsy testing on CSF samples **DOES NOT MEET COVERAGE CRITERIA**.

9) Cell capture-enumeration assays of CTCs (e.g., CELLSEARCH® CTC) **DO NOT MEET COVERAGE CRITERIA**.

III. Table of Terminology

Term	Definition
AACC	American Association for Clinical Chemistry
ALK	*Anaplastic lymphoma receptor tyrosine kinase*
AMP	The Association for Molecular Pathology
AR	*Androgen receptor*
AR-V7	*Androgen receptor splice variant 7*
ASCO	American Society of Clinical Oncology
BRAF	*B-Raf proto-oncogene*
BRCA1	*Breast cancer type 1 susceptibility gene*
BRCA1/2	*Breast cancer type 1/2 susceptibility gene*
BRCA2	*Breast cancer type 2 susceptibility gene*
CAM	Cell adhesion molecule
CAP	College of American Pathologists
CF	Cell-free
cfDNA	Cell-free tumour deoxyribonucleic acid
CGP	Comprehensive somatic genomic profiling
CLIA ’88	Clinical Laboratory Improvement Amendments of 1988
c-MET	*Cellular mesenchymal epithelial transition*
CNS	Central nervous system
CRC	Colorectal cancer
CRPC	Castration-resistant prostate cancer
CSCO	Chinese Society of Clinical Oncology
CSF	Cerebrospinal fluid
CSF-CTC	Circulating tumor Cells in cerebrospinal fluid
CTCs	Circulating tumor cells
ctDNA	Circulating tumor deoxyribonucleic acid
CTLA-3	Cytotoxic T-lymphocyte-associated protein 3
DLX1	Distal-less 1
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
EAU	European Association of Urology
EGFR	*Epidermal growth factor receptor*
EpCAM	Epithelial cell adhesion molecule

Term	Definition
ER+ MBCs	Estrogen receptor-positive metastatic breast cancer
ERCC1	*Excision repair cross-complementation group 1*
ER-CTCs	Estrogen receptor-negative circulating tumor cells
ESMO	European Society for Medical Oncology
ESTRO	European Society of Urogenital Radiology
EV	Extracellular vesicle
ExoRNA	Exosome ribonucleic acid
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
GC	Gastric cancer
gDNA	Genomic deoxyribonucleic acid
HCC	Hierarchical condition category
HDL	High-density lipoprotein
hENT1	*Human equilibrative nucleoside transporter 1*
HER2	Human epidermal growth factor receptor 2
HOXC6	Homeobox C6
IASLC	International Association for the Study of Lung Cancer
InDels	Insertions/Deletions
KLK3	*Kallikreins 3*
KRAS	*Kirsten rat sarcoma viral oncogene homolog*
LAG-3	Lymphocyte-activation gene 3
LM	Leptomeningeal metastasis
MDX	Molecular diagnostics
MET	*MET Proto-Oncogene*
MRI	Myotubularin 1
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MSI-H	Microsatellite instability-high
NACB	National Academy of Clinical Biochemistry
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
NGS	Next-generation sequencing
NIH	National Institute of Health
NK	Natural killer
NRAS	*Neuroblastoma rat sarcoma*
NSCLC	Non-small cell lung cancer
PCR	Polymerase chain reaction
PD-L1	Programmed death-ligand 1
PFS	Progression-free survival
PIK3CA	*Phosphatidylinositol 3-Kinase*

Term	Definition
PSA	Prostate-specific antigen
RET	*Rearranged during transfection*
RGQ	Rapid gas quenching
RNA	Ribonucleic acid
RNases	Ribonucleases
RRM1	*Ribonucleotide reductase, M1 subunit*
RT-PCR	Reverse transcriptase polymerase chain reaction
SIOG	International Society of Geriatric Oncology
SNVs	Single nucleotide variants
tDNA	Tissue deoxyribonucleic acid
TEXs	Tumor-derived exosomes
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TKIs	Tyrosine kinase inhibitors
TMB	Tumor mutational burden
TOP1	*DNA topoisomerase 1*
TOP2A	*DNA topoisomerase 2 alpha*
TOP2B	*DNA topoisomerase 2 beta*
TP	*Tumour protein*
TUBB3	*Tubulin beta 3 class III*
UcfDNA	Urinary cell-free deoxyribonucleic acid
utDNA	Urine-derived tumor deoxyribonucleic acid
XRCC1	*X-ray repair cross-complementing 1*

IV. Scientific Background

The science of noninvasive disease monitoring has advanced greatly since circulating cell-free DNA (cfDNA) was first reported in body fluids by Mandel and Metais. Since then, the evolution of sensitive cfDNA detection technologies has enabled the development of liquid biopsies with many clinical applications. For example, in oncology, the use of liquid biopsy allows for patient stratification, screening, monitoring treatment response and detection of minimal residual disease after surgery or recurrence. Liquid biopsies have grown in importance because the genetic profile of tumors can affect how well patients respond to a certain treatment. However, this characterization is currently achieved through a biopsy despite the inherent problems in procurement of tissue samples and the limitations of tumor analyses. For example, the invasive nature of a biopsy poses a risk to patients and can have a significant cost (Brock et al., 2015).

Tumor sampling from some cancer types also remains difficult resulting in inadequate amount of tissue for genetic testing (Brock et al., 2015). In the case of advanced or metastatic non-small cell lung cancers (NSCLC), as many as 69% of cases do not have accessible tissue (Douillard et al., 2009). Even when tissue can be collected, preservation methods such as formalin fixation can cause false positive results for genetic tests (Quach et al., 2004). Finally, due to tumor heterogeneity, biopsies often suffer from sample bias (Bedard et al., 2013). Liquid biopsies are

becoming more popular as they provide an opportunity to genotype in a less invasive and expensive manner. However, the low sensitivity (between 60-80%) and higher number of false negative cases compared to traditional tissue biopsy are limitations associated with liquid biopsies (Sequist & Neal, 2022).

Approaches to Liquid Biopsy Analysis

Circulating tumor cells (CTCs)

According to Brock et al. (2015), CTCs are cells shed into the vasculature from a primary tumor and may constitute seeds for subsequent growth of additional tumors (metastasis) in distant organs (Brock et al., 2015). CTCs generally confer the advantage of containing RNA, DNA, and protein from tumor cells including both nuclear and cytoplasmic biomarkers, which is not attainable from ctDNA or exosomes (Yu et al., 2021). They have been detected in various metastatic carcinomas (Mavroudis, 2010) but are extremely rare in healthy subjects and patients with nonmalignant diseases (Brock et al., 2015). Clinical evidence indicates that patients with metastatic lesions are more likely to have CTCs amenable to isolation but their frequency is low, often ~1-10 CTCs per mL of whole blood (Miller et al., 2010). As 1 mL of blood contains ~7×10^6 white blood cells and ~5×10^9 red blood cells, technologies capable of reproducibly isolating a single CTC from the background of all other blood components are fundamental. While such levels of sensitivity are challenging, there are several novel developments in this area, including positive selection, negative selection, physical properties or even enrichment-free assays to efficiently isolate these rare CTCs (Alix-Panabieres & Pantel, 2013). However,Bettegowda et al. (2014)) stated that an advantage of ctDNA is that it can be analyzed from bio-banked biofluids, such as frozen plasma (Bettegowda et al., 2014).

Typically, CTCs are defined as cells with an intact viable nucleus, cytokeratin positive, epithelial cell adhesion molecule (EpCAM) positive and with the absence of CD45 (Brock et al., 2015). Unfortunately, EpCAM and other markers are not always expressed on CTCs (Grover et al., 2014). In addition, non-tumor epithelial cells are known to circulate in the blood of patients with prostatitis or patients undergoing surgery (Brock et al., 2015; Murray et al., 2013). The heterogeneity of CTCs is a major challenge from a technical standpoint. This has led to alternative strategies of CTC enrichment such as the CTC-iChip which does not rely on tumor antigen expression (Brock et al., 2015; Karabacak et al., 2014).

Sequencing the genetic material from CTCs has demonstrated that the majority are not cancer cells, even when the isolated cell(s) fit the phenotypic criteria of being a CTC. One study by Marchetti et al. (2014) developed a protocol to recover the CTC enriched samples from the cartridge of the Veridex platform and found that from 37 NSCLC patients, the *EGFR* mutation allele abundance ranged between 0.02% and 24.79% with a mean of 6.34%. Brock et al. (2014) concluded that the number of CTCs found in the blood is therefore highly dependent on how the platform defines a cell as a CTC (Brock et al., 2015; Marchetti et al., 2014). The CellSearch CTC test, a Food and Drug Administration (FDA) approved actionable CTC test, requires that samples are processed within 96 hours of collection after being drawn into the *Cellsave* preservative tube. This test does not analyze the molecular genetics of the tumor; rather *Cellsave* is a platform for CTC numeration. A positive test (more than five detected CTCs for metastatic breast and prostate cancer and more than three CTCs for metastatic colorectal cancer per 7.5 mL of blood) is

associated with decreased progression-free survival and decreased overall survival in these patients (Aggarwal et al., 2013).

Overall, although CTCs have produced some promising results in evaluating prognosis of patients with varying cancers, further studies are needed to assess the clinical utility of these biomarkers (Adamczyk et al., 2015; Bidard et al., 2016; Foukakis & Bergh, 2022; Ignatiadis & Dawson, 2014).

Cell-free DNA (cfDNA)

There is currently an intensive research effort to understand the utility of cfDNA in various clinical fields, such as cancer research, non-invasive prenatal testing and transplant rejection diagnostics (Brock et al., 2015). In a systematic review and meta-analysis of 20 studies and 2012 cases covering assessment of *EGFR* mutational status in NSCLC, Luo et al. (2014)found a sensitivity of 0.674, a specificity of 0.935, and area under the curve of 0.93. The authors concluded that detection of *EGFR* mutation by cfDNA is of adequate diagnostic accuracy and cfDNA analysis could be a promising screening test for NSCLC (Luo et al., 2014).

In a study, Jiang et al. (2015)observed that most cfDNA in plasma is reportedly fragmented, around 150-180 bp in length with a higher prevalence of tumor associated mutations in the shorter fragments. Per authors, when analyzing the mutation abundance with massively parallel sequencing, a significant correlation was found between mutations and fragments less than 150 bp. Notably, the size of the majority of cfDNA fragments overlaps well with the size of histone DNA (Jiang et al., 2015).

A direct comparison of mutation detection on cfDNA *vs.* CTCs showed a higher abundance of the mutation on the cfDNA from the same patient; moreover, recent large studies comparing the effectiveness of cfDNA analysis to tissue biopsy in NSCLC showed the clinical value of the liquid biopsy approach (Douillard et al., 2014). This positive result led to an approval to use cfDNA analysis for *EGFR* mutation analysis for IRESSA in Europe (in patients where a tumor sample was not evaluable), making it the first *EGFR* tyrosine kinase inhibitor for which cfDNA testing is included in the label. Although promising, challenges remain when using cfDNA to characterize the mutation status of a tumor. In addition to the low copy number of mutant alleles, the median half-life of cfDNA in circulation ranges from 15 minutes to a few hours (Brock et al., 2015).

Brock et al. (2015), in their review, observed that the total concentration of cfDNA in the blood of cancer patients varies considerably with tumor specific mutations ranging from undetectable (less than 1 copy per 5 mL of plasma) to patients with over a hundred thousand copies of the mutation per mL of plasma. The authors note that "the challenge of how to maximize the yield of the cfDNA and pair this with a platform sensitive enough to detect rare variants in the background of wild-type DNA remains. Optimally, the ability to detect mutations in plasma should not be limited to a subpopulation of patients with very high mutant copy numbers in circulation" (Brock et al., 2015). This has been proven to be challenging in early stage cancers (Yu et al., 2021).

While many analytical platforms report the mutation load with an allelic frequency compared to

the wild-type DNA platforms relying solely on the allelic frequency without recording the number of mutations have limitations. This is because the allelic frequency of a gene is affected by the amount of wild-type DNA not related to the tumor. Therefore, it is important to consider the processes that affect the amount of wild-type DNA in circulation (Brock et al., 2015). For example, exercise increases cfDNA levels almost 10-fold (Breitbach et al., 2014). Other pre-analytical variables such as blood collection, the cellular process leading to its release, and extraction protocols affect the amount and size range of cfDNA fragments in a sample (Devonshire et al., 2014).

Exosomes

In the last few years, the exosome field has grown exponentially impacting various areas of research. Studies demonstrating that exosomes are actively released vesicles (carrying RNA, DNA, and protein) and can function as intercellular messengers. Yanez-Mo et al. (2015) highlights these developments in a review outlining the biological properties of exosomes and other extracellular vesicles (EVs). However, Gould and Raposo (2013) observed that the exosome field still lags behind as the standardization of extracellular vesicle (EV) types are not yet firmly established. The majority of exosomes range in size from 30-200 nanometers (nm) in diameter and are isolated from all bio-fluids, including serum, plasma, saliva, urine and cerebrospinal fluid (Brock et al., 2015).

Due to the size of an exosome, on average just over 100 nanometers, the entire transcriptome cannot be packaged inside every vesicle. By way of comparison, retrovirus particles with a similar size can package only around 10 kb, so it is likely that a single vesicle of that size carries only a limited number of transcripts. However, exosomes are extremely abundant (10^{11} per mL of plasma) and when isolating the vesicle fraction, most of the transcriptome can be detected (Brock et al., 2015). Per Huang et al. (2013)and Kahlert et al. (2014), exosomal RNA can be used for mutation detection as well as global profiling of most types of RNA, and the profile alone (without mutation characterization) can be utilized for diagnostics (Brock et al., 2015). In the study 'Immune modulation of T-cell and NK (natural killer) cell activities by TEXs (tumor-derived exosomes)', Whiteside (2013)observed that exosome investigations have focused on the important physiologic and pathophysiologic functions of these vesicles in micro-metastasis, angiogenesis and immune modulation and as a means for detection of tumor specific mutations in bio-fluids (Whiteside, 2013). Consequently, in 2012, interest in this new field increased when the National Institute of Health (NIH) dedicated the large strategic Common Fund to study these new entities of extracellular RNA. The goal of this effort is to better understand how exosomes can be utilized for biomarkers and therapeutics as well as understanding this new mechanism of intercellular communication (NIH, 2017).

Mutation detection and RNA profiling

Analysis of nucleic acids present in bodily fluids can provide a better understanding of the disease, as summarized in Table below.

Analysis capability	Examples	CTCs	cfDNA	Exosomes

Mutations	Point mutations, InDels, amplifications, deletions, translocations	Yes	Yes	Yes
Epigenetic modifications	Methylation patterns	Yes	Yes	Yes
RNA transcription profiles	Levels/activity of mRNA, microRNA, long non-coding RNA, RNA splice variants	Yes	No	Yes
Phenotypic studies of cells from the tumor	Cell morphology, protein localization, *in vivo* studies	Yes	No	No
Inflammatory response, stromal and other systemic changes	Inflammatory RNA and protein markers	No	No	Yes
Analysis of RNA as well as DNA and protein profiles from tumor cells	Separate or in combination	Yes	No	Yes
Can utilize bio-banked samples	Frozen plasma, urine and other bio-fluids	No	Yes	Yes

CTCs, circulating tumor cells; cfDNA, cell-free DNA; InDels, insertions/deletions. (Brock et al., 2015)

RNA profiling from biofluids is also difficult. However, since exosomes contain RNA, it was possible to separate the fragile RNA from the large amounts of RNases and PCR inhibitors. As cell-free RNA in blood is immediately degraded, RNAs in serum and plasma were either protected inside vesicles, in protein complexes or associated with HDL particles (Brock et al., 2015). The levels of these microRNAs are tightly regulated in normal cells, and dysregulation has been implicated in several human diseases, e.g., cardiovascular (Thum & Condorelli, 2015) and neurological, and is strongly linked to cancer development and progression. However, microRNAs represent only a minor fraction of the transcriptome. By contrast, the nucleic acids in exosomes can be isolated and the entire transcriptome examined (Brock et al., 2015).

The most significant hurdle for all forms of liquid biopsy remains the relative rarity of nucleic acid derived from a tumor against the background of normal material found in most patient samples. In fact, the majority of cell, cell-free nucleic acids, microRNAs and exosomes in a liquid biopsy will have originated from normal cells with numbers fluctuating as a consequence of biological variations (Brock et al., 2015).

Furthermore, although liquid biopsy was first introduced with serum, other liquid media, such as urine and cerebrospinal fluid (CSF), have been used to evaluate other conditions. Cell-free DNA is not necessarily confined to blood, and other media have been proposed.

Urine

Urine's primary advantage over blood is that it is non-invasive, allowing for more convenient testing. Urinary cell-free DNA (UcfDNA) has been proposed as a biomarker for the detection and diagnosis of certain cancers, particularly bladder and prostate cancer (Lu & Li, 2017). An

example of this is SelectMDX. SelectMDX evaluates two mRNA cancer-related biomarkers (HOXC6 and DLX1 with *KLK3* as a reference gene) to assist a clinician in deciding to continue routine screening or to order a prostate biopsy. This test is considered a "non-invasive urine test" (a liquid biopsy) and reports a binary result of "increased risk" or "very low risk" (MDx, 2018). Van Neste et al. evaluated this test at a 0.90 area under curve in a validation cohort. The authors concluded that the mRNA signature was one of the most significant components of the validation results (Van Neste et al., 2016). Shore et al. (2019) assessed the effect of SelectMDX results on clinical decision making and found that out of 253 patients SelectMDX evaluated as "negative," only 12% underwent a biopsy (Shore et al., 2019).

Xu et al. (2021) assessed the diagnostic value of urinary exosomes for urological tumors. The authors performed a systematic review and meta-analysis of 16 studies with a total of 3224 patients. Diagnostic value was calculated based on the number of true positives, false positives, true negatives, and false negatives. The sensitivity of using urinary exosomes for the diagnosis of urological tumors was 83% and the specificity was 88%. Sensitivity and specificity results were similar regardless of urinary exosome content type and tumor type. The authors conclude that "urinary exosomes may serve as novel non-invasive biomarkers for urological cancer detection" (Xu et al., 2021).

Cerebrospinal Fluid (CSF)

CSF is a colorless, clear liquid produced by the choroid plexus. CSF acts to control flow of molecules to the central nervous system (CNS). Due to the tight control of the CSF, it may play a significant role in assessing several conditions. CSF is traditionally used to evaluate conditions such as meningitis, but it has also been used to assess central nervous system cancers, such as leptomeningeal metastases (Demopoulos, 2022; Johnson, 2021). In addition to widely-known measures of pathology in CSF (opening pressure, total protein, glucose, cell count with differential), circulating tumor cells in CSF have also been proposed as markers for epithelial tumors (Demopoulos, 2022).

Lin et al. (2017) evaluated the diagnostic accuracy of circulating tumor cells in CSF (CSF-CTC) in patients with leptomeningeal metastasis (LM). 30 of 95 total patients were diagnosed with LM based on a combination of CSF cytology and MRI. CSF-CTCs were detected in 43 patients (median 19.3 CSF-CTC/mL). Based on receiver operating curve analysis, the optimal cutoff was found to be 1 CSF-CTC/mL, identifying patients at a rate of 93% sensitivity, 95% specificity, positive predictive value 90%, and negative predictive value 97% (Lin et al., 2017). Diaz et al. (2022)studied the clinical utility of CSF-CTC by evaluating how CSF-CTC quantification was able to predict the outcome of LM. The authors performed a single institution retrospective study of 101 LM patients with solid tumors. The CSF-CTC count significantly predicted survival continuously ($p=0.0027$). The authors conclude that "CSF-CTCs quantification predicts survival in newly diagnosed LM, and outperforms neuroimaging" and suggest CSF-CTC can be used for LM prognosis and to assess disease burden (Diaz et al., 2022).

Mathios and Phallen (2022) published a review paper noting "significant strides" towards understanding the molecular mechanisms of brain cancer. Research advances in the field include a focus on the "tumor microenvironment" and identifying molecular biomarkers with liquid-based analyses (such as CSF in liquid biopsy). While it is a rapidly advancing area of research,

clinical utility is currently limited, that is, there are currently "no approved noninvasive tests that are clinically useful" for gliomas. The authors point to Cristiano et al. (2019) as an example of a study that analyzed genome-wide cfDNA fragment features (in a variety of cancers); the authors were able to distinguish patients with cancer from non-cancer patients (as well as isolate the tissue of origin). In another glioma-specific study, Mouliere et al. (2018) detected 5 of 13 patients' brain tumors (38%) using a cfDNA fragmentation-based approach to analyze cfDNA fragments and copy number alterations in CSF. In conclusion, the authors note that, despite recent excitement over promising studies, liquid biopsy approaches to brain cancer are still "in their infancy" (Mathios & Phallen, 2022).

Proprietary Testing

FDA approval of use of the Roche Cobas *EGFR* Mutation Test in plasma was based on evaluation of plasma samples from the ENSURE study (Wu et al., 2015), a multicenter, open-label, randomized, Phase III study of stage IIIB/IV NSCLC patients. 98.6% of the patients enrolled (214/217) had a plasma sample available for testing. The agreement between the Cobas *EGFR* Mutation Test in plasma and tissue was evaluated for detection of *EGFR* mutations. In 76.7% of tissue-positive specimens, plasma was also positive for an *EGFR* mutation. Plasma was negative for *EGFR* mutation in 98.2% (95.4%, 99.3%) of tissue-negative cases. The patients whose plasma results were positive for exon 19 deletion and/or an L858R mutations treated with erlotinib had improved progression-free survival (PFS) compared to those treated with chemotherapy (FDA, 2016).

Another commercially available, FDA-approved test is Guardant360 by Guardant Health Inc. Guardant360 is a gene panel that sequences 74 genes (including 18 amplifications and 6 fusions) associated with NSCLC and reports the percentage of cfDNA (Guardant, 2022). The manufacturer purports that this genetic test will allow providers to make better treatment decisions based on the mutations present in the patient (Health, 2023). The gene panel was analytically validated, with 99.8% accuracy on 1000 consecutive samples (Lanman et al., 2015).

FoundationOne has also created a proprietary FDA-approved test that examines cell-free DNA. FoundationOne's liquid CDx test evaluates 324 genes using circulating cell-free DNA and is FDA-approved to report short variants in 311 genes(FoundationOne, 2022, 2023). A prior version of this test (covering 62 genes) was evaluated based on 2666 reference samples. The assay reached >99% sensitivity of short variants of allele frequencies of >0.5%, >95% sensitivity of allele frequencies 0.25%-0.5%, and >70% sensitivity of allele frequencies 0.125%-0.25%. Out of 62 healthy volunteers, no false positives were detected (Clark et al., 2018).

Biodesix is another laboratory that offers a liquid biopsy panel. Biodesix offers two tests; one called GeneStrat, tests *EGFR*, *ALK*, *ROS1*, *RET*, *BRAF*, and *KRAS* (Biodesix, 2023). Sensitivities of 78%-100% for *EGFR*, *ALK*, and *KRAS* with the GeneStrat test were shown in multiple validation studies (Mellert et al., 2017). GeneStrat also detected over 88% of *RET* or *ROS1*-positive patients (Mellert et al., 2018). Biodesix also offers GeneStrat NGS, a broad 52 gene panel also evaluated through blood-based liquid biopsy technology.

Other firms that offer liquid biopsy testing include ResolutionBio (now part of Agilent) which offers Agilent Resolution ctDx FIRST ("companion diagnostic to KRAZATI™ (adagrasib) for

the detection of *KRAS* G12C in non-small cell lung cancer [NSCLC]") and Agilent Resolution ctDx LUNG, which focuses on actionable genes for lung cancer such as *EGFR* and *ALK*; Circulogene (tests *BRAF, EGFR, KRAS, ALK, ROS1,* PD-L1, and MSI), Neogenomics (InvisionFirst, 37-gene panel including 10 actionable genes), and Biocept (CNSide™). As liquid biopsy is a rapidly emerging field, it is possible that many more tests will find their way into the clinical setting (Biocept, 2023; Circulogene, 2023; Neogenomics, 2022; ResolutionBio, 2021).

Clinical Utility and Validity

Seeberg et al. (2015) conducted a prospective study to assess the prognostic and predictive value of CTCs in 194 patients with colorectal liver metastasis referred to surgery. 153 patients underwent a resection (41 patients had an unresectable tumor), and CTCs were detected in 19.6% of patients. Patients with unresectable tumors had a 46% CTC positivity rate compared to 11.7% for resectable tumors. Patients with two or more CTCs experienced reduced time to relapse/progression. Two or more CTCs was a strong predictor of progression and mortality in all subgroups of patients. The authors concluded that "CTCs predict nonresectability and impaired survival. CTC analysis should be considered as a tool for decision-making before liver resection in these patients" (Seeberg et al., 2015),

Groot et al. (2013) performed systematic review and meta-analysis to investigate the prognostic value of CTCs in patients with resectable colorectal liver metastases or widespread metastatic colorectal cancer (CRC). The results of 12 studies representing 1,329 patients were suitable for pooled analysis. The overall survival and progression-free survival were worse in patients with CTCs, with hazard ratios of 2.47 for overall survival rate and 2.07 for progression-free survival. The authors concluded that "the detection of CTCs in peripheral blood of patients with resectable colorectal liver metastases or widespread metastatic CRC is associated with disease progression and poor survival" (Groot Koerkamp et al., 2013).

Zhang et al. (2012) conducted a meta-analysis of published literature on the prognostic value of CTC in breast cancer. Forty-nine eligible studies enrolling 6,825 patients were identified. The presence of CTC was significantly associated with shorter survival in the total population and the prognostic value of CTC was significant in both early and metastatic breast cancer. The authors concluded that "the detection of CTC is a stable prognosticator in patients with early-stage and metastatic breast cancer. Further studies are required to explore the clinical utility of CTC in breast cancer" (Zhang et al., 2012).

Pinzani et al. (2021) assessed that the clinical validity of CTCs has been demonstrated in cancer screening, prognosis, and monitoring treatment responses. In the original article by Cabel et al. (2017), using the Cellsearch® technique in early non-metastatic cancer has reported low CTC detection rates (5-30% depending on cancer type), with limited specificity since "some circulating epithelial cells can be found in individuals with inflammatory disease or even in some healthy individuals." However, in the preliminary report of another study, it was found that a CTC count >25 could "distinguish lung cancer from benign lesions in patients with abnormal lung imaging. CTC count was also shown to be an "independent prognostic factor in non-small cell lung cancer and small cell lung cancer;" despite this, CTCs are rare in the non-metastatic setting, and thus cannot be completely utilized as an independent prognostic factor in the

localized setting. With respect to the independent cancers, Cabel et al. (2017) summarizes the clinical validity of CTC detection in **Figure 1**. (Gregory et al., 2013) (Gregory et al., 2013) (Gregory et al., 2013)

On the clinical utility of CTC, Cabel et al. (2017) initially stated "the clinical utility of CTC detection (i.e. does it improve patient outcome) has yet to be demonstrated before it can be implemented in routine clinical practice." In recent time, it was seen that specific CTC features may have clinical utility in "[predicting] the sensitivity to specific immunotherapies," and in the case of ER+ MBCs, ER-CTCs can develop and reflect "acquisition of therapy resistance by the primary tumor" (Pinzani et al., 2021).

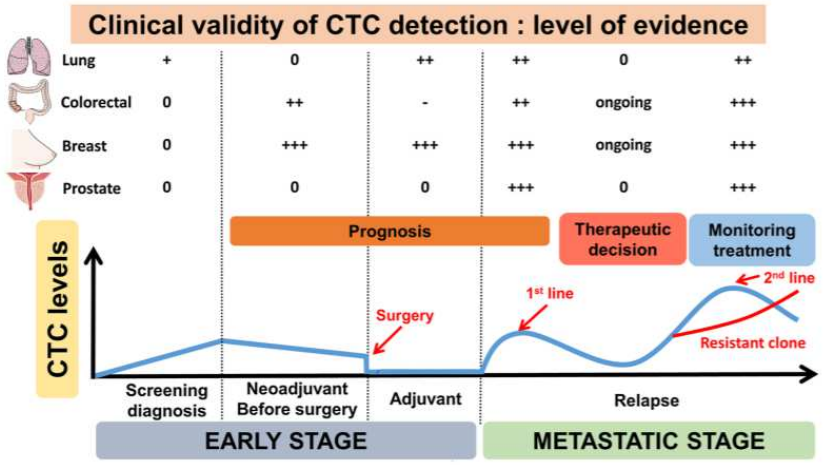

Figure 1. Clinical validity of circulating tumor cells (CTC): level of evidence according to clinical settings (Cabel et al., 2017).

Oxnard et al. found that: "Sensitivity of plasma genotyping for detection of T790M was 70%. Of 58 patients with T790M-negative tumors, T790M was detected in plasma of 18 (31%). ORR and median PFS were similar in patients with T790M-positive plasma (Objective response rate [ORR], 63%; progression-free survival [PFS], 9.7 months) or T790M-positive tumor (ORR, 62%; PFS, 9.7 months) results. Although patients with T790M-negative plasma had overall favorable outcomes (ORR, 46%; median PFS, 8.2 months), tumor genotyping distinguished a subset of patients positive for T790M who had better outcomes (ORR, 69%; PFS, 16.5 months) as well as a subset of patients negative for T790M with poor outcomes (ORR, 25%; PFS, 2.8 months) (Oxnard et al., 2016)." The authors concluded that "upon availability of validated plasma T790M assays, some patients could avoid a tumor biopsy for T790M genotyping" (Oxnard et al., 2016).

A review by Sacher et al. genotyped 180 patients with NSCLC using plasma droplet PCR (plasma ddPCR). This was done to validate the plasma droplet PCR technique, and the study identified 115 *EGFR* mutations and 25 *KRAS* mutations. The plasma ddPCR was measured to have 82% sensitivity for the *EGFR* 19 del, 74% for L858R, 77% for T790M, and 64% for *KRAS*. The positive predictive value was 100% for every mutation apart from T790M at 79%. The authors concluded that the technique "detected *EGFR* and *KRAS* mutations rapidly with the high specificity needed to select therapy and avoid repeat biopsies". The authors also noted that this assay "may also detect *EGFR* T790M missed by tissue genotyping due to tumor heterogeneity

in resistant disease" (Sacher et al., 2016).

Kim et al. (2017) evaluated the clinical utility of Guardant360. This study used the Guardant360 panel to detect mutations in patients with metastatic NSCLC and other cancers. Somatic mutations were detected in 59 patients, 25 of which had actionable mutations. Out of the 73-patient NSCLC cohort, 62 were found to have somatic mutations and 34 had actionable mutations. After these genetic findings were identified, molecularly matched therapy was provided to 10 patients with gastric cancer (GC) and 17 with NSCLC. Response rate was 67% in GC and 87% in patients with NSCLC, while disease control rate was 100% for both types (Kim et al., 2017).

Odegaard et al. (2018) validated the Guardant360 cell-free DNA sequencing test and aimed to "demonstrate its clinical feasibility". The authors found that the test could detect variants down to "0.02% to 0.04% allelic fraction/2.12 copies with ≤0.3%/2.24-2.76 copies". Clinical validation in a cohort of over 750 patients demonstrated high accuracy and specificity, with positive percent agreement (with PCR) of 92%-100% and negative percent agreement of over 99%. In terms of feasibility, the authors performed the test in 10593 patients and found the technical success rate to be over 99.6% and the clinical sensitivity to be 85.9%. The authors also noted that 16.7% of these mutations were targetable with FDA-approved treatments (with 72% with "treatment or trial recommendations") with as many as 34.5% of non-small cell cancer samples having a targetable mutation (Odegaard et al., 2018).

Aggarwal et al. (2019) evaluated the utility of plasma-based sequencing in improving mutation detection in patients with non-small cell lung cancer. The authors first performed next-generation sequencing (NGS) on tissue, then plasma-based sequencing. 229 patients had concurrent sequencing, and NGS alone detected 47 targetable mutations. Addition of plasma sequencing brought that number to 82 targetable mutations. Furthermore, 36 of 42 patients that received "plasma next-generation sequencing–indicated therapy" achieved a "complete or a partial response or stable disease". The authors concluded that "adding plasma next-generation sequencing testing to the routine management of metastatic non–small cell lung cancer appears to increase targetable mutation detection and improve delivery of targeted therapy" (Aggarwal et al., 2019).

Leighl et al. (2019) evaluated the utility of "comprehensive cell-free DNA analysis" to identify genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancer (NSCLC). 282 patients were included. Tissue genotyping (current standard of care) identified a guideline-recommended biomarker in 60 patients, whereas cell-free DNA identified a relevant biomarker in 77 patients. Concordance between the two methods was 80% (48 biomarkers detected in both methods). For FDA-approved targets (*EGFR*, *ALK*, *ROS1*, *BRAF*), concordance was >98.2% with 100% positive predictive value for cell-free DNA. Cell-free DNA was also found to have a faster median turnaround time (9 days compared to 15 for tissue genotyping), and "guideline-complete" (assessment of all eight guideline-recommended biomarkers [*EGFR*, *ALK*, *ROS1*, *BRAF*, *RET*, *MET* amplification and exon 14 skipping, and HER2]), was significantly more likely (268 patients vs 51) (Leighl et al., 2019).

Dudley et al. (2019) have developed a novel high-throughput sequencing method that uses urine-derived tumor DNA (utDNA) known as utDNA CAPP-Seq (uCAPP-Seq) to detect bladder

cancer. This technique was used to analyze samples from 118 patients with early-stage bladder cancer and 67 healthy adults. "We detected utDNA pretreatment in 93% of cases using a tumor mutation-informed approach and in 84% when blinded to tumor mutation status, with 96% to 100% specificity" (Dudley et al., 2019). These results show that utDNA can be used to diagnose early-stage bladder cancer with high sensitivity and specificity.

Wang et al. (2018) performed a meta-analysis to determine the diagnostic performance of cell-free DNA (both blood and urine) assays in bladder cancer. 11 studies encompassing 802 patients were included. The authors evaluated cell-free DNA assays at the following statistics: "sensitivity 0.71, specificity 0.78 positive likelihood ratio 3.3, negative likelihood ratio 0.37, diagnostic odds ratio 9, and area under curve 0.80. No publication bias was identified. The authors concluded that "cell-free DNA has a high diagnostic value in bladder cancer" (Wang et al., 2018).

cfDNA can hopefully be used to indicate prognoses of personalized peptide vaccine therapy in patients with NSCLC. Waki et al. (2021) identified that cfDNA integrity "decreased after the first cycle of vaccination" and that those with "high prevaccination cfDNA integrity survived longer than those with low prevaccination integrity (median survival time (MST): 17.9 versus 9.0 months, respectively; hazard ratio (HR): 0.58, p= .0049)," showing that monitoring cfDNA levels could contribute to quantifying treatment success and predicting patient lifespans.

For exosome-based liquid biopsy, Yu et al. (2021) have proposed a synergistic alternative of combining cfDNA and exosomal RNA to "increase the sensivity of mutation detection… the exosome component enables a combination of exosomal RNA, cfDNA, and disease specific proteins… the unique composition of the exosome compartment makes these vesicles particularly amenable for multi-analyte testing, since they carry cancer-informative DNA, RNA, proteins, lipids, oligosaccharides, and metabolites. In one study, a high sensitivity (92%) for *EGFR* mutations was found for utilizing exosomal RNA and ctDNA together and remained high in a subpopulation that's been difficult for ctDNA assays to detect (88% sensitivity). ExoRNA and ctDNA combined analyses on *BRAF, KRAS,* and *EGFR* mutations in exosomes and respective ctDNA have also better correlated the biomarkers with treatment outcomes when compared to ctDNA alone (Yu et al., 2021).

Lee et al. (2021) analyzed the clinical utility of ctDNA to reliably detect *EGFR* in ctDNA. The authors compared *EGFR* analysis results between tissueDNA (tDNA) and ctDNA from 554 NSCLC cases. ctDNA analysis detected *EGFR* mutation in 57.3% of cases. ctDNA detection correlated with metastatic stage and disease progression ($p<0.001$). The authors followed up after an average of 41.09 month and found that, "survival analysis revealed ctDNA status and M stage ($p < 0.001$) to be independent predictors of overall survival in the multivariate analysis." The authors conclude that ctDNS is clinically useful for *EGFR* analysis, but note the possibility of false negatives and recommend using tDNA to confirm ctDNA results in some situations (Lee et al., 2021). Syeda et al. (2021) evaluated the use of ctDNA as a biomarker for melanoma. The authors measured changes in ctDNA and survival following "BRAF, MEK, or BRAF plus MEK inhibitor therapy" in patients participating in two clinical trials. The $BRAF^{V600}$-mutant was measured in ctDNA before and during treatment. "Elevated baseline $BRAF^{V600}$ mutation-positive ctDNA concentration was associated with worse overall survival outcome." The authors conclude that $BRAF^{V600}$-mutation ctDNA analysis can be used as a biomarker to predict clinical outcomes (Syeda et al., 2021).

V. Guidelines and Recommendations

National Comprehensive Cancer Network (NCCN)

NCCN guidelines for non-small cell lung cancer (NSCLC) strongly advise "broader molecular profiling with the goal of identifying rare driver mutations for which effective drugs may already be available, or to appropriately counsel patients regarding the availability of clinical trials. Broad molecular profiling is a key component of the improvement of care of patients with NSCLC." Furthermore, the NCCN states that "Data suggest that plasma genotyping (also known as plasma testing or liquid biopsy) may be considered at progression instead of tissue biopsy to detect whether patients have T790M; however, if the plasma biopsy is negative, then tissue biopsy is recommended" (NCCN, 2023b).

However, the NCCN goes on to state that cell-free or circulating tumor DNA testing should not be used in lieu of histologic tissue diagnosis. The NCCN notes that specificity is generally very high for cell-free tumor testing but is lacking in sensitivity (up to 30% false-negative rate) and that standards for testing have not been well established. The use of cell-free or circulating tumor DNA may be considered in specific clinical situations, such as if a patient is medically unfit for an invasive tissue sampling or if there is insufficient material for a molecular analysis following pathologic confirmation of an NSCLC diagnosis (but only if "follow-up tissue-based analysis is planned for all patients in which an oncogenic driver is not identified." The NCCN notes that "recent data suggest that plasma cell-free/circulating tumor DNA testing can be used to identify EGFR, ALK, and other oncogenic biomarkers that would otherwise not be identified in patients with metastatic NSCLC" (NCCN, 2023b).

For NSCLC, the NCCN provides the following specific recommendations for liquid biopsy:

> "The use of cell-free/circulating tumor DNA testing can be considered in specific clinical circumstances, most notably:
>
> - If a patient is medically unfit for invasive tissue sampling
> - In the initial diagnostic setting, if following pathologic confirmation of a NSCLC diagnosis there is insufficient material for molecular analysis, cell-free/circulating tumor DNA can be used; however, follow-up tissue-based analysis for all patients in which an oncogenic driver is not identified should be planned (see NSCL-18 for oncogenic drivers with available targeted therapy options).
> - In the initial diagnostic setting, if tissue-based testing does not completely assess all recommended biomarkers owing to tissue quantity or testing methodologies available, consider repeat biopsy and/or cell-free/circulating tumor DNA testing.
> - In the initial diagnostic setting, if the feasibility of timely tissue-based testing is uncertain, concurrent cfDNA testing may aid in biomarker evaluation for treatment selection, provided negative results are considered per above limitations."

The NCCN lists "comprehensive germline and somatic profiling to identify candidates for additional targeted therapies" as part of the workup for recurrent stage IV (M1) breast cancer." They go on to specifically note that "tissue or plasma-based circulating tumor DNA (ctDNA) assays may be used. Tissue-based assays have greater sensitivity, but ctDNA may reflect tumor

heterogeneity more accurately." The NCCN also states that assessment of the PIK3CA mutation may be performed through liquid biopsy if the tumor is HR-positive, HER2 negative, and if therapy with alpelisib plus fulvestrant is being considered. Finally, for the management of breast cancer with liquid biopsy techniques, the NCCN states that "the clinical use of Circulating Tumor Cells (CTC) or circulating DNA (ctDNA) in metastatic breast cancer is not yet included in the NCCN Guidelines for Breast Cancer for disease assessment and monitoring", though the sentence that follows would indicate that this statement refers to a count of CTCs, not their use for genotyping: "Patients with persistently increased CTC after 3 weeks of first-line chemotherapy have a poor PFS and OS" (NCCN, 2022c).

The NCCN states that AR-V7 testing in CTCs "can be considered to help guide selection of therapy in the post-abiraterone/enzalutamide metastatic CRPC [castration-resistant prostate cancer] setting." The NCCN does not comment on any particular liquid medium over another (e.g., urine, CSF, serum). However, the NCCN does specify the use of circulating DNA for rucaparib treatment, stating that "the preferred method of selecting patients for rucaparib treatment is somatic analysis of BRCA1 and BRCA2 using a circulating tumor DNA sample" (NCCN, 2023a). SelectMDx is also acknowledged by the NCCN; "the panel believes that SelectMDx score is potentially informative in patients who have never undergone biopsy, and it can therefore be considered in such men" (NCCN, 2022j).

With regards to circulating tumor DNA (ctDNA) in colon cancer, the NCCN "panel believes that there are insufficient data to recommend the use of multigene assays, Immunoscore, or post-surgical ctDNA to estimate risk of recurrence or determine adjuvant therapy" (NCCN, 2022e).

NCCN guidelines for small cell lung cancer do not address use of CTCs or ctDNA for patient management (NCCN, 2023c).

For neuroendocrine tumors, NCCN notes that CTCs have been studied as prognostic markers, but state that more research is required. There is no single biomarker available that is satisfactory as a diagnostic, prognostic, or predictive marker (NCCN, 2022h).

For a primary CNS lymphoma, the NCCN remarks that cerebrospinal fluid analysis may "possibly" include gene rearrangement evaluation. For leptomeningeal metastases, the NCCN notes that assessment of CTCs in CSF "increases sensitivity of tumor cell detection and assessment of response to treatment" (NCCN, 2022d).

For pancreatic adenocarcinomas, the NCCN acknowledges that circulating cell-free DNA is being investigated as a biomarker for screening. The NCCN also notes that if tumor tissue is not available, cell-free DNA testing may be considered (NCCN, 2022i).

For esophageal, esophagogastric junction cancers, and gastric cancers, the NCCN states "testing using a validated NGS-based [next generation sequencing] genomic profiling assay performed in a CLIA-approved laboratory may be considered for some patients. A negative result should be interpreted with caution, as this does not exclude the presence of tumor mutations or amplifications" (NCCN, 2022f). The NCCN does not comment on the usage of liquid biopsies, ctDNA, or CTCs for testing for hepatobiliary cancers (NCCN, 2022g).

For acute myeloid leukemia, the NCCN notes that "morphologically detectable," circulating leukemic blasts from peripheral blood may be used to detect molecular abnormalities (NCCN, 2022a).

For bladder cancer, the NCCN mentions RT-PCR testing for FGFR2/3 gene alterations but does not specify whether this can be done through a liquid biopsy or cell-free DNA. The only comment made is that the laboratory should be CLIA-approved (NCCN, 2022b).

American Society of Clinical Oncology (ASCO)

In 2016, ASCO published updated recommendations for the use of tumor markers in treatment of metastatic breast cancer. ASCO found that although CTCs may be prognostic, they are not predictive for clinical benefit when used to guide or influence decisions on systemic therapy for metastatic breast cancer. ASCO recommends clinicians to not use these markers as adjunctive assessments (Van Poznak et al., 2015). Similarly, ASCO recommended against use of CTCs to guide decisions about adjuvant systemic therapy for women with early stage invasive breast cancer (Andre et al., 2019).

In 2019, ASCO stated that clinicians "should not use circulating biomarkers as a surveillance strategy for detection of recurrence in patients who have undergone curative-intent treatment of stage I-III NSCLC or SCLC". ASCO states that further data is required to validate this approach (Schneider et al., 2019).

In 2018, ASCO and the College of American Pathologists (CAP) released a joint review on "circulating tumor DNA analysis in patients with cancer". In it, they note that apart from the assays that have received "regulatory appeal", most assays have "insufficient evidence" for both clinical validity and clinical utility. They note discordant results between circulating DNA assays and tissue genotyping. Furthermore, they remark on the lack of evidence for use in monitoring therapy effectiveness, diagnosing early-stage cancer, or cancer screening.

However, they point to evidence that well-validated assays may support initiation of targeted therapy (Merker et al., 2018).

National Academy of Clinical Biochemistry (NACB), now known as the American Association for Clinical Chemistry (AACC)

In 2010, the NACB issued practice guidelines for the use of tumor markers in liver, bladder, cervical, and gastric cancers. It found that CTCs had "questionable" clinical utility in the assessment of liver cancer and did not recommend their use (Sturgeon et al., 2010).

The NACB published an updated guideline in 2020. For liver cancer, they note circulating cell-free serum DNA as "undergoing evaluation" for "predictive marker for distant metastasis of hepatitis C virus–related HCC." The plasma proteasome is also undergoing evaluation for "assessment of early HCC in patients with chronic viral chronic hepatitis; assessment of metastatic potential of HCC." Finally, circulating methylated DNA is undergoing evaluation for HCC screening, detection, and prognosis. No other circulating tumor markers for bladder, cervical, and gastric cancers were mentioned (Sturgeon et al., 2020).

College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP)

An expert panel was convened to review and update the CAP-IASLC-AMP Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors. This panel consists of practicing pathologists, oncologists, and a methodologist.

The panel states there is "insufficient evidence to support the use of circulating cell-free plasma DNA (cfDNA) molecular methods for the diagnosis of primary lung adenocarcinoma". According to the panel, there is also "insufficient evidence to support the use of circulating tumor cell (CTC) molecular analysis for the diagnosis of primary lung adenocarcinoma, the identification of EGFR or other mutations, or the identification of EGFR T790M mutations at the time of EGFR TKI-resistance"(College of American Pathologists, 2018; Lindeman et al., 2018).

However, the panel acknowledges that "In some clinical settings in which tissue is limited and/or insufficient for molecular testing, physicians may use a cell-free plasma DNA (cfDNA) assay to identify EGFR mutations" (Lindeman et al., 2018).

In 2021, the IASLC published an updated consensus statement on liquid biopsy testing. They note that liquid biopsy "includes a variety of methodologies for circulating analytes. From a clinical point of view, plasma circulating tumor DNA is the most extensively studied and widely adopted alternative to tissue tumor genotyping in solid tumors, including NSCLC" (Rolfo et al., 2021)

The following recommendations were presented in a consensus statement:

1. In clinical practice, ctDNA collection, sample handling, and automated processing should be performed using standardized and clinically validated procedures to reduce operator variability and false-negative results.
2. Because of the growing number of guideline-recommended oncogene targets to be assessed in advanced NSCLC, testing of plasma ctDNA should be performed by a clinically validated NGS platform rather than single-gene, PCR-based approaches, both in treatment-naive patients and those associated with multiple mechanisms of acquired resistance (MOR) to targeted agents. Where plasma NGS is not available owing to technical and economic constraints, single-gene or low multiplex-based approaches may represent appropriate alternatives. Use of limited PCR analysis for EGFR mutations as the initial step in molecular assessment, for example, remains highly relevant in areas of the world where the EGFR mutation rate is high. Nevertheless, single-gene testing should not be considered complete, and if negative, serial testing for additional actionable biomarkers must be pursued.
3. The benefit of tissue and plasma NGS is now established in several clinical practice settings. It is anticipated, owing to broad-based coverage of requisite oncogenes, decreased turnaround times, and emerging data on cost effectiveness, that in the near future, NGS will become increasingly available worldwide. Implementation of a multidisciplinary MTB to assist clinicians in treatment decision-making is advisable, as described previously.
4. In patients with oncogene-addicted NSCLC, liquid biopsy is emerging as not only complementary to tissue-based analysis but also acceptable as the initial approach ("plasma

first") for biomarker evaluation at the time of diagnosis and for monitoring the efficacy of targeted therapies. Finally, a plasma-first approach is appropriate for identification of MOR to targeted therapies in many clinical settings.
5. Indications for liquid biopsy in patients with nononcogene-addicted NSCLC are less well defined at this time, although there are several promising areas of investigation. As noted previously, bTMB is an emerging biomarker, pending completion of ongoing prospective randomized trials and refinement of methodology.

American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology

These joint guidelines from these societies were published regarding molecular biomarkers for colorectal cancer. Despite the potential of liquid biopsy for assessment of tumor recurrence and treatment resistance, the technique "awaits robust validation and further studies to determine their clinical utility" (Sepulveda et al., 2017).

European Society for Medical Oncology (ESMO) and Chinese Society of Clinical Oncology (CSCO)

These guidelines state that liquid biopsy can be used as "the initial test for the detection of a T790M mutation [for EGFR in NSCLC], and if tests are negative, a re-biopsy should be attempted if feasible" (Wu et al., 2018).

European Association of Urology (EAU), European Society for Radiotherapy and Oncology (ESTRO), European Society of Urogenital Radiology (ESUR), International Society of Geriatric Oncology (SIOG)

The joint guidelines on prostate cancer state that "In asymptomatic men with a prostate-specific antigen level between 2–10 ng/mL and a normal digital rectal examination, use one of the following tools for biopsy indication:

- risk-calculator;
- imaging;
- an additional serum, urine or tissue-based test."

These joint guidelines acknowledged SelectMDX as a test to select for repeat biopsies, but the guidelines noted SelectMDX as having an "uncertain role" and "probably not cost-effective" (EAU, 2021).

American Society of Colon and Rectal Surgeons (ASCRS)

The ASCRS released clinical practice guidelines for the management of colon cancer. The guidelines state that "the use of multigene assays, CDX2 expression analysis, and ctDNA may be used to complement multidisciplinary decision-making for patients with stage II or III colon cancer" (Vogel et al., 2022).

VI. Applicable State and Federal Regulations

DISCLAIMER: If there is a conflict between this Policy and any relevant, applicable government policy for a particular member [e.g., Local Coverage Determinations (LCDs) or National Coverage Determinations (NCDs) for Medicare and/or state coverage for Medicaid], then the government policy will be used to make the determination. For the most up-to-date Medicare policies and coverage, please visit the Medicare search website: http://www.cms.gov/medicare-coverage-database/overview-and-quick-search.aspx. For the most up-to-date Medicaid policies and coverage, visit the applicable state Medicaid website.

Food and Drug Administration (FDA)

There are four FDA-approved liquid biopsy tests as of January 10, 2023. The cobas EGFR Mutation Test v2 from Roche Diagnostics is an assay purported to detect epidermal growth factor receptor (EGFR) gene mutations in NSCLC patients. The test is intended as a companion diagnostic test for the cancer drug Tarceva (FDA, 2016), and a similar test for the T790M mutation has been produced by the same company. A second test is the Cell Search® Circulating Tumor Cell (CTC) Test, which is used to predict and analyze outcomes for individuals with metastatic breast, prostate, or colon cancer(CellSearch, 2023). A third test is Guardant360® CDx, which detects ctDNA and other common genetic errors in order to help in the choice of a therapeutic or treatment (Health, 2023). Lastly, FoundationOne® Liquid CDx is an FDA-approved liquid biopsy test that detects ctDNA and may be able to assist a provider in determining the type of treatment that will be most effective (FoundationOne, 2022).

Many labs have developed specific tests that they must validate and perform in house. These laboratory-developed tests (LDTs) are regulated by the Centers for Medicare and Medicaid (CMS) as high-complexity tests under the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88). LDTs are not approved or cleared by the U. S. Food and Drug Administration; however, FDA clearance or approval is not currently required for clinical use.

VII. Applicable CPT/HCPCS Procedure Codes

CPT	Code Description
81162	BRCA1 (BRCA1, DNA repair associated), BRCA2 (BRCA2, DNA repair associated) (eg, hereditary breast and ovarian cancer) gene analysis; full sequence analysis and full duplication/deletion analysis (ie, detection of large gene rearrangements)
81163	BRCA1 (BRCA1, DNA repair associated), BRCA2 (BRCA2, DNA repair associated) (eg, hereditary breast and ovarian cancer) gene analysis; full sequence analysis
81164	BRCA1 (BRCA1, DNA repair associated), BRCA2 (BRCA2, DNA repair associated) (eg, hereditary breast and ovarian cancer) gene analysis; full duplication/deletion analysis (ie, detection of large gene rearrangements)
81194	NTRK (neurotrophic receptor tyrosine kinase 1, 2, and 3) (eg, solid tumors) translocation analysis
81210	BRAF (B-Raf proto-oncogene, serine/threonine kinase) (eg, colon cancer, melanoma), gene analysis, V600 variant(s)

CPT	Code Description
81235	EGFR (epidermal growth factor receptor) (eg, non-small cell lung cancer) gene analysis, common variants (eg, exon 19 LREA deletion, L858R, T790M, G719A, G719S, L861Q)
81275	KRAS (Kirsten rat sarcoma viral oncogene homolog) (eg, carcinoma) gene analysis; variants in exon 2 (eg, codons 12 and 13)
81276	KRAS (Kirsten rat sarcoma viral oncogene homolog) (eg, carcinoma) gene analysis; additional variant(s) (eg, codon 61, codon 146)
81309	PIK3CA (phosphatidylinositol-4, 5-biphosphate 3-kinase, catalytic subunit alpha) (eg, colorectal and breast cancer) gene analysis, targeted sequence analysis (eg, exons 7, 9, 20)
81405	Molecular pathology procedure, Level 6 (eg, analysis of 6-10 exons by DNA sequence analysis, mutation scanning or duplication/deletion variants of 11-25 exons, regionally targeted cytogenomic array analysis)
81406	Molecular pathology procedure, Level 7 (eg, analysis of 11-25 exons by DNA sequence analysis, mutation scanning or duplication/deletion variants of 26-50 exons)
81479	Unlisted molecular pathology procedure
86152	Cell enumeration using immunologic selection and identification in fluid specimen (eg, circulating tumor cells in blood)
86153	Cell enumeration using immunologic selection and identification in fluid specimen (eg, circulating tumor cells in blood); physician interpretation and report, when required
0011M	Oncology, prostate cancer, mRNA expression assay of 12 genes (10 content and 2 housekeeping), RT-PCR test utilizing blood plasma and urine, algorithms to predict high-grade prostate cancer risk Proprietary test: NeoLAB™ Prostate Liquid Biopsy Lab/Manufacturer: NeoGenomics Laboratories
0091U	Oncology (colorectal) screening, cell enumeration of circulating tumor cells, utilizing whole blood, algorithm, for the presence of adenoma or cancer, reported as a positive or negative result Proprietary test: FirstSightCRC Lab/Manufacturer: CellMax Life
0155U	Oncology (breast cancer), DNA, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) (eg, breast cancer) gene analysis (ie, p.C420R, p.E542K, p.E545A, p.E545D [g.1635G>T only], p.E545G, p.E545K, p.Q546E, p.Q546R, p.H1047L, p.H1047R, p.H1047Y), utilizing formalin-fixed paraffin-embedded breast tumor tissue, reported as PIK3CA gene mutation status Proprietary test: therascreen® PIK3CA RGQ PCR Kit Lab/Manufacturer: QIAGEN
0177U	Oncology (breast cancer), DNA, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) gene analysis of 11 gene variants utilizing plasma, reported as PIK3CA gene mutation status Proprietary test: therascreen® PIK3CA RGQ PCR Kit Lab/Manufacturer: QIAGEN

CPT	Code Description
0179U	Oncology (non-small cell lung cancer), cell-free DNA, targeted sequence analysis of 23 genes (single nucleotide variations, insertions and deletions, fusions without prior knowledge of partner/breakpoint, copy number variations), with report of significant mutation(s) Proprietary test: Resolution ctDx Lung™ Lab/Manufacturer: Resolution Bioscience
0229U	BCAT1 (Branched chain amino acid transaminase 1) and IKZF1 (IKAROS family zinc finger 1) (eg, colorectal cancer) promoter methylation analysis Proprietary test: Colvera® Lab/Manufacturer: Clinical Genomics Pathology Inc
0317U	Oncology (lung cancer), four-probe FISH (3q29, 3p22.1, 10q22.3, 10cen) assay, whole blood, predictive algorithm-generated evaluation reported as decreased or increased risk for lung cancer Proprietary test: LungLB® Lab/Manufacturer: LungLife AI®
0332U	Oncology (pan-tumor), genetic profiling of 8 DNA-regulatory (epigenetic) markers by quantitative polymerase chain reaction (qPCR), whole blood, reported as a high or low probability of responding to immune checkpoint–inhibitor therapy Protietary test: EpiSwitch® CiRT (Checkpoint-inhibitor Response Test) Lab/Manufacturer: Next Bio-Research Services, LLC
0333U	Oncology (liver), surveillance for hepatocellular carcinoma (HCC) in high-risk patients, analysis of methylation patterns on circulating cell-free DNA (cfDNA) plus measurement of serum of AFP/AFP-L3 and oncoprotein des-gamma-carboxy-prothrombin (DCP), algorithm reported as normal or abnormal result Protietary test: HelioLiver™ Test Lab/Manufacturer: Fulgent Genetics, LLC
0337U	Oncology (plasma cell disorders and myeloma), circulating plasma cell immunologic selection, identification, morphological characterization, and enumeration of plasma cells based on differential CD138, CD38, CD19, and CD45 protein biomarker expression, peripheral blood Protietary test: CELLSEARCH® Circulating Multiple Myeloma Cell (CMMC) Test Lab/Manufacturer: Menarini Silicon Biosystems, Inc
0338U	Oncology (solid tumor), circulating tumor cell selection, identification, morphological characterization, detection and enumeration based on differential EpCAM, cytokeratins 8, 18, and 19, and CD45 protein biomarkers, and quantification of HER2 protein biomarker–expressing cells, peripheral blood Protietary test: CELLSEARCH® HER2 Circulating Tumor Cell (CTC-HER2) Test Lab/Manufacturer: Menarini Silicon Biosystems, Inc
0343U	Oncology (prostate), exosome-based analysis of 442 small noncoding RNAs (sncRNAs) by quantitative reverse transcription polymerase chain reaction (RT-qPCR), urine, reported as molecular evidence of no-, low-, intermediate- or high-risk of prostate cancer Protietary test: miR Sentinel™ Prostate Cancer Test Lab/Manufacturer: miR Scientific, LLC

CPT	Code Description
0356U	Oncology (oropharyngeal), evaluation of 17 DNA biomarkers using droplet digital PCR (ddPCR), cell-free DNA, algorithm reported as a prognostic risk score for cancer recurrence Proprietary test: NavDx® Lab/Manufacturer: Naveris, Inc
0368U	Oncology (colorectal cancer), evaluation for mutations of APC, BRAF, CTNNB1, KRAS, NRAS, PIK3CA, SMAD4, and TP53, and methylation markers (MYO1G, KCNQ5, C9ORF50, FLI1, CLIP4, ZNF132 and TWIST1), multiplex quantitative polymerase chain reaction (qPCR), circulating cell-free DNA (cfDNA), plasma, report of risk score for advanced adenoma or colorectal cancer Proprietary test: ColoScape™ Colorectal Cancer Detection Lab/Manufacturer: DiaCarta Clinical Lab

Procedure codes appearing in policy documents are included only as a general reference tool for each policy. They may not be all-inclusive.

VIII. Evidence-based Scientific References

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IX. Review/Revision History

Effective Date	Summary
12/01/2024	Initial Policy Implementation