Using all our genomes: Blood-based liquid biopsies for the early detection of cancer

Eddie Adams¹  |  Gregory D. Sepich-Poore¹,²  |  Sandrine Miller-Montgomery¹  |  Rob Knight²,³,⁴,⁵

¹ Micronoma, Inc., San Diego, California, USA
² Department of Bioengineering, University of California San Diego, La Jolla, California, USA
³ Department of Pediatrics, University of California San Diego, La Jolla, California, USA
⁴ Department of Computer Science and Engineering, University of California San Diego, La Jolla, California, USA
⁵ Center for Microbiome Innovation, University of California San Diego, La Jolla, California, USA

Correspondence
Eddie Adams, Micronoma, Inc., San Diego, CA, USA.
Email: eddie.adams@micronoma.com

Funding information
National Cancer Institute of the U.S. National Institutes of Health, Grant/Award Number: F30 CA243480;
National Cancer Institute of the U.S. National Institutes of Health, Grant/Award Number: R01 CA255206

Abstract
The pursuit of highly sensitive and specific cancer diagnostics based on cell-free nucleic acids isolated from minimally invasive liquid biopsies has been an area of intense research and commercial effort for at least two decades. Most of these tests detect cancer-specific mutations or epigenetic modifications on circulating DNA derived from tumor cells (ctDNA). Although recent FDA approvals of both single and multianalyte liquid biopsy companion diagnostic assays are proof of the tremendous progress made in this domain, using ctDNA for the diagnosis of early-stage (stage I/II) cancers remains challenging due to several factors such as low mutational allele frequency in circulation, overlapping profiles in genomic alterations among diverse cancers, and clonal hematopoiesis. This review discusses these analytical challenges, interim solutions, and the opportunity to complement ctDNA diagnostics with microbiome-aware analyses that may mitigate several existing ctDNA assay limitations.

KEYWORDS
cancer detection, cancer screening, cell-free DNA, circulating tumor DNA, early detection, microbiome

1  |  INTRODUCTION —THE NEED FOR EARLY DETECTION OF CANCER

Cancer is the second leading cause of death globally, responsible for an estimated 9.6 million deaths in 2018 and untold suffering for patients and their families.¹ Longitudinal survival statistics have overwhelmingly demonstrated that early detection of cancer, when the malignant neoplasm is confined to the tissue of origin (TOO), is associated with improvement of patient outcomes and...
reduced patient costs.\textsuperscript{2,3} For instance, early-stage detection of colorectal cancer yields a 90\% 5-year survival rate, compared to only 14\% survival with late-stage detection.\textsuperscript{4–6} Similarly, nearly all patients with breast and prostate cancers diagnosed at early stages can expect to live at least 5-years post-diagnosis.\textsuperscript{4} Highly aggressive cancer types may benefit even more from early detection. Pancreatic ductal adenocarcinoma carries a long-term survival rate of less than 8.5\%, but documented cases of incidental pancreatic cancer diagnoses at early stages have shown 5-year survival rates over 80\%, nearly a 10-fold improvement.\textsuperscript{7} These survival and cost benefits of early-stage detection have motivated public cancer screening programs.\textsuperscript{8} For example, in the United States, invasive squamous cervical cancer incidence and deaths have declined 70\% since 1950 due to early detection of precancerous and cancerous lesions through Pap smear testing.\textsuperscript{9} However, not all cancer types are easily accessible for screening, leading to later stage detection with higher morbidity and mortality. Lung cancer, the most prevalent cancer worldwide and one of the most aggressive cancers to treat, is plagued by late diagnosis, and has a corresponding 5-year survival rate of less than 15\%.\textsuperscript{10,11} These data demonstrate that early detection of cancer can tremendously improve patients’ clinical outcomes and accelerate the course of therapeutic intervention to address malignant processes before genomic lesions accumulate and tumor metastases occur.

2 THE PROMISE OF LIQUID BIOPSY FOR CANCER DIAGNOSTICS

Conventional cancer diagnostics and management rely on obtaining images and biopsies of primary tumors or their metastases to characterize their molecular subtype and genomic aberrations.\textsuperscript{12} Large-scale sequencing efforts, such as The Cancer Genome Atlas (TCGA) or Pan-Cancer Atlas of Whole Genomes, have catalogued the multimomic changes underlying tissue-specific malignant transformation and have revealed shared and distinct features between cancer types. When compared to new samples, these cancer atlases can inform prognoses and, at times, suggest tailored therapeutic options for patients based on their molecular profiles, offering the opportunity to provide personalized cancer medicine.

Many challenges exist, however, with obtaining routine tissue biopsies in certain anatomical regions due to local vasculature, delicate tissue structures, and other surgical complexities. Genetic heterogeneity between multiple malignant lesions may also limit prognostic conclusions or therapy choices when sampling genomic aberrations from only one site. Moreover, collection of all lesions in circumstances of metastasis is usually unfeasible due to impacts on patient morbidity and quality of life. Collecting longitudinal biopsies of solid tumors to characterize the response to treatment further risks compounded tissue damage, particularly since subsequent biopsies may reveal nonmalignant tissue.

“Liquid biopsies” attempt to address several of these tissue biopsy limitations and are based on the premise that cell-free nucleic acids (cfNAs) enter circulation through cell turnover or cell death. Circulating nucleic acids can be obtained from a routine and easily accessible blood sample, and because multiple malignant lesions are connected by systemic blood flow, sampling of cfNAs potentially captures all genetic alterations from multiple tumors without performing tissue biopsies on each. Paired with TCGA and Pan-Cancer Atlas of Whole Genomes maps of cancer type-specific alterations and matched patient survival, cfNAs could be used to prognose patients and tailor their treatments.\textsuperscript{13–15} Liquid biopsies are not restricted to blood and could include urine, saliva, or other biofluids, depending on the targeted cancer.\textsuperscript{16–19} The minimally invasive nature of liquid biopsies is highly advantageous and permits successive, serial sampling while maintaining strong concordance with the genomic and epigenomic lesions identified in matched solid tumor biopsies.\textsuperscript{20–22}

Current development efforts to create liquid biopsy diagnostics for cancer encompass a wide array of analytes, including circulating tumor-derived DNA (ctDNA), RNA, microRNAs, circular DNAs, and RNAs, exosomes, and intact circulating tumor cells. However, the main research and commercial focus of liquid biopsy-based assay development has focused on ctDNA due to its stability and interpretability when compared against tumor mutation profiles.\textsuperscript{17,23–25} There are numerous sources of ctDNA, ranging from tumor cell apoptosis and necrosis to secretion of exosomes or other vesicular structures.\textsuperscript{26} Because each genome equivalent (GE) of ctDNA should bear the genetic mutations of the tumor cell where it was derived, deep sequencing of blood-borne ctDNA can provide a minimally invasive means of detecting and characterizing early-stage malignant processes.\textsuperscript{27,28}

The presence of cell-free DNA (cfDNA) fragments in blood was first reported by Mandel and Métais in 1948, but it was not until 46 years later in 1994 that two seminal reports by Sorenson et al. and Vasioukhin et al. set the stage for modern uses of blood-based liquid biopsies for oncological indications.\textsuperscript{29–31} An increase in serum cfDNA concentration from patients with established cancers prompted Sorenson and colleagues to test for mutated KRAS sequences in plasma cfDNA of patients with pancreatic ductal adenocarcinoma.\textsuperscript{30,32–34} Using allele-specific primers, Sorenson et al. detected mutated KRAS sequences
in plasma cfDNA and showed that the KRAS mutation was identical to that found in the patients’ tumors, thereby demonstrating that the mutant KRAS fragment in plasma was of tumor origin.\textsuperscript{30} Similarly, Vasioukhin et al. demonstrated PCR-based identification of NRAS point mutations in plasma of patients with myeloid disorders. Noting that the presence of RAS mutations in patients with myeloid disorders was a poor prognostic factor, the authors suggested the opportunity of plasma sequencing to diagnose and prognose a patient without an invasive bone marrow biopsy. They also predicted that “since the presence or absence of mutations might reflect the success of clinical remission by chemotherapy, the easy accessibility of plasma for a detection test would be very useful in monitoring the disease,” prescient in light of subsequent development of the field.\textsuperscript{31} Parallel advances in cfDNA analysis for noninvasive prenatal testing further demonstrated the promise of liquid biopsy for early-stage cancer detection when incidental identification of cancers in presymptomatic pregnant women was reported by two groups.\textsuperscript{35,36} In these studies, fetal aneuploidy analysis via high-throughput sequencing of maternal plasma cfDNA identified copy number variations that could not be attributed to the fetal genome. Further examination of these women via orthogonal technologies, such as magnetic resonance imaging, revealed the presence of various cancers, unintended yet serendipitous demonstrations that cfDNA analysis could detect cancers in the absence of overt clinical symptoms.

Nearly 30 years later, blood-based liquid biopsies in oncology are now becoming routine for management of established disease in clinical research settings.\textsuperscript{37} Detection of mutant ctDNA alleles, either alone or in combination with other circulating biomarkers, has revolutionized cancer diagnostics; however, significant biological and technical hurdles remain to attain the requisite sensitivity for reliable detection of early-stage cancers. In this review, we discuss current applications of liquid biopsy in oncology and the analytical challenges germane to using liquid biopsies for early-stage cancer detection.

3 | USE OF LIQUID BIOPSY IN CANCER MANAGEMENT

3.1 | Disease prognosis

Leon and colleagues first showed in 1977 that total cfDNA concentrations (i.e., ctDNA from cancer cells + cfDNA from noncancer cells) were higher in cancer patients than healthy controls using a competitive radioimmunoassay. They analyzed the serum of 173 patients with various cancer types and 55 healthy individuals, reporting that the average DNA concentration in cancer patients was $180 \pm 38 \text{ ng/mL}$ (50,279 haploid GEs/mL, assuming 3.58 pg/haploid genome) and $13 \pm 3 \text{ ng/mL}$ (3631 GEs) in healthy controls.\textsuperscript{32} This finding has been reproduced in plasma cfDNA across numerous cancers and has served as a simple, easy to measure, diagnostic indicator. Other studies have also shown prognostic utility of cfDNA amounts. For example, Sirera et al. measured human telomerase reverse transcriptase in cfDNA of stage IIIB and IV non-small cell lung cancer (NSCLC) patients via real-time PCR, and found that a high pretreatment human telomerase reverse transcriptase level was a poor prognostic indicator for time to progression and overall survival.\textsuperscript{38}

In addition to using total or per-gene cfDNA concentrations as prognostic indicators, quantifying ctDNA has proved to be very powerful. In a prospective study of 230 patients with resected stage II colon cancer, Tie et al. sought to determine if ctDNA could be used to triage patients who should receive postoperative adjuvant chemotherapy from those who would not benefit from the treatment. Using next-generation sequencing (NGS) of plasma ctDNA, the authors found that 100% of patients who had detectable ctDNA at their first postoperative visit (4-10 weeks after surgery) relapsed within 3 years while only 10% of the ctDNA negative group relapsed, thereby defining a group—ctDNA-positive patients—who were at extremely high risk of radiologic recurrence when not treated with adjuvant chemotherapy.\textsuperscript{39} The prognostic value of ctDNA concentration has also been demonstrated in lung, breast, melanoma, and ovarian cancers.\textsuperscript{40-44} This information empowers clinicians to determine which patients may benefit from indication-specific therapeutic options and to more effectively tailor the amount and duration of the selected therapy.

3.2 | Monitoring treatment efficacy and resistance

Longitudinal genomic profiling of tumors during and after patient treatment is a significant challenge for traditional tissue biopsy due to the difficulty in obtaining enough tissue for analysis, risk of permanent tissue damage from repeated sampling, and tumor heterogeneity precluding representative sampling.\textsuperscript{45,46} These problems are exacerbated if the tumors are located in anatomically difficult-to-access or dangerous locations.\textsuperscript{22} Liquid biopsy-based assays have proved an attractive alternative to traditional biopsy by being minimally invasive and, in theory, representative of all tumors that access circulation. These characteristics enable serial monitoring of tumor fate during
therapy and real-time evolution of resistance mechanisms. A landmark study by Murtaza and colleagues showcased this application of liquid biopsy, analyzing serial plasma samples by whole-exome sequencing to track the genomic evolution of different metastatic cancers in response to therapy. Exome sequencing of plasma cfDNA was performed for patients with advanced breast, ovarian, and lung cancers before and after treatment, spanning multiple treatments over the course of 1-2 years. This led to the identification of mutant alleles associated with the emergence of therapy resistance, such as an activating mutation in PIK3CA following treatment with paclitaxel; a truncating mutation in RB1 following treatment with cisplatin; and the T790M resistance-conferring mutation in EGFR following gefitinib treatment. The ability to detect these mutations through minimally invasive, serial liquid biopsies provide important insights into selection pressures exerted on tumors during therapy and may suggest combination therapies that pre-empt or more effectively combat evolved resistance. The use of this technique to assess treatment response and emerging resistance has been applied extensively in NSCLC and has shown similar value in colorectal cancer, metastatic melanoma, and gastric cancers.

A recent study further suggested that liquid biopsies can provide molecular insights not observable from a single lesion tissue biopsy. Parikh and colleagues conducted a direct comparison of liquid versus tissue biopsy for detecting acquired resistance and tumor genomic heterogeneity in gastrointestinal cancers. In this study, plasma cfDNA from a cohort of 42 patients with molecularly characterized gastrointestinal cancers with progressive disease despite targeted therapy were analyzed. Sequence analysis identified at least one validated resistance mutation in 76% of patients, with 53% of patients exhibiting multiple resistance mutations. In total, 78 different resistance mutations were identified. Whole exome sequencing analysis of matched post-progression single lesion tissue biopsies (possible for 23 patients) identified resistance mutations in 11 out of 23 (48%) patients, whereas cfDNA analysis identified at least one resistance mutation in 20 out of 23 (87%) patients. In five cases, where multiple biopsies or rapid autopsies could be performed, multiple resistance mutations were identified within distinct metastatic lesions. These results indicate that acquired resistance to targeted therapy in gastrointestinal cancer is highly heterogeneous and demonstrate that liquid biopsy can identify multiple resistance mechanisms across distinct metastatic loci within the same patient—a very advantageous feature when we consider that more than 60% of solid tumor-related deaths are attributed to metastatic disease rather than advanced local disease.

### 3.3 Treatment selection—Companion diagnostics

Blood-based liquid biopsies for cancer first became medically reimbursable with the 2016 FDA approval of the cobas EGFR Mutation Test v2, which analyzes plasma cfDNA from patients with metastatic NSCLC not eligible for tissue biopsy. The cobas assay is a real-time PCR test that qualitatively detects 42 defined mutations of the EGFR gene in exons 18-21, exon 19 deletions, and the T790M resistance mutation, all of which can affect efficacy of the receptor tyrosine kinase inhibitor erlotinib. In Phase III studies utilizing the cobas assay, patients whose plasma ctDNA were positive for exon 19 deletion and/or L858R substitution mutations had improved progression-free survival with erlotinib treatment compared to those treated with a nontargeted combination of gemcitabine plus cisplatin. The test has received further approval as a companion diagnostic with Iressa (gefitinib)—another EGFR inhibitor—for the first-line treatment of patients with NSCLC harboring exon 19 deletions or the exon 21 L858R substitution mutation, as well as with Tagrisso (osimertinib), a third-generation EGFR tyrosine kinase inhibitor that selectively targets T790M mutation, which often emerges as an evolutionary consequence of first-line EGFR inhibitor treatment.

Another single gene qPCR assay—Qiagen’s therascreen PIK3CA RGQ PCR liquid biopsy assay—received regulatory approval in 2019 for the detection of 11 mutations in the phosphatidylinositol 3-kinase catalytic subunit alpha (PIK3CA) gene present in plasma samples of patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative, PIK3CA-mutated, advanced, or metastatic breast cancer. This test aids clinicians in identifying breast cancer patients who may be eligible for treatment with the PIK3CA inhibitor, alpelisib, in combination with fulvestrant, a selective estrogen receptor degrader, based on PIK3CA mutations.

FDA approval of the single gene cobas EGFR and therascreen PIK3CA assays marked a significant clinical advance in liquid biopsies and signaled a paradigm shift in how cancer biopsies are collected. More complex assays were approved by the FDA in 2020: the NGS-based Guardant360 CDx and FoundationOne Liquid CDx companion diagnostic assays for solid tumors. These assays use targeted hybridization-based capture to enrich ctDNA sequences from prepared plasma cfDNA NGS libraries to provide genomic alteration data on numerous analytes from a single blood draw. The Guardant360 assay was approved as a companion diagnostic for osimertinib in NSCLC using a limited panel of EGFR alterations but also received approval for tumor mutation profiling.
wherein single nucleotide variants and insertions and deletions (indels) in 54 genes are detected; additionally, copy number variations in two genes and fusions in four genes are reported. The FoundationOne Liquid CDx assay was approved as a companion diagnostic for three lung cancer therapies and a prostate cancer therapy; moreover, analogous to Guardant360, the FDA approved Foundation’s assay for tumor profiling, wherein genetic alterations in 324 genes are detected to help a physician develop personalized therapeutic options for the patient. The scale of Foundation Liquid CDx’s analyte panel makes it possible to perform comprehensive genomic profiling of the entire exonic region of cancer-relevant genes in advanced-stage cancers through a simple blood draw. This represents a significant technical advance and one that facilitates tumor analysis in cases where insufficient DNA or poor DNA quality is available by tissue biopsy.\textsuperscript{58,59}

4 | COMMERCIAL LIQUID BIOPSIES FOR POPULATION SCREENING OR EARLY DETECTION

The success of liquid biopsy-based detection of advanced cancers and the aforementioned incidental detection of cancers in asymptomatic women undergoing noninvasive prenatal testing has spurred significant investment in clinical and commercial efforts in early-stage cancer detection (Table 1). Early detection tests, if sensitive enough, could be applied to symptomatic patients to substantially reduce the time between clinical presentation and disease diagnosis, thereby increasing the premetastasis window of opportunity for therapeutic intervention. If specific enough, these tests could also be employed as routine (e.g., yearly) screening tests in healthy individuals to identify instances of asymptomatic cancer.

To date, the only blood-based liquid biopsy assay that has obtained FDA approval for cancer diagnosis is Epigenomics’s Epi proColon colorectal cancer screening assay.\textsuperscript{60} This is a PCR assay for the qualitative detection of methylated \textit{Septin9} ctDNA isolated from 3.5 milliliters of patient plasma, since methylation of certain CpG motifs in the promoter region of the \textit{SEPT9\_v2} transcript has been associated with colorectal cancer but not healthy tissue. Specifically, the Epigenomics assay utilizes bisulfite treatment of isolated ctDNA and methylation-specific primers to detect the presence of methylated \textit{Septin9}.\textsuperscript{51} The test obtained FDA approval in April 2016 and is indicated to screen adults of 50 years or older, defined as the average risk for colorectal cancer, who are unable or unwilling to undergo routine screening tests (e.g., flexible sigmoidoscopy, colonoscopy, or stool tests). As patient participation in conventional colorectal screening programs is suboptimal, a minimally invasive blood test may boost patient testing and concomitant identification of premetastatic colorectal cancer lesions. However, despite the regulatory approval of the Epi proColon assay, the Centers for Medicare and Medical Services recently ruled that the Epi proColon assay does not meet the sensitivity (≥74%) and specificity (≥90%) thresholds established by two existing assays—fecal immunochemical tests and the multianalyte stool DNA test Cologuard—to qualify for national reimbursement coverage, a decision that will undoubtedly reduce clinical uptake and utilization of this first blood-based ctDNA cancer diagnostic.\textsuperscript{62}

In contrast to the single biomarker Epi proColon assay, an array of multianalyte blood-based liquid biopsy cancer diagnostics are currently in development (Table 1). For example, CancerSEEK (Thrive Earlier Detection) is a blood test that combines ctDNA and protein biomarkers to screen for eight types of cancer, five of which have no existing approved screening tests (ovarian, liver, stomach, pancreatic, and esophageal cancer).\textsuperscript{63} The CancerSEEK assay utilizes multiplex PCR analysis of ctDNA derived from 7.5 milliliters of patient plasma to achieve detection of driver mutations at 2001 genomic loci across 16 genes. Amplicon sequencing data are then combined with the protein concentrations of eight cancer-associated protein biomarkers (cancer antigen 125 (CA-125), carcinoembryonic antigen, cancer antigen 19-9, prolactin, hepatocyte growth factor, osteopontin, myeloperoxidase, and tissue inhibitor of metalloproteinases 1) to arrive at a cancer classification. In a test of 1005 patients previously diagnosed with stage I-III breast, colorectal, gastric, liver, lung, esophageal, ovarian, or pancreatic cancer, a median of 70% of patients tested positive for cancer via CancerSEEK. Sensitivities ranged from 33% for breast cancer to 98% for ovarian cancer with excellent (>99%) specificity overall. By leveraging machine learning (ML), Cohen et al. developed a classification system based on sequencing and proteomics data that could localize the source of the cancer to two anatomic sites in a median of 83% of CancerSEEK-positive patients, or to a single anatomic site or single organ in a median of 63% of the CancerSEEK-positive patients. TOO determination is a critical component of any early cancer detection platform, as circulating genomic and/or proteomic signatures of tumors may be apparent well-before localized tumors can be pinpointed radiologically. However, since the CancerSEEK study population consisted largely of symptomatic cancers, it will be of great interest to see if future embodiments of this method can provide both a means of cancer detection and TOO localization when extended to asymptomatic individuals with early-stage disease; alternatively, as shown in the second generation CancerSEEK DETECT-A study, TOO localization may be
| Company          | Cancer application(s)                              | Patient status                  | Product name          | Technology description                                                                 |
|------------------|---------------------------------------------------|---------------------------------|-----------------------|---------------------------------------------------------------------------------------|
| Biocept          | Lung and breast cancer mutation detection         | Previously diagnosed           | Target Selector™     | Multiplex PCR amplification via "Switch-Blocker” technology: suppression of wild-type gene sequences focuses PCR on mutant sequences of interest. Amplification is followed by ampiclon sequencing. | 98 |
| Clinical Genomics| Colorectal cancer recurrence                     | Previously diagnosed and treated| Colvera®             | Methylation-specific PCR-based detection of methylated BCA1 and IKZF1 ctDNA post bisulfite treatment. | 99 |
| Delfi Diagnostics| Multicancer screening                            | No prior diagnosis of cancer    | TBD                  | cfDNA whole-genome NGS followed by fragmentation analysis.                            | 91 |
| Epigenomics      | Colorectal cancer detection                      | No prior diagnosis of cancer    | Epi proColon®        | Methylation-specific PCR-based detection of methylated Septin9 post bisulfite treatment. | 60 |
| Foundation Medicine (Roche) | Tumor mutation profiling                       | Previously diagnosed           | FoundationOne®       | Hybridization probe enrichment of ctDNA mutations followed by NGS.                   | 58 |
| Freenome         | Colorectal cancer detection                      | No prior diagnosis of cancer    | PreEmpt CRC™         | Multianalyte detection (ctDNA, cRNA, proteins) with machine learning classification.     | 100 |
| Grail            | Multicancer screening                            | No prior diagnosis of cancer    | Galleri™             | Bisulfit sequencing: bisulfit treatment of cfDNA, targeted methylation hybridization probe enrichment of ctDNA followed by NGS. | 56 |
| Guardant Health  | Multicancer screening (with a first indication in colorectal cancer) | No prior diagnosis of cancer    | LUNAR-2              | Multiomic analysis of cfDNA incorporating methylation, mutation, and fragmentomic analyses. | 101 |
| Inivata          | Tumor mutation profiling                         | Previously diagnosed           | InVisionSeq™         | Tagged-amplicon deep sequencing (TAmp-Seq).                                          | 102 |
| Micronoma        | Lung cancer diagnosis                            | No prior diagnosis of cancer    | Oncobiota™           | Metagenomic sequencing of total cfDNA followed by machine learning-based disease classification. | 93 |
| Natera           | Colorectal cancer molecular residual disease, recurrence monitoring | Previously diagnosed and treated | Signatera™           | Personalized mutation detection: mutations present in patient’s tumor(s) are used to design custom ctDNA sequencing panels. | 103 |
| Nucleix          | Lung cancer diagnosis                            | No prior diagnosis of cancer    | Lung EpiCheck®       | Methylation-specific PCR-based detection of methylated markers postbisulfite treatment. | 104 |
| Thrive (Exact Science) | Multicancer screening                          | No prior diagnosis of cancer    | CancerSEEK           | Multiplex amplicon sequencing + proteomic biomarker detection.                         | 63 |
| VolitionRX       | Lung, colorectal, and prostate cancers; single cancer, ELISA-based screening | No prior diagnosis of cancer    | Nu-Q™                | ELISA-based quantification of nucleosome-bound cfDNA.                                 | 105 |

Each of these tests employs cfDNA, either alone or in combination with other circulating biomarkers, to detect cancer, perform tumor mutation profiling, or detect cancer recurrence.
better addressed by orthogonal means such as through PET-CT. Although the DETECT-A study used an earlier version of CancerSEEK that did not employ ML, Thrive, since acquired by Exact Sciences, recently completed an unpublished 3000 subject clinical trial (Detecting Cancers Earlier Through Elective Plasma-based CancerSEEK Testing [ASCEND]), consisting of 1000 subjects with known or suspected cancer and 2000 noncancer controls. The results from the ASCEND trial should clarify how their assay incorporating ML works with early-stage cancers.

In contrast to Thrive’s assay, which combines driver gene mutations and cancer-associated proteomic markers to arrive at a diagnosis, GRAIL Inc. (recently acquired by Illumina) uses differential DNA methylation of genomic CpG sites to discriminate among different cancers and cancer versus noncancer samples. GRAIL has set an ambitious goal to accurately screen for more than 50 unique cancer types from a single sample through targeted bisulfite sequencing analysis of ctDNA methylation patterns. DNA methylation-based biomarkers have been explored in many disease areas but may prove particularly useful in liquid biopsy-based cancer diagnostics as a means of determining which ctDNA fragments are truly tumor-derived. While most driver mutations in oncogenes (e.g., TP53, KRAS) are common among cancers regardless of their TOO, CpG methylation profiles are highly specific to tissues and tumors-derived therefrom, potentially enabling a more exact diagnosis of cancer. In addition, there are millions of CpG sites throughout the human genome whose methylation states (methylated versus unmethylated) can comprise a cancer-specific signature whereas canonical ctDNA mutations are limited in copy number/genome and, therefore, impose a sensitivity limitation for detection.

GRAIL has dedicated considerable resources toward constructing a database of DNA methylation patterns from thousands of individuals with different cancer types, healthy noncancer controls, and noncancer controls with other medical conditions. By applying ML algorithms to these data, GRAIL has developed classifiers to distinguish cancer-associated methylation patterns from noncancer patterns and has used these classifiers in large-scale clinical studies. In a recent publication detailing the results of their clinical validation study featuring liquid biopsy analyses for over 50 cancer types, GRAIL’s classifier was able to predict the TOO in 96% of samples with an apparent cancer-signature (344/359) and, of these, 93% (321/344) were accurate classifications, a compelling demonstration of the diagnostic power of epigenetic signatures in liquid biopsies. Cancer diagnostic tests based on alternative ctDNA methylation status (i.e., 5-methylcytosine and 5-hydroxymethylcytosine) have been described by others with similar degrees of success in identifying the TOO.

Although GRAIL’s assay specificity (99.3%) was impressive, the reported sensitivities for early-stage (I and II) cancers leave room for improvement, particularly for what is intended to serve as a population-wide cancer screening diagnostic. Stage I cancers were detected with a sensitivity of just 18% (95% confidence interval (CI), 13-25%) and stage II cancers 43% (CI, 35-51%). Such low sensitivities for stage I and II cancers are neither surprising nor unique to GRAIL’s assay, as ctDNA-based diagnostic assays have reported similar difficulties in detecting early-stage cancers that partially stem from the paucity of cancer-derived DNA in circulation at any given time. One potential exception is the pan-cancer screening assay, PanSeer, developed by Singlera Genomics and colleagues that analyzes DNA methylation patterns at 10,613 CpG sites across 477 genomic regions and can reportedly identify the presence of certain cancers (colorectal, esophageal, liver, lung, and stomach) through liquid biopsy up to 4 years prior to conventional diagnosis methods. Since reporting these results in June of last year, Singlera has raised $150 million USD in Series B financing to expand PanSeer testing with a large prospective study of healthy individuals to determine if their screening assay can reduce cancer deaths in a cost-effective manner.

5 | TECHNICAL AND BIOLOGICAL HURDLES FOR EARLY DETECTION VIA ctDNA

Much has been written about the technical difficulties associated with early-stage ctDNA cancer diagnostics. Principal among these difficulties is that small, localized tumors, which do not exhibit ample cell turnover, do not release sufficient amounts of DNA into the circulation. As discussed above, plasma from cancer patients contains on average more cfDNA than healthy controls, but, in nearly all cases where cfDNA concentrations have been measured, the cancer cohorts consisted of patients whose tumors were already diagnosed by conventional means. Thus, it is difficult to ascertain from the existing literature how much ctDNA may be present in asymptomatic individuals harboring tumors below the limit of detection of existing imaging modalities.

Furthermore, there may be a substantial difference between how much ctDNA an early-stage diagnostic can theoretically detect versus what it needs to detect to make a substantial improvement in patient care. Diamandis and Fiala have argued that tumors ≤5 millimeters in diameter are an optimal size for early and curable cancer detection. Using breast cancer as a model, they review data showing that approximately 6% of tumors of 5 millimeters in diameter progress but are poorly detected by mammography.
in 26% of cases. They further suggest that liquid biopsy detection of a tumor 10 millimeters in diameter would not represent a diagnostic improvement, as more than 90% of breast cancer tumors 10 millimeters in diameter or larger are currently detected by mammographic screening. Likewise, low-dose computed tomography can identify potential cancerous lung nodules with diameters as small as 4 millimeters. Thus, if a liquid biopsy assay can diagnose tumors in the 5-10 mm diameter range (approximately 0.06–0.5 cm³ or 6 ×10⁶ to 1 ×10⁸ cells/tumor), per Diamandis and Fiala’s assessment, it would represent a true advance in diagnostic capability.

But is it reasonable to expect liquid biopsy assays to detect tumors of such small size and cellularity? Diamandis and Fiala argue that overcoming this challenge is highly unlikely based on recent estimates comparing ctDNA amounts and tumor volumes. For instance, Abbosh et al. measured the mutant allele frequency (MAF) in patients with early-stage lung cancers and showed that 10 cm³ (27-millimeter diameter) tumors had an average MAF of just 0.1%, or 1 mutant DNA copy per 1000 nonmutant copies. Extrapolating this measurement, Diamandis and Fiala calculate that an early-stage tumor of 5-millimeter diameter would contribute only 1 mutant DNA GE per every 160,000 GE in circulation. Assuming that an asymptomatic patient with early-stage cancer have a ctDNA concentration similar to that of healthy individuals, or approximately 6200 haploid GE per 4 milliliters of plasma from a 10-milliliter blood draw, then one would need to draw and analyze over 100 milliliters of blood to detect a tumor 5 millimeters in diameter—an unlikely scenario. These calculations are consistent with a recent mathematical model of ctDNA shedding. Support for these calculations are also found in Phallen et al. and Cohen et al., wherein successful instances of liquid biopsy detection of early-stage cancers were observed only in symptomatic patients with MAFs more than 0.01%, suggesting that ctDNA-based detection requires at least a 10- to 12.5-millimeter diameter tumor for enough ctDNA to be present in the circulation, which is not an improvement over conventional screening methods. Nonetheless, caveats to this mathematical argument may exist. If ctDNA concentration in early stage, asymptomatic individuals correlates more strongly with tumor metabolic activity than size, then small tumors may still shed sufficient DNA for detection. In addition, the MAF measured in the above studies could be a function of the sensitivity of the techniques employed, as technique-dependent differences in MAF for various cancers and stages have been previously observed.

Another major complicating factor in ctDNA analysis that has drawn a lot of attention in recent years is a phenomenon referred to as clonal hematopoiesis (CH). In CH, white blood cells (WBCs) that constitute the main source of cfDNA in circulation accumulate somatic mutations in specific oncogenes (e.g., KRAS, JAK2, and TP53) and copy number alterations in genes associated with hematological malignancies as people age. While only about 1% of all patients with CH develop a hematological malignancy, the cfDNA originating from clonal leukocytes can be a confounding factor in ctDNA analysis when CH-derived mutations are falsely interpreted as originating from the tumor under investigation. For example, in a study of patients with advanced EGFR-mutant NSCLC, Oxnard and colleagues found that most JAK2 mutations identified in plasma cfDNA were traced to peripheral blood cells from these same patients. More recently, Razavi et al. carried out an extensive sequencing study to fully characterize the source of mutations found in ctDNA. To this end, they performed ultra-deep sequencing (60,000X depth) of 508 genes in ctDNA isolated from 124 patients with various metastatic cancers, genomic DNA isolated from patient-paired WBCs, and, when available, matched tumor tissue. The same analyses were performed on 47 noncancer controls (i.e., cfDNA and paired WBCs) and collectively revealed the confounding nature of CH on ctDNA analyses. Specifically, 82% of the mutations found in the cfDNA of noncancer control subjects and 53% of the mutations found in ctDNA of cancer patients were also identified in their respectively matched WBC-derived genomic DNA, providing solid evidence that CH is a major reservoir of cfDNA mutations and tremendously diminishes assay specificity. Since aging is a risk factor for both CH and carcinogenesis, Razavi et al. strongly recommended that any ctDNA assay incorporate a paired analysis of patient-derived WBC genomic DNA to ensure that leukocyte-originating mutations are not misinterpreted as tumor-derived signals. It remains to be seen, however, how these findings may affect recent regulatory approvals for Guardant Health’s and Foundation Medicine’s ctDNA-based tumor mutation profiling. Epigenetic changes with age that could potentially confound methylome-based ctDNA diagnostics also are unknown.

6 | THE POTENTIAL FOR MICROBIOME-BASED LIQUID BIOPSY DIAGNOSTICS IN CANCER

As detailed above, extensive effort has been applied to identify, distinguish, and quantify tumor-derived DNA from nontumor, wild-type DNA sequences to provide a sensitive and specific diagnosis of cancer. Although CH poses a significant challenge to the specificity of ctDNA-based diagnostics, alternative ctDNA analysis methods based on differential DNA methylation or DNA fragmentation patterns are being actively explored.
potential orthogonal source of cfDNA comprises nonhuman species, derived from bacteria, viruses, fungi, and phages that live on and within the human body. Despite many claims that tumors are sterile, recent evidence has shown overlapping immunohistochemistry, immunofluorescence, electron microscopy, and sequencing data of microbes in tumors in approximately 10 cancer types. These cancer types appear to harbor distinct microbiomes from each other, suggesting a diagnostic opportunity. Moreover, historical observations between clinical bacteremias and subsequently diagnosed colorectal cancers, despite no or minimal presenting symptoms of those cancers, have been described since the 1970s and were validated in a meta-analysis of more than 13,000 patients in Hong Kong. Recent work by Poore et al. suggests that broadening the scope of cancer-associated DNA sequences to include circulating, cell-free microbial DNA (mbDNA) may provide an attractive means of bypassing several problems posed by CH and the low MAF inherent to early-stage tumors while also facilitating TOO classification. Prompted by studies detailing that some cancer types are intimately associated with microbial activity, Poore and colleagues characterized the extent and taxonomic diversity of microbial nucleic acids found across a range of human tumors in a comprehensive computational study. To accomplish this, the authors analyzed all treatment-naive whole-genome and transcriptome studies from TCGA for their bacterial, viral, and archaeal nucleic acid content. The TCGA dataset consisted of 18,116 samples (4831 whole-genome sequencing and 13,285 RNA-seq) from 10,481 patients across 33 different tumor types and included non-neoplastic tumor-adjacent tissue and patient blood samples. Of the 6.40 x 10^12 sequencing reads in TCGA, 7.2% were classified as nonhuman, of which 35.2% could be taxonomically assigned to bacteria, viruses, or archaea using a comprehensive reference microbial database of 59,974 total microbial genomes. To account for microbial nucleic acid contaminants that may have been introduced during the TCGA cohort DNA and RNA extractions, an in silico decontamination pipeline featuring statistical contaminant inference and historical known extraction kit contaminant removal was employed, discarding up to 91.3% of microbial taxa from the data in the most stringent analyses. The decontaminated datasets were then used to train stochastic gradient-boosting ML models (using a 70–30% train-test split for all cancers) to discriminate between and within types and stages of cancer. The trained models could effectively discriminate one cancer versus all others (n = 32 tested cancer types) and tumor versus normal adjacent tissue (n = 15 types of cancer with sufficient samples) solely using mbDNA and/or microbial RNA. Because colon cancer is epidemiologically linked to clinical bacteremia, Poore et al. explored TCGA blood-derived normal samples (cancer patient whole blood or buffy coat DNA preparations; n = 1866 whole-genome sequencing samples across 20 cancer types) and found cancer type-specific mbDNA signatures. Additionally, the ML models trained on this blood-derived mbDNA could classify stage Ia-IIc cancers with high sensitivity and specificity. Remarkably, ML models trained solely on mbDNA features correctly classified many cancers that lacked all genomic alterations reported by the Guardant360 and FoundationOne liquid biopsy assays, demonstrating that mbDNA signatures can be truly orthogonal, cancer genome-independent biomarkers for cancer classification. To further validate this finding and extend it to a more standardized liquid biopsy format, plasma-derived cell-free microbial DNA (cf-mbDNA) from 100 patients with stage III-IV lung (n = 25), prostate (n = 59), or melanoma (n = 16) cancers were compared to cf-mbDNA from 69 HIV-negative, healthy patients. Isolating cf-mbDNA with a commercially available cfDNA extraction kit with dozens of experimental contamination controls, the authors applied the same TCGA microbial detection pipeline and ML steps as before with the exception that, due to the small sample sizes, nested leave-one-out iterative ML was performed instead of train-test splits. Again, cf-mbDNA-based signatures resulted in accurate discrimination between cancer types and cancer versus healthy, with lung and prostate cancer showing the highest and second-highest performance, respectively. Specifically, 22 of 25 lung cancer samples were correctly classified via leave-one-out iterative ML with five false positives, yielding a sensitivity and specificity of 88 and 93%, respectively. Commercial efforts are now underway to further explore cancer-associated cf-mbDNA signatures for diagnostic applications with an initial focus on lung cancer, for which, effective, early-stage diagnostics are desperately needed. It will be critical to expand the breadth of controls to include nonhealthy, noncancer complications of the lungs, such as chronic obstructive pulmonary disease, sarcoidosis, interstitial fibrosis, and other conditions, where microbial involvement may overlap substantially with that of lung cancer, potentially decreasing the specificity of the cf-mbDNA signature for cancer. The development of cf-mbDNA-based diagnostics is nascent, but it is not difficult to imagine their use to complement other cancer liquid biopsies and potentially mitigate problems associated with existing ctDNA analyses (Figure 1). Because cf-mbDNA is wholly independent of the human genome, the accumulation of somatic mutations in CH that decreases ctDNA assay specificities cannot alter the specificity of cf-mbDNA signatures.
Assaying cf-mbDNA may improve liquid biopsy sensitivities as well. Whereas ctDNA-based assays are limited by the frequency of targeted mutant alleles (often 1:100-1:1000 ratios of mutant:normal alleles), cf-mbDNA typically comprises 1–3.5% of the reads in a shotgun metagenomic dataset in our experience, independent of cancer stage. This is a rich data source that, when coupled with ML, can provide sensitive and specific cancer-specific signatures containing hundreds of features. Furthermore, because mbDNA is truly orthogonal to the human genome, mbDNA signatures can be coupled with a range of cancer-associated host biomarkers—such as the driver mutations discussed above, methylomics, miRNAs, circular DNAs or RNAs, or proteomic markers—to generate new kinds of multianalyte, multispecies assays that leverage the full breadth of circulating biomarkers in cancer.

7 | OUTLOOK

The last five years have demonstrated tremendous advances in the development and regulatory approval of blood-based liquid biopsy assays for minimally-invasive monitoring of cancer. Single gene assays have been quickly
supplanted by comprehensive genomic profiling panels; single modality assays have substituted for multi-omic ones; and single timepoint tests have been augmented with longitudinal sampling strategies that query the time-dependent mutation status of critical cancer genes and identify recurrent disease. While consistent detection of early-stage cancers via ctDNA may always be a challenge due to the limited quantities of tumor-derived DNA in circulation, circulating microbial DNA signatures may offer a new and host genome-independent means of diagnosing cancer by itself or in combination with host-centric approaches. Combinations of these technologies may provide better cancer detection at the earliest stages of disease.

Further technical developments notwithstanding, where do liquid biopsy assays for early cancer detection have the potential to impact patient care and health care costs most immediately? We suggest that liquid biopsy assays can have an instrumental role in clinically triaging high-risk patients downstream of imaging or other clinical tests with high false positive rates for cancer. Here we consider lung cancer as a useful example. The National Lung Screening Trial (NLST) previously demonstrated that low-dose computed tomography (LDCT) reduced lung cancer deaths by 20% by improving rates of early-stage lung cancer detection in a high-risk population (age 55-80 years old with ≥ 30 pack-year smoking history and smoking cessation <15 years). Based on this data, medically reimbursable lung cancer screening with annual LDCT scans was implemented in 2015 and since then the screening guidelines have been revised to include individuals aged 50-80 years old with ≥ 20 pack-year smoking history and smoking cessation <15 years. One consequence of LDCT screening for lung cancer, however, is the large number of unnecessary invasive procedures performed on patients with benign pulmonary masses due to LDCT’s high false positive rate for presumptive cancers: in the NLST trial, 39% of patients had a positive screening test (detection of a mass) and 96% of those positive screens were false positives. This false positive rate arises from the fact that it is difficult to distinguish a malignant mass (cancer) from non-malignant pulmonary nodules (such as granulomas) or benign tumors (carcinoids and hamartomas) by radiologic image analysis alone.

In turn, high false positive screening rates lead to unnecessary invasive procedures, higher systemic healthcare costs, and create morbidity and mortality risks to patients that counteract the potential benefits of screening. For example, 24% of invasive procedures performed during the NSLT revealed benign masses and 1.24% of patients with benign procedures died within 90 days of their operation. Considering the recently updated U.S. Preventive Services Task Force recommendation for annual LDCT screening, which extended screening eligibility up to 14.5 million individuals, the absolute number of unnecessary invasive procedures performed annually (and the burden of their associated complications) is expected to increase. This circumstance provides a window of opportunity for liquid biopsy diagnostics, wherein LDCT screening can be paired with minimally-invasive molecular assays to rule in or out the need for invasive biopsy of lung masses of unknown significance. Specifically, LDCT would enable detection of virtually all anomalous pulmonary masses ≥4 mm in size, irrespective of their clinical importance, whereas highly specific and sensitive molecular assays can differentiate between likely benign versus likely malignant masses that warrant surgical intervention.

We contrast this triage model—wherein a liquid biopsy assay is developed to address a specific clinical question—with the broader effort to utilize ctDNA detection within the context of a population-based molecular screen for multiple cancers, such as GRAIL’s Galleri test. While noble in pursuit, recent data from the Circulating Cell-free Genome Atlas study continue to demonstrate that, for most cancers tested, the paucity of ctDNA in circulation for early-stage malignancies restricts the utility of these tests to advanced Stage III and IV cancers (overall sensitivity by stage, stage I: 16.8%, stage II: 40.4%, stage III: 77.0%, stage IV: 90.1%; specificity fixed at 99%). It is important to emphasize that these validation results reflect the assay’s performance with patients already clinically diagnosed with cancer, and thus, one may anticipate the sensitivities for Stage I and Stage II cancers to decrease in population screens with asymptomatic individuals and individuals presenting with benign lesions, precancerous lesions, or non-cancer complications.

In summary, the ultimate litmus test for a liquid biopsy is whether it adds value to clinical practice by detecting cancers earlier than conventional diagnostic means while avoiding large numbers of false positives or false negatives. Existing data on the quantity of ctDNA in circulation and clinical trials of it as a diagnostic modality suggest that patient triaging opportunities are realizable but that we are not there yet with population-level screening performance. Given the complexity of cancer genome, epigenome, proteome, and microbiome landscapes, a one-size-fits-all test for population-level screening may not be realistic, just as existing conventional screens vary by cancer type. Nonetheless, we maintain an optimistic hope that novel combinations of multi-analyte, multispecies targets can address this goal in the future, providing sufficient sensitivity and specificity for early cancer detection that translates to better patient survival and quality of life.
ACKNOWLEDGMENTS
The figure was created with BioRender.com. We also acknowledge the patients and their families who have helped contribute toward a better understanding of this field. G.D.S.-P. supports by a fellowship from the National Cancer Institute of the U.S. National Institutes of Health under award number F30 CA243480. R.K. is supported by the National Cancer Institute of the U.S. National Institutes of Health under award number R01 CA255206.

CONFLICT OF INTEREST
E.A., G.D.S.-P., and S.M.-M are employees of Micronoma, Inc., an in vitro diagnostics company founded to commercialize the microbiome-based diagnostic approach described in this article. G.D.S.-P. and R.K. are inventors on a US patent application (PCT/US2019/059647) submitted by The Regents of the University of California and licensed by Micronoma that covers methods of diagnosing and treating cancer using microbial biomarkers in blood and cancer tissues. S.M-M, G.D.S.-P., and R.K. are founders of Micronoma and all authors report stock interest in the company. R.K. is a member of the scientific advisory board for GenCirq, holds an equity interest in GenCirq, and can receive reimbursements for expenses up to US$5000 per year.

ORCID
Eddie Adams https://orcid.org/0000-0003-0664-6235
Gregory D. Sepich-Poore https://orcid.org/0000-0002-3443-3715
Sandrine Miller-Montgomery https://orcid.org/0000-0003-4969-9505
Rob Knight https://orcid.org/0000-0002-0975-9019

REFERENCES
1. F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, A. Jemal, CA: A Cancer J. Clin. 2018, 68, 394.
2. D. Crosby, N. Lyons, E. Greenwood, S. Harrison, S. Hiom, J. Moffat, T. Quallo, E. Samuel, I. Walker, Early Detection and Diagnosis Roadmap Steering Group. Lancet Oncol. 2020, 21, 1397.
3. R. Etzioni, N. Urban, S. Ramsey, M. McIntosh, S. Schwartz, B. Reid, J. Radich, G. Anderson, L. Hartwell, Nat. Rev. Cancer 2003, 3, 243.
4. SEER Cancer Statistics Review, 1975-2017 SEER, https://seer.cancer.gov/csr/1975_2017/index.html. Accessed January 2021.
5. A. G. Zauber, Dig. Dis. Sci. 2015, 60, 681.
6. R. L. Siegel, K. D. Miller, A. G. Sauer, S. A. Fedewa, L. F. Buttery, J. C. Anderson, A. Cercek, R. A. Smith, A. Jemal, CA: A Cancer J. Clin. 2020, 70, 145.
7. A. L. Blackford, M. I. Canto, A. P. Klein, R. H. Hruban, M. Goggins, JNCI J. Nat. Cancer Inst. 2020, 112, 1162.
8. K. M. Murphy & R. H. Topel The value of health and longevity (2005). https://www.nber.org/papers/w11405. (accessed: April 2021).
9. O. Adegoke, S. Kulasingam, B. Virnig, J. Women's Health (Larchmt) 2012, 21, 1031.
10. C. Allemani, H. K. Weir, H. Carreira, R. Harewood, D. Spika, X-Si Wang, F. Bannon, J. V. Ahn, C. J. Johnson, A. Bonaventure, R. Marcos-Gragera, C. Stiller, G. Azevedo E Silva, W. Q. Chen, O. I. Oggunbiyi, B. Rachet, M. J. Soeberg, H. You, T. Matsuda, M. Bielksa-Lasota, H. Storm, T. C. Tucker, M. P. Coleman, Lancet 2015, 385, 977.
11. R. M. Hoffman, R. Sanchez, Med. Clin. North Am. 2017, 101, 769.
12. V. T. DeVita, T. S. Lawrence, S. A. Rosenberg DeVita. Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology. Wolters Kluwer, PA 2019.
13. Z. Wang, M. A. Jensen, J. C. Zenklusen, Methods Mol. Biol. 2016, 1418, 111.
14. I. Mallona, A. Sierco, M. A. Peinado, Methods Mol. Biol. 2018, 1766, 123.
15. Y. Mamatjan, S. Agnihotri, A. Goldenberg, P. Tonge, S. Mansouri, G. Zadeh, K. Aldape, J. Mol. Diagn. 2017, 19, 881.
16. D. W. Crescon, S. V. Bratman, S. M. Chan, L. L. Siu, Nature Cancer 2020, 1, 276.
17. A. Baassiri, F. Nassar, D. Mukherji, A. Shamseddine, R. Nasr, S. Temraz, Int. J. Mol. Sci. 2020, 21, 1398.
18. G. De Rubis, S. Rajeev Krishnan, M. Beavawy, Trends Pharmacol. Sci. 2019, 40, 172.
19. E. Heitzer, I. S. Haque, C. E. S. Roberts, M. R. Speicher, Nat. Rev. Genet. 2019, 20, 76.
20. L. Jahangiri, T. Hurst, Cancers (Basel) 2019, 11, 1938.
21. L. S. Schwartzberg, H. Horinouchi, D. Chan, S. Chernilo, M. L. Tsai, D. Isla, C. Escrui, J. P. Bennett, K. Clark-Langone, C. Svedman, P. Tomasini, G. Alexander, F. L. Baehner, T. Bauer, A. Bergamaschi, J. Crown, D. Davison, D. A. Eberhard, N. Gabrail, J. Han, W. Irvin, M. Lopatin, J. Osiri, B. T. Sumrall, NPJ Prec. Oncol. 2020, 4, 15.
22. M. J. Overman, J. Modak, S. Kopetz, R. Murthy, J. C. Yao, M. E. Hicks, J. L. Abbuzzese, A. L. Tam, J. Clin. Oncol. 2013, 31, 17.
23. Z. Zhang, T. Yang, J. Xiao, EBioMedicine 2018, 34, 267.
24. J. V. Carter, N. J. Galbraith, D. Yang, J. F. Burton, S. P. Walker, S. Galandiu, Br. J. Cancer 2017, 116, 762.
25. J. L. Robinson, A. Feizi, M. Uhlén, J. Nielsen, Cell Rep. 2019, 26, 2622.
26. A. R. Thierry, S. El Messaoudi, P. B. Gahan, P. Anker, M. Stroun, Cancer Metastasis Rev. 2016, 35, 347.
27. J. Phallen M. Sausen, V. Adleff, A. Leal, C. Hruban, J. White, V. Anagnostou, J. Fiksel, S. Cristiano, E. Papp, S. Speir, T. Reinert, M. -, B. W. Orntoft, B. D. Woodward, D. Murphy, S. Parpart-Li, D. Riley, M. Nesselbush, N. Sengamalay, A. Georgiadis, Q. K. Li, Sci. Transl. Med. 2017, 9, eaan2415.
28. E. P. Diamandis, C. Fiala, F1000Res 2017, 6, 2129.
29. P. Mandel, P. Metais, C. R. Seances Soc. Biol. Fis. 1948, 142, 241.
30. G. D. Sorenson D. M. Pribish, F. H. Valone, V. A. Memoli, D. J. Bzik, S. L. Ya, Cancer Epidemiol. Biomarkers Prev. 1994, 3, 67.
31. V. Vasioukhin, P. Anker, P. Maurice, J. Lyautey, C. Lederrey, M. Stroun, Br. J. Haematol. 1994, 86, 774.
32. S. A. Leon, B. Shapiro, D. M. Sklaroff, M. J. Yaros, Cancer Res. 1977, 37, 646.
33. B. Shapiro, M. Chakrabarty, E. M. Cohn, S. A. Leon, Cancer 1983, 51, 2116.
34. A. Maebo, Nihon Kyobu Shikkan Gakkai Zasshi 1990, 28, 1085.
Wolfgang, M. G. Goggins, M. D. Molin, T. L. Wang, R. Roden, A. P. Klein, J. Patak, L. Dobyn, J. Schaefer, N. Silliman, M. Popoli, Science (New York, N.Y.) 2018, 359, 926.

64. A. M. Lennon, A. H. Buchanan, I. Kinde, A. Warren, A. Honushfsky, A. T. Cohan, D. H. Ledbetter, F. Sanfilippo, K. Sheridan, D. Rosica, C. S. Adonizio, H. J. Hwang, K. Lahouelj, J. D. Cohen, C. Douville, A. A. Patel, L. N. Hagmann, D. D. Roltson, N. Malani, S. Zhou, C. Bettegowda, D. L. Diehl, B. Urban, C. D. Still, L. Kann, J. I. Woods, Z. M. Salvati, J. Vadakara, Science 2020, 369, eabb9601.

65. Thrive Earlier Detection Corp. Detecting Cancers Earlier Through Elective Plasma-based CancerSEEK Testing—Ascertaining Serial Cancer Patients to Enable New Diagnostic (accessed: February 2021).

66. M. C. Liu, G. R. Oxnard, E. A. Klein, M. V. Seiden, M. C. Liu, G. R. Oxnard, E. A. Klein, D. Smith, D. Richards, T. J. Yeatman, A. L. Cohn, R. Lapham, J. Clement, A. S. Parker, M. K. Tummala, K. McIntyre, M. A. Sekeres, A. H. Bryce, R. Siegel, X. Wang, D. A. Berry. Ann. Oncol. 2020, 31, 745.

67. N. Michel. DNA Methylation and Complex Human Disease. Elsevier, Amsterdam, Nethelands 2016.

68. A. P. Feinberg, R. Ohlsson, S. Henikoff, Nat. Rev. Genet. 2006, 7, 21.

69. M. P. Hanley, M. A. Hahn, A. X. Li, X. Wu, J. Lin, J. Wang, A. H. Choi, Z. Ouyang, Y. Fong, G. P. Pfeifer, T. J. Devers, D. W. Rosenberg, Oncogene 2017, 36, 5035.

70. S. Y. Shen, R. Singhania, G. Fehringer, A. Chakravarthy, M. H. A. Roehrl, D. Chadwick, P. C. Zucca, A. Borgida, T. T. Wang, T. Li, O. Kis, Z. Zhao, A. Sprefacio, T. D. S. Medina, Y. Wang, D. Roulois, I. Ettayebi, Z. Chen, S. Chow, T. Murphy, A. Arruda, G. M. O’kane, J. Liu, M. Mansour, J. D. Mcpherson, C. O’Brien, N. Leigh, P. L. Bedard, N. Fleschner, G. Liu, M. D. Minden, S. Gallinger, A. Goldenberg, T. J. Pugh, M. M. Hoffman, S. V. Bratman, R. J. Hung, D. D. De Carvalho, Nature 2018, 563, 579.

71. S. Y. Shen, J. M. Burgener, S. V. Bratman, D. D. De Carvalho, Nat. Procot. 2019, 14, 2749.

72. G. D. Guler, Y. Ning, C. J. Ku, T. Phillips, E. McCarthy, C. K. Ellison, A. Bergamaschi, F. Collin, P. Lloyd, A. Scott, M. Antone, W. Wang, K. Chau, A. Ashworth, S. R. Quake, S. Levy, Nat. Commun. 2020, 11, 5270.

73. X. Chen, J. Gole, A. Gore, Q. He, M. Lu, J. Min, Z. Yuan, X. Yang, Y. Jiang, T. Zhang, C. Suo, X. Li, L. Cheng, Z. Zhang, H. Niu, Z. Li, Z. Xie, H. Shi, X. Zhang, M. Fan, X. Wang, Y. Yang, J. Dang, C. Mcconnell, J. Zhang, J. Wang, S. Yu, W. Ye, Y. Gao, K. Zhang, R. Liu, Li Jin, Nat. Commun. 2020, 11, 3475.

74. Singlera Genomics, Singlera Genomics raises $150 million in Series B financing to promote the development and commercialization of early cancer screening products. https://www.prnewswire.com/news-releases/singlera-genomics-raises-150-million-in-series-b-financing-to-promote-the-development-and-commercialization-of-early-cancer-screening-products-30192505.html (accessed: March 2021).

75. A. Babayan, K. Pantel, Genome Med. 2018, 10, 21.

76. C. Fiala, E. P. Diamandis, Clin. Chem. Lab. Med. 2020, 58, 1651.

77. A. Bardelli, K. Pantel, Cancer Cell 2017, 31, 172.

78. N. Pashayan, P. D. P. Pharoah, Science 2020, 368, 589.

79. A. K. Mattoo, C. Bettegowda, S. Zhou, N. Papadopoulos, K. W. Kinzler, B. Vogelstein, Sci. Transl. Med. 2019, 11, aay1984

80. National Lung Screening Trial Research Team... N. Engl. J. Med. 2011, 365, 395.

81. S. Avanzini, D. M. Kurtz, J. J. Chabon, E. J. Moding, S. S. Hori, S. S. Gambhir, A. A. Alizadeh, M. Diehn, J. G. Reiter, Sci. Adv. 2020, 6, eabc4308.

82. M. Elaizezy, S. A. Joostes, Comput Struct Biotechnol J 2018, 16, 370.

83. S. Volik, M. Alcaide, R. D. Morin, C. Collins, Mol. Cancer Res. 2016, 14, 898.

84. J. Baumul, B. Levy, Clinical Cancer Research: An Official Journal of the American Association for Cancer Research 2018, 24, 4352.

85. H. T. Chan, S. Nagayama, M. Y. Chinn, M. Otaki, R. Hayashi, K. Kiyotani, Y. Fukunaga, M. Ueno, Y. Nakamura, S. K. Low, Molecular Oncology 2020, 14, 1719.

86. R. Okamura, D. E. Piccioni, A. Boichard, S. Lee, R. E. Jimenez, J. K. Sicklick, S. Kato, R. Kurzrock, Int. J. Cancer 2021, 148, 2839.

87. D. P. Steensma, Blood Adv 2018, 2, 3404.

88. Y. Hu, B. C. Ulrich, J. Supplee, Y. Kuang, P. H. Lizotte, N. B. Feeney, N. M. Guibert, M. M. Awad, K. K. Wong, P. A. Jânné, C. P. Paweletz, G. R. Oxnard, Clin. Cancer Res. 2018, 24, 4437.

89. P. Razavi, B. T. Li, D. N. Brown, E. Hubbell, R. Shen, W. Abida, K. Juluru, I. D. Bruijn, D. H. De Velder, M. Verheij, A. Cats, C. J. A. Punt, G. R. Vink, N. C. T. Van Grieken, M. Koopman, R. J. A. Fijnenman, J. S. Johansen, H. J. Nielsen, G. A. Meijer, C. L. Andersen, R. B. Scharpf, V. E. Veluculescu, Nature 2019, 570, 385.

90. G. D. Sepich-Poore, L. Zitvogel, R. Straussman, J. Hasty, J. A. Wargo, R. Knight, Science 2021, 371, eaaaw3616.

91. G. D. Poore, E. Kopylova, Q. Zhu, C. Carpenter, S. Fraraccio, S. Wendro, T. Kosioleak, S. Janssen, J. Metcalf, S. J. Song, J. Kanbar, S. Miller-Montgomery, R. Heaton, R. Mckay, S. P. Patel, A. D. Swafford, R. Knight, Nature 2020, 579, 567.

92. D. Nejmam, I. Livyatan, G. Fiks, N. Gavert, Y. Zwang, L. T. Geller, A. Rotter-Maskowitz, R. Weiser, G. Malle, E. Gigi, A. Melts, G. M. Douglas, I. Kamer, V. Gopalakrishnan, T. Dadosh, S. Levin-Zaaimd, S. Avnet, T. Atlan, Z. A. Cooper, R. Arora, A. P. Cogdill, MdAW Khan, G. Ologun, Y. Bussi, A. Weinberger,... R. Straussman, Science 2020, 368, 973.

93. R. S. Klein, R. A. Recco, M. T. Catalano, S. C. Edberg, J. I. Casey, N. H. Steigbigel, N. Engl. J. Med. 1977, 297, 800.

94. T. N. Y. Kwong, X. Wang, G. Nakatsu, T. C. Chow, T. Tipoe, R. Z. W. Dai, K. K. K. Tsoi, M. C. S. Wong, G. Tse, M. T. V. Chan, F. K. L. Chan, S. C. Ng., J. C. Y. Wu, W. K. K. Wu, J. Yu, J. J. Y. Sung, S. H. Wong, Gastroenterology 2018, 155, 383.

95. American Lung Association, Lung cancer fact sheet. www.lung.org/lung-health-diseases/lung-disease-lookup/lung-cancer/resource-library/lung-cancer-fact-sheet. Accessed: May 2021.

96. Biocept. Our Technology–Biocept. https://biocept.com/technology (accessed: April 2021).
99. G. P. Young, S. K. Pedersen, S. Mansfield, D. H. Murray, R. T. Baker, P. Rabbitt, S. Byrne, L. Bambacas, P. Hollington, E. L. Symonds, *Cancer Med.* 2016, 5, 2763.

100. Freenome Holdings Inc. Prevention of Colorectal Cancer Through Multimomics Blood Testing. https://clinicaltrials.gov/ct2/show/NCT04369053 (accessed: April 2021).

101. Guardant Health, Inc. Evaluation of the ctDNA LUNAR Test in an Average Patient Screening Episode. https://clinicaltrials.gov/ct2/show/NCT04136002 (accessed: April 2021).

102. T. Forshew, M. Murtaza, C. Parkinson, D. Gale, D. W. Y. Tsui, F. Kaper, S. J. Dawson, A. M. Piskorz, M. Jimenez-Linan, D. Bentley, J. Hadfield, A. P. May, C. Caldas, J. D. Brenton, N. Rosenfeld, *Sci. Transl. Med.* 2012, 4, 136ra68.

103. T. Reinert, T. V. Henriksen, E. Christensen, S. Sharma, R. Salari, H. Sethi, M. Knudsen, I. Nordenstoft, H. T. Wu, A. S. Tin, M. Heilskov Rasmussen, S. Vang, S. Shchegrova, A. Fryendahl Boll Johansen, R. Srinivasan, Z. Assaf, M. Balciglu, A. Olson, S. Dashner, D. Hafez, S. Navarro, S. Goel, M. Rabinowitz, P. Billings, S. Sigurjonsson, L. Dyrskjot, R. Swnerton, A. Aleshin, S. Laurberg, A. Husted Madsen, A. S. Kannerup, K. Stribolt, S. Vang, S. Shchegrova, A. Fryendahl Boll Johansen, R. Srinivasan, Z. Assaf, M. Balciglu, A. Olson, S. Dashner, D. Hafez, S. Navarro, S. Goel, M. Rabinowitz, P. Billings, S. Sigurjonsson, L. Dyrskjot, R. Swnerton, A. Aleshin, S. Laurberg, A. Husted Madsen, A. S. Kannerup, K. Stribolt, S. Palmelund Krag, L. H. Iversen, K. Gotschalck Sunesen, C. HoJ Lin, B. G. Zimmermann, C. Lindbjerg Andersen, *JAMA Oncol.* 2019, 5, 1124.

104. M. Gaga, J. Chorostowska-Wynimko, I. Horváth, M. C. Tammemagi, D. Shitrit, V. H. Eisenberg, H. Liang, D. Stav, D. L. Faber, M. Jansen, Y. Raviv, V. Panagoulias, P. Rudzinski, G. Izbicki, O. Ronen, A. Goldhaber, N. Arber, I. Haas, Q. Zhou, *Eur. Respir. J.* 2021, 57, 2002682.

105. J. F. Rahier, A. Druez, L. Faugeras, J. P. Martinet, M. Géhenot, E. Josseaux, M. Herzog, J. Micallef, F. George, M. Delos, T. De Ronde, A. Badaoui, L. D’hondt, *Clin Epigenetics* 2017, 9, 53.

106. M. A. Sánchez-Romero, J. Casadesús, *Nat. Rev. Microbiol.* 2020, 18, 7.

107. S. Adhikari, P. D. Curtis, *FEMS Microbiology Reviews* 2016, 40, 575.

108. R. Sadeh, I. Sharkia, G. Fialkoff, A. Rahat, J. Gutin, A. Chapple-boim, M. Nitzan, I. Fox-Fisher, D. Neiman, G. Meier, Z. Kamari, D. Yaish, T. Peretz, A. Hubert, J. E. Cohen, A. Salah, M. Temper, A. Grinshpun, M. Maoz, S. Abu-Gazala, A. Ben Ya’acov, E. Shteyer, R. Safadi, T. Kaplan, R. Shemer, D. Planer, E. Galun, B. Glaser, A. Zick, Y. Dor, N. Friedman, *Nat. Biotechnol.* 2021, 39, 586.

109. P. Burnham, M. S. Kim, S. Agbor-Enoh, H. Luikart, H. A. Valantine, K. K. Khush, I. De Vlaminck, *Sci. Rep.* 2016, 6, 27859.

110. P. A. Gammage, C. Frezza, *BMC Biol.* 2019, 17, 53.

111. M. J. L. Ma, H. Zhang, P. Jiang, S. T. K. Sin, W. K. J. Lam, S. H. Cheng, W. L. Lee, W. Gai, O. Y. O. Tse, W. Peng, J. Wong, R. Raghpahuty, R. S. M. Wong, D. Sahota, T. Y. Leung, K. C. A. Chan, R. W. K. Chiu, Y. M. D. Lo, *Clin. Chem.* 2019, 65, 1161.

112. J. Zhu, F. Zhang, M. Du, P. Zhang, S. Fu, L. Wang, *Sci. Rep.* 2017, 7, 10968.

113. P. Kumar, L. W. Dillon, Y. Shibata, A. A. Jazaeri, D. R. Jones, A. Dutta, *Mol. Cancer Res.* 2017, 15, 1197.

114. P. Jiang, T. Xie, S. C. Ding, Z. Zhou, S. H. Cheng, R. W. Y. Chan, W. S. Lee, W. Peng, J. Wong, V. W. S. Wong, H. L. Y. Chan, S. L. Chan, L. C. Y. Poon, T. Y. Leung, K. C. A. Chan, R. W. K. Chiu, Y. M. D. Lo, *Genome Res.* 2020, 30, 1144.

115. M. W. Snyder, M. Kircher, A. J. Hill, R. M. Daza, J. Shendure, *Cell* 2016, 164, 57.

116. E. A. Klein, D. Richards, A. Cohn, M. Tummala, R. Lapham, D. Cosgrove, G. Chung, J. Clement, J. Gao, N. Hunkapiller, A. Jamshidi, K. N. Kurtzman, M. V. Seiden, C. Swanton, M. C. Liu, *Ann. Oncol.* 2021, 32, 1167.

117. A. H. Krist, K. W. Davidson, C. M. Mangione, M. J. Barry, M. Cabana, A. B. Caughey, E. M. Davis, K. E. Donahue, C. A. Doubeni, M. Kubik, C. S. Landefeld, L. Li, G. Ogedegbe, D. K. Owens, L. Pfert, M. Silverstein, J. Stevermer, C. W. Tseng, J. B. Wong, *JAMA* 2021, 325, 962.

How to cite this article: E. Adams, G. D. Sepich-Poore, S. Miller-Montgomery, R. Knight. *VIEW. 2022*, 3, 20200118. https://doi.org/10.1002/VIW.20200118