Cell-free DNA approaches for cancer early detection and interception

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ABSTRACT

Rapid advancements in the area of early cancer detection have brought us closer to achieving the goals of finding cancer early enough to treat or cure it, while avoiding harms of overdiagnosis. We evaluate progress in the development of early cancer detection tests in the context of the current principles for cancer screening. We review cell-free DNA (cfDNA)-based approaches using mutations, methylation, or fragmentomes for early cancer detection. Lastly, we discuss the challenges in demonstrating clinical utility of these tests before integration into routine clinical care.

INTRODUCTION

The early detection of cancer is intended to identify a malignant transformation in asymptomatic individuals at average risk or elevated risk of developing cancer, that is measurable by a screening test, but not advanced enough to cause clinical symptoms or to be detected during usual clinical care. In this way, tumors can be intercepted earlier than current efforts. The underlying premise of early detection and interception efforts is that finding cancers at early stages, or even high-risk premalignant lesions, allows for curative or earlier treatment leading to reduced mortality and morbidity. For example, the National Lung Screening Trial has shown that early detection and interception of lung cancer through surgical resection reduces overall mortality in this high-risk population.[1] The actualization of early cancer detection and interception, however, is not simple. The challenge is 'bringing to treatment those with previously undetected disease and… avoiding harm to those persons not in need of treatment'.[2] Currently only five cancer types—breast, cervical, colorectal, lung, and prostate—have widely accepted screening tests and only four have national screening recommendations in the USA (online supplemental table S1).[3] While these five cancer types represent some of the more commonly diagnosed in men and in women in the USA,[4] most cancer types remain without a screening test or endorsed screening protocol. In addition, for those cancers with demonstrated benefits of early detection, there remains substantial room for improving the accessibility of screening approaches worldwide and the benefit-to-risk ratios. To this end, the concept of blood-based, cell-free DNA (cfDNA)-based early cancer detection testing holds promise to close the cancer screening gap in underserved populations.

The cfDNA molecules present within the circulation were described as early as 1948, with the majority of cfDNA being derived from dying white blood cells (WBCs), vascular endothelial cells, and hepatocytes.[5 6] However, for individuals with cancer, circulating tumor-derived DNA (ctDNA) can be shed into the bloodstream, providing an opportunity for early cancer detection through liquid biopsy approaches. Recent technological advancements in the characterization of ctDNA,[5 7–9] including those to inform therapy selection through detection of *EGFR* gene mutations or other actionable changes in panels of genes[10–12] have demonstrated the feasibility and clinical utility of blood-based tests to guide the clinical management of patients with cancer, typically at late stages. Indeed, based on the findings of recent studies in advanced non-small cell lung cancer,[13–16] a plasma-first approach has been proposed and incorporated into clinical guidelines in lung cancer.[17 18]

Despite these initial successes, the use of cfDNA approaches for early cancer detection has been more challenging because it requires the ability to detect hallmarks or evidence of cancer in the circulation of individuals with no known diagnosis or symptoms of disease. Two important events opened the field in this regard: the realization that widespread chromosomal abnormalities that arise during tumorigenesis can be detected in the circulation of patients with cancer using whole-genome sequencing of cfDNA[19 20]

and the detection of cancer in women who had undergone non-invasive prenatal testing through detection of chromosomal changes first believed to be of fetal origin but subsequently determined to originate from hematological or solid cancers in the mothers.[21 22] The stage of the cancers detected in these individuals ranged from stage II to IV, suggesting that signals from even localized cancers could be detected in the blood of asymptomatic/presymptomatic individuals. The intensive research efforts that ensued intially led to two sets of approaches that identified either mutations or methylation changes in cfDNA and could detect individuals with asymptomatic cancer, although at high cost and with low sensitivity to detect stage I disease.[9 23–26]

With proof-of-concept established that signals of cancers can be detected from blood of asymptomatic/presymptomatic individuals, a second wave of questions naturally arose regarding clinical validity: Can early-stage (stage I) cancers be detected reliably? Can cancer signals be traced to the organ of origin? And even thornier clinical utility questions emerged: Can finding early cancers in the blood benefit patients—especially since a positive benefit/harm ratio for screening has not yet been demonstrated for all cancer types?[27–30] Health economics questions arose, too. Can a test be done affordably to enable widespread population access? Or is a narrower high-risk population the best to target? Further research is needed to determine both the optimal frequency and the cost-effectiveness of testing, to ensure that individuals have the most favorable outcomes from testing balanced with their or society's willingness to cover the costs of these approaches. Considering the promise and the constraints, we have arrived at a challenging crossroad. Before we actualize the detection of early-stage cancers, we should consider the principles that guide testing for early cancer detection and how the emerging technologies measure up to those principles.

The principles of screening

In 1968 Wilson and Jungner published a set of principles to guide the selection of diseases amenable to screening[2] that are largely considered the gold standard in the assessment of screening decisions. These principles have been reevaluated more than once (online supplemental table S2), as technology has made it easier to detect diseases, including cancers, in earlier, preclinical stages using molecular diagnostic tests that characterize genomic rather than pathological alterations. Here we apply the most recent iteration of the principles of screening[31] as early detection tests come of age. We will focus primarily on the disease and test principles (the principles for screening programs are beyond the scope of this article; see online supplemental table S3).

The principles state that the disease or condition, in this case cancer, should be an important health problem, with well-understood epidemiology and natural history, a detectable preclinical phase, and clearly defined target population for testing. A prudent approach with any new

technology would be to use it for the group most likely to benefit—in this case, those who are most likely to develop cancer. Doing so intrinsically improves the potential ratio of benefits to harms. Once evaluations in targeted elevated risk populations prove successful, incremental expansions of the target population can be undertaken informed by risk and harm data. This is consistent with guidance from the WHO regarding the use of pilot screening programs to gather real-world data to inform more widespread screening based on evidence.[32 33] The United States Preventive Services Task Force already recommends screening for four cancer types (lung, colorectal, breast and cervical), so applying new screening technologies to one or more of these cancer types is a reasonable initial approach.

According to the principles, a cancer screening test should be fit for purpose, have good performance characteristics, be acceptable and affordable to the target screening population, and have clinical utility. The emergence of new technologies for the analysis of cfDNA circulating in the blood holds great promise to improve performance and access of current screening and may enable screening for other cancers for which no approaches are currently available. Because the amount of tumor-derived ctDNA present in patients with early-stage cancer can be relatively small compared with the total amount of cfDNA, looking for evidence of cancer in the blood of asymptomatic individuals is very much a needle-in-a-haystack problem. Methods and techniques have become available to find that 'needle'; the challenge is to do so with robust performance characteristics in early-stage cancers and at affordable costs.

Today, there are three basic approaches to the detection of cancer by analyses of cfDNA (table 1). These approaches include testing for (1) somatic mutations, (2) methylation profiles, or (3) whole-genome fragmentation profiles (figure 1).

Cancer screening using somatic mutations

DNA sequencing of tumor DNA has been widely used in the development of companion diagnostic tests to select appropriate targeted therapies for individual patients. The great majority of Food and Drug Administration-approved companion diagnostic tests are based on sequencing tumor samples for the presence of specific mutations (eg, *EGFR* T790M mutation) to guide treatment selection.[33] Use of mutation-based ctDNA detection approaches for screening asymptomatic individuals, however, has not gained traction, largely because the amount of ctDNA is very small (often <1% of the total cfDNA) in early-stage cancer. Such an approach to asymptomatic screening would necessitate sequencing very deeply (>10,000×) and using error-reducing sequencing approaches (eg, molecular identifiers combined with redundant sequencing and consensus calling) to reliably determine if rare mutated-DNA fragments are present in cfDNA. Using whole-genome sequencing for such approaches would be prohibitively expensive.

Table 1 Current cell-free DNA-based approaches for the detection of cancer

Approach	Methodology	Relative abundance of target signals	Assay	Number of targets	Performance	Cohort size	Tumor informed	Extent of clinical evaluation	Relative cost of approach	References
Mutation analysis	Capture-based targeted, multiplex PCR, genome-wide or whole-exome next generation sequencing	Few to hundreds of known mutations or genome-wide	Elio Plasma Resolve	33	VAF≥95% LoD 0.1%–0.5%	n=75	No	Clinical validity, case–control, prospective cohort	$$$–$$$$	32
			FoundationOne Liquid	324	VAF≥95% LoD 0.4%	n>30 cancer types	No			12
			Guardant360	74	VAF>80% LoD 0.25%	n>1500	No			34
			MRDetect	Genome-wide	VAF LoD 10^{-5}, >85% at 20×, >99% at 50×	n=98	Yes			73
			Signatera	16	VAF>95% LoD 0.01%	n=49	Yes			35 36
			CancerSeek (DETECT-A)	16	Specificity: 98.9% Sensitivity: 27%	n=9,911	No			26
			RaDaR	48	VAF=95% LoD 0.001%	n=88, n=17	Yes			37 38
DNA methylation profiling	Bisulfite conversion-sequencing or DNA methylation immunoprecipitation-sequencing	Thousands to tens of thousands of CpG methylation differences	Galleri (PATHFINDER)	Targeted methylation	Specificity: 99.1% Sensitivity: 29%	n=6,621	No	Clinical validity, case–control, prospective cohort	$$$–$$$$	81
			cfMeDIP-seq	Anti-5mc	Lung AUC: 0.971 AML AUC: 0.98 PDAC AUC: 0.92	n=388	No			24 59
Fragmentation profiling	Low-coverage whole-genome, next-generation sequencing	Millions of fragmentation differences	DELFI	Genome-wide	AUC: 0.86 - 0.98 across cancer types	n=423	No	Proof-of-concept, clinical validity, case–control, prospective-retrospective cohort	$–$$	61–63
			End Motifs	Motif diversity score	AUC 0.86	n=89	No			70
			GALYFRE	Genome-wide	Specificity: 95% Sensitivity: 66.9%	n=661	No			71
		Mutational signatures	GEMINI	Genome-wide	AUC 0.90	n=109	No			41
			Pointy	Genome-wide	AUC 0.96	n=405	No			42

AML, acute myeloid leukemia; AUC, area under the curve; LoD, limit of detection; PDAC, pancreatic ductal adenocarcinoma; VAF, variant allele fraction.
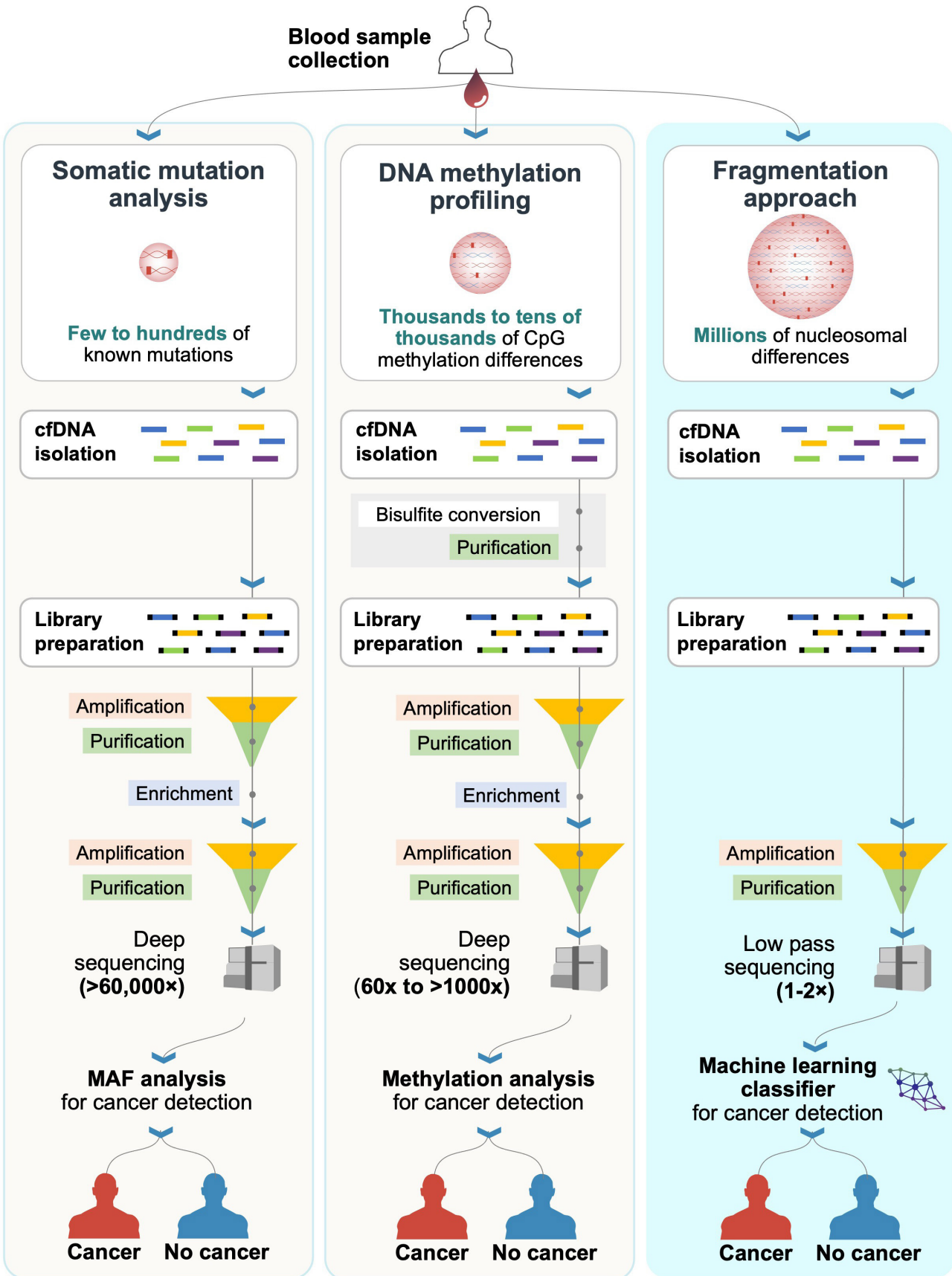
Figure 1 Liquid biopsy approaches for cancer detection. Cell-free DNA technologies evaluating mutations or methylation are often costly, multistep, time consuming processes prior to data analyses. Low-coverage whole-genome sequencing approaches can minimize arduous laboratory protocols at low-cost with broadly applicable bioinformatic pipelines. cfDNA, cell-free DNA; MAF, mutant allele frequency.

To get a sense of the challenge, DNA sequencing for a specific mutation at 60,000-fold unique coverage depth will only reveal on average 30 tumor-derived heterozygous mutant molecules if the ctDNA amount is 0.1%. Furthermore, sequencing this deeply would require that the investigated sample contains 60,000 unique DNA fragments covering all genomic loci. To reliably reach this number in a testing environment, one would have to analyze cfDNA from a substantial volume of blood (>20 mL). Given the challenges of performing such deep sequencing on a genome-wide scale, most approaches explored up to now have been restricted to sequencing of predefined gene panels (eg, selected DNA fragments with genes frequently mutated in the target cancers) by either capture-based or multiplex-PCR–based approaches, all of which increase the cost and effort of such methods (table 1, figure 1).[9 12 26 32 34–40] Further refinement of somatic mutation-based approaches to early cancer detection are needed to make these more affordable for implementation on a population scale. Beyond individual mutations, genome-wide mutational signatures, such as C:G>A:T changes, are known to be increased in certain regions of tumors of individuals with a smoking exposure and may improve cancer detection.[41–43]

Another hurdle associated with somatic mutation-based approaches is determining whether the source of the alteration is from the tumor or WBCs. If somatic mutations were found in asymptomatic individuals, additional sequencing or imaging would be needed to determine the source of the mutation and its clinical relevance.[26] Leal *et al* reported that more than half of mutations in the *TP53* gene detected in the blood were derived from WBCs highlighting the challenges of pinpointing the cellular origin of plasma mutations.[44] Consequently, targeted somatic mutation analysis as a standalone approach lacks the utility needed for widespread use in cancer screening of asymptomatic individuals. It comes as no surprise that approaches relying on somatic mutations are now being combined with other biomarkers.[45]

Cancer screening using methylation profiles

Methylation of promoter DNA sequences is a common mechanism used to regulate gene expression.[46 47] Because cancer development and progression are frequently associated with aberrant patterns of DNA methylation,[48 49] and PCR and next-generation sequencing (NGS)-based methods with high analytical sensitivity are available, methylation profiling has been developed as a means to detect cancer.[50 51] Methylation profiling evaluates epigenetic profiles of cells that have lysed and distributed their contents into circulation. It measures the methylation status of subsets of the ~28 million CpG sites in the genome.[52] These sites are not equally distributed across the genome, and many are concentrated in ~30,000 CpG islands that are located near the promoters of genes and have a critical role in regulating gene expression.[52] Since the genes expressed in each cell type are different, these patterns of differential methylation can be used to identify the cell of origin of circulating cfDNA molecules.

Methylation screening approaches range from measuring a single gene to using a panel of predefined methylation sites that have been shown to be differentially methylated in cancer cells and WBCs.[53–56] PCR-based approaches to detect methylation require preselection of a set of target genes and cannot be done on an unselected genome-wide basis.[57] A broader enrichment for methylation sites involves hybrid capture of small regions of the genome containing a subset of CpG sites, typically using bisulfite conversion, amplification, and sequencing to determine the methylation status at each site, and application of an algorithm to determine the presence of cancer-derived DNA in the sample and the cell of origin of the cancer-derived DNA molecule (figure 1).[25 58] A more comprehensive approach to methylation profiling for cancer detection uses immunoprecipitation of cell-free methylated DNA to isolate the methylated DNA regions throughout the genome and then analyses of these by NGS (table 1).[24 59] More recently, methylation approaches coupled with mutation detection from the same DNA molecule have been developed.[60]

Cancer screening using whole-genome sequencing

Evaluation of cfDNA fragmentation patterns, known as 'fragmentomes', represent the most recent advancement in cfDNA-based screening modalities.[61] These emerging new approaches focus on the physical characteristics of cfDNA fragments, which vary markedly across the genome in cfDNA derived from tumor cells compared with WBCs.[61–63] Early observations suggested that tumor-derived cfDNA is shorter overall when compared with non-cancer cfDNA molecules.[64] More recent findings showed that mutated tumor-derived DNA from different regions in the genome could be smaller or larger than wild-type non-cancer cfDNA.[61] Additionally, these studies revealed that germline mutations as well as variants related to clonal hematopoiesis, which can confound mutation-based analyses, harbor cfDNA fragment size distributions that are similar to non-cancer cfDNA and therefore should not influence fragment size based approaches.[44 61 65] This previously unappreciated knowledge provides a robust foundation for genome-wide fragmentome based approaches focused on early cancer detection.

The intracellular and extracellular DNA fragmentation processes associated with apoptosis and necrosis involve enzymes including caspases and endonucleases that cleave chromosomal DNA molecules into short fragments. These cleavage sites are non-random and determined in part by the association of the DNA molecules with the nucleosomal proteins, which protect the DNA from endonuclease digestion.[66] On a genome-wide level, cfDNA fragmentation has been shown to reflect broader patterns of chromatin structure.[66] The cellular genome is organized into two basic patterns known as open and closed chromatin that is mechanistically linked to the

regulation of gene expression. The regions of open and closed chromatin are highly conserved and characteristic of the unique gene expression profile in each cell type. The three-dimensional chromatin organization is quite different in the open and closed chromatin and controls binding of the RNA transcription complex to the template DNA. This three-dimensional structure also affects the binding of other large protein molecules and complexes, such as the endonucleases and caspases, to the DNA resulting in reproducible patterns of cleavage in each cell type. Consequently, the cleavage patterns determined by the basal chromatin organization can be used to differentiate between cfDNA derived from different cell types, and between cancer and normal cells. The result is that the cfDNA fragmentome is both characteristic of the cell type/organ from which it originates, and distinct between cancer and normal cells.[61]

Historically, only a few approaches had leveraged genome-wide cfDNA analyses, including those to assess changes in chromosomal copy number,[19] nucleosomal footprints,[66] fragment lengths,[67] transcription start sites[68] or transcription factor binding sites.[69] Genome-wide analyses of cfDNA fragmentomes led to the realization that changes in cfDNA size and coverage throughout the genome using machine learning models could be used for early detection.[61] Additional analyses from whole genome sequencing of cfDNA have included end motifs,[70] and fragment end position aberrancy.[71] These recent advances in cfDNA fragmentation biology provide orthogonal approaches to detect cancer beyond existing methods.

The majority of cfDNA fragment-based approaches rely on whole-genome next-generation sequencing data, unlike mutation analyses that mainly use targeted tumor-specific gene panels with deep coverage (table 1, figure 1).[9 12 32 34 72] More recently, whole-genome sequencing to detect mutations has emerged[73] but this genome-wide sequencing approach requires tumor tissue, WBCs, and plasma biomaterials to be sequenced at high coverage (~35×), making it more suitable for identification of minimal residual disease rather than early cancer detection. Variations to this method have used whole-exome tumor-tissue sequencing to identify personalized tumor-specific targets that are subsequently analyzed in plasma after therapeutic intervention.[35 37 38 74] These evolving mutation-based approaches, while effective, require substantial logistics, including in obtaining both tissue and blood specimens, as well as operational costs that may limit clinical feasibility. Alternatively, emerging advancements of fragmentomics may lower such barriers to entry not only in the setting of minimal residual disease but for early detection screening (table 1).

To this end, a recent cfDNA fragmentation approach has been described that involves the extraction of cfDNA from plasma, construction of whole-genome libraries, low-coverage whole-genome sequencing, and application of a machine learning algorithm to predict the presence of cancer DNA (figure 1). This approach has been tested in a multi-cancer cohort,[61] a hepatocellular carcinoma cohort,[63] as well as a large lung cancer cohort.[62] Studies so far have shown that fragmentation-based detection has promising sensitivity for the detection of localized early-stage cancers (stage I/II).[61 62] Because fragmentation profiles can be determined through low-coverage whole-genome sequencing and there are potentially millions of fragment changes in the cfDNA originating from a cancer cell, the cfDNA fragmentation approach has potential to be widely available at low cost, satisfying the principle in that a screening test be accessible and acceptable to the target screening population (online supplemental table S2). Moreover, whole-genome fragmentation analyses do not require expensive gene-capture panels or bisulfite conversion of DNA prior to sequencing, a step during which substantial amounts of the DNA sample are lost (figure 1). In addition, all tumor-derived cfDNA is potentially assessed for the test, inherently increasing analytical sensitivity, compared with approaches that use only specific regions of the tumor DNA as input. That said, the steps involved in fragmentation analysis, including blood sample collection, cfDNA extraction, DNA sequencing, and machine-learning models, must be reproduced reliably for the test to remain robust, and it remains uncertain how much process variation can be accommodated in fragmentation analyses.

Impact of inflammatory conditions on cfDNA

Non-malignant diseases that involve the host immune system, such as chronic inflammation or autoimmunity, have demonstrated an increase in cfDNA levels in the blood.[75 76] The elevated release of cfDNA into circulation could lead to a larger 'haystack' of cfDNA molecules that may affect the ability for a few low-quantity ctDNA molecules to be detected by mutation-based cancer tests. In fact, most cfDNA-focused mutation studies exclude individuals with inflammatory conditions, while only a few studies evaluate mutations in conditions such as chronic liver diseases or neoplasia from inflammatory bowel disease.[77 78]

By contrast, the use of cfDNA fragmentation-based approaches in individuals with inflammatory or autoimmune diseases have been reported for patients with liver or lung cancer.[62 63 70] Jiang *et al* showed that fragment end motifs in healthy individuals and those with hepatitis B virus infection (with or without cirrhosis) are more similar to each other than to motifs of persons with hepatocellular carcinoma.[70] In parallel, the development of an approach that assesses genome-wide fragment position through an information-weighted fraction of aberrant fragments value also observed that profiles from non-cancer individuals were similar to those from individuals harboring the hepatitis B virus with or without cirrhosis.[71] Recently, Foda *et al* evaluated genome-wide cfDNA fragmentomes in cancer-free individuals with viral hepatitis or liver cirrhosis and observed low fragmentome scores comparable to those individuals without inflammation or cancer.[63] In a study of a prospectively collected population

of patients at high risk of developing lung cancer, cfDNA fragmentomes of individuals with or without autoimmune diseases were not statistically significant, and levels of circulating inflammatory markers C-reactive protein and interleukin 6 did not correlate with fragmentome-based scores in non-cancer individuals, suggesting that genome-wide fragmentation is not skewed by the presence of chronic inflammation.[62] In summary, the current literature suggests that inflammatory diseases leading to increased cfDNA levels may have an impact on detection of cfDNA mutations but fragmentation approaches appear robust in this setting.

Proof-of-principle early cancer detection studies

Currently, multiple efforts are ongoing to develop liquid biopsy screening tests that effectively enable multi-cancer early detection (MCED) (table 2). Liquid biopsy early cancer screening in this setting necessitates a high specificity to reduce potentially adverse clinical intervention from false positives as well as to minimize overdiagnosis. To achieve such standards of test performances, cfDNA assay features have leveraged the biological oncogenic process such as mutations, protein biomarkers, methylation, and fragmentation features from plasma.[61 79 80] The CancerSeek initial proof-of-concept approach across eight cancer types from a total of 1005 individuals introduced the potential applicability of mutations and protein biomarkers as a way to detect cancers early.[79] While preliminary data from the CancerSeek approach was promising, clinical translation through a prospective interventional study known as DETECT-A (Detecting cancers Earlier Through Elective mutation-based blood Collection and Testing) resulted in a sensitivity of 27% for all cancer types at a specificity of 98.9% with a positive predictive value (PPV) of 19.4% (tables 1 and 2).[26] Methylation based clinical trials such as the PATHFINDER study revealed a sensitivity of 29% at a specificity of 99.1% with a PPV of 38% (tables 1 and 2).[81] Both DETECT-A and the PATHFINDER PPVs highlight the existing challenges for pan-cancer early detection tests as opposed to scenarios of high-risk screening that may benefit from a liquid biopsy prescreen. More recently, the SYMPLIFY study assessed individuals with signs and symptoms that may be indicative of cancer using the Galleri MCED test (ISRCTN10226380) (table 2).[82] Interestingly, this analysis consisted of 5461 individuals with symptoms and resulted in a sensitivity of 66.3% (95% CI 61.2 to 71.1) at a specificity of 98.4% (95% CI 98.1 to 98.8) with a PPV of 75.5% (95% CI 70.5 to 80.1). It is important to note that, while the assay applied in PATHFINDER and SYMPLIFY is the same, SYMPLIFY examined patients with symptoms such that the study population is different and must be considered when evaluating performances and future applications.

To improve early cancer detection performance from these intial studies, multiple clinical trials are ongoing focused on detection of a variety of cancer types (Table 2), with the goal of achieving sensitivities in early stage

disease that could clinically useful. One approach has been to integrate subsets of features including mutations, methylation, and genome-wide fragment characteristics in these analyses. Jamshidi and colleagues use a pan-feature comprised of a 507 targeted gene panel that evaluated single nucleotide variants (SNV) and white-blood cell corrected-SNVs, whole-genome methylation (30× coverage), and whole-genome sequencing (30× coverage) subcategorized to independently assess the features of copy number alterations (CNA), white-blood cell corrected CNA, fragment endpoints, fragment lengths, and allelic imbalance.[80] One limitation of this study was that fragment features were not combined together and compared with methylation as was reported to an extent in previous research from the same group.[83] Bruhm et al., recently combined genome-wide fragmentation features and regional mutational signatures from low-coverage whole genome sequencing to show an increased performance for cancer detection compared to either method alone.[41] Orthogonal analyses as well as new approaches that reflect the underlying cfDNA biology and machine learning are still warranted to further confirm and extend such initial findings. Lastly, combining cfDNA features and emerging radiomics approaches may further improve detection especially in the context of high-risk screening.

Establishing clinical utility for early cancer detection tests

The National Cancer Institute of the USA defines clinical utility as 'the likelihood that a test will, by prompting an intervention, result in an improved health outcome'.[84] In the case of early cancer detection, the intervention that the test should inform/prompt is whether to proceed to confirmatory diagnostic testing and subsequent intervention or to continue in the future with the recommended regular screening. Because high sensitivity and high specificity are uncommon in a single test, tests are often divided into those that have high-enough sensitivity to effectively 'rule-out' the presence of cancer without a false-positive rate that causes substantial clinical burden and those that have high-enough specificity to 'rule-in' cancer at a sensitivity high enough to prove clinically useful. The ultimate and most clinically important objective of cancer screening is to reduce the likelihood that a screened individual dies of cancer. As the ultimate goal of cancer screening is to reduce cancer specific mortality, the ultimate endpoint would be cancer specific survival. As it easily can take over a decade to reliably evaluate this endpoint, as a rule, intermediate endpoints like a stage shift or detecting high-risk precursor lesions are used as alternatives. Care then must be taken that any intermediate endpoints used are representative of ultimate outcome.[30 85–88] For some cancer types, like prostate and perhaps breast cancer, a shift in stage at diagnosis could rather reflect the ability of the screening test to detect indolent cancers that otherwise would not become symptomatic or impact the survival of the patient. As early cancer detection tests come of age, how do we safeguard against overdiagnosis of cancer? According to the

Table 2 Clinical trials evaluating new approaches focused on early cancer detection through screening

NCT	Sponsor	Trial name	Estimated enrollment	Biomarker	Condition	Age eligibility (years)	Status
NCT05366881	Adela	CAMPERR	7,000	cfDNA	MCED	≥40	Recruiting
NCT04972201	Burning Rock	PROMISE	2,305	cfNA	MCED	40–75	Recruiting
NCT03869814	ClearNote Health	MT1410	6,500	5-hmC ctDNA	MCED	45–75	Recruiting
NCT04825834	DELFI Diagnostics	L101	2,500	ctDNA	Lung cancer	≥50	Recruiting
NCT05306288	DELFI Diagnostics	CASCADE	15,000	ctDNA	Lung cancer	≥50	Recruiting
NCT04144751	Exact Sciences	Blue-C	24,000	Blood-based	Colorectal cancer	≥40	Active, not recruiting
NCT04369053	Freenome	Preempt	25,000	ctDNA	Colorectal cancer	45–84	Active, not recruiting
NCT05516927	Freenome	Sanderson Study	8,000	Multiomics	MCED	≥30	Recruiting
NCT05227261	Gene Solutions	K-DETEK	3,000	ctDNA	Lung, breast, liver, colorectal, and gastric cancers	≥40	Recruiting
ISRCTN10226380	Grail	SYMPLIFY	6,238	cfDNA	MCED in symptomatic individuals	≥18	Completed
NCT04241796	Grail	Pathfinder	6,662	ctDNA	MCED	≥50	Completed
NCT03085888	Grail	Strive	99,481	cfNA	MCED	≥18	Active, not recruiting
NCT03934866	Grail/UCL	Summit	13,035	cfNA	Lung cancer	55–77	Active, not recruiting
NCT03774758	Guardant Health	17–22915	590	ctDNA	Lung cancer	≥40	Recruiting
NCT04136002	Guardant Health	Eclipse	20,000	ctDNA	Colorectal cancer	45–84	Recruiting
NCT05181826	Helio Health	ELITE	1,200	ctDNA	Cancer-free, benign, cancer	≥18	Recruiting
NCT05199259	Helio Health	Liver-1	1,200	ctDNA	Hepatocellular carcinoma	≥18	Recruiting
NCT03694600	Helio Health	CLiMB	1,600	cfDNA	Liver cirrhosis, hepatocellular carcinoma	21–84	Recruiting
NCT05432128	Singlera Genomics	2019YFC1315803	600	ctDNA	Lung cancer	18–75	Recruiting
NCT05485077	Singlera Genomics	KYS-2022007	18,000	cfDNA	Colorectal cancer	≥40	Recruiting

cfDNA, cell-free DNA; cfNA, cell-free nucleic acids; ctDNA, circulating tumor-derived DNA; MCED, multi-cancer early detection.

principles of screening (online supplemental table S2), do we adequately understand the natural histories of the types of cancer likely to be detected? Stage shift per se may not translate into improved outcomes for some cancer types, and other factors, such as the availability of effective treatments, matter more. Indeed, as the principles of screening point out (online supplemental table S2), there should be 'follow-up care that will modify the natural history and clinical pathway for the disease or condition….' With respect to early cancer detection testing, how would we determine the magnitude of stage shift needed for each cancer type that would result in a survival benefit? Are there interventions available that could modify the natural history and clinical pathway for every type of cancer that could be detected early? If not, how should we measure the clinical utility of early detection for these less treatable cancer types? The answers to these questions are even more pertinent in the case of ctDNA screening tests that can identify multiple different types of cancer at once. Addressing these important points may require the conduct of randomized controlled trials of testing versus no testing, with endpoints that include both 5-year cancer-specific survival and overall survival, along with parallel studies to measure quality of life of those tested and untested, and cost-effectiveness of testing and no-testing strategies.

Some have argued that the endpoint for early cancer detection tests should be all-cause mortality,[89] because overall mortality is what matters most to patients, it minimizes the effects of misclassification of causes of death, and showing actual lives saved is the most direct demonstration that early cancer detection saves lives. Few cancer screening trials have demonstrated significant improvements in all-cause mortality. The National Lung Screening Trial is the only single-cancer screening trial to demonstrate a significant reduction in all-cause mortality in a population with heavy smoking histories.[1] With median follow-up of 6.5 years, the rate of death from any cause was 6.7% lower (95% CI 1.2 to 13.6; p=0.02) in the arm that underwent low-dose computed tomography (n=26,722) versus chest radiography (n=26,732). The multi-cancer Prostate Lung Colorectal Ovarian trial (n=154,887; median follow-up of 17 years) demonstrated a small but significant reduction in overall mortality (2%; p=0.036) for those in the screening arm versus the usual care arm, particularly for male participants.[90] Because reduced overall mortality is harder to demonstrate in clinical trials, the use of such as cancer-specific mortality becomes more attractive.

CONSIDERATIONS FOR THE FUTURE
The promise of cfDNA-based approaches to cancer early detection is apparent, but the journey from discovery to clinical implementation is, and should be, methodical and thoughtful. Tests for cancers for which there is established strong evidence that earlier detection leads to improved clinical outcomes need to have strong sensitivity to be useful as adjunct or alternative screening approaches to established approaches. They also must be valid across the at-risk populations, and affordable to ensure their accessibility. The health economics of liquid biopsy screening will loom large as a concern until these tests become more affordable to more people.

Exactly how these tests will integrate into routine practice remains to be seen and will likely differ by test, depending on the intended use for which each test is validated (eg, as a novel screening pathway without precedent, a pathway parallel to an existing, established screening pathway, or a screening step preceding an existing, established diagnostic pathway). How each test fits into an overall cancer screening paradigm will affect how cost-effectiveness is weighed. At the moment there is a lack of empirical data on cost-effectiveness of these tests. Future studies should take into account the costs and healthcare resource utilization associated with use or disuse of the test, as well as the course of action taken following positive or negative test results. Factors such as static versus variable uptake of test use, as well as uptake of existing diagnostic procedures driven by test use can also affect cost-effectiveness.

Tests for multiple cancers face considerably more challenges and questions that need to be addressed, especially if the cancers potentially detected include those that are uncommon or rare, or hard to treat. In many cases, there is no established clinical approach to a generalized cancer signal, and strategies to accompany these tests with a short list of the most likely tumor sites have yet to be clinically evaluated for their capacity to reduce the burden and risks of work-up of those signals. While at first it may seem intuitive what next step should be taken, the reality is that the history of cancer screening is one where the effort to streamline the evaluation of initial positives has been vexing.

Appropriate management strategies for many of the potential cancers detected by a multi-cancer test should be clearly defined (eg, best evidence-based therapies or interventions), as the evidence suggests that these tests identify a subset of disease that is generally more aggressive.[91] Whether standard therapeutic approaches to this subset are optimal is a question only clinical studies can answer. Proof of concept for multicancer tests, demonstrating that they both provide benefits in terms of reducing cancer mortality and do not drive harm through false assurances when their results are negative (eg, by leading people to forego other recommended screening tests) are challenges that still need to be addressed. While we focused on cfDNA-based approaches, other blood-based multiomics approaches that take advantage of protein or RNA markers are also in development. Ultimately, combinations of DNA and non-DNA approaches may be implemented if shown to optimize test performance and clinical utility.

CONCLUSIONS

Great strides have been made in the development of cfDNA-based early cancer detection tests. Initial studies providing proof-of-principle evidence that these tests can detect cancer signals in asymptomatic or pre-symptomatic individuals have given way now to clinical trials designed to establish the clinical utility of these tests. New approaches, including cfDNA fragmentation-based methods, may allow for widespread accessibility of these tests in appropriate screening populations worldwide. Many areas of investigation remain to be addressed before these tests are fully integrated into routine practice in a way that optimizes patient outcomes.

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REFERENCES

1. The National Lung Screening Trial Research Team. Reduced lung-cancer mortality with low-dose computed Tomographic screening. *N Engl J Med* 2011;365:395–409.
2. Wilson JM, Jungner YG. Principles and practice of mass screening for disease. *Bol Oficina Sanit Panam* 1968;65:281–393.
3. United States preventive services task force. Published recommendations for cancer. Available: https://www.uspreventive servicestaskforce.org/uspstf/topic_search_results?topic_status=P&category%5B%5D=15&searchterm= [Accessed 5 Aug 2022].
4. American Cancer Society. Cancer facts & figures 2022. Available: https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2022.html [Accessed 5 Apr 2023].
5. Mandel P, Metais P. Nuclear acids in human blood plasma. *C R Seances Soc Biol Fil* 1948;142:241–3.
6. Moss J, Magenheim J, Neiman D, et al. Comprehensive human cell-type methylation Atlas reveals origins of circulating cell-free DNA in health and disease. *Nat Commun* 2018;9:5068.
7. Stroun M, Anker P, Lyautey J, et al. Isolation and characterization of DNA from the plasma of cancer patients. *Eur J Cancer Clin Oncol* 1987;23:707–12.
8. Stroun M, Anker P, Maurice P, et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989;46:318–22.
9. Phallen J, Sausen M, Adleff V, et al. Direct detection of early-stage cancers using circulating tumor DNA. *Sci Transl Med* 2017;9:eaan2415.
10. Office of the Commissioner. FDA APPROVES first blood test to detect gene Mutation associated with non-small cell lung cancer [U.S. Food and Drug Administration]. Available: https://www.fda.gov/news-events/press-announcements/fda-approves-first-blood-test-detect-gene-mutation-associated-non-small-cell-lung-cancer [Accessed 5 Apr 2023].
11. Aggarwal C, Thompson JC, Black TA, et al. Clinical implications of plasma-based Genotyping with the delivery of personalized therapy in metastatic non-small cell lung cancer. *JAMA Oncol* 2019;5:173.
12. Woodhouse R, Li M, Hughes J, et al. Clinical and Analytical validation of Foundationone liquid Cdx, a novel 324-gene cfDNA-based comprehensive Genomic profiling assay for cancers of solid tumor origin. *PLoS One* 2020;15:e0237802.
13. Leighl NB, Page RD, Raymond VM, et al. Clinical utility of comprehensive cell-free DNA analysis to identify Genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancer. *Clin Cancer Res* 2019;25:4691–700.
14. Pritchett MA, Camidge DR, Patel M, et al. Prospective clinical validation of the Invisionfirst-lung circulating tumor DNA assay for molecular profiling of patients with advanced Nonsquamous non-small-cell lung cancer. *JCO Precis Oncol* 2019;3:PO.18.00299.
15. Garcia-Pardo M, Czarnecka K, Law JH, et al. Plasma-first: accelerating lung cancer diagnosis and molecular profiling through liquid biopsy. *Ther Adv Med Oncol* 2022;14:17588359221126151.
16. Cui W, Milner-Watts C, O'Sullivan H, et al. Up-front cell-free DNA next generation sequencing improves target identification in UK first line advanced non-small cell lung cancer (NSCLC) patients. *Eur J Cancer* 2022;171:44–54.
17. Aggarwal C, Rolfo CD, Oxnard GR, et al. Strategies for the successful implementation of plasma-based NSCLC Genotyping in clinical practice. *Nat Rev Clin Oncol* 2021;18:56–62.

18 Rolfo C, Mack P, Scagliotti GV, *et al*. Liquid biopsy for advanced NSCLC: A consensus statement from the International Association for the study of lung cancer. *J Thorac Oncol* 2021;16:1647–62.

19 Leary RJ, Sausen M, Kinde I, *et al*. Detection of Chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. *Sci Transl Med* 2012;4:162ra154.

20 Chan KCA, Jiang P, Zheng YWL, *et al*. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and Tumoral heterogeneity by Massively parallel sequencing. *Clin Chem* 2013;59:211–24.

21 Osborne CM, Hardisty E, Devers P, *et al*. Discordant noninvasive Prenatal testing results in a patient subsequently diagnosed with metastatic disease. *Prenat Diagn* 2013;33:609–11.

22 Bianchi DW, Chudova D, Sehnert AJ, *et al*. Noninvasive Prenatal testing and incidental detection of occult maternal malignancies. *JAMA* 2015;314:162.

23 Church TR, Wandell M, Lofton-Day C, *et al*. Prospective evaluation of Methylated Sept9 in plasma for detection of asymptomatic colorectal cancer. *Gut* 2014;63:317–25.

24 Shen SY, Singhania R, Fehringer G, *et al*. Sensitive tumour detection and classification using plasma cell-free DNA Methylomes. *Nature* 2018;563:579–83.

25 Liu MC, Oxnard GR, Klein EA, *et al*. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Annals of Oncology* 2020;31:745–59.

26 Lennon AM, Buchanan AH, Kinde I, *et al*. Feasibility of blood testing combined with PET-CT to screen for cancer and guide intervention. *Science* 2020;369:eabb9601.

27 Buys SS, Partridge E, Black A, *et al*. Effect of screening on ovarian cancer mortality: the prostate, lung, colorectal and ovarian (PLCO) cancer screening randomized controlled trial. *JAMA* 2011;305:2295–303.

28 Andriole GL, Crawford ED, Grubb RL, *et al*. Prostate cancer screening in the randomized prostate, lung, colorectal, and ovarian cancer screening trial: mortality results after 13 years of follow-up. *JNCI Journal of the National Cancer Institute* 2012;104:125–32.

29 Pinsky PF, Prorok PC, Yu K, *et al*. Extended mortality results for prostate cancer screening in the PLCO trial with median follow-up of 15 years. *Cancer* 2017;123:592–9.

30 Menon U, Gentry-Maharaj A, Burnell M, *et al*. Ovarian cancer population screening and mortality after long-term follow-up in the UK collaborative trial of ovarian cancer screening (UKCTOCS): a randomised controlled trial. *Lancet* 2021;397:2182–93.

31 Dobrow MJ, Hagens V, Chafe R, *et al*. Consolidated principles for screening based on a systematic review and consensus process. *CMAJ* 2018;190:E422–9.

32 Al Zoughbi W, Fox J, Beg S, *et al*. Validation of a circulating tumor DNA-based next-generation sequencing assay in a cohort of patients with solid tumors: A proposed solution for decentralized plasma testing. *Oncologist* 2021;26:e1971–81.

33 Center for Devices, Radiological Health. *List of Cleared or Approved Companion Diagnostic Devices (In Vitro and Imaging Tools)*. U.S. Food and Drug Administration, Available: https://www.fda.gov/medical-devices/in-vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-in-vitro-and-imaging-tools [accessed 5 Apr 2023].

34 Lanman RB, Mortimer SA, Zill OA, *et al*. Analytical and clinical validation of a Digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. *PLoS One* 2015;10:e0140712.

35 Coombes RC, Page K, Salari R, *et al*. Personalized detection of circulating tumor DNA Antedates breast cancer metastatic recurrence. *Clin Cancer Res* 2019;25:4255–63.

36 Sethi H, Salari R, Swenerton R, *et al*. Analytical validation of the Signatera RUO assay, a highly sensitive patient-specific Multiplex PCR NGS-based non-invasive cancer recurrence detection and therapy monitoring assay. Available: https://www.natera.com/wp-content/uploads/2020/11/Oncology-Clinician-Analytical-Validation-of-the-Signatera-RUO-Assay-AACR-2018.pdf [Accessed 17 May 2023].

37 Gale D, Heider K, Ruiz-Valdepenas A, *et al*. Residual ctDNA after treatment predicts early relapse in patients with early-stage non-small cell lung cancer. *Ann Oncol* 2022;33:500–10.

38 Flach S, Howarth K, Hackinger S, *et al*. Liquid biopsy for minimal residual disease detection in head and neck squamous cell carcinoma (LIONESS)-A Personalised circulating tumour DNA analysis in head and neck squamous cell carcinoma. *Br J Cancer* 2022;126:1186–95.

39 Campbell MR. Update on molecular companion diagnostics - a future in personalized medicine beyond Sanger sequencing. *Expert Rev Mol Diagn* 2020;20:637–44.

40 Cohen JD, Douville C, Dudley JC, *et al*. Detection of low-frequency DNA variants by targeted sequencing of the Watson and crick strands. *Nat Biotechnol* 2021;39:1220–7.

41 Bruhm DC, Mathios D, Foda ZH, *et al*. Single molecule genome-wide Mutation profiles of cell-free DNA for non-invasive detection of cancer. *Nat Genet* 2023; 55(8):1301-1310. doi: 10.1038/s41588-023-01446-3.

42 Wan JCM, Stephens D, Luo L, *et al*. Genome-wide mutational signatures in low-coverage whole genome sequencing of cell-free DNA. *Nat Commun* 2022;13:4953:4953.:.

43 Alexandrov LB, Ju YS, Haase K, *et al*. Mutational signatures associated with tobacco smoking in human cancer. *Science* 2016;354:618–22.

44 Leal A, van Grieken NCT, Palsgrove DN, *et al*. White blood cell and cell-free DNA analyses for detection of residual disease in gastric cancer. *Nat Commun* 2020;11:525.

45 Campos-Carrillo A, Weitzel JN, Sahoo P, *et al*. Circulating tumor DNA as an early cancer detection tool. *Pharmacol Ther* 2020;207:107458.

46 Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;16:6–21.

47 Weber M, Hellmann I, Stadler MB, *et al*. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 2007;39:457–66.

48 Baylin SB, Esteller M, Rountree MR. Aberrant patterns of DNA methylation, Chromatin formation and gene expression in cancer. *Hum Mol Genet* 2001;10:687–92.

49 Pfeifer GP. Defining driver DNA methylation changes in human cancer. *Int J Mol Sci* 2018;19:1166.

50 Constantin N, Sina AAI, Korbie D, *et al*. Opportunities for early cancer detection: the rise of ctDNA methylation-based Pan-cancer screening Technologies. *Epigenomes* 2022;6:6.

51 Mio C, Damante G. Challenges in promoter methylation analysis in the new era of Translational oncology: a focus on liquid biopsy. *Biochim Biophys Acta Mol Basis Dis* 2022;1868:166390.

52 Lander ES, Linton LM, Birren B, *et al*. Initial sequencing and analysis of the human genome. *Nature* 2001;409:860–921.

53 US Food & Drug Administration. Epi Procolon test Premarket approval. 2016. Available: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P130001

54 Potter NT, Hurban P, White MN, *et al*. Validation of a real-time PCR-based qualitative assay for the detection of Methylated Sept9 DNA in human plasma. *Clin Chem* 2014;60:1183–91.

55 Jensen SØ, Øgaard N, Ørntoft M-BW, *et al*. Novel DNA methylation biomarkers show high sensitivity and specificity for blood-based detection of colorectal cancer-a clinical biomarker discovery and validation study. *Clin Epigenetics* 2019;11:158.

56 Jensen SØ, Øgaard N, Nielsen HJ, *et al*. Enhanced performance of DNA methylation markers by simultaneous measurement of sense and Antisense DNA strands after cytosine conversion. *Clin Chem* 2020;66:925–33.

57 Barros-Silva D, Marques CJ, Henrique R, *et al*. Profiling DNA methylation based on next-generation sequencing approaches: new insights and clinical applications. *Genes (Basel)* 2018;9:429.

58 Klein EA, Richards D, Cohn A, *et al*. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. *Ann Oncol* 2021;32:1167–77.

59 Shen SY, Burgener JM, Bratman SV, *et al*. Preparation of cfMeDIP-Seq libraries for Methylome profiling of plasma cell-free DNA. *Nat Protoc* 2019;14:2749–80.

60 Wang Y, Douville C, Cohen JD, *et al*. Detection of rare mutations, copy number alterations, and methylation in the same template DNA molecules. *Proc Natl Acad Sci USA* 2023;120.

61 Cristiano S, Leal A, Phallen J, *et al*. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* 2019;570:385–9.

62 Mathios D, Johansen JS, Cristiano S, *et al*. Detection and characterization of lung cancer using cell-free DNA Fragmentomes. *Nat Commun* 2021;12:5060.

63 Foda ZH, Annapragada AV, Boyapati K, *et al*. Detecting liver cancer using cell-free DNA Fragmentomes. *Cancer Discov* 2023;13:616–31.

64 Underhill HR, Kitzman JO, Hellwig S, *et al*. Fragment length of circulating tumor DNA. *PLoS Genet* 2016;12:e1006162.

65 van 't Erve I, Medina JE, Leal A, *et al*. Metastatic colorectal cancer treatment response evaluation by ultra-deep sequencing of cell-free DNA and matched white blood cells. *Clinical Cancer Research* 2023;29:899–909.

66 Snyder MW, Kircher M, Hill AJ, *et al*. Cell-free DNA comprises an in vivo Nucleosome footprint that informs its tissues-of-origin. *Cell* 2016;164:57–68.

67 Mouliere F, Chandrananda D, Piskorz AM, *et al*. Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci Transl Med* 2018;10:eaat4921.

68 Ulz P, Thallinger GG, Auer M, *et al*. Inferring expressed genes by whole-genome sequencing of plasma DNA. *Nat Genet* 2016;48:1273–8.

69 Ulz P, Perakis S, Zhou Q, *et al*. Inference of transcription factor binding from cell-free DNA enables tumor subtype prediction and early detection. *Nat Commun* 2019;10:4666.

70 Jiang P, Sun K, Peng W, *et al*. Plasma DNA end-motif profiling as a Fragmentomic marker in cancer, pregnancy, and transplantation. *Cancer Discov* 2020;10:664–73.

71 Budhraja KK, McDonald BR, Stephens MD, *et al*. Genome-wide analysis of aberrant position and sequence of plasma DNA fragment ends in patients with cancer. *Sci Transl Med* 2023;15:eabm6863.

72 Newman AM, Bratman SV, To J, *et al*. An Ultrasensitive method for Quantitating circulating tumor DNA with broad patient coverage. *Nat Med* 2014;20:548–54.

73 Zviran A, Schulman RC, Shah M, *et al*. Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nat Med* 2020;26:1114–24.

74 Marsico G, Sharma G, Perry M, *et al*. Abstract 3097: Analytical development of the Radartm assay, a highly sensitive and specific assay for the monitoring of minimal residual disease. *Cancer Res* 2020;80(16_Supplement):3097.

75 Mondelo-Macía P, Castro-Santos P, Castillo-García A, *et al*. Circulating free DNA and its emerging role in autoimmune diseases. *J Pers Med* 2021;11:151.

76 Szpechcinski A, Chorostowska-Wynimko J, Struniawski R, *et al*. Cell-free DNA levels in plasma of patients with non-small-cell lung cancer and inflammatory lung disease. *Br J Cancer* 2015;113:476–83.

77 Marchio A, Amougou Atsama M, Béré A, *et al*. Droplet Digital PCR detects high rate of Tp53 R249S Mutants in cell-free DNA of middle African patients with hepatocellular carcinoma. *Clin Exp Med* 2018;18:421–31.

78 Kinugasa H, Hiraoka S, Nouso K, *et al*. Liquid biopsy for patients with IBD-associated Neoplasia. *BMC Cancer* 2020;20:1188.

79 Cohen JD, Li L, Wang Y, *et al*. Detection and localization of surgically Resectable cancers with a multi-Analyte blood test. *Science* 2018;359:926–30.

80 Jamshidi A, Liu MC, Klein EA, *et al*. Evaluation of cell-free DNA approaches for multi-cancer early detection. *Cancer Cell* 2022;40:1537–49.

81 Schrag D, McDonnell CH, Nadauld L, *et al*. 903O A prospective study of A multi-cancer early detection blood test. *Annals of Oncology* 2022;33:S961.

82 Nicholson BD, Oke J, Virdee PS, *et al*. Multi-cancer early detection test in symptomatic patients referred for cancer investigation in England and Wales (SYMPLIFY): a large-scale, observational cohort study. *The Lancet Oncology* 2023;24:733–43.

83 Melton C, Freese P, Bagaria S, *et al*. Optimized early cancer detection from whole-genome sequencing of cell-free DNA. 2019.

84 National Cancer Institute. NCI dictionary of Genetics terms. 2012. Available: https://www.cancer.gov/publications/dictionaries/genetics-dictionary/def/clinical-utility [Accessed 5 Apr 2023].

85 Owens L, Gulati R, Etzioni R. Stage shift as an Endpoint in cancer screening trials: implications for evaluating Multicancer early detection tests. *Cancer Epidemiol Biomarkers Prev* 2022;31:1298–304.

86 Katalinic A, Eisemann N, Kraywinkel K, *et al*. Breast cancer incidence and mortality before and after implementation of the German Mammography screening program. *Int J Cancer* 2020;147:709–18.

87 Park S, Oh C-M, Cho H, *et al*. Association between screening and the thyroid cancer "epidemic" in South Korea: evidence from a nationwide study. *BMJ* 2016;355:i5745.

88 Ahn HS, Kim HJ, Welch HG. Korea's thyroid-cancer "Epidemic"--Screening and Overdiagnosis. *N Engl J Med* 2014;371:1765–7.

89 Carr D, Kent DM, Welch HG. All-cause mortality as the primary Endpoint for the GRAIL/national health service England multi-cancer screening trial. *J Med Screen* 2022;29:3–6.

90 Pinsky PF, Miller EA, Zhu CS, *et al*. Overall mortality in men and women in the randomized prostate, lung, colorectal, and ovarian cancer screening trial. *J Med Screen* 2019;26:127–34.

91 Bredno J, Lipson J, Venn O, *et al*. Clinical correlates of circulating cell-free DNA tumor fraction. *PLoS One* 2021;16:e0256436.