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# Liquid Biopsy by Next-Generation Sequencing: a Multimodality Test for Management of Cancer

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## Abstract

**Purpose of Review** While liquid biopsy is still relatively a new concept, the advent of next-generation sequencing (NGS) technologies has recently generated a revolution in the field and will be the focus of this review.

**Recent Findings** Circulating tumor DNA (ctDNA) derives from tumor cells and provides information about the genetic alterations of tumors. However, ctDNA concentration in plasma can be below the level of detection by conventional methods; therefore, screening for actionable genetic information is challenging. Clinical trials exploring targeted and untargeted sequencing to improve the outcomes of ctDNA detection are showing promising results, having reached a limit of detection as low as 0.001% of ctDNA in a background of normal circulating DNA.

**Summary** Most of the challenges related to the sensitivity of detection of ctDNA have been defeated by dint of NGS-based approaches. Despite all the efforts, these methods are still expensive, time-consuming, and require advanced skills for appropriate interpretation. Nevertheless, the technology is rapidly improving, and the expectations for the implementation of liquid biopsy into the clinical practice in the near future are high.

**Keywords** Liquid biopsy · Circulating tumor DNA · Next-generation sequencing · Targeted sequencing · Untargeted sequencing

## Introduction

Mandel and Metais were the first to report in 1948 the detection of cell-free DNA (cfDNA) circulating in the blood of cancer free individuals [1]. This finding triggered a series of subsequent studies aimed at understanding the mechanisms behind the generation of cfDNA. Studies conducted between 1968 and 1973 on blood and synovial fluid samples suggested that DNA released from damaged cells in body fluids contributed to higher levels of nucleic acid in individuals with pathologic rheumatologic conditions [2–5]. In 1976, Leon et al. showed significantly higher concentrations of free DNA in the blood of patients with cancer compared to normal controls [6]. The origin of circulating DNA remained enigmatic until 1989, when Stroum et al. showed that circulating DNA in cancer patients originates from tumor cells due to decreased stability [7]. In 1994, point mutations in the *NRAS* gene were first detected in DNA obtained from the plasma of patients with myelodysplastic syndrome and acute myeloid leukemia [8]. Additional advances were reported in the ensuing two decades [9, 10]. Thierry et al. provided one of the earliest clinical validation studies using circulating tumor DNA (ctDNA) to detect *BRAF* V600E and *KRAS* mutations in patients with metastatic colorectal cancer with 100% and 92% analytic sensitivity and specificity, respectively, by comparison to results from biopsy samples [11]. As such, there has been a steady increase in the recognition of the value of liquid biopsy (LB) approaches for the diagnosis and surveillance of cancer (Fig. 1).

Malignant neoplasms and their genetic profile are known to evolve continuously and acquire new mutations and treatment resistance leading to variability, particularly between the

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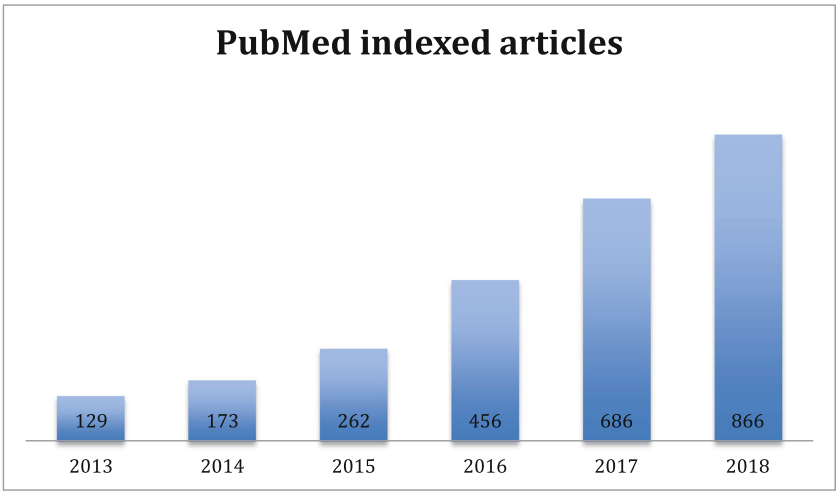


**Fig. 1** Timeline of liquid biopsy–related publications in the previous six years

primary tumor site and metastatic sites. Moreover, the stochastic nature of clonal evolution results in intra-tumoral heterogeneity and engenders "molecular sampling limitations" in conventional surgical biopsy (SB) sampling [12–14]. Furthermore, SB is an invasive procedure associated with multiple complications particularly for patients on anti-angiogenic medication. Finally, the risk of "cancer cells seeding" has been well-documented as a potential complication of SB [15].

In view of inherent barriers of traditional biopsies, the potential of "liquid biopsy" has continued to be an attractive venue for cancer biomarker testing. Furthermore, the advent of targeted therapy and immunotherapy was the major reason behind the urge of improving molecular biomarker testing in general and LB in particular, especially in the current era of high-throughput clinical tests [16, 17]. Currently, the Food and Drug Administration (FDA) has approved at least 86 drugs specifically targeting biomarker genes for more than 46 liquid and solid malignancies [18, 19•]. As of date, the FDA has approved 51 genetic tests, covering at least 561 genes related to therapeutic, prognostic purposes, as well as tumor profiling. However, the clinical applications of genetic biomarkers in LB are not only limited to therapeutic purposes but also extend to multiple other practices. These applications include the following: (1) cancer screening and early detection, particularly because the amount of cfDNA has been shown to be higher in patients with malignancy compared to healthy individuals [20, 21]; (2) investigations of cancers of unknown primary [22]; (3) risk-stratification and tumor staging [23••]; (4) tumor genotyping and assessment of clonal evolution; (5) therapy selection [24]; (6) monitoring of treatment response [25, 26]; (7) detection of emergence of treatment resistance [27, 28]; and (8) detection of minimal residual disease [29].

In this review, we focus on the state of the art in the field of LB and highlight ongoing efforts to incorporate this class of tests in the management of cancer patients. Our discussion will be concentrated on next-generation sequencing (NGS) technologies, including targeted sequencing as well as whole-exome and whole-genome sequencing, as new and robust tool for the evaluation of circulating tumor DNA.

## Biological Considerations

Liquid biopsy is based on detection of circulating tumor DNA (ctDNA), circulating tumor cells (CTC), circulating free RNA (cfRNA), and circulating extracellular vesicles (EVs) in plasma [18, 30–34]. The focus of this review will be on DNA-based assays utilizing cfDNA.

Multiple previous studies reported different results with respect to the levels of mutant cfDNA in the circulation of cancer patients, varying from 0.01 to 90% [35]; these discrepancies might be related to the testing method, the disease, as well as the characteristics of cfDNA including mechanisms of secretion, half-life, and elimination processes. The type and location of the primary neoplastic mass are crucial in determining the level of cfDNA in cancer patients [36]. Tumors presently known to be associated with high levels of cfDNA include gastrointestinal tract, breast, pancreas, liver, and cutaneous cancers. On the other hand, central nervous system, kidney, prostate, and thyroid tumors are associated with lower amounts of cfDNA in the circulation even in advanced stages of disease [23••, 37]. Bettegowda et al. related these differences to the presence of a capsule and/or a blood-brain barrier surrounding the latter organs. Furthermore, in 2012, Heitzer et al. attempted to quantify the cfDNA in the plasma of patients with colorectal cancer; mutant DNA fragments were not detected even when using ultrasensitive platforms in advanced metastatic cases; here, the authors attributed these findings to the short life of circulating DNA [38]. In contrast to these results, another study published in 2017 by Schmiegel et al.

showed a concordance rate of 92% for *KRAS* mutation detection in plasma versus solid tissue for stage IV colorectal cancer patients using BEAMing technique [39]. Along with the controversial results in the literature, it is essential to remember that liquid biopsy is a reflection of a more thorough genetic profile of the malignant lesion as opposed to tissue biopsy which is subject to tumor heterogeneity; hence, discordant results between the two methods are not surprising.

## Regulations for Liquid Biopsy Applications

cfDNA testing was approved by the FDA in 2016 for *EGFR* mutations (deletion of exon 19 and L858R substitution in exon 21) in patients with non-small cell lung cancer (NSCLC). The detection of these mutations may help in identifying patients eligible for Erlotinib treatment, which was approved in 2013 as a first-line treatment for NSCLC with activating *EGFR* mutations. However, in their algorithm, the FDA recommended a conventional tumor biopsy when the targeted DNA alteration is not detected in the blood [40•]. The approved test (cobas *EGFR* Mutation Test v2) is a real-time PCR assay, which can also detect the T790M *EGFR* mutation that leads to therapy resistance in patients undergoing treatment with Erlotinib; in the latter cases, the treatment should be switched to another approved EGFR tyrosine kinase inhibitor, osimertinib, a third-generation inhibitor. Another cfDNA-based test was approved by the FDA in April 2016, the Epi proColon (Epigenomics AG) for the methylation status of the *SEPT9* promoter for colorectal cancer screening [41]. In June 2016, the US Preventive Services Task Force conducted a study to update the recommendation for colorectal cancer screening and included the SEPT9 DNA among the studied testing methods. Review of supporting literature for SEPT9 was limited, and the test was found to have a sensitivity < 50% for screening [42]; hence, it was not included in the updated screening strategy recommended by the USPSTF [43]. Recently, a large study was performed to evaluate SEPT9 in colorectal cancer in 558 patients to confirm a sensitivity of 61% and specificity of 93% for diagnosing CRC and 100% sensitivity for the prediction of post-surgery recurrence in case of persistence of high levels of SEPT9 [44]. The current regulatory landscape of liquid biopsy testing remains in development as proficiency testing methods, and structured checklists become available to the laboratories offering these assays.

## Technical Aspects for Liquid Biopsy

Levels of cfDNA can be increased in cancer [7] as well as in a vast variety of physiologic [45] and non-cancerous pathologic conditions including pregnancy, inflammatory disease, and sepsis [46, 47]. Furthermore, ctDNA concentration in body fluids can be below the amounts of detection by conventional methods, e.g., as low as 0.01% [48–50] of the total cfDNA in early stages of disease and as high as 99% in advanced metastatic diseases [23••]. Moreover, ctDNA is highly fragmented and can be as small as 85 base pairs [38]; Thierry et al. studied the correlation between the size of ctDNA and the tumor stage and proved that the two variants vary in opposite ways resulting in very small fragments of DNA in advanced cases [51]. For all these reasons, detection of altered DNA sequences among extensive amounts of wild-type sequences is challenging and requires highly sensitive analytical methods to achieve high sensitivity and accuracy of detection. (Table 1). There are numerous different NGS platforms based on distinct sequencing technologies; however, they are all based on the same principle of sequencing rare and minute segments of DNA repeatedly to optimize the depth and consequently provide an accurate DNA mapping. Sequencing does not target specific mutations, but rather, it studies all the possible genetic alterations; hence, it provides the opportunity to uncover new mutations for potential targeted therapy and the possibility to monitor the treatment response for tumors without known associated genetics alterations. Moreover, cancer is a multigene disease that involves multiple oncogenes and tumor suppressors; thus, a thorough insight into the whole genome alterations through sequencing would provide a better understanding of the pathogenesis of diseases.

Two groups of deep sequencing techniques exist, the *untargeted* techniques including whole genome, whole exome, and whole methylome sequencing, as well as FAST-seq and the *targeted* techniques for single or multiple gene mapping (Table 2). The sensitivity and specificity of gene mapping vary in opposite to the panel size, in other words, whole genome and whole-exome sequencing, uncover new genetic findings but have a limit of detection of 5% minor allele frequency (MAF); hence, they provide interesting data for research practice, while smaller cancer panels can detect mutations down to 0.001% MAF with lower costs and less time rendering the later more relevant for clinical practice [68]. On the other hand, every cancerous lesion harbors multiple neoplastic clones that define the heterogeneity of malignant tumors; each clone is characterized by different size, genetic assortment, and potentials for DNA secretion leading to different percentages of the circulating genetic alterations in the plasma. From this perspective, untargeted approaches will divulge higher percentage of total ctDNA, since it maps the entire tumor genome; however, targeted sequencing detects predefined genetic aberrations that are not always actively secreted in the plasma, engendering a higher risk of false negative. Moreover, whole-exome and whole-genome sequencing (WES and WGS) show evidence of novel genetic mutations in the plasma not identified in the tumor biopsy; these discrepancies are related to the presence of metastatic clones

**Table 1** List of available methods for ctDNA analysis

| Method | Technology |
|---|---|
| Conventional PCR | Mass spectrometry |
| | SCODA |
| | ND-NaME-PrO |
| | cSMART |
| Digital PCR | Beaming |
| | ddPCR |
| | COLD-PCR |
| | Microfluidic digital-PCR |
| Real-time PCR | ARMS-Scorpions PCR |
| | Clamping PCR |
| | MS-PCR |
| | AS-PCR |
| | AS-NEPB-PCR |
| | PNA-LNA |
| | Ampli-Seq |
| Targeted sequencing | Safe- SeqS |
| | CAPP-Seq |
| | iDES- CAPP-Seq |
| | E-TAmSeq |
| | MCTA-Seq |
| | Bias-Corrected Targeted NGS |
| Untargeted sequencing | WES |
| | WGS |
| | FAST-SeqS |
| | Whole methylome sequencing |

*ARMS* amplification refractory mutation system, *BEAMing* beads, emulsion, amplification, magnetics, *CAPP-Seq* cancer personalized profiling by deep sequencing, *ddPCR* droplet digital PCR, *SAFE-SeqS* safe-sequencing system, *TAm-Seq* tagged-amplicon deep sequencing, *SCODA* sequence-specific synchronous coefficient of drag alteration, *ND-NaME-PrO* nuclease-assisted minor-allele enrichment using overlapping probes, *cSMART* circulating single molecule amplification and re-sequencing, *ddPCR* droplet-digital PCR, *COLD-PCR* coamplification at lower denaturation temperature PCR, *PARE* personalized analysis of rearranged ends, *MS-PCR* methylation specific, allele-specific-PCR (AS-PCR), *AS-NEPB-PCR* allele-specific non-extendable primer blocker, *PNA-LNA* peptide nucleic acid–locked nucleic acid clamp PCR, *CAPP-Seq* cancer personalized profiling by deep, *iDES-enhanced* integrated digital error suppression, *TAmSeq* tagged amplicon deep sequencing, *E-TAmSeq* enhanced tagged amplicon deep sequencing, *MCTA-seq* methylated CpG tandems amplification and sequencing, *WGS* whole-genome sequencing, *WES* whole-exome sequencing

and provide actionable information for therapeutic and prognostic purposes. For instance, *ESR1* mutation which can induce resistance to aromatase inhibition can be initially absent in the primary tumor but present in cfDNA of patients with metastatic breast cancer [69••]. These findings highlight the value of WES and WGS of cfDNA in metastatic disease for the identification of new targetable genetic alterations. Briefly, the choice of the category and the platform depend on the clinical application of the testing, the expected fraction of ctDNA in the body fluid, and previous information about the tumor genome. Targeted techniques particularly targeted sequencing platforms can identify DNA alterations as low as 0.01%, and as such, they are ideal for cancer screening, early detection, and disease monitoring [23••]. On the other hand, untargeted sequencing approaches permit mutation profiling and insight into the genomic alterations in an agnostic manner that permits baseline evaluation and follow-up detection of clonal evolution and ensuing treatment resistance.

Overall, the lower limit of detection for the different sequencing techniques are as follows: 20% for Sanger sequencing, 5 to 10% for pyrosequencing [68], and 1 to 5% for next-generation sequencing [70]; however, practically, the lower limit of detection is related to the clinical application and should be as low as 0.01% for minimal residual disease monitoring [23••]. To fulfill the right objectives of liquid biopsy, researchers have developed multiple new strategies to enrich tumor DNA when the available altered DNA fragments are present in minute amounts to reach higher sensitivity levels with lower variant allele frequency (VAF).

## Untargeted Sequencing Platforms

Somatic copy number alterations (SCNAs) are widely present in all human cancers. Since their identification requires low sequencing depth, multiple researchers have investigated untargeted sequencing data for the detection of these aberrations. Heitzer et al. performed low-coverage WGS on plasma samples from patients with prostate cancer to identify ploidy status and copy number profile [63]. They demonstrated that the reduction of the sequencing depth to an average of three million reads with 0.1× whole-genome sequencing did not adversely affect the sensitivity or the specificity of the method. SCNAs from specimen harboring at least 10% tumor DNA were identified with a sensitivity of > 80% and specificity of 80%; aneuploidy was detected with ctDNA concentration as low as 1%. The most attractive feature of using shallow depth of sequencing was the tremendous decrease of the procedure time, reported to be limited to 12 h, as compared to two days for regular deep sequencing [69••]. Interestingly, another group performed an integrated analysis of whole-genome sequencing by pairing NGS results with personalized analysis of rearranged ends (PARE) and digital karyotyping (DK); the objective of combining these approaches is to perform a comprehensive analysis of chromosomal copy number alterations as well as rearrangements in the plasma of advanced colorectal and breast cancer patients. First, at levels of 0.75% ctDNA, the combined approaches methodology achieved a sensitivity of > 90% and a specificity of > 99% for the detection of tumor; second, the group confirmed that the identification of SCNA required less sequencing than chromosomal rearrangements at the same levels of ctDNA [64]. Unfortunately, SCNA are

**Table 2** Comparison of technological sequencing platforms used for circulating tumor DNA analysis

| Method | Technology | Approach | Type of alteration detected | Minimum allele frequency | Sensitivity | Refs |
|---|---|---|---|---|---|---|
| Targeted sequencing | Tam-seq | Amplicon based sequencing | SNVs, indels | 2% | 97% | [52] |
| | eTAm-Seq | Amplicon based sequencing | SNVs, indels | 0.13% | 100% | [53, 54] |
| | SAFE-seq | Amplicon based sequencing | SNVs, indels | 0.1% | | [55, 56] |
| | CAPP-seq | Hybrid capture | SNVs, indels, CNVs, SVs | 0.01% | 100% | [57] |
| | iDES-CAPP-seq | Hybrid capture | SNVs, indels, CNVs, SVs | 0.004% | 100% | [58] |
| | Digital sequencing | Hybrid capture | | 0.1% | 85% | [59] |
| | MCTA-Seq | | | 0.25% | 94% | [60] |
| | Bias-corrected targeted sequencing | | Point mutations, CNVs, rearrangements | 0.4% | | [50] |
| Untargeted sequencing | Whole-exome sequencing | | SNVs, indels, CNVs, SVs | 5% | | [61, 62] |
| | Whole-genome sequencing | | SNVs, indels, CNVs, SVs | 1–10% | 80% | [63] |
| | ULP-WGS | PARE, DK, WES | (SCNAs) | 0.75% | 90% | [64] |
| | Whole methylome | BS-sequencing | | | 43–81% | [65] |
| | Digital karyotyping | | CNVs | 0.001–0.01 | 95% | [66] |
| | FAST-seq | | Genome-wide CNVs | > 10% | Low sensitivity | [67] |

*TAm-Seq* tagged-amplicon deep sequencing, *eTAm-Seq* enhanced tagged amplicon sequencing, *CAPP-Seq* cancer personalized profiling by deep sequencing, *iDES-CAPP* integrated digital error suppression cancer personalized profiling by deep sequencing, *Safe-Seq* safe-sequencing system, *DS* digital sequencing, *MCTA-Seq* methylated CpG tandems amplification and sequencing, *ULP-WGS* ultralow-pass whole-genome sequencing, *PARE* personalized analysis of rearranged ends, *DK* digital karyotyping, *BS* bisulfite-sequencing, *SCNAs* somatic copy number alterations, *SNVs* single nucleotide variant, *indels* insertion/deletion, *CNVs* copy number variant, *SVs* structural variant

common findings of tumors and are observed in nearly all cancer genomes; therefore, although WES of these targets provides fast and sensitive results at lower depth of coverage, it cannot provide information about the origin of the tumor; accordingly, WES for SCNAs should not be incorporated as a screening tool for cancer. Adalteinsson et al. applied SCNAs to develop an ultralow-pass whole-genome sequencing (ULP-WGS) analytical approach named ichorCNA for estimating the percentage of tumor in cfDNA. This approach can be used to investigate the presence or absence of ctDNA and to guide the decision to perform whole exome versus whole-genome sequencing. Furthermore, the quantitative estimate of tumor fraction by ULP-WGS can be used to calibrate the depth of sequencing to reach statistical power for identifying mutations in cell-free DNA. This technique yielded appropriate results to guide the calibration of the sequencing depth in metastatic diseases and consequently provided the opportunity to perform shallow sequencing (0.1× coverage) instead of deep sequencing (high coverage) in qualified cases [71].

## Targeted Sequencing

Currently, at least nine platforms for targeted sequencing and multiple genetic biomarkers exist and are available for research and clinical practice. Most target enrichment methods for amplification of low-frequency allele that were developed before the era of NGS utilized the hybridization capture (HC) method; HC consists of sending target specific probes to capture randomly sheared exons followed by PCR amplification of the captured sequences [72]. Nevertheless, PCR amplification artifacts can be the source of potential error particularly when the target is a low-frequency DNA variant typically seen in ctDNA. Hence, in an attempt to eliminate these biases, unique identifier barcodes (UIB) have been developed; different barcodes are used to mark a specific target DNA sequence; as a result, every group of DNA reads coupled with the same identifier originate from the same DNA sequence, resulting in reduction of errors and improvement of the sequencing accuracy [73•].

Next-generation sequencing was first introduced in the liquid biopsy field in 2012 by Forshew et al. who developed the Tagged-Amplicon Deep Sequencing (TAm-Seq) technology. The latter is characterized by the usage of short amplicons, two-step amplification, and barcodes to efficaciously amplify small amounts of fragmented cfDNA. Compared to digital PCR, Tam-Seq showed sensitivity and specificity of 97% for the detection of single nucleotide variants (SNVs) and indels for an allele frequency of at least 2% in plasma of patients with advanced ovarian cancer [52]. Five years later, the same team designed a more sophisticated and sensitive version of the same technology that can detect gene fusions. This assay is the enhanced tagged amplicon sequencing (eTAm-Seq) that was first developed to study 36 genes associated with non-small cell lung cancer (NSCLC). The assay yielded excellent results, the limit of detection of ctDNA was 0.25% of VAF for SNVs and indels with > 99% and 92% sensitivity respectively,

and as for the ALK and ROS1 gene fusions, eTAm-Seq had a sensitivity up to 100% for VAF of 0.13% [53, 54].

EGFR mutation testing in plasma was granted FDA approval for non-small cell lung cancer treatment based on numerous studies of the different PCR platforms [74]. Allele-specific arrayed primer extension (AS-APEX) showed the highest concordance rate of 97% between plasma and tumor tissue for EGFR alterations detection [75]. NGS studies yielded low sensitivity results (50%) for the detection of T790M mutation of *EGFR* in plasma of patients who experienced failure of treatment with TKI. Moreover, several resistance mechanisms to TKI other than T790M exist including amplification of *NRAS* and *MET* as well as *PIK3CA* E545K mutation, these alterations were identified in tumor samples of patients in the same study, but not in the plasma samples [76–78]. Hence, the latter study showed a limited role for NGS in treatment follow-up of patients with lung cancer [79]. On the other hand, cancer personalized profiling by deep sequencing (CAPP-Seq) is an ultrasensitive targeted sequencing platform that was introduced by Newman et al. in 2014 for cases of NSCLC. In CAPP-Seq, recurrently known mutated regions in a particular cancer are selected and targeted with biotinylated probes; this step is followed by a probe-based hybridization capture to identify the tumor DNA and reduce the background noise from normal cfDNA. The method detected *EGFR* and *KRAS* in 100% of patients with advanced stage NSCLC for a MAF > 0.1% and 0.01% with corresponding specificities of 99% and 96% respectively [57]. Two years later, the same group developed integrated digital error suppression (iDES) which is an approach to remove highly stereotypical background artifacts of sequencing; this method was combined to CAPP-seq and yielded a sensitivity of detection of *EGFR* mutations of 0.004% MAF without compromising the specificity [58]. iDES-CAPP-seq is today the most sensitive targeted sequencing method for the detection of single nucleotide variant (SNVs), insertion/deletion (indels), copy number variant (CNVs), and structural variant (SVs).

Safe-sequencing system (Safe-Seq), developed by Kinde et al. in 2011, is another ultra-sensitive method of MPS. In this technique, randomly sheared DNA fragments will bind to particular UIDs before running PCR amplification; this step is followed by a solid phase during which probes specific to the DNA of interest will retain the targeted DNA sequences and remove the undesired fragments; finally, another PCR amplification will follow to create the so-called UID families before running the regular DNA sequencing. The resulted sequences will be named supermutants which consist of UID families in which ≥ 95% of the sequences harbor exactly the similar mutation [80]. Safe-seq has been applied for treatment follow-up of gastrointestinal tumors with high sensitivity of detection of ctDNA with mutant allele concentration as low as 0.1% [55, 56].

Digital sequencing (DS) is another targeted sequencing platform that makes usage of the unique molecular indices for the detection of low-frequency alleles. A large study including 510 patients with stage III and stage IV solid tumors was conducted by Lanman et al. The group studied 54 clinically actionable genes using the DS technique; they reported an analytical sensitivity of 85% for MAF as low as 0.1% [59].

Epigenetics markers particularly DNA methylation are also implicated in tumorigenesis and are the hallmarks for many cancer types; *TET1*, *TET2*, *TET3*, and TDG antimethylation proteins are associated with leukemias as well as other solid tumors, and hypomethylation of LINE-1 retrotransposons was described in lung tumors [81, 82]. cfDNA has been reported to harbor not only genetic markers but also epigenetic alterations. Methylated CpG tandems amplification and sequencing (MCTA-Seq) platform is based on the nested PCR principle for the detection of hypermethylated CpG islands. The technique begins with a bisulfite conversion step which converts unmethylated cytosines to uracils, followed by semi-specific amplification, then selective amplification of the methylated CpG islands sites, and finally sequencing to detect the methylated DNA sequences. Wen et al. used MCTA-Seq on cfDNA to identify four genes as markers of Hepatocellular carcinoma including RGS10, STSIA6, *RUNX2*, and *VIM*. The sensitivity of detection was 94% and the specificity 89% for a MAF of 0.25% in the plasma [60]. However, the initiating step of Bisulfite treatment on which this method is based is known to affect the quality of DNA; hence, it may have repercussion on the accuracy of the resulting genetic information leading to false negative and false positive outcomes [83].

Tumor mutational burden (TMB) is the measurement of the total number of nonsynonymous mutations per coding area of a tumor genome; it is conventionally determined using WES. Due to the high costs, complexity of data analysis, and long turnaround time of untargeted sequencing, targeted techniques are being evaluated as an alternative for the calculation of TMB [84]. Multiple large-scale NGS clinical trials and assays have been conducted so far including "MOSCATO 01/MATCHR" for WES, "NEO liquid assay," "Guardant Health Guardant 360," "CAPP-Seq," and "Foundation Medicine bTMB" to validate the calculation of TMB on blood samples [85]. Most of the projects involve cases of NSCLC patients who might benefit from immunotherapy [86]. The two major projects conducted at Gustave-Roussy are MOSCATO 01/MATCHR which showed a concordance rate of 92% between WES and targeted sequencing on plasma samples; however, in the same study, the concordance of ctWES and tWES was only 53% [87]. To improve these outcomes, Chaudhuri et al. applied CAPP-Seq to genotype the plasma of NSCLC patients, they formulated an equation to compare the CAPP-seq mutation burden to whole-exome mutation burden results and reported a significant positive correlation ($r = 0.93$); however, this method was validated on only five cases of NSCLC included in the study [88]. The largest projects conducted are phase II and phase III POLAR/OAK which were performed on 794

NSCLC patients; they were designed to compare the concordance levels between blood tumor mutational burden (bTMB) and tumor mutational burden (tTMB) as well as to assess the efficiency of bTMB assay on the survival after treatment with either chemotherapy or immunotherapy. The study yielded significant improvement of the survival of the patients receiving immunotherapy versus chemotherapy independently of the TMB levels. The comparison between targeted sequencing of bTMB and tTMB on 279 patients examining a panel of 394 genes with a ctDNA of 1% and VAF as low as 0.5% showed an agreement of 81%; yet the concordance rate dropped to 17% when testing included only a panel of 62 genes [89]. This finding highlights the great impact of selecting the appropriate gene panel for adequate TMB results in targeted sequencing to generate accurate results; Chalmers et al. confirmed the importance of sequencing at least 1 Mb of the genome, as well as selecting the appropriate genomic area to be included in the designed panel for successful results [86]. Based on the recent successful results yielded by the POLAR/OAK projects for TMB calculation, Foundation Medicine did a step forward and requested FDA approval to incorporate Foundation ACT (FACT) liquid biopsy for the TMB measurement.

The enthusiastic solicitation to conduct liquid biopsy projects in the last decade focused on cases of advanced stages of cancer; however, survival rates rely significantly on screening efficacy and timing of diagnosis; hence, the recent liquid biopsy trials are shifting to accommodate the urgent need for cancer screening and early diagnosis. Cohen et al. designed CancerSEEK a cost-effective sequencing platform that targets 16 genes with complementary testing of eight protein biomarkers to identify the eight most common types of neoplasms. This diagnosis tool was studied on 1005 symptomatic patients with non-metastatic stage I to III disease; the highest sensitivity of detection (98%) was observed with ovarian and liver neoplasms, and the overall median detection rate of stage I cancer was as low as 43%. The localization of the tissue of origin was accurate in 63% of the patients; it was concluded based on a statistical algorithm obtained from the protein biomarkers results combined with the demographics of the patients [90]. In this study, the recruited patients manifested symptoms related to their disease; hence, the assay is a diagnostic but not a screening tool. On the other hand, the Circulating Cell-free Genome Atlas Study (CCGA) study for breast cancer cases included symptomatic and asymptomatic patients with stage I to IV diseases. Whole-genome bisulfite sequencing (WGBS) used by this group yielded impressive results for high-stage cases; however, the detection rate of asymptomatic disease was limited to 10% [91]. The attempts to use cfDNA to serve as a screening tool remain futile and inapplicable in clinical practice, a successful implementation of liquid biopsy in early detection of cancer will need more appropriately outlined clinical trials.

## Conclusion

Liquid biopsy has overturned the world of precision medicine particularly with the advent of next-generation sequencing. New methods and platforms have emerged yielding impressive results that opened the horizon for new applications of cfDNA testing. Most of the challenges related to the sensitivity of detection have been defeated in the last few years by dint of NGS-based approaches. Despite all the efforts, these methods are still expensive, time-consuming, and require advanced skills and training for appropriate interpretation. Nevertheless, the technology is rapidly and continuously improving, and the expectations for the implementation of liquid biopsy into the clinical practice in the near future are high.

### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance


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