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LEADING ARTICLE

Monitoring Melanoma Using Circulating Free DNA

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Abstract
Genetic material derived from tumours is constantly shed into the circulation of cancer patients both in the form of circulating free nucleic acids and within circulating cells or extracellular vesicles. Monitoring cancer-specific genomic alterations, particularly mutant allele frequencies, in circulating nucleic acids allows for a non-invasive liquid biopsy for detecting residual disease and response to therapy. The advent of molecular targeted treatments and immunotherapies with increasing effectiveness requires corresponding effective molecular biology methods for the detection of biomarkers such as circulating nucleic acid to monitor and ultimately personalise therapy. The use of polymerase chain reaction (PCR)-based methods, such as droplet digital PCR, allows for a very sensitive analysis of circulating tumour DNA, but typically only a limited number of gene mutations can be detected in parallel. In contrast, next-generation sequencing allows for parallel analysis of multiple mutations in many genes. The development of targeted next-generation sequencing cancer gene panels optimised for the detection of circulating free DNA now provides both the flexibility of multiple mutation analysis coupled with a sensitivity that approaches or even matches droplet digital PCR. In this review, we discuss the advantages and disadvantages of these current molecular technologies in conjunction with how this field is evolving in the context of melanoma diagnosis, prognosis, and monitoring of response to therapy.

Key Points

Circulating free DNA can provide non-invasive, real-time information about a patient's tumour burden and subsequent response to therapy.

Development of sensitive, targeted, next-generation sequencing technologies that detect tumour mutations in circulating free DNA is revolutionising the monitoring of cancer patients.

1 Introduction

Liquid biopsy involves the extraction, detection, and analysis of DNA, RNA, proteins, vesicles, or cells derived from biofluids such as blood, urine, saliva, pleural effusions, and cerebrospinal fluid (CSF). The development of liquid biopsies for genomic profiling of solid tumours as a means of detecting actionable mutations, monitoring cancer progression/evolution, and predicting response to therapy in a non-invasive manner is a rapidly growing field (reviewed in [1–6]).

A major biomarker of tumour-related genetic changes is circulating free DNA (cfDNA), specifically, the tumour-derived circulating tumour DNA (ctDNA) fraction (reviewed in [7–11]). cfDNA is highly fragmented DNA with a size distribution of ~ 130–170 bp [12–14], which is equivalent to the size of nuclease-cleaved nucleosomes, and may arise from multiple mechanisms, including apoptosis, necrosis, and active secretion (reviewed in [15–17]). cfDNA can be found in many biofluids, including blood, urine, CSF, saliva, and stool. Levels of ctDNA, which often increase with tumour volume, can predict response to targeted and immunotherapies, can be used to monitor residual disease

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and tumour heterogeneity, and can reveal expanding therapy-resistant tumour clones (reviewed in [18–21]).

One major challenge associated with the clinical application of ctDNA arises from the variability in patient ctDNA levels that is associated with cancer stage, tumour burden and location, response to therapy, vascularity and cellular turnover. Thus, levels of ctDNA relative to the total cfDNA pool of an individual can vary from < 0.01 to > 50% [22]. Coupled with the fact that cfDNA is not abundant and has a short half-life of only a few hours, the reliable and sensitive detection of ctDNA remains challenging, especially in patients with early-stage cancer (reviewed in [23]). For blood-derived cfDNA, a number of studies have sought to optimise the yield and stability of cfDNA by comparing a range of collection tubes and other factors during blood collection [24–30] and a range of commercial cfDNA purification kits [31–37].

The methodology used to detect and analyse the genomic alterations in cfDNA, along with the strengths and weaknesses of these various approaches are the subject of a number of recent reviews [4, 10, 20, 38–40]. Cancer-associated alterations in cfDNA include single nucleotide variations (SNVs), rearrangement of genomic sequences, copy number variations (CNVs), microsatellite instability, loss of heterozygosity and DNA methylation (reviewed in [11]). The most common methods used to detect cfDNA can be classified into standard polymerase chain reaction (PCR)-based, digital PCR, whole-exome or targeted deep sequencing. The primary focus of this review is the application of digital PCR and targeted next-generation sequencing (NGS) gene panels to identify and monitor tumour-associated genetic changes in cfDNA isolated from cancer patients with an emphasis on cutaneous melanoma (Fig. 1). Other sources of circulating nucleic acids that have the potential to complement detection of cfDNA are also highlighted.

2 Biomarkers and Genetics of Melanoma

The high tumour mutation burden (TMB; i.e. the number of somatic mutations found in the genome of a single tumour) of cutaneous melanoma has been well documented, with the most significant driver mutations in *BRAF, NRAS*, *NF1*, and *KIT* genes [41–50]. Prior to 2010, the typical 1-year survival for stage IV melanoma patients was only 25% [51]. With the introduction of tyrosine kinase inhibitors targeting the mitogen-activated protein kinase (MAPK) pathway in patients with $BRAF^{V600}$-mutated melanoma, and antibodies against immune checkpoints such as programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), the 1-year survival for patients receiving combination dabrafenib and trametinib, combination encorafenib and binimetinib, combination vemurafenib and cobimetinib, single-agent PD-1 antibodies, and a combination of PD-1 and CTLA-4 antibodies has improved to 72%, 75%, 75%, 73%, and 73%, respectively [52–56]. However,

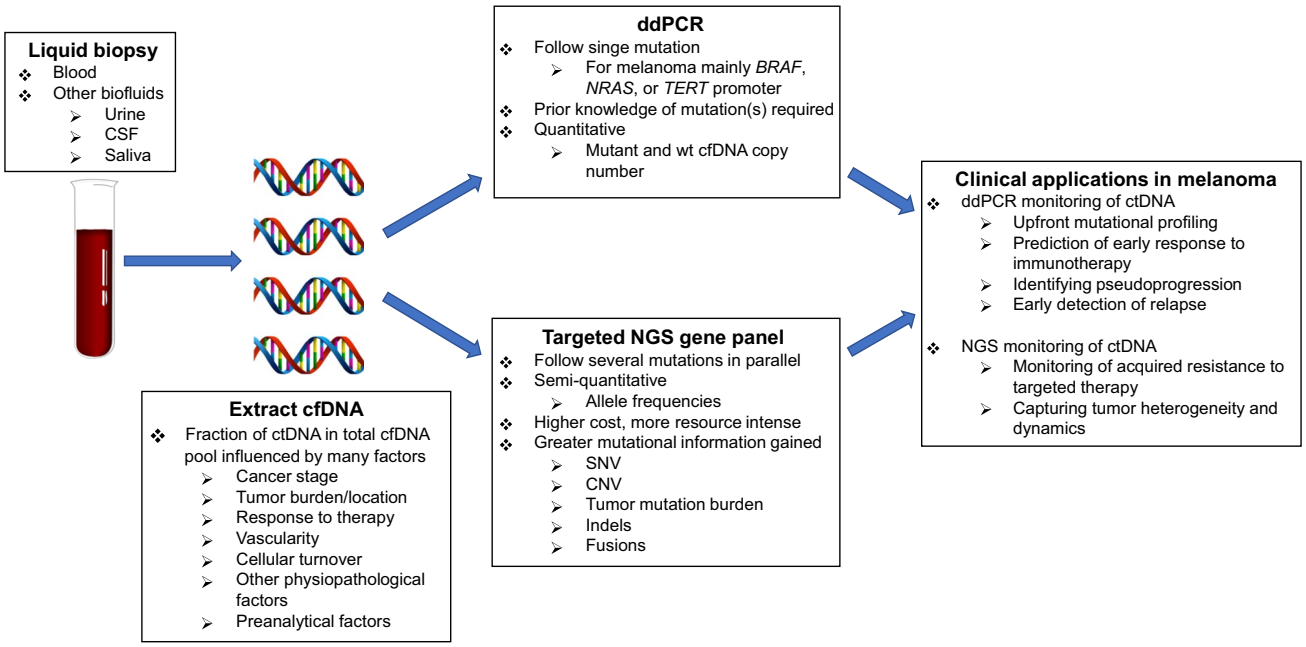

Fig. 1 Comparison of ddPCR and NGS platforms for the monitoring of ctDNA in melanoma. *cfDNA* circulating free DNA, *CNV* copy number variation, *ctDNA* circulating tumour DNA, *CSF* cerebrospinal fluid, *ddPCR* droplet digital polymerase chain reaction, *indels* insertions or deletions, *NGS* next-generation sequencing, *SNV* single nucleotide variation

major limitations exist for both MAPK and immune checkpoint inhibitors, including the emergence of drug resistance in the majority of patients within 12 months for MAPK inhibitors and low but durable response rates for single-agent immune checkpoint inhibitors at 10–40% [5, 57–63]. Thus, the identification of biomarkers that can predict and monitor patient responses remains a critical unmet need, and the use of liquid biopsies and specifically cfDNA to inform patient selection and monitor patient response to treatment is a rapidly evolving area of research (reviewed in [4, 5, 10, 64, 65]).

3 PCR-Based Methods to Detect ctDNA in Melanoma

The use of PCR-based methods, particularly droplet digital (dd) PCR and BEAMing (beads, emulsion, amplification and magnetics), for the detection of ctDNA as a biomarker in metastatic melanoma and other cancers has been well documented (reviewed in [10, 66]). The potential clinical applications of PCR-based analysis of ctDNA in melanoma as a predictive biomarker, measuring tumour heterogeneity/dynamics, identifying resistance driver mutations for targeted therapy, evaluating early response to therapy, and monitoring the development of resistance to therapy, have been highlighted in many studies (reviewed in [64]). These allele-specific PCR-based methods mainly detect single driver mutations in key genes such as *BRAF* and *NRAS*. They require prior knowledge of the driver mutation, which typically comes from sequencing of the primary solid tissue biopsy using targeted pan-cancer panels that analyse mutations in genes such as *BRAF* and *NRAS* [48].

The use of ddPCR-based detection of *BRAF* or *NRAS* mutations in ctDNA from metastatic melanoma patients has illustrated an inverse correlation between ctDNA copy number and response to either targeted or immunotherapy [67–76]. A direct correlation between ctDNA levels, based on ddPCR-detection, and tumour burden has also been reported [77, 78]. The potential for ddPCR-based detection of ctDNA to replace tumour genotyping of melanomas has been postulated both for detecting major driver mutations in *BRAF* and *NRAS* [79] as well as other mutations, such as in the *TERT* promoter [80], which can be found in up to 70% of all melanomas [81].

The majority of studies involving ddPCR-based detection of ctDNA have been based on the isolation of ctDNA from plasma. One of the major limitations is tracking patients with brain metastases, which occur in 50–75% of all stage IV melanoma patients [82–84]. The low detectability of ctDNA seen in patients with predominant brain metastases suggests that the blood–brain barrier may significantly impact the release of ctDNA into the circulation (reviewed in [10]), and in fact, plasma ctDNA has been shown not to be a reliable indicator of melanoma brain metastases [71]. There are two studies which were able to successfully detect ctDNA using ddPCR in the CSF of melanoma patients with metastasis to the central nervous system [85, 86]. Detection of CSF ctDNA may provide greater sensitivity than conventional cytology and appears to reflect central nervous system-tumour burden and is an indicator of response to therapy [85, 86].

The limitations of only being able to detect one to two mutations simultaneously and the need for specialised equipment encountered with ddPCR and more traditional fluorescence-based PCR have been addressed in a recent report [87]. Alternative approaches to ddPCR have also been investigated. The combination of surface-enhanced Raman spectroscopy (SERS) nanotags combined with PCR was shown to provide similar sensitivity to ddPCR in simultaneously detecting *BRAF*, *NRAS*, and *KIT* mutations in ctDNA from melanoma patients [87]. The potential of nanoparticle-based sensing of mutations in ctDNA, without the need for PCR amplification, has also been highlighted [88]. Whether these approaches truly approach the same sensitivity, specificity, reproducibility, and accuracy in ctDNA quantification as ddPCR requires further validation.

A fully automated integrated platform from Biocartis (Belgium), designated Idylla, for multiplex real-time PCR-based detection of major *NRAS*, *BRAF*, *KRAS*, or *EGFR* mutations in either ctDNA from blood or genomic DNA from tissue has been developed. This platform has been validated using mainly tissue from melanoma patients, and showed > 95% concordance with other methods of detection, and as such, represents a feasible diagnostic platform given the ease of use, automation, and fast 1-day turnaround [89–93].

These targeted ctDNA mutation detection methods, which rely on screening previously identified tumour mutations, can be used for the real-time monitoring of patient response to therapy. However, they provide no information on tumour evolution and cannot identify newly acquired somatic mutations that may confer resistance to therapy and which may be targetable with alternative therapies. Furthermore, 20–30% of melanoma patients who have rare mutations or no identifiable mutations on standard tissue mutation testing platforms provide additional challenges regarding ctDNA mutation detection. The identification of a broader number of mutations with the detection of acquired resistance markers requires the use of NGS approaches, as discussed in the next section.

4 NGS-Based Methods to Detect ctDNA in Melanoma

The use of NGS in the context of melanoma and other cancers, whether in the form of whole-genome, whole-exome, or targeted gene panels, is well documented for tissue-derived

samples [38, 49, 94–100]. These NGS approaches are able to interrogate mutations in many genes and provide increasing sensitivity as the sequencing becomes more targeted. The drive to complement or replace tissue-based sequencing with sequencing of ctDNA for both initial diagnosis and longitudinal assessment of cancer patients on therapy is now a major focus in the field. The less invasive sampling required for ctDNA has obvious advantages over the more invasive tissue collection, with which serial sampling may not be possible in many cases.

The use of fragmented and low copy number ctDNA as a template for NGS, compared with the more abundant higher molecular weight genomic DNA used in tissue-based NGS, has been a major limiting factor, and this has been largely overcome with advancements in sequencing technology (reviewed in [40]). All of these advancements have aimed to improve the detection sensitivity and detection specificity of monitoring rare ctDNA mutations within the larger pool of total cfDNA. However, there are continuing limitations due to varying cfDNA quality and quantity arising from non-standardised pre-analytical workflows used for both blood collection and subsequent extraction of cfDNA (reviewed in [40]).

The feasibility of whole-genome and whole-exome sequencing using ctDNA as a template to identify somatic mutations, which can subsequently be used to guide choice and response to therapy, has been demonstrated in a number of cancers, including melanoma [76, 101–109]. The value of using whole-genome and whole-exome sequencing, given their turnaround time, cost, and the actual need for such a broad approach, has come into question with the advent of more targeted NGS gene panels compatible with cfDNA (Table 1). The whole-genome or whole-exome sequencing approach can still be used initially to design patient-specific targeted NGS gene panels for longitudinal monitoring of therapy (reviewed in [19]).

4.1 Targeted NGS Gene Panels to Guide and Monitor Response to Therapy

Targeted NGS gene panels are typically based on proprietary technology for sequencing library preparation and are compatible with one of the three current NGS platforms: Thermofisher Ion Torrent (USA); Illumina (USA); or Roche (Switzerland). Whether pan cancer (covering major solid tumour cancers, including lung, breast, prostate, colorectal and melanoma) or cancer specific, NGS cancer panels generally include common somatic driver mutations such as those in *EGFR*, *BRAF*, *NRAS*, *KRAS*, *PDGFRA*, and *KIT*. In the case of melanoma, this can guide the choice of initial targeted MAPK inhibitor therapy, i.e. patients with $BRAF^{V600}$-mutant melanoma can be effectively treated with combination inhibitors targeting mutant BRAF and its downstream effector MEK [110]. Further inclusion of known somatic resistance mutations plus oncogenic targets in downstream signalling pathways [63] can identify pathways of resistance to first-line therapy and guide the choice of alternative therapies. A practical application of NGS targeted gene panels for the parallel analysis of several genes in genotyping primary melanoma patients has been documented using genomic DNA [111]. Such a targeted panel could also be applied in the analysis of ctDNA, to provide diagnostic/prognostic information and monitor resistance during treatment of patients with stage III/IV melanoma.

4.1.1 Targeted NGS Gene Panels Developed for Genomic DNA and Applied to cfDNA

The application of targeted NGS gene panels in the analysis of ctDNA can identify dominant and therapeutically targetable genetic mutations. Initially, targeted NGS gene panels based on library preparation and sequencing technology developed for tissue-derived genomic DNA templates were used in studies employing ctDNA as a template (Table 1, rows 1–5). These consisted of off-the-shelf and custom-designed pan-cancer panels [86, 112–115] as well as a custom-designed melanoma panel [109].

In one study, a custom melanoma-specific NGS gene panel was designed based on whole-genome sequencing analysis of cfDNA (and tissue) from pre- and post-treatment (targeted or immune checkpoint inhibition) samples to identify a signature of SNVs in non-coding and coding regions associated with progression on therapy (Table 1, row 2) [109]. The targeted NGS gene panel was then used to monitor response to therapy based on increases in mutant allele frequency of the signature SNVs within cfDNA over several time points post-therapy. Interestingly, a *TERT* promoter mutation identified by whole-exome sequencing could not be incorporated into the targeted NGS gene panel based on the repetitive sequence of this region [109].

For one of the custom pan-cancer NGS gene panels used for melanoma (Table 1, row 4) [114], the panel design attempted to incorporate known melanoma driver and BRAF inhibitor resistance mutations, including mutations in *BRAF*, *NRAS*, *KRAS*, *MAP2K1*, and *CDKN2A* [63]. The feasibility of this panel was then highlighted using ctDNA from melanoma patients, who had previously received (or were still receiving) targeted or immune checkpoint therapy, to measure mutant allele frequency of the targeted genes. This showed a high concordance with tissue samples even though the tissue samples had been collected earlier than the plasma and during this interval patients had received one or more therapies [114].

Table 1 Targeted next-generation sequencing cancer gene panels compatible with cfDNA

Gene panel	Design	Developed for	Panel type	Number of genes	Mutation type	Sensitivity (MAF %)	Reported	Cancer tested
Thermofisher Ion AmpliSeq cancer hotspot panel v2	Off-the-shelf	gDNA	Pan cancer	50	SNV, indel	5	[86, 112]	Melanoma, lung
Thermofisher Ion AmpliSeq	Custom	gDNA	Melanoma	91	SNV	5	[109]	Melanoma
Thermofisher Ion AmpliSeq	Custom	gDNA	Pan cancer	6	SNV, indel	5	[113]	Lung, colorectal, melanoma
Illumina TruSeq	Custom	gDNA	Pan cancer	61	SNV, indel	1	[114]	Melanoma
KAPA Biosystems	Custom	gDNA	Pan cancer	398	SNV, indel, fusion, CNV	Not reported	[115]	Gastrointestinal
Thermofisher Oncomine pan-cancer cell-free assay	Off-the-shelf	cfDNA (and cfRNA)	Pan cancer	52	SNV, indel, fusion, CNV	0.1	None	Covers 18 cancers including melanoma
Thermofisher Ion AmpliSeq HD	Custom	cfDNA	Variable	Variable	SNV, indel, fusion, CNV	0.1	None	
ArcherDX Archer RevealctDNA28	Off-the-shelf	cfDNA	Pan cancer	28	SNV	1	None	
Roche Avenio ctDNA targeted, expanded and surveillance kits	Off-the-shelf	cfDNA	Pan cancer	17, 77, 197	SNV, indel, fusion, CNV	0.1	[140]	Lung
Guardant Health Guardant360	Off-the-shelf	cfDNA	Pan cancer	73	SNV, indel, fusion, CNV	0.1	[116, 141–149]	Urinary bladder, lung, breast, melanoma, colorectal, gastrointestinal
Foundation Medicine Foundation-ACT	Off-the-shelf	cfDNA	Pan cancer	62	SNV, indel, fusion, CNV	0.5	[147, 150]	Breast, lung, gastrointestinal, colorectal, prostate
Natera Inc Signatera	Custom	cfDNA	Variable	Variable (minimum 16)	SNV, CNV	0.01	[151–153]	Lung, breast
CellMax Life LBx Liquid Biopsy	Off-the-shelf	cfDNA	Pan cancer	73	SNV, indel, fusion, CNV	0.1	None	

cfDNA circulating free DNA, *cfRNA* circulating free RNA, *ctDNA* circulating tumour DNA, *CNV* copy number variation, *gDNA* genomic DNA, *indel* insertion or deletion, *MAF* mutant allele frequency, *pan cancer* covers major solid tumour cancers, *SNV* single nucleotide variation, *variable* can design gene panel of choice

4.1.2 Targeted NGS Gene Panels Developed for cfDNA

The subsequent development of cfDNA-optimised targeted NGS gene panels for deep sequencing of ctDNA looks to revolutionise the use of liquid biopsies as a diagnostic tool in cancer therapy (Table 1, rows 6–13). Many of these panels have the reported ability to detect mutant allele frequencies as low as 0.01%. All recommend around 20–30 ng of starting cfDNA for high-quality library preparation, and this amount of cfDNA can be commonly obtained from 10 ml of blood (4–5 ml of plasma). Less cfDNA can be used as the input, but will result in a subsequent decrease in the limit of detection. For instance, if the limit of mutation detection was a 0.01% frequency in 100 ng input cfDNA, 10 ng input cfDNA would only allow for detection of mutations occurring at a 0.1% frequency.

Several targeted NGS gene panels developed for cfDNA are now available for research and include those from Thermofisher, Roche, and ArcherDX (USA) (Table 1). Thermofisher is the only company which currently provides the

option of custom-designing cfDNA-optimised NGS gene panels in the form of their new AmpliSeq HD technology (Table 1). The AmpliSeq HD technology can also be used for genomic DNA from tissue sources. Thermofisher also offer cancer-type–specific off-the-shelf cfDNA panels for breast, colorectal, and lung cancer in their Oncomine range. To date, no melanoma-specific cfDNA-optimised NGS gene panels are available, though the off-the-shelf panels do include a subset of melanoma somatic driver and resistance mutations.

The potential of targeted ctDNA sequencing is evident from a growing number of clinically focused companies who have embraced this approach, including Guardant Health (USA), CellMax Life (USA), and Foundation Medicine (USA), who have all developed off-the-shelf pan-cancer cfDNA-optimised NGS gene panels (Table 1). Of particular note is the use of the Guardant360 platform (Table 1) to analyse ctDNA from a cohort of > 20,000 cancer patients across several cancers, including melanoma, illustrating the ability of this platform to distinguish primary driver from secondary emerging clonal resistance mutations [116]. Another clinically focused company, Natera (USA), offers an individualised NGS panel, known as Signatera, which is custom-designed after whole-exome sequencing of cancer tissue (Table 1). The additional tissue sequencing step of Signatera adds significantly to the turnaround time and diagnostic cost, but provides patient specificity.

NGS of cfDNA can detect low frequency variants, but the clinical relevance of these variants, which may reflect clonal hematopoiesis, remains unclear [117]. In addition, detection of low frequency variants in NGS of cfDNA can lead to discordance when comparing different NGS platforms [118]. Therefore, although the concept of identifying multiple actionable mutations in the circulation to guide therapeutic decisions is an attractive concept, the results from these tests must be used with caution and comparison of NGS tests across large numbers of patients with cancer needs to be done to improve clinical utility.

4.2 Analysis of ctDNA as a Predictor of Response to Immunotherapy

The use of ctDNA as a predictor of response to immunotherapy has largely relied on detection using ddPCR. Several studies involving metastatic melanoma patients, based on ddPCR detection of a single mutation in *BRAF* or *NRAS*, have shown that elevated ctDNA levels at baseline and on immunotherapy correlate with a poor prognosis [71, 78]. Lee et al. used a combination of baseline and early on treatment ctDNA levels to predict response to immunotherapy, where undetectable ctDNA early during treatment was associated with improved objective response and overall survival. Furthermore, ctDNA was able to accurately and rapidly differentiate between melanoma patients receiving immune checkpoint inhibitors displaying pseudoprogression, defined as initial growth followed by eventual tumour response, and true disease progression [72].

An alternative to ddPCR-based approaches is the measure of TMB based on targeted NGS gene panels. High TMB within a particular tumour often correlates with a greater number of neoepitopes, leading to greater immunogenicity and a likely greater chance of responding to immunotherapy (reviewed in [119, 120]). The use of whole-exome sequencing and targeted NGS gene panels, such as the Guardant360 panel (Table 1), have shown promising results for a range of cancers, including melanoma, in assessing response to immunotherapies based on measuring TMB [121, 122]. A recent retrospective analysis of two large clinical trials in non-small cell lung cancer demonstrated concordance between tumour and blood TMB, with high mutation burden in plasma associated with clinically significant improvement in progression-free survival from anti-PD-L1 [123]. However, the assays used in matching tumour and plasma, despite overlap, identified non-identical variants, which likely originated from the samples themselves as opposed to technical variation.

Therefore, several factors in the use of cfDNA and targeted NGS gene panels in quantifying TMB still need to be addressed in more detail. Such factors include whether the depth of sequencing coverage of targeted NGS gene panels is sufficient for an accurate determination of TMB especially for mutations of low allelic frequency present in the tumour, and whether the cfDNA reflects the TMB of an individual or a number of tumours.

4.3 Factors to Consider when Choosing Allele-Specific PCR or NGS

One important consideration when selecting a molecular test is the cost effectiveness and turnaround time. In one study, the detection of $BRAF^{V600E}$ in melanoma using allele-specific PCR-based monitoring of ctDNA was compared to NGS targeted gene panel-based monitoring of genomic DNA [124]. The turnaround time was reported to be faster at 2.9 ± 1.1 days for PCR compared to 4.7 ± 1.6 days for NGS [124]. The cost was also significantly higher for NGS, at US$270 per sample compared to US$40 per sample for allele-specific PCR [124]. The advantage of NGS lies in the ability to capture much greater mutational information compared to allele-specific PCR techniques such as ddPCR (Fig. 1). Thus, although NGS can provide significant additional mutation data, the value of this data, which may constitute non-clinically relevant mutations, to the clinical management of cancer patients needs to be considered relative to the increase in cost and extensive bioinformatic resources needed for NGS analysis.

Allele-specific PCR, such as ddPCR, is a quantitative technique, whereas NGS is only semiquantitative (Fig. 1). Quantitating ctDNA can be influenced by multiple factors which are likely to induce an increased release of non-tumour DNA into the plasma. These factors include physiopathological factors, such as inflammation, autoimmune diseases, pregnancy and physical exercise (reviewed in [125]), or preanalytical factors primarily during blood collection (reviewed in [126]). This needs to be taken into consideration when using NGS or ddPCR to measure mutant allele frequency, since it depends on the number of wild-type cfDNA copies derived from normal cells.

5 Other Sources of Circulating Nucleic Acid

In an effort to increase both the sensitivity of detection and to capture the evolution of cancer-related genomic changes in the circulation, other sources of nucleic acid, both DNA and RNA, are being investigated. These alternative sources of DNA and RNA include circulating free RNA (cfRNA), extrachromosomal circular DNA, circulating tumour cells (CTCs), circulating endothelial cells, tumour educated platelets and extracellular vesicles such as exosomes (reviewed in [8, 127–132]).

Given that there is a requirement for specialised workflows to isolate cells or vesicles prior to the extraction of DNA or RNA and the fact that isolation methods are yet to be standardised (reviewed in [132, 133]), these alternate sources of tumour markers have not been thoroughly explored. The particular difficulty of isolating and assessing the genomic profile of melanoma-derived CTCs has been addressed [134, 135]. These issues stem from the apparent diversity of melanoma CTCs, which require further characterisation of specific surface markers to enable immunoaffinity-based purification of these CTC subpopulations. A subsequent report appears to have overcome some of these issues in identifying an RNA signature from CTCs isolated from melanoma patients on immunotherapy which may be a predictor of early response [136]. A recent observation has highlighted that prostate tumour-derived large extracellular vesicles (oncosomes) contain significant levels of circulating tumour genomic DNA with identifiable genomic alterations [137]. Given the limitations of detecting ctDNA in early-stage cancers, the search for other diagnostic/prognostic DNA/RNA-based biomarkers from these alternative sources is certainly worth further investigation.

6 Conclusions and Future Directions

Conventional tissue biopsy for genotyping in cancer diagnosis is still considered to be the gold standard. However, this approach has its limitations when faced with limited tumour tissue and often depends on sample tissue collected from a section of a single tumour and thus does not provide a true representation of the heterogeneous tumour burden. Furthermore, the availability of tissue for monitoring treatment over time is an issue. Liquid biopsy-based genotyping makes it possible to assess both tumour heterogeneity and to provide an accurate assessment of TMB [123]. In particular, the use of cfDNA from biofluids in conjunction with molecular technologies such as ddPCR or targeted NGS can provide non-invasive real-time information about a patient's tumour burden and subsequent response to therapy.

The increasing sensitivity (detecting as low as 0.01% mutant allele frequency) of targeted NGS gene panels for the detection of cfDNA in cancer patients is now comparable with the sensitivity of such common digital PCR approaches as ddPCR. Given the capacity for multiplexing, currently not possible with ddPCR, and for designing custom targeted NGS gene panels optimised for cfDNA (as offered by Thermofisher's new AmpliSeq HD technology), the likelihood is that targeted NGS gene panels will supersede ddPCR both in a research and diagnostic setting. This is now feasible in a diagnostic context given that a typical workflow for targeted NGS gene panels can be completed in 2–3 days. Importantly, the increasing availability of cfDNA standards for NGS (Seracare, USA, and Horizon Discovery, UK) containing cancer-relevant somatic mutations of known allele frequencies allows validation and standardisation of targeted NGS gene panels.

One of the main issues which still needs to be resolved to fully incorporate NGS technology into ctDNA analysis is the need to address whether NGS methods are truly as specific as PCR-based methods such as ddPCR and BEAMing. Applications such as upfront mutational profiling [80], prediction of early response [67, 70, 71], pseudoprogression [72] and early detection of relapse (during treatment and in stage III melanoma) [69, 73] have important clinical implications for the management of patients. To date, optimised ctDNA-based NGS targeted gene panels for melanoma remain to be fully evaluated for their effectiveness for future use in the clinic.

Finally, several important considerations that need to be addressed with cfDNA analysis include: the limited ability to detect cfDNA in early-stage cancers, including melanoma; and the fact that ctDNA arising from a tumour may not be detectable in cfDNA because of the site of the tumour [138, 139]. Therefore, analysis of other biofluids for cfDNA and new technologies that enrich for cfDNA is warranted. The rapid development of sensitive technologies that accurately detect tumour mutations in circulating nucleic acids is revolutionising the monitoring of cancer patients, and although tissue biopsies still provide essential diagnostic information, new targeted and immune-based therapies require real-time,

longitudinal monitoring of tumour evolution via non-invasive and serial liquid biopsies.

Compliance with Ethical Standards

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Conflict of interest Russell J. Diefenbach, Jenny H. Lee, and Helen Rizos declare that they have no conflicts of interest that might be relevant to the contents of this manuscript.

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