# Plasma DNA analysis in prostate cancer – Opportunities for improving clinical management

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## Abstract

### BACKGROUND

Molecular characterization of tumor could be important for clinical management. Plasma DNA obtained non-invasively as a liquid biopsy could be widely applicable for clinical implementation in biomarker-based treatment strategies.

### CONTENT

Prostate cancer is a disease with variable clinical outcomes and molecular features and therefore presents multiple opportunities for biomarker-based treatment optimization. Tissue analysis may not be representative of the lethal clone in localized disease or of intra-patient, inter-metastases heterogeneity; fresh tissue is often challenging to obtain by biopsy of metastasis whereas archival samples may not represent current disease and may be of insufficient quality. Plasma DNA is of variable tumor to normal fraction that requires accurate estimation using sensitively measured genomic events. In plasma with sufficient tumor content, the spectrum of genomic aberrations closely resembles tissue and could be used to molecularly characterize patients in real time. In this review we discuss the opportunities for improving the clinical management using plasma DNA analysis in different clinical scenarios across the disease spectrum, from detection of prostate cancer, disease relapse, to treatment response prediction, response assessment and interrogation of treatment resistance in metastatic prostate cancer. Combinational strategies may incorporate other modalities, including circulating tumor cells, circulating miRNA and extracellular vesicles analysis, which could help to achieve more accurate characterization.

### SUMMARY

There are many promises of plasma DNA analysis changing clinical management. Also, there are existing challenges that need to be addressed to implement clinically qualified tests, such as accurate, fit-for-purpose assay design, technical reproducibility and prospective validation in large cohorts of patients.

## Introduction to circulating tumor DNA

Plasma DNA or cell-free DNA (cfDNA) is fragmented, extracellular DNA collected non-invasively by liquid biopsy, and has been used to detect circulating fetal DNA in pregnant women and tumor DNA in cancer patients. Tumor apoptotic and necrotic cells are digested mainly by phagocytes, and cell debris including nuclear material such as DNA is released into the circulation. Host cfDNA fragments are between 143bps and 166bps *(1) (2)*; some studies suggest that tumor-derived cfDNA is shorter than normal cfDNA (as exemplified by comparisons of the fragment length of DNA harboring mutant compared with wild-type alleles); this introduces the opportunity to enrich tumor DNA using size-selection based approaches *(3) (4) (5)*. Moreover specific patterns in plasma DNA length can inform on cfDNA nucleosome occupancies that correlate with the nuclear architecture, gene structure, and expression observed in cells, suggesting that this information could be used to identify what cell type cfDNA originated from *(6) (7)*. Intact tumor cells can also be detected in circulation in advanced cancer patients (i.e. circulating tumor cells, CTCs) and these have been proposed as a source of plasma DNA. There is a correlation between the number of CTCs and the amount of circulating tumor DNA in metastatic prostate cancer *(8)*; however, tumor DNA equivalent to several 100 to 1000s of genomes is routinely extracted from 10mls of blood from men with advanced prostate cancer in which fewer than 10 CTCs are detected. This difference could be a result of CTC capture efficiency by current technologies or more rapid degradation of CTCs, but could also suggest different mechanisms of release.

The early clinical application of cfDNA was in fetal medicine to detect aneuploidy such as trisomy 21 and other congenital disorders *(9)*. Similarly, circulating tumor DNA (ctDNA) or plasma tumor DNA in cancer patients can be quantified and characterized *(10)*. This could be important as acquiring tissue from metastatic sites, especially repeatedly, is challenging and tissue biopsies may fail to represent the genomic landscape of multiple metastases. Therefore, liquid biopsies can provide a solution to non-invasively profile a cancer's genome in real-time, improving monitoring of treatment response, detection

and interrogation of disease recurrence and molecularly-driven treatment prediction. Also, with improved sensitivity, early detection of cancer could become possible. Plasma DNA, given its relatively uniform fragment size, is amenable to next-generation sequencing and the main challenges in its analysis compared to tissue studies are the relatively low DNA inputs (~10-20ng/ml) and variable tumor DNA fraction (<1% to >90%). These two factors are linked: as more tumor DNA enters circulation, both the tumor-to-normal fraction and the total amount of DNA that is extracted per ml of plasma increase. Overall, the proportion of plasma DNA that is tumor in origin varies by tumor volume, sites and number of metastases, disease status, and cancer biology. A report from the TRACERx study, a multi-center, multi-region sequencing project to interrogate lung tumor evolution, has demonstrated that radiologically-defined primary non-small cell lung cancer volume is associated with mean clonal variant allelic frequency (VAF), an indicator of circulating tumor DNA fraction; for example, a volume of $10cm^3$ predicted a VAF of 0.1% *(11)*. Moreover, the presence of plasma tumor DNA was associated with different histopathological subtypes. For example, in stage I non-small cell lung cancer, ctDNA was detected in over 90% of squamous cell carcinoma, compared to <20% in adenocarcinoma *(11)*. Analysis of earlier stage patients may therefore require a combinatorial approach of larger blood volumes and more sensitive assays. For example, amplicon-based or customized target enrichment using improved biochemistry of random molecular barcoding and optimized, error-correcting analysis on ultra-deep sequencing (i.e. >10,000X) can potentially improve the sensitivity of rare mutation and indel detection on ctDNA *(12-15)*.

Epigenetic information, such as DNA methylation change, can be extracted from plasma DNA using modified next-generation sequencing protocols to obtain information additional to the genomic status *(16)*. DNA methylation, the addition of a methyl group to cytosine, is a modification that occurs at thousands of sites across the genome and is tissue-of-origin and cancer specific. This information can therefore be exploited for cancer detection, diagnosis of tumor type, estimation of tumor cfDNA fraction,

and assessment of treatment response *(17) (18)*. For example, a pilot study has shown detection of *GSTP1* methylation in blood was prognostic and could be used as a response surrogacy marker in metastatic castration-resistant prostate cancer (mCRPC) *(19)*. In colorectal cancer, several individual methylation markers, such as exon 1 of the vimentin gene, have been tested in plasma DNA for diagnostic purpose *(20) (21)*. Additionally, targeted sequencing of informative CpG methylation markers in hepatocellular carcinoma have shown high diagnostic fidelity *(22)*. Epigenetic information could also potentially be integrated with genomic analyses to improve the clinical utility of liquid biopsies.

Multiple technologies are converging to accelerate the development of a ctDNA clinical test. This includes the optimization of pre-analytical factors involved in sample collection, including plasma collection tubes that minimize ex vivo leukocyte degradation (that presents as fragments >1000 base pairs in length and can interfere with quantification, analysis and VAF or copy number assessment due to dilution of tumor DNA) and minimize inter-sample variability. Next-generation sequencing (NGS) of whole genomes or exomes has been optimized for research purposes but most tests for clinical use are focused on selected recurrent and informative targets, and include either custom NGS panels covering hot-spot mutations and copy number aberrations or targeted approaches such as droplet digital PCR (ddPCR) and the use of Beads, Emulsions, Amplification and Magnetics (BEAMing) *(23) (24) (25) (26)*. ddPCR and BEAMing may be more economical, amenable to high-sensitivity testing and have a more rapid turnaround than targeted NGS. However, the limited number of targets tested could limit their broader applicability. ctDNA as a liquid biopsy is currently limited by a number of inherent challenges. Firstly, information related to protein, mRNA and other epigenetic modifiers, such as histone methylation or acetylation, will be missed and approaches to capture the information could improve the value of blood analyses relative to tissue. Secondly, plasma DNA is a mixture of tumor DNA from different clones and/or metastatic sites and it is unclear whether it is possible to determine the

metastatic site of origin or the relative contribution of individual metastases. This explains challenges for interrogating the subclonal architecture and rare, private genomic lesions at the resolution achieved with intact cell analysis. Thirdly, the fact that variable contributions from normal tissue DNA also introduces inter-patient and sample differences and requires higher sensitivity approaches.

# Molecular characterization of circulating tumor DNA in prostate cancer

Prostate cancer is a highly heterogeneous disease with variable clinical outcomes. A number of recurrent genomic changes have been reported either in isolation or in combination to improve prognostication or prediction of response to therapy of an individual's cancer. Detection of copy number alterations from primary prostate tumor samples has been shown to be a strong prognostic indicator linked with more aggressive disease and increased risk of relapse after local intervention *(27)*. More recently, comprehensive analysis of localized prostate cancer identified additional DNA methylation markers, along with genetic aberrations, as being strongly linked with disease recurrence *(28)*. In metastatic disease, detection in plasma DNA of genomic alterations involved in specific pathways could allow molecularly-driven treatment selection, especially for the majority of patients for whom tumor tissue is not available or is of insufficient quality.

**Estimating tumor fraction**

Given the highly variable admixture of tumor to normal DNA, estimation of tumor fraction is a key first step to characterizing the tumor genomic landscape. Detection of copy number changes requires plasma tumor DNA fractions above a critical threshold. Interrogating resistance needs recognition of the tumor-driven denominator. Conceptually, to estimate ctDNA fraction one can track a genomic change that occurs early in carcinogenesis (pre-branching) and is therefore present in every cancer cell in that individual. In several cancers, the allelic frequency of common and recurrent hot-spot point mutations has been used to track tumor DNA *(29) (30)*. Proof-of-concept analyses in metastatic breast cancer have used structural variants or somatic mutations identified in tumor tissue, and droplet digital PCR or amplicon-based targeted deep sequencing to quantify and define circulating tumor DNA levels *(29)*.

This could be further optimized and personalized to track patient-specific mutations identified by multi-regional sequencing *(11)*. Prostate cancer does not have commonly recurrent, clonal point mutations and thus requires a broader approach. One strategy is to quantitate a panel of genomic changes that have occurred at an early stage of prostate cancer and if truncal events would be present in all metastasizing cells. Two such events that could be used to track tumor content in prostate cancer are mono-allelic deletions associated with ETS gene family rearrangements (primarily involving the oncogenes *ERG* or *ETV1* that fuse with an androgen-regulated promoter) and *NKX3.1* deletion on chromosome 8p, strongly linked with prostate cancer development. Either alteration occurs in more than 50% of advanced prostate cancer patients, and has been shown to be clonal in mCRPC *(31) (32)*. As coverage estimations for quantitating mono-allelic deletions are unreliable in plasma samples with relatively lower tumor fractions, alternative approaches such as leveraging information of germline heterozygous SNPs could be used to measure tumor reads harboring the deletions *(8)*.

Another approach is to estimate tumor fraction by using VAF from mutation calls in whole exome or very broad targeted next-generation sequencing *(33)*. This approach requires adjustment for loss of heterozygosity (LOH) for every mutation call, or a conservative assumption that LOH co-occurs with all mutations. This could under-estimate tumor fraction if LOH is assumed when it is not present, or conversely over-estimate if a mutation is in an amplified region. The accuracy of using deletions or mutations to quantitate tumor fraction will be dependent on that aberration being present in all, or at least the majority, of clones represented in circulation; emergence of a clone that harbors aberrations not included in the data will be missed. A third approach is to use the magnitude of genome-wide copy number aberrations to estimate tumor fraction. This could be especially suited for very advanced prostate cancer *(34)*. ichorCNA, a software applicable to shallow whole genome sequencing (WGS), estimates tumor ploidy and tumor fraction. This could represent a very economical approach that could be widely implemented across plasma samples but may not be amenable to detect tumor fractions

below 8-10%, and it can serve as a triage to select samples with higher tumor fraction applicable for further analysis *(35)*.

**Plasma DNA based molecular stratification**

Analysis of plasma DNA from mCRPC patients shows similar prevalence of major genomic sub-types as in tissue studies *(33)*. These include somatic mutations in *TP53* (42%-56%), *AR* (10% -15%), *APC* (8%-12%), and *PTEN* (6%-10%). Systematic comparison of targeted sequencing data from liquid biopsy and tissue has shown a high degree of concordance with multiple shared mutations (usually the most abundant in both biopsy and plasma), mutations restricted to plasma tumor DNA (putatively private to metastases that were not biopsied) and less abundant mutations private to the biopsied metastasis not detected in plasma tumor DNA (the latter could be reduced by increasing the sensitivity of ctDNA assays) *(36)*. This finding is important for future plasma DNA clinical implementation, as most of the drivers or actionable lesions can be detected from circulation in mCRPC patients.

The androgen receptor gene (*AR*) is very rarely detected as mutated or gained in cancers that have not become resistant to endocrine therapies but is aberrant in 30-70% of mCRPC. Obtaining tissue biopsies from men immediately at development of mCRPC is challenging as a result of the low metastatic volume and tumor biopsies may miss *AR* gained tumor. Analysis of plasma DNA has been used to identify *AR* copy number gain or somatic point mutations across the mCRPC spectrum. A large number of point mutations have been identified in plasma tumor DNA, including *AR* c.2226 G>T associated with a W742C amino acid change that results in bicalutamide becoming an agonist and that is no longer detected after bicalutamide discontinuation and initiation of the next line of effective treatment *(8) (37)*. Detection of *AR* copy number gain or one of the two most common and functionally relevant mutations (*AR* c.2105T>A and c.2632A>G resulting in L702H and T878A amino acid

196 changes), in plasma prior to initiation of treatment with the 2nd line endocrine agents abiraterone or
197 enzalutamide is strongly associate with worse outcome *(25) (33) (38)*. Interestingly, an L702H change
198 results in activation by glucocorticoids (including prednisone) and has only been detected in patients
199 previously treated with prednisone *(8)*. In contrast, *AR* aberrant patients receiving taxanes do not have
200 a worse outcome than *AR* normal, introducing the opportunity to select mCRPC patients for *AR*
201 targeting drugs versus taxanes based on ctDNA analysis *(39)*. A similar observation has been made for
202 the AR splice variants lacking ligand binding domain and composite biomarkers that assess *AR* mRNA,
203 gene, and protein could improve prediction of resistance to abiraterone or enzalutamide *(40)*. More
204 recently, studies have reported that *TP53* aberrations are associated with a worse outcome that is
205 independent of tumor fraction, *AR* aberrations and other variables *(41)*. The detection of circulating
206 biomarkers is likely as tumor fraction increases: this could introduce a bias of higher tumor fraction
207 (associated with worse prognosis) contributing to the observation of worse outcome, suggesting
208 complexity for predicting treatment resistance *(38)*.
209
210 Plasma DNA analysis could also explain mechanisms of resistance through analysis of sequential
211 samples identifying genomic changes associated with emergent clones. This approach identified
212 emergence or an increased VAF of the aforementioned L702H and T878A amino acid changes in
213 progression samples from mCRPC patients treated with abiraterone (in combination with prednisone)
214 *(38)*. PARP inhibition is being clinically evaluated in mCRPC patients with an underlying DNA repair
215 gene defect. Plasma DNA analysis of pre-treatment and progression samples from patients treated with
216 PARP inhibitors has identified multiple (even in a single patient) reversion *BRCA2* mutations that
217 restore protein function and lead to resistance to PARP inhibitors *(42) (43)*. These genomic changes
218 that are associated with resistance are often detected in plasma tumor DNA prior to clinical or
219 radiological progression and could allow earlier treatment change. Also it appears that more mutations
220 were detected in plasma than in a matched tumor biopsy.

221

## Translational application of ctDNA in prostate cancer

Although ctDNA analysis has the potential for improving patient care there is presently no plasma-based test yet that is supported by level 1 evidence for implementation into clinical practice in prostate cancer management. Here we highlight applications in different tumor types and discuss their potential role in prostate cancer clinical management (**Figure 1**).

### Early detection of prostate cancer

Cancer detection and diagnosis at a pre-symptomatic stage could radically improve cancer mortality rates but remains challenging. Improved screening of men for prostate cancer will have major public health benefits – current practices using prostate specific antigen (PSA) result in over-diagnosis of non-lethal disease and over-treatment of several thousand men every year *(44)*. Diffusion-weighted pelvic magnetic resonance imaging (MRI) and targeted screening of germline genetic high-risk men are strategies being explored, in combination with PSA, to minimize false positive detection rate *(45) (46) (47)*.

The major challenges for a plasma DNA test in this setting are as follows: balancing high specificity and sensitivity in detecting plasma tumor DNA, low ctDNA abundance, and the lack of prior information on the unique molecular features of each individual tumor. In general, cancer screening needs to reflect cell-of-origin in order to inform clinicians to make actionable plans. Different tumors harbor distinct methylation features, and most changes are tissue-specific. 'CancerLocator' uses methylation status from low-coverage whole genome bisulfite sequencing on plasma DNA from lung, breast and colorectal patients and healthy volunteers to identified undiagnosed tumors *(17)*. Similar approaches using a custom targeted panel to capture informative CPG sites in hepatocellular carcinoma also showed

promising results for cancer detection in patients with liver diseases *(22)*. Targeted error correction sequencing (TEC-seq) was developed also to address the technical hurdle of rare genetic alternation detection without prior tumor information [13]. An ongoing prospective, multi-centre trial (ClinicalTrial.gov Identifier: NCT02889978) commercially-sponsored by GRAIL aims to systemically tackle the challenges of early diagnosis by large-scale, multi-centre plasma collection and centralized analysis using NGS-based approaches *(48)*. Tests for early cancer detection, especially of non-indolent aggressive disease, will need to minimize over-treatment and balance the risks of unnecessary anguish for men who do not require further treatment. This test could be targeted at specific groups, for example based on germline risk factors.

**Risk stratification and detection of minimal residual disease and relapse**

Detection of ctDNA shortly after surgical resection or radiotherapy treatment to the primary could be used to stratify patients who require additional systemic treatment. The feasibility of this has been shown in multiple cancer types, including breast, colorectal, and lung tumors – these studies suggest that ctDNA detected shortly after surgery more sensitively predicts tumor relapse than currently used clinicopathological parameters: the risk for relapse in ctDNA positive compared to ctDNA negative has been reported as greater than 6 fold in multiple studies across tumor types *(11) (30) (49)*. This could have important utility in prostate cancer where the risk of relapse is highly variable and could allow selection of adjuvant systemic treatment for the relatively low proportion of patients who would derive maximum benefit. A number of randomized clinical trials in this setting are collecting plasma to evaluate the relationship of ctDNA with treatment response and long-term benefit (examples: ClinicalTrial.gov Identifier: NCT01411332, and NCT01411345). Similarly, analysis of sequential samples from men in follow-up could detect early relapse and avoid life prolonging treatment. In these settings plasma DNA analysis would have to improve on, alone or in combination on serum PSA readings.

**Prediction of treatment outcome and response assessment in metastatic disease**

The first plasma-based test to receive approval from the regulatory authorities for clinical use in cancer patients is the Cobas EGFR Mutation test used to identify *EGFR* mutations, exon 19 deletion or exon 20 insertions for the selection of patients with metastatic non-small cell lung cancer that stand to benefit from EGFR-targeted therapy.

In mCRPC, mismatch repair deficiency occurs in <2% of patients *(50) (51)*; given immunotherapy has shown increased efficacy and PD1 blockade has received regulatory approval for use in this molecularly-defined subgroup of patients, there is an indication to test for MMR gene defects in mCRPC patients *(52)*. DNA repair genes are more common, occurring up to 20% of mCRPC patients. Ongoing trials are selecting mCRPC patients with an underlying germline or somatic DNA repair defects for treatment with agents targeting DNA repair mechanisms, most notably PARP inhibitors *(53)*. The majority of trials are utilizing archival formalin fixed paraffin embedded (FFPE) tissue or a fresh tissue biopsy for patient selection. Major efforts are underway to concurrently develop a ctDNA-based test. The main challenge remains the accurate detection of mono-allelic (in combination with pathogenic deactivating mutations) and bi-allelic deletions in ctDNA with a highly variable and often low (<0.1) tumor-to-normal fraction.

Given CRPC metastases primarily involve bone, quantitative imaging assessment of response or early progression is challenging. Serum PSA is often used in clinical practice to guide decisions on continuing or stopping treatment for disease progression. However, given PSA expression is exquisitely androgen-regulated, absolute levels and changes may not entirely reflect disease behavior and in fact PSA has not met the requirements for a surrogate biomarker of overall survival *(54)*. CTC dynamics

have been shown to strongly associate with treatment benefit across multiple therapeutic strategies. Comprehensive evaluation of CTC change before and after the treatment indicated that a drop in CTC number in week 13 is strongly linked with prolonged survival *(54)*. These results are encouraging for liquid biopsy assessment in this setting but the absence of and costs for detection of CTC could limit this application in earlier disease states. ctDNA change in metastatic breast cancer reflective of treatment response had superior sensitivity to CTC and CA15-3 *(29)*. Preliminary data in mCRPC indicated that plasma DNA change in sequential plasma samples from mCRPC reflects treatment response *(43)*. Future studies could further assess this.

**Combinations with other modalities**

A constellation of other emerging circulating biomarkers such as circulating microRNA (miRNA), and extracellular vesicles (EV) have shown potential for future clinical translation. CTC can be detected and isolated using different technologies but the challenge of detecting rare intact cells could limit implementation of CTC analysis in patients with lower tumor volume. Combination biomarkers that analyze both ctDNA and CTC could have higher resolution than ctDNA alone, especially as they can study expression. A recent example is the combined analysis of AR aberrations including genomic aberrations in plasma DNA and increased AR splice variant mRNA expression or nuclear protein expression in CTC *(40) (55)*. Circulating miRNAs, short non-coding RNAs released into the circulation, are known to be resistant to RNase digestion and could be quantified in prostate cancer for diagnostic and prognostic purposes *(56) (57) (58)*. EV contains tumor material and can be another source of cancer-specific information in the circulation. Early findings reveal genetic aberrations specific to metastatic prostate cancer in large EVs *(59)*. It is possible that these circulating tumor markers (CTC,

circulating miRNA, EV) could be integrated with plasma DNA analysis to facilitate better clinical decisions.

## Conclusion

Plasma DNA analysis reports in prostate cancer have to date shown promising clinical utility in cohorts where the aims of analyses were defined after sample collection or prospectively but did not influence treatment. Also, the majority of studies have performed using tests conducted in a research setting in specialist labs. Implementation of a test into clinical practice requires level 1 evidence that prospectively demonstrates improved outcomes as a result of testing. This requires an analytically validated assay that is fit-for-purpose, a clinical question that needs addressing, strong biological supporting data and clinical associations shown in retrospective studies, and prospective trials where the test is implemented in the pre-defined patient population.

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**Figure legends: Applications of plasma DNA analysis in prostate cancer**

# ctDNA Applications in prostate cancer clinical management