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Circulating tumor DNA for early cancer detection

Clare Fiala[1], Vathany Kulasingam[2,3], Eleftherios P. Diamandis[1, 2,3 *].

[1] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada

[2] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

[3] Department of Clinical Biochemistry, University Health Network, Toronto, Ontario, Canada

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*To whom the correspondence should be addressed:
Eleftherios P. Diamandis, MD, PhD
Mount Sinai Hospital and University Health Network
60 Murray St. Box 32, Floor 6, Rm L6-201.
Toronto, ON, MST 3L9,
Canada Tel. (416) 586-8443
E-mail: Eleftherios.diamandis@sinaihealthsystem.ca

Abstract

BACKGROUND: Cancer cells release circulating tumor DNA (ctDNA) into the bloodstream which can now be quantified and examined using novel high throughput sequencing technologies. This has led to the emergence of the 'liquid biopsy' which proposes to analyze this genetic material and extract information on a patient's cancer using a simple blood draw.

CONTENT: Circulating tumor DNA (ctDNA) has been detected in many advanced cancers. It has also been proven to be a highly sensitive indicator of relapse and prognosis. Sequencing the genetic material has also led to the discovery of mutations targetable by existing therapies. While ctDNA screening is more expensive, it is showing promise against circulating tumor cells and traditional cancer biomarkers. ctDNA has also been detected in other bodily fluids including cerebral spinal fluid, urine, saliva and stool.

The utility of ctDNA for early cancer detection is being studied. However, a blood test for cancer faces heavy obstacles such as extremely low ctDNA concentrations in early stage disease and benign mutations caused by clonal hematopoiesis, causing both sensitivity and specificity concerns. Nonetheless, companies and academic labs are highly active in developing such a test.

CONCLUSION: At this time, circulating tumor DNA is unlikely to perform at the high level of sensitivity and specificity required for early diagnosis and population screening. However, circulating tumor DNA in blood and other fluids has important clinical applications for cancer monitoring, prognosis and selection of therapy that require further investigation.

IMPACT STATEMENT: ctDNA testing is poised to usher in a new era of precision, individualized cancer treatment. Many promising results have been reported in monitoring for disease relapse, mutation profiling and treatment response. Nevertheless, problems with

sensitivity, specificity and clonal hematopoiesis have plagued the development of ctDNA tests to test asymptomatic patients for cancer.

Introduction

In the current cancer diagnostic pathway, a tumor is detected by imaging, prompted by a screening program, from clinical presentation or sometimes just by chance. Next, the patient usually undergoes a tissue biopsy. The sample is analyzed by a pathologist to determine if it is cancerous. If it is malignant and accessible, the tumor is usually resected surgically. The pathology analysis, combined with targeted DNA sequencing, is used to determine the need for, and type, of adjuvant chemotherapy.

Though tumor biopsies in some shape or form have been used for the last hundred years and form the basis of modern cancer treatment, they are by no means a perfect tool. Even with more sophisticated technology, such as needle biopsies, to remove tiny amounts of tissue, these procedures are still uncomfortable and invasive. Tumors are extremely heterogenous so sequencing only small lesions, as in the current paradigm, will not produce a comprehensive picture of all the mutations. Moreover, some tumors such as brain or lung neoplasms are not easily accessible, so a biopsy cannot be performed. In these cases, there is no knowledge of mutations to inform the optimum course of treatment. Finally, while biopsies cannot be repeated often due to their high morbidity, cancer is continuously mutating and evolving, meaning treatment can be based on data that are not timely or even relevant.

Recently, much attention has been focused on the emergence of a new cancer biomarker: circulating tumor DNA (ctDNA). Since 1946, it has been known that normal cells naturally

release DNA fragments resulting in cell free DNA (cfDNA) circulating in the blood stream **(1)**. In 1994, it was discovered that some cfDNA had mutations that originated from neoplasms **(2,3)**. Tumor cells (from primary tumors, micrometastases and overt metastases) release DNA into the circulation, known as circulating tumor DNA (ctDNA) **(4, 5)**. The mechanisms of this release are still uncertain; however, tumor cells are thought to secrete their DNA during necrosis or apoptosis **(6)**.

ctDNA is extremely fragmented and disordered, often into a laddering pattern of 170-180 base pairs **(7)**. Tissue damaging therapies to treat the cancer such as surgery, radiation and chemotherapy further disfigure the ctDNA **(8)**. Renal dysfunction can further confound data **(9)** as ctDNA is cleared through the kidneys and liver **(10)**. In the last decade, the advent of very sensitive, high throughput screening technologies such as BEAMing, Safe-SeqS, TamSeq and ddPCR means it is possible to isolate, quantify and sequence this DNA, bringing ctDNA applications even closer to the clinic.

Clinical Applications of ctDNA Liquid Biopsies

ctDNA can be harvested from a simple blood draw. It has a half-life of up to three hours meaning it must be processed rapidly **(11).** There is two to four times more ctDNA in plasma than serum, so generally, whole blood is centrifuged immediately after collection **(12)**. The ctDNA in the plasma is then analyzed/sequenced, forming a liquid biopsy.

This new biomarker is poised to confer many advantages over conventional biomarkers **(13, 14).** Assuming all regions of the tumor secrete ctDNA at the same rate, sequencing the genetic material will provide a far more comprehensive overview of all the mutations present in the tumor than just sequencing small lesions. This information can then be used to inform more

targeted treatment. Since liquid biopsies only involve drawing blood, they can be repeated far more often to provide an up to date overview of the mutations, ensuring consistent personalized treatment.

A groundbreaking study by Bettegowda et al. showed that ctDNA is detectable in more than 75% of advanced pancreatic, ovarian, colorectal, bladder, liver, gastroesophageal, breast, melanoma, liver and head and neck cancers **(15)**. Many more investigations have been performed and ctDNA has shown promise in managing many different cancers. Thus, the literature about the utility of ctDNA in monitoring for relapse, treatment progress and disease burden is rapidly expanding.

The accurate and early detection of metastases is crucial to ensure the assessment of response to treatment. Monitoring treatment response is also imperative as it enables clinicians to discontinue ineffective therapies, thus minimizing side effects and unnecessary costs. It also provides an opportunity to introduce novel therapeutics to target the new mutations detected in the tumor. ctDNA is showing a distinct advantage over conventional imaging methods as it provides longer lead times by detecting chemotherapy resistance or residual disease earlier. ctDNA screening to identify patients at high risk of relapse would provide high translational value in creating personalized adjuvant therapy protocols.

There have been extensive investigations about the utility of ctDNA for predicting relapse in individuals diagnosed with colorectal cancer. In a 2017 longitudinal study of 27 colorectal cancer patients, the presence of ctDNA immediately after surgical resection was strongly correlated with relapse. All 14 of the patients who eventually relapsed had ctDNA detectable after their resections, while all those who were ctDNA-negative post-surgery remained cancer free **(16).** Furthermore, ctDNA was detected an average of 9.4 months earlier in

the plasma of the individuals who relapsed before the tumors were visible by computerized tomography, providing a significant lead time to introduce new treatments. Similar results were observed by Tie et al, whose team used massively parallel sequencing-based assays to sequence plasma samples from 164 postoperative stage II colon cancer patients. Only 16 (9.8 %) of the patients with negative ctDNA following resection relapsed. In patients treated with adjuvant chemotherapy, the presence of ctDNA after completion was also associated with lower recurrence-free survival rates **(17).**

Another study of colorectal cancer used matched tumor samples to create personalized liquid biopsies. They used up to six assays per individual to detect specific somatic variants (SSVs) in 11 mid to late stage patients. The SSVs were both 100% sensitive and 100% specific in detecting postsurgical relapse. This approach provided 2–15 (average 10) months' lead time on detection of metastatic recurrence compared to typical post-surgical screening, providing compelling evidence of the ability of ctDNA to closely monitor patients' responses to treatment **(18)**.

A similar investigation was recently performed with a sub cohort of 24 early stage non-small cell lung cancer (NSCLC) patients. In summary, Abbosh et al. sequenced and compared tumor and healthy tissue from the primary surgical resections to identify the single nucleotide variants (SNVs) associated with cancer. They then used this a priori knowledge of mutations to design personalized ctDNA panels to check for relapse by scanning each patients' blood for mutated ctDNA. These liquid biopsies were able to detect relapse and therapy resistance in patients, an average of 70 days before tumors were observable on computed tomography (CT) scans, though in four cases the lead time was over 6 months **(19)**.

In breast cancer, a 2013 ground-breaking proof of concept study of 52 women with metastatic disease was able to correlate ctDNA with treatment response to systemic therapy. Dawson et al. used targeted sequencing to detect mutations in PIK3CA and TP53. While cancer associated mutations were only detected in 57% of patients' tumors, these mutations were in turn detected in the plasma of all but one (97 %) of this sub cohort. ctDNA levels were further shown to be proportional with tumor size, as detected on radiographic imaging, and a significant correlation between the number of ctDNA copies in the blood and patient outcomes was observed **(20)**.

Garcillas- Murillas et al showed the utility of ctDNA monitoring in early stage breast cancer. The group created personalized tumor-specific dPCR assays for 55 patients who received neoadjuvant chemotherapy with curative intent and predicted relapse far more sensitively than conventional clinical imaging methods, providing an average lead time of 7.9 months **(21)**.

The utility of ctDNA in monitoring disease prognosis, tumor burden and treatment response has been shown in several other malignancies including melanoma **(22, 23)**, head and neck cancer **(24),** lymphoma **(25)**, prostate **(26)**, liver **(27)**, gastric **(28)**, gynaecologic **(29)**, kidney **(30)** and pancreatic cancers **(31)**.

Though the technology is still in its' infancy, ctDNA analysis has shown translational value in detecting mutations that can be targeted by existing treatments. Chaudhuri et al used ctDNA deep sequencing (CAPP-seq) analysis to create personalized profiles of 40 stage I – III lung cancer patients who received chemotherapy with curative intent, and 54 healthy controls. 94% of patients who experienced recurrence had detectable ctDNA in the first post chemotherapy blood sample. Significantly, 53% of patients had mutation profiles targetable by immune checkpoint blockade or tyrosine kinase inhibitors **(32)**.

In colorectal cancer, KRAS mutations were identified in the ctDNA of 69% of patients and PIK3CA mutations in 17% of patients. These mutations are actionable by the multi-kinase inhibitor regorafenib, showing that ctDNA can help clinicians select personalized, targeted chemotherapies **(33)**.

More recently, a similar study was performed with 605 mid to late stage cancer patients with 28 different solid tumor types (61% lung cancer). Shu et al sequenced tumor specimens from biopsies and then used targeted next generation sequencing to sequence matched plasma samples. Somatic mutations were detected in the ctDNA of 87% of patients. The most frequently mutated tumor suppressor genes were *TP53* (18.1%), *APC* (3.3%) and *DNMT3A* (2.5%), and *EGFR* (11.9%), *KRAS* (3.7%) and *PIK3CA* (3.0%) were the most commonly mutated oncogenes. 71% of patients had at least one clinically actionable mutation (either with a drug approved or in clinical trials) **(34)**.

In 2016, therapeutically targetable and driver resistance mutations were detected in the ctDNA of advanced NSCLC cancer patients using massively parallel digital exome sequencing. Thompson et al detected 275 variants (SNVs, indels and fusions) in 102 patients without prior knowledge of primary tumor mutations. In total, mutations were detected in 45 genes, with EGFR mutations making up 20% of total variants. The group detected eleven KRAS mutations associated with resistance to EGFR inhibitors while also finding ten actionable ERBB2 mutations shown to be sensitive to targeted therapies. Furthermore, the variants were cross referenced against available treatments with auspicious results: 70% of patients had a relevant clinical trial, 55% patients had a potential off-label targeted therapy, and 31% of patients had variants targetable by an FDA-approved treatment. This study further compared the utility of sequencing tissue versus plasma samples for detecting variants in a 50-patient subgroup. ctDNA

plasma sequencing detected a mean number of 2.8 variants per patient while only 1.5 variants were detected per patient in tumor DNA screening, showing the advantage of ctDNA in encompassing tumor heterogeneity **(35)**.

Only one ctDNA "liquid biopsy" test has been approved so far by the Food and Drug Administration (FDA). The cobas EGFR Mutation Test v2 (Roche Molecular Systems) was approved in June 2016 and is intended to help clinicians decide if patients may benefit from erlotinib by screening for exon 19 deletion or exon 21 (L858R) substitution mutations in EGFR. (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm504488.htm) Consequently, ctDNA-based diagnostics are currently rarely used in the clinic.

The description and clinical utility of ctDNA technologies for prognosis, detection of relapse, selection of treatment, disease/ tumor burden and early detection can be found in **Table 1.**

ctDNA Compared to Alternatives

ctDNA sequencing for monitoring disease progression is showing advantages over traditional biomarkers. While conventional biomarker tests are far less expensive and much less labour -intensive than ctDNA testing, they have many weaknesses, mainly low sensitivity and specificity. Commonly used biomarkers such as CA-125, CEA and PSA are also found in the serum of patients without cancer, though usually in lower concentrations **(36, 37, 38)**. Furthermore, they are not elevated in a significant portion of patients with advanced cancer **(39, 40)**. By its' nature, ctDNA could not be found unless there is a tumor present and levels of ctDNA have been strongly correlated with disease burden. Improvements in sequencing would increase ctDNA testing sensitivity and specificity, as further decreasing the cost. From 2009 to

2014 alone, the cost of sequencing per genome dropped from $100,000 to $5,000 (including labour and reagents) **(41).** ctDNA has a shorter half-life compared to some traditional biomarkers that can stay in the circulation for weeks, meaning sequencing this material provides a highly current snapshot of the disease (**42**).

ctDNA is not just limited to blood-based applications. It has been detected in other readily available biological fluids which have the advantage of being closer to the carcinoma, and thus likely to contain more ctDNA.

Sequencing ctDNA from cerebral spinal fluid (CSF) is promising for managing brain cancer. While lumbar punctures are more invasive than venipunctures, the blood brain barrier dramatically reduces the amount of ctDNA in the blood, making the detection of brain cancer in the blood infeasible. However, ctDNA CSF biopsies for brain cancer have far lower morbidity than performing a traditional needle or open biopsy, which benefits patients. A 2017 study was able to detect histone H3 mutations in 8 pediatric patients with diffuse midline glioma. Their test sensitivity was 87.5% and specificity (100%) was validated through immunohistochemical staining and Sanger sequencing of available concordant tumor tissue specimens **(43).** Another study identified ctDNA in 100% (n=21) of all medulloblastomas, ependymomas, and high-grade gliomas that were adjacent to the CSF space **(44)**. This suggests that cancer DNA in CSF could be useful in managing of patients with primary tumors of the brain or spinal cord.

ctDNA in urine has also been found to be a useful biomarker in urologic malignancies **(45).** ctDNA was studied in the urine of a 68-patient sub cohort with non-invasive bladder transitional cell carcinoma who had undergone transurethral resection. Urinary cellular FGFR3 mutation was found to have a 73% sensitivity and 87% specificity in the diagnosis of cancer recurrence after resection **(46)**.

In lung adenocarcinoma, Pu et al. detected EFGR mutations in both saliva and plasma samples of 17 patients, finding a 100% concordance between saliva and tumor samples **(47)**. Saliva is also being investigated as a potentially valuable biomarker for head and neck cancers **(48)**.

Gathering ctDNA from stool has also been shown as a viable alternative to blood testing in colorectal cancer. A comparison between a plasma methylated test or a multi-marker stool test for SEPT9 in colorectal cancer patients demonstrated that sDNA test had significantly greater sensitivity than the ctDNA test (87% vs 60% sensitivity) **(49)**.

In the discussion of alternatives to ctDNA, it is integral to note continuing research in circulating tumor cells (CTCs). CTCs invasculate into the blood stream from primary tumors to eventually form metastases **(50, 51)**. They have been detected in many cancers **(52)**, including bladder **(53)**, gastric **(54)**, prostrate **(55, 56)** ovarian **(57)**, colon **(58)**, breast **(59)** and lung carcinomas **(60)**.

Extremely sensitive methods are required to detect these cells as there is one circulating cancer cell per billion of normal cells **(61)**. The Food and Drug Administration (FDA) has only approved one test for the quantification of CTCs. The CELLSEARCH CTC (Menarini-Silicon Biosystems) test is designed for patients with metastatic breast, prostate and colorectal cancers. The test enumerates CTCs of epithelial origin in whole blood using immunofluorescence. (https://www.cellsearchctc.com/)

An investigation of 19 patients with early breast cancer indicated that both CTC counts and ctDNA (*HER2* amplification) were predictors of metastatic disease **(62)**. In 2013, a study of metastatic colon cancer found a significant correlation between the presence of CTCs and the

detection of circulating DNA fragments containing tumor-specific mutations such as those in *KRAS* **(63)**.

The Dawson et al study also compared quantities of CTCs and CA-15.3 with imaging results and found ctDNA provided the greatest correlation with disease burden as well as the earliest indicator of response to treatment **(20)**.

ctDNA was often present in patients without detectable CTCs, indicating more research needs to be conducted to understand the relationship between these biomarkers **(15)**.

ctDNA Testing For Early Detection

Early cancer detection is often viewed as a new frontier in ctDNA technology. The paradigm behind using ctDNA to detect cancer is that tumors will secrete genetic material into the circulation before they are visible on imaging or produce symptoms. The development of this technology could eliminate the need for frequent imaging which carries the risk of radiation exposure. Detecting cancer early means that not only is the tumor smaller, but it is less complex, so it can be effectively treated with a milder therapy **(64).** It is also far less likely to have metastasized. Thus, the knowledge that early diagnosis leads to increased survival is the backstay of current screening programs.

Routine mammograms and PSA tests are stalwarts of the efforts to diagnose cancer early. However, both methods have serious flaws. Mammography can miss small tumors and breast cancer propagates rapidly. This means a woman who is reassured by a negative result one year can return the next to find she has a metastatic tumor. Prostate specific antigen (PSA) tests have the opposite flaw: low specificity. This leads to unnecessary overtreatment as well as distress. Consequently, both cancers could benefit from improved screening technologies (**65, 66, 67**).

Demonstrating any ctDNA test for early cancer detection has nearly 100% specificity and sensitivity is fundamental due to the perils associated with false negatives and false positives **(68).** Not only would patients and clinicians be deceptively reassured by a false negative blood test result, the carcinoma would likely not be treated until it became obvious on imaging or caused physical symptoms. Moreover, at that time, the tumor would be much larger, with more complex mutations and possibly even metastasized. False positives could cause distress, expensive additional tests to correct the result and even lead to overtreatment. Finally, even if clinicians receive a true positive result for an early tumor, it can be difficult to determine its location as the same mutations can occur across several cancers.

Despite these challenges, several cancers have been identified as attractive targets for early diagnosis via ctDNA screening. Pancreatic cancer has extremely high mortality and surgical resection is the only curative treatment. However, it is generally detected in its' late stages and the vast majority of patients (80% – 85%) present with inoperable advanced or metastatic cancer, causing a dismal 4% - 7% 5-year survival rate **(69)**. However, pancreatic cancer is extremely slow developing. There is an average 11.7-year period from the acquisition of the initiating mutation to a full tumor and another 6.8 years needed to develop the first metastatic sub clone **(70)**. Its' mutations have been widely studied; KRAS, CDKN2A, TP53 and SMAD4 are known to be commonly altered **(71)**. A liquid biopsy test to detect pancreatic cancer in asymptomatic individuals could save thousands of lives.

Lung cancers such as Small-Cell Lung Cancer (SCLC) are also good targets. Lung cancer has the highest mortality rates of all cancers and there are no good treatment options at late stages. SCLC patients have almost universal inactivation of TP53, a mutation potentially targetable with existing therapies. A 2016 study of 51 SCLC patients and 123 healthy controls

detected TP53 mutations in the plasma of 59.1% of late stage patients but only 35.7% of early stage patients **(72).**

Slightly more promising results were reported by Bettegowda in 2014. The group used digital PCR and found ctDNA in less than 50% of patients with primary brain, renal, prostate and thyroid cancers. In patients with localized tumors, the detection rate was 50% (breast), 48 % (pancreatic), 53% (gastroesophageal) and 73 % (colorectal) **(15).**

In NSCLC, ctDNA was detected in 100% of patients with stage II–IV disease and in 50% of patients with stage I cancer, using CAPP -Seq, one of the most sensitive sequencing methods available due to its' combined approach of isolating ctDNA, enhanced sample preparation and extremely targeted deep sequencing **(73)**. This approach is economically viable; with cost around $500 to process each sample, including materials and labour **(74)**.

Challenges of Using ctDNA for Early Diagnosis

A prior knowledge of the tumor mutations is essential for the functioning of the most sensitive ctDNA sequencing techniques. Most studies garner this knowledge by sequencing tissue biopsy samples and designing personalized tests. This labour-intensive approach is required for each patient. Thus, these methods could not be used in detection of disease; by the nature of this process there would be no prior biopsy sample to work off.

Currently, exome sequencing is the only method for sequencing ctDNA without knowing the tumor mutations. This method is extremely expensive, especially to sequence at the depth required for primary tumors. Consequently, there are limited studies using this technique in the literature.

Murtaza et al performed an unguided search for deleterious mutations in blood samples by sequencing the exome of a small cohort (n =6) of patients with advanced ovarian, lung and breast cancers. In 5 of the cases, exome sequencing revealed specific results such as an activating mutation in PIK3CA after treatment with paclitaxel or a resistance-conferring mutation in EGFR following treatment with gefitinib **(75)**.

Even if exome sequencing methods become less prohibitively expensive, fundamental problems still remain in using ctDNA for early detection. Cancer is an extremely heterogenous disease with layers of mutations. However, new discoveries about clonal hematopoiesis have shown that not all mutations are cancer-causing or lead to tumors.

Spontaneous mutations occur in a person's cells throughout their lifetime and most do not have deleterious or even noticeable effects. However, mutations during gestation can cause developmental disabilities, while accumulating somatic mutations can progressively cause aging and cancer. The rate of mutation is positively correlated with age **(76)**. In a study of 26 136 cancer-free individuals, the frequency of somatic mutations increased with age, from 0.23% under 50 years to 1.91% between 75 and 79 years **(77)**. In those under 50 years of age, the frequency of detectable clonal mosaicism in peripheral blood is low (less than 0.5%) but rapidly rises to 2-3% in the elderly **(78)**.

Genovese et al performed a massive study of 12380 individuals aged 19 -93. They used whole exome sequencing on peripheral blood cells and followed subjects for 2 -7 years. They observed clonal hematopoiesis in 10% of people over 65 years of age, and only 1% in those under 50. Significantly, they found some of the genes which are mutated in patients with myeloid cancers are also mutated in healthy individuals and do not cause cancer. Subsequently,

they found the risk of an individual with clonal haematopoiesis developing cancer was only 1.0% per year **(79).**

Another study reported mutations in individuals who remained cancer-free over a 6 year follow up period. KRAS2 was mutated with a frequency of 1.2% and p53 was mutated 3.6% in healthy volunteers **(80)**. In 2016, Fernandez-Cuesta reported an even higher mutation rate: 11% of healthy controls had detectable cfDNA p53 mutations **(72)**. Mutations of p53 in normal individuals were also reported by Newman et al **(81)**.

Looking Forward

In spite of all these challenges, at least one company (GRAIL) was founded in 2016 to develop a blood test for early stage cancer in asymptomatic individuals, to detect tumors so tiny, they cannot be viewed on imaging Their current plan is to sequence ctDNA from thousands of blood samples at an incredible depth. (https://grail.com/about).

In August 2016, GRAIL launched the Circulating Cell-Free Genome Atlas Study (CCGA) in an attempt to characterize the mutational heterogeneity of cancer as well as distinguish benign and cancerous mutations. They aim to collect contemporaneous blood and tumor samples from 10 500 patients with early stage malignancies and blood samples from 4500 healthy controls. The estimated completion date is September 2023. (https://clinicaltrials.gov/show/NCT02889978)

Since February 2017 GRAIL has also been enrolling participants in their STRIVE breast cancer study, an observational, longitudinal investigation of 120,000 women undergoing routine mammography. Blood samples will be collected and the cfDNA sequenced, and the women will

be followed for five years to see if they receive a cancer diagnosis. (https://clinicaltrials.gov/ct2/show/NCT03085888).

GRAIL released its' first abstracts in June 2017 during the Annual Meeting of the American Society of Clinical Oncology. (http://www.globenewswire.com/news-release/2017/06/03/1007917/0/en/GRAIL-Announces-Data-From-Early-Research-Analyzing-its-High-Intensity-Sequencing-Approach.html). They sequenced tumor tissue and ctDNA from blinded blood and tissue from 124 patients with metastatic breast, NSCLC and castration resistant prostate cancer. The main novelty is their high throughput approach; sequencing the cfDNA at 60 000x raw depth using a 508-gene panel. They detected at least one variant in plasma that was previously detected in tumor tissue in 90% of the patients.72% of the actionable mutations that were detected in tumor tissue were also detected in plasma **(82**).

GRAIL also acknowledges the challenge of clonal haematopoiesis in its twin abstract studying mutations in 151 metastatic cancer patients and 47 healthy volunteers. It found that both groups had mutations in their blood, most commonly in DNMT3A, TET2, PPM1D, and TP53, and the rate of mutations increased with age. This makes developing their proposed ctDNA test even more challenging **(83)**.

The development of a plasma test for early stage cancer detection is a noble cause and could save millions of lives. However, more research is required to investigate the challenges that this test must surmount. A standardized process e.g. reagents, equipment and procedure must be established to homogenize the collection of ctDNA **(42)**.

Other challenges may be more difficult to overcome. Regardless of the sensitivity of the sequencing technology, there simply may not be enough ctDNA in a sample from an early stage cancer patient to allow detection.

Very small tumors, for example those with 5-mm diameter, are unlikely to cause clinical symptoms or be visible on imaging. While the detection of smaller tumors e.g. 1 mm diameter is the goal of some institutions, these tumors are more likely to be benign and may be overtreated **(84)**. Five mm diameter tumors have only a 6% chance of progressing and are also very localized, less complex and easier to cure **(85)**. However, only 26% of these tiny malignancies are detected on imaging **(86)** e.g. mammography which has a detection limit of 4 mm lesions **(87)**.

Assuming a spherical neoplasm, and based on published reports **(88)**, we recently calculated that a tumor volume of 1 cm^3 or 12.5 mm in diameter **(89)** will release into the circulation enough ctDNA to represent 0.01% of all circulating DNA **(90)**. We have further shown that this fraction of ctDNA is equivalent to 1 mutant genome equivalent per 4 ml of plasma (10 ml blood draw). These data led us to conclude that ctDNA could be used to detect tumors of approximately 10 mm in diameter but not smaller **(89)**. A visual representation of these data can be found in **Figure 1**.

These predictions were recently verified by two high-profile reports on the use of ctDNA for early cancer diagnosis. Phallen et al and Cohen et al reported good sensitivities and specificities for detecting early stage cancers but all of their patients had fractional ctDNA greater than 0.01% and all patients were symptomatic **(90, 91)**.

Based on current data, it is unlikely ctDNA has high enough sensitivity or specificity for use in early detection in asymptomatic patients. Clearly this is a very active area of investigation which promises to produce data that are superior to those delivered by the classical cancer biomarkers.

References

1. Mandel P. and Métais P. Les acides nucléiques du plasma sanguin chez l'homme. C. R. Séances Soc. Biol. 1948; 142: 241–43.

2. Vasioukhin V, Anker P, Maruice P, Lyautey J, Lederrey C, and Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br. J. Haematol. 1994; 86: 774–9.

3. Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ and Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood.Cancer Epidemiol. Biomarkers Prev. 1994; 3: 67–71.

4. Crowley E, Di Nicolantonio E, Loupakis F, and Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat. Rev. Clin. Oncol. 2013; 10: 472-84.

5. Heitzer E, Ulz P, and Gegil JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin. Chem. 2015; 61: 112-23.

6. Choi JJ, Reich, CF.III, and Pietsky DS. The role of macrophages in the in vitro generation of extracellular DNA from apoptotic and necrotic cells. Immunology. 2005; 115: 55-62.

7. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001; 61: 1659-65.

8. Alix-Panabieres C and Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov. 2016; 6: 479-91.

9. Kirsch C, Weickmann S, Schmidt B, and Fleischhacker M. An improved method for the isolation of free-circulating plasma DNA and cell-free DNA from other body fluids. Ann. N. Y. Acad. Sci. 2008; 1137: 135–9.

10. Elshimali YI, Khaddour H, Sarkissyan M, Wu Y, and Vadgama JV. The clinical utilization of circulating cell free DNA (CCFDNA) in blood of cancer patients. Int. J. Mol. Sci. 2013; 14: 18925–58.

11. Meng S, Tripathy D, Frenkel EP, et al. Circulating tumor cells in patients with breast cancer dormancy. Clin. Cancer Res. 2004; 10: 8152–62.

12. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, and Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin. Chem. 2003; 49: 1028–9.

13. Jiang P and Lo YM. The long and short of circulating cell-free DNA and the ins and outs of molecular diagnostics. Trends Genet. 2016; 32: 360-71.

14. Chan KC and Lo YM. Circulating tumour-derived nucleic acids in cancer patients: potential applications as tumour markers. Br. J. Cancer. 2016; 96: 681-5.

15. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci. Transl. Med. 2014; 6: 224ra24.

16. Scholer LV, Reinert T, Orntoff MW, et al. Clinical implications of monitoring circulating tumor DNA in patients with colorectal cancer. Clin. Cancer Res. 2017; 23: 5437-45.

17. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci. Transl. Med. 2016; 8: 356ra92.

18. Reinert T, Scholer LV, Thompsen R, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2016; 65: 625-34.

19. Abbosh C, Birkbak NJ, Wilson GA, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. Nature. 2017; 545: 446 – 51.

20. Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. N. Engl. J. Med. 2013; 368: 1199-201.

21. Garcillas-Murillas I, Schiavon G, Weigelt B, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci. Transl. Med. 2015; 7: 302ra133.

22. Gray ES, Rizos H, Reid AL et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget. 2015; 6: 420000-18.

23. Lee RJ, Gremel G, Marshall A, et al. Circulating tumor DNA predicts survival in patients with resected high risk stage II/III melanoma. Ann. Oncol. 2017. Epub.

24. Van Ginkel JW, Huibers MMH, Van Es RJJ, de Bree R and Willems SM. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. BMC Cancer. 2017; 17: 428.

25. Scherer F, Kurtz DM, Newman AM, et al. Distinct biological subtypes and patterns of genome evolution in lymphoma revealed by circulating tumor DNA. Sci. Transl. Med. 2016; 8: 364ra24.

26. He W and Bishop KS. The potential use of cell-free-circulating-tumor DNA as a biomarker for prostate cancer. Expert Rev Mol. Diagn. 2016; 8: 839-52.

27. Ono A, Fujimoto A, Yamamoto Y, et al. Circulating Tumor DNA Analysis for Liver Cancers and Its Usefulness as a Liquid Biopsy. Cell Mol. Gastroenterol. Hepatol. 2015; 1: 516-34.

28. Hamakawa T, Kukita Y, Kurokawa Y, et al. Monitoring gastric cancer progression with circulating tumour DNA. Br. J. Cancer. 2015; 1: 352-56.

29. Pereira E, Camacho-Vanegas O, Anand S, et al. Personalized circulating tumor DNA biomarkers dynamically predict treatment response and survival in gynecologic cancers. PLoS One. 2015; 10: e0145754.

30. Fenner A. Kidney cancer: Tracking and therapy selection using ctDNA. Nat. Rev. Urol. 2017; 14: 389.

31. Cheng H, Liu C, Jiang J et al., Analysis of ctDNA to predict prognosis and monitor treatment responses in metastatic pancreatic cancer patients. Int. J. Cancer. 2017; 140: 2344-50.

32. Chaudhuri HH, Chabon JJ, Lovejoy AF, et al. Early detection of molecular residual disease in localized lung cancer by circulating tumor DNA profiling. Cancer Discov. 2017; 7: 1394-1403.

33. Tabernero J, Lenz HJ, Siena S, et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: a retrospective, exploratory analysis of the CORRECT trial. Lancet Oncol. 2015; 16: 937-48.

34. Shu Y, Wu X, Tong X, et al. Circulating tumor DNA mutation profiling by targeted next generation sequencing provides guidance for personalized treatments in multiple cancer types. Sci. Rep. 2017; 7: 583.

35. Thompson JC, Yee SS, Troxel AL, et al. Detection of therapeutically targetable driver and resistance mutations in lung cancer patients by next generation sequencing of cell-free circulating tumor DNA. Clin. Cancer Res. 2016; 22: 5772-82.

36. Mazzucchelli R, Colanzi P, Pomante R, Muzzonigro G and Montironi R. Prostate tissue and serum markers. Adv Clin. Pathol. 2000; 4:111–20.

37. Ruibal Morell A. CEA serum levels in non-neoplastic disease. Int J Biol Markers. 1992; 7: 160–66.

38. Sikaris KA. CA125—A test with a change of heart. Heart Lung Circ. 2011; 20: 634–40.

39. Ballehaninna UK, and Chamberlain RS. Serum CA. 19-9 as a biomarker for pancreatic cancer—A comprehensive review. Indian J. Surg. Oncol. 2011; 2: 88–100.

40. Wanebo HJ, Rao B, Pinsky CM, et al. Preoperative carcinoembryonic antigen level as a prognostic indicator in colorectal cancer. N Engl. J Med. 1978; 299: 448–51.

41. Leung F, Kulasingam V, Diamandis EP, Hoon DS, Kinzler K, Pantel K, and Alix-Panabieres, C. Circulating tumor DNA as a cancer biomarker: fact or fiction? Clin. Chem. 2016; 62: 1054–60.

42. Wang R, Li X, Zhang H, and He J. Cell-free circulating tumor DNA analysis for breast cancer and its clinical utilization as a biomarker. Oncotarget. 2017; 8: 75742-55.

43. Huang TY, Piunti A, Lulla R, et al. Detection of Histone H3 mutations in cerebrospinal fluid-derived tumor DNA from children with diffuse midline glioma. Acta. Neuropathol. Commun. 2017; 5: 28.

44. Wang Y, Springer S, Zhang M, et al. Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord. Proc. Natl. Acad. Sci. U.S.A. 2015; 112: 9704-9.

45. Di Meo A, Bartlett J, Cheng Y, Pasic MD and Yousef GM. Liquid biopsy: a step forward towards precision medicine in urologic malignancies. Mol. Cancer. 2017; 16: 80.

46. Couffignal C, Desgrandchamps F, Mongiat-Artus P, et al. The diagnostic and prognostic performance of urinary FGFR3 mutation analysis in bladder cancer surveillance: a prospective multicenter study. Urology. 2015; 86: 1185–90.

47. Pu D, Liang H, Wei F, et al. Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: A pilot study. Thorac. Cancer. 2016; 7:428–36.

48. Van Ginkel JH, Slieker FJB, de Bree R, van Es RJJ, Van Cann EM, and Willems SM. Cell-free nucleic acids in body fluids as biomarkers for the prediction and early detection of recurrent head and neck cancer: A systematic review of the literature. Oral Oncol. 2017; 75: 8-15.

49. Berger BM and Ahlquist DA. Stool DNA screening for colorectal neoplasia: biological and technical basis for high detection rates. Pathology. 2012; 44:80–8.

50. Ignatiadas M, Lee M, and Jeffrey SS. Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. Clin. Cancer Res. 2015; 21: 4786-800.

51. Millner LM, Linder MW and Valdes R. Jr. Circulating tumor cells: a review of present methods and the need to identify heterogeneous phenotypes. 2013; 43: 295-304.

52. Lianidou ES. Gene expression profiling and DNA methylation analyses of CTCs. Mol. Oncol. 2016; 10: 431-42.

53. Naoe M, Ogawa Y, Morita J, et al. Detection of circulating urothelial cancer cells in the blood using the CellSearch System. Cancer. 2007; 109: 1439-45.

54. Wolfrum F, Vogel I, Fändrich F, and Kalthoff H. Detection and clinical implications of minimal residual disease in gastro-intestinal cancer. Langenbecks Arch. Surg. 2005; 390:430-41.

55. Markou A, Lazaridou M, Paraskevopoulos P, et al. Multiplex gene expression profiling of in vivo isolated circulating tumor cells in high-risk prostate cancer patients. Clin. Chem. 2018; 64: 297-306.

56. Makarovskiy AN, Ackerley W 3rd, Wojcik L, Halpert GK, Stein BS, Carreiro MP, and Hixson DC. Application of immunomagnetic beads in combination with RT-PCR for the detection of circulating prostate cancer cells. J. Clin. Lab. Anal. 1997; 11: 346-50.

57. Choy A and McCulloch PG. Detection and enumeration of circulating tumour cells in colorectal cancer. Br. J. Surg. 1993; 80: 1490.

58. Giannopoulou L, Kasimir-Bauer S, and Lianidou ES. Liquid biopsy in ovarian cancer: recent advances on circulating tumor cells and circulating tumor DNA. Clin. Chem. Lab. Med. 2018; 56: 186-197.

59. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N. Engl.J. Med. 2004; 351: 781-91.

60. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin. Cancer Res. 2004; 10: 6897-904.

61. Zhang Z, Ramnath N and Nagrath S. Current status of CTCs as liquid biopsy in lung cancer and future directions. Front Oncol. 2015; 5: 209.

62. Shaw JA, Brown J, Coombes RC, et al. Circulating tumor cells and plasma DNA analysis in patients with indeterminate early or metastatic breast cancer. Biomark. Med. 2011; 5: 87-91.

63. Heitzer E, Auer M, Hoffman EM, et al. Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer. Int. J. Cancer. 2013; 133: 346-56.

64. Aravanis AM, Lee M, and Klausner RD. Next-Generation sequencing of circulating tumor DNA for early cancer detection. Cell. 2017; 168: 571-4.

65. Diamandis, EP. Cancer dynamics and the success of cancer screening programs. Clin. Chem. Lab Med. 2016; 54: e211-2.

66. Diamandis, EP., Li M. The side effects of translational omics: overtesting, overdiagnosis, overtreatment. Clin. Chem. Lab Med. 2016; 54: 389-96.

67. Esserman, L., Shieh, Y. and Thompson, I. Rethinking screening for breast cancer and prostrate cancer. JAMA. 2009; 302; 1685-92.

68. Wentzensen N, and Wacholder S. From differences in means between cases and controls to risk stratification: a business plan for biomarker development. Cancer Discov. 2013; 3: 148-57.

69. Vincent A, Herman J, Schulick R, Hruban RH, and Goggins M. Pancreatic cancer. Lancet. 2011; 378: 607-20.

70. Takai E and Yachida SD. ctDNA as a liquid biopsy target for detection of pancreatic cancer. World J. Gastroenterol. 2016; 22: 8480-88.

71. Bailey P, Chang DK, Nones K, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature. 2016; 531: 47-552.

72. Fernandez -Cuesta F, Perdomo S, Avogbe PH, et al. Identification of circulating tumor DNA for the early detection of small-cell lung cancer. EBioMedicine. 2016;10: 117-23.

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

74. Spellman PT and Gray JW. Detecting cancer by monitoring circulating tumor DNA. Nat. Med. 2014; 20: 474-5.

75. Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013; 497; 108-12.

76. Martincorena I, and Campbell PJ. Somatic mutation in cancer and normal cells. Science. 2015; 349: 1483-9.

77. Jacobs KB, Yeager M, Zhou W, et al. Detectable clonal mosaicism and its relationship to aging and cancer. Nat. Genet. 2012; 44: 651-8.

78. Laurie CC, Laurie CA, Rice K, et al. Detectable clonal mosaicism from birth to old age and its relationship to cancer. Nat. Genet. 2012; 6: 642-50.

79. Genovese G, Kahler AK, Handsaker RE, et al. Clonal hematopoiesis and blood- cancer risk inferred from blood DNA sequence. N. Engl. J. Med. 2014; 371: 2477-87.

80. Gormally E, Vineis E, Matullo G, et al. TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: a prospective study. Cancer Res. 2006; 66: 6871-6.

81. Newman, AM., Lovejoy, AF., Klass, DM, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol. 2016; 34: 547-55.

82. Razavi R, Li BT, Abida W, et al. Performance of a high-intensity 508-gene circulating-tumor DNA (ctDNA) assay in patients with metastatic breast, lung, and prostate cancer. J Clin Oncol. 2017;35: Ahead of print.

83. Rasavi P, Li BT, Hou C, et al. Cell-free DNA (cfDNA) mutations from clonal hematopoiesis: Implications for interpretation of liquid biopsy tests. J. Clin. Oncol. 2017; 35: Ahead of print.

84. Garber K. Ontario institute offers new model of cancer research. J. Natl. Cancer Inst. 2008; 100: 980–82.

85. Countercurrents Series, Narod SA. Disappearing breast cancers. Curr. Oncol. 2012; 19: 59–60.

86. Weedon-Fekjaer H, Lindqvist BH, Vatten LJ, et al. Breast cancer tumor growth estimated through mammography screening data. Breast Cancer Res. 2008; 10: R4.

87. Schwaederle M, Husain H, Fanta PT, et al.: Detection rate of actionable mutations in diverse cancers using a biopsy-free (blood) circulating tumor cell DNA assay. Oncotarget. 2016; 7: 9707–17.

88. Del Monte U. Does the cell number 109 still really fit one gram of tumor tissue? Cell Cycle. 2009; 8: 505-6.

89. Diamandis EP, and Fiala C. Can circulating tumor DNA be used for direct and early stage cancer detection? F1000 Res. 2017; 6: 2129.

90. Phallen J, Sausen M, Adleff V, et al. Direct detection of early-stage cancers using circulating tumor DNA.Sci Transl. Med. 2017;9: eaan2415.

91. Cohen JD, Li L, Wang Y, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018;359:926-930

Table 1: Potential applications of ctDNA in cancer diagnostics and management.

Application	Summary	Potential for clinical utility	References
Prognosis	• Undetectable ctDNA in the blood stream after surgery are correlated with improved prognosis and smaller chances of relapse • Strength and type of chemotherapy can be informed by ctDNA analysis showing likelihood of relapse	Excellent	13-31, 70, 88,89
Monitoring treatment efficacy and early relapse detection	• Increased number of mutations or rising ctDNA concentration can indicate treatment failure, resistance and relapse	Excellent, maybe in combination with protein markers	13-20, 23-29
Selection of treatment	• Knowledge of mutations in the ctDNA informs choice of therapy (personalized treatments) • ctDNA avoids tumor heterogeneity issues by providing overview of all the mutations in the tumor (assuming all tumor cells secrete DNA at the same rate)	Excellent	17, 28, 30-33, 73
Tumor size/ disease burden	• Larger amount of ctDNA in blood denotes advanced tumor stage and volume • Blood testing does not carry the risk of radiation exposure or poor accuracy of imaging and can be repeated more often than traditional biopsies.	Excellent, especially in combination with imaging	15, 17,18, 20-29, 88, 89
Detection of cancer in asymptomatic individuals /population screening	• Most studies show poor sensitivity, especially for early stage tumors • For small tumors, there is not enough ctDNA present to allow for an accurate test result • Threshold appears to be 1cm diameter tumors	Under intense investigation; could be used in combination with protein biomarkers	13, 30, 70, 88, 89

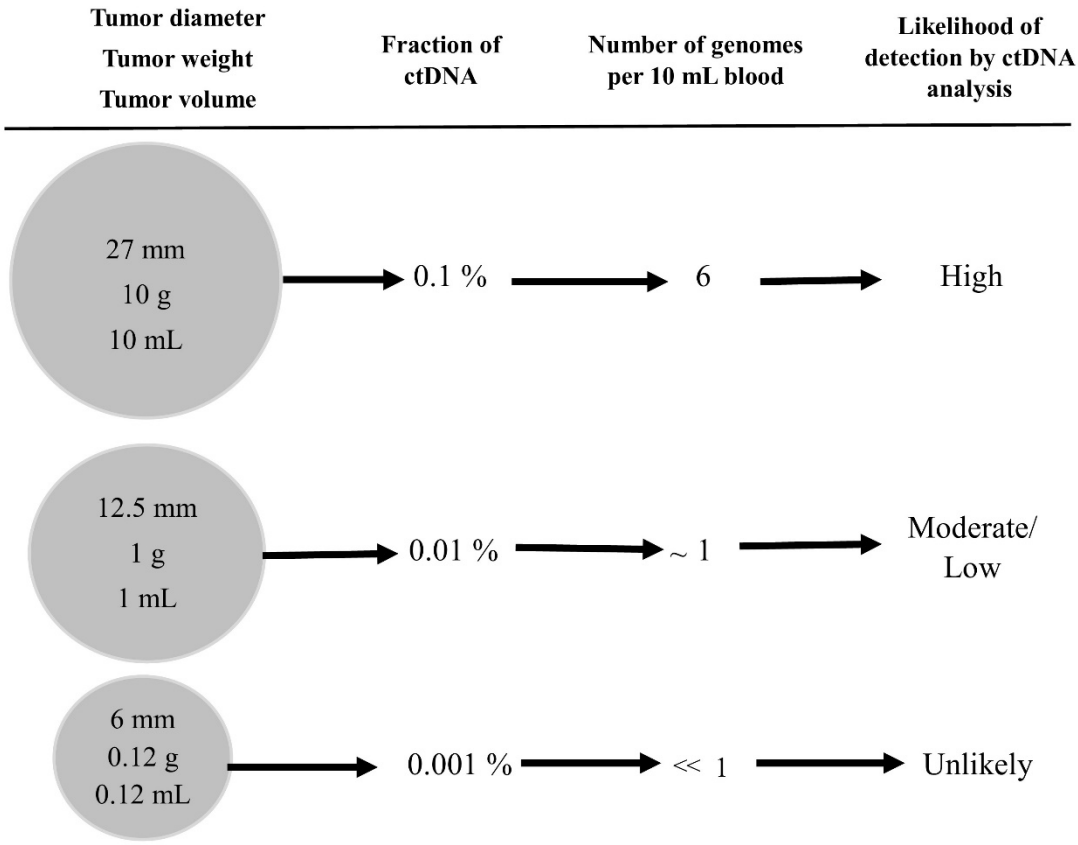

Figure 1: Likelihood of detecting tumors of various sizes through ctDNA quantification.