Clinical Background

Introduction

The incidence and prevalence of certain cancers are undeniably and remarkably increasing. There is a great number of complexities to the causes and difficulties in treating cancer. As it is known, there are a number of factors that play into what causes cancer or predisposes one to cancer which include, but are not limited to: biological, environmental, and lifestyle factors. DNA derived from tumor cells, also known as circulating tumor DNA (ctDNA), may be the answer to how we confer treatment options and monitor patients, in light of tumor subtypes, therapy resistance, and genetic alterations that occur in tumors.

In the presence of a tumor, circulating tumor DNA is released into the plasma either actively, through the secretion of vesicles, or passively, through cell necrosis and apoptosis (Figure 1) (1).

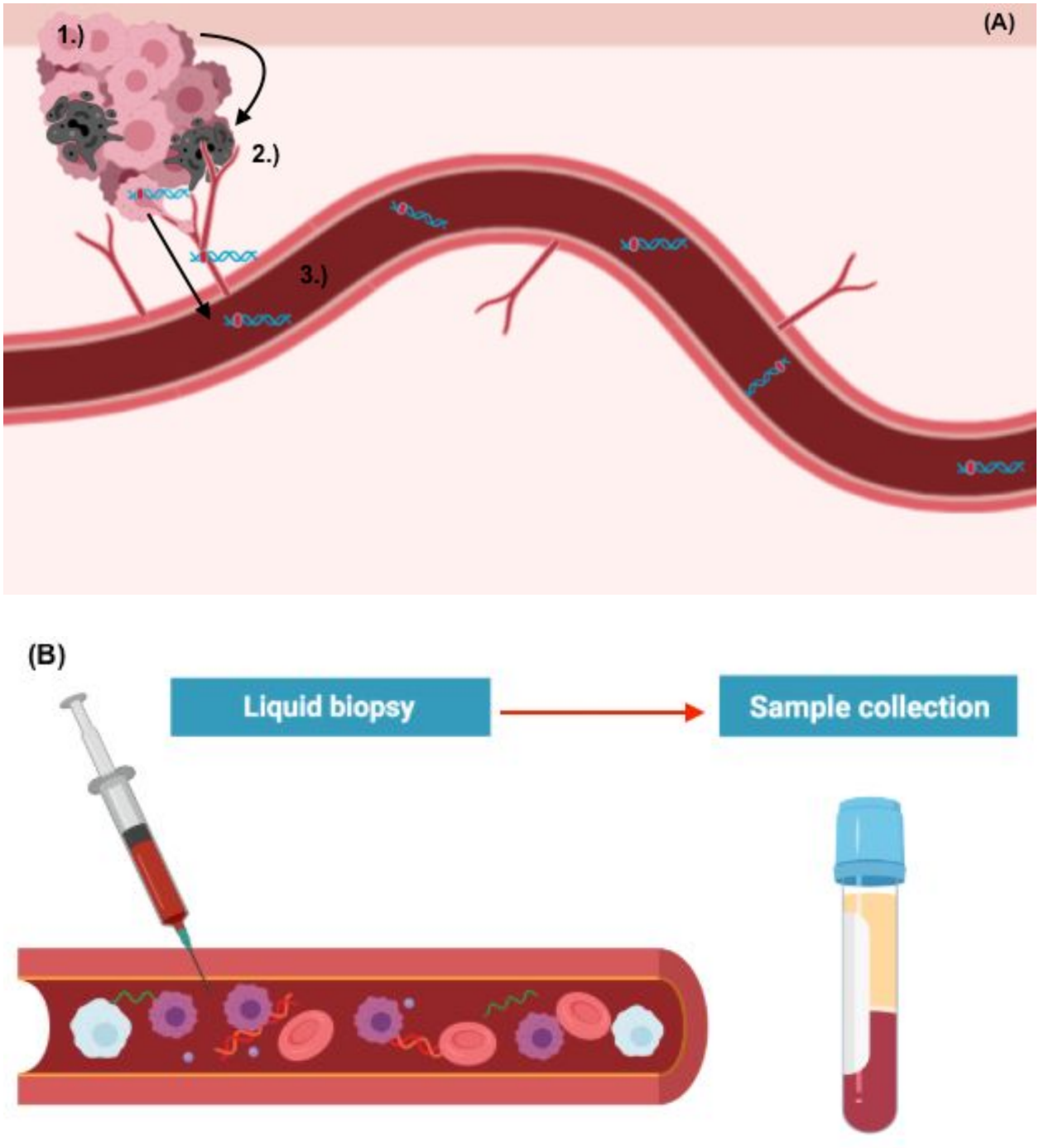


Figure 1:(A) In the presence of tumor cell proliferation, ctDNA is excised into the plasma through apoptosis. (B) A sample of patient's blood is taken via liquid biopsy. The ctDNA is then separated from the rest of the plasma contents for further analysis.

The ctDNA can then be extracted from the plasma from a blood draw as seen in Figure 1b. The amount of ctDNA present in the plasma and the rate in which it is released into the plasma is correlated with the size, vascularity, and location of the tumor(1). Upon blood draw, ctDNA can be analyzed for point mutations, epigenetic modifications, large or small chromosomal indels, translocations, amplifications, or deletions since it is genetic material derived from a direct source (2). Many challenges exist when obtaining a biopsy, such as its invasive nature, but liquid biopsy of ctDNA reconcile these obstacles to aid in the areas of screening, tumor staging, mutation profiling, therapy selection, and therapy resistance or response (1). Additionally, liquid biopsies can be easily repeated for routine tests that become necessary after initial diagnosis. Often, the original tissue biopsy is no longer viable or has already been exhausted.

The ease of a standard sized liquid blood draw could be readily obtained to perform the desired assessment of equal value. Therefore, the use of liquid biopsies for ctDNA detection allows for widespread implementation in various settings to help fill current methodological gaps in which cancer biopsies tend to be necessary. To this regard, ctDNA drives the development and implementation of liquid biopsies in cancer diagnostics. However, with all the exciting possibilities offered by ctDNA in a plethora of cancers, research has deviated from targeting specific cancer niches, and instead moving towards applications in all types of oncological treatments. One example of how ctDNA liquid biopsies have already begun filling such roles, for areas of identified unmet clinical needs, is its implementation in non-small cell lung cancer

(NSCLC). The first liquid biopsy to be FDA approved using ctDNA for the diagnosis of NSCLC resulted from the gap in the ability to obtain pleural biopsies, as well as the amount deemed safe for extraction (3). Therefore, the first FDA approved device using ctDNA extracted from liquid biopsies to diagnose and treat NSCLC aided in obtaining a substantial biopsy without removing too much tissue; subsequently, eliminating the need to perform an invasive procedure that may ultimately expose the patient to certain risks such as infection. Furthermore, current biopsies for those with lung cancer lean towards being costly, invasive, and risky — resulting in the monumental developmental demand for obtaining a liquid biopsy instead (3).

Unmet Clinical Needs: *Ovarian Cancer*

Ovarian cancer remains one of the leading causes of death in women from gynecologic malignancy. This cancer results from genetic alterations in oncogenes and tumor suppressor genes involved in growth and cell apoptotic pathways. Proto-oncogenes c-myc, K-ras and Akt are often mutated or amplified in several types of ovarian carcinoma (4). Mutation of p53 tumor suppressor gene is frequently found to be mutated in over 50% of early-stage and advanced stage ovarian cancer. A family history of cancer is the strongest risk factor for developing ovarian cancer. Risk of invasive epithelial ovarian cancer increases by 50% amongst women with first-degree relatives with a history of ovarian cancer (5) . However, 40% of ovarian cancer cases are caused by inherited mutations in BRCA1 and BRCA2. These mutations are more prevalent in certain ethnic groups such as those of Ashkenazi Jewish descent. It's recommended for post-menopausal women with BRCA1, BRCA2 mutations to undergo bilateral salpingo-oopherectomy which decreases risk of ovarian cancer by 80% (4). Reliable screening modalities for genetic alterations in oncogenic drivers, tumor suppressor p53 gene, and

BRCA1/BRCA2 genes must be developed for purposes of genetic counseling and treatment selection.

Liquid biopsy offers a wide range of benefits, which translates to its potential uses in key applications, as aforementioned, that current standards of care methods are not well suited for. One application is the ability to detect cancer at an early stage when treatment results in increased successful outcomes during prognosis of cancer. For example, ovarian cancer itself currently does not have a specific screening method (6). For invasive epithelial ovarian cancer, the five year survival rates dramatically decrease from 92% in stage one to 75% in late stage 2 cancer (7). Due to the lack of screening methods, only about 20% of ovarian cancers are detected in the early stages; however, when patients are diagnosed early on, it has been found that over 94% of these patients have prognoses beyond the five year mark (8).

It is possible that intervention with screening via ctDNA may increase the survival rates of patients. Furthermore, treatment options for ovarian cancer are limited to surgical resection, chemotherapy, and palliative care. According to Vaughan et. al, instead of treating invasive epithelial ovarian cancer as one disease, as it is being treated now, it needs to move in a direction where it is based off of the many subtypes and molecular profiles of the tumor present (9). In other words, lack of treatment options is due to the limited knowledge regarding stratification of ovarian tumor drivers, thus leading to this problematic single treatment approach. Another gap in the current standard of care for ovarian cancer that could be bridged by ctDNA is in post-operative monitoring and tracking therapeutic response. In this single treatment model, surgery, chemotherapy, and/or radiation may not be conducive to a patient's tumor in the presence of intratumoral heterogeneity and possible recurrence (9). Here, we see how ctDNA has the

potential ability to both analyze tumor molecular profiles and allow for clinical management specifically tailored to the patient by monitoring treatment response.

Unmet Clinical Needs: *Colorectal Cancer*

Genomic and epigenetic mutations are common in the development of colorectal cancer (CRC). The most pertinent pathway of carcinogenesis is the chromosomal instability pathway. In this pathway, genomic alterations include activation of proto-oncogene K-Ras and inactivation of tumor suppressor genes APC, TP53 and loss of heterozygosity on the long arm of chromosome 18 (10). Molecular profiling of the CRC tumor can help determine epigenetic silencing of mismatch repair genes MLH1, MSH2. These genetic alterations can be detected during the initiation of adenoma, before mutation of APC and TP53 genes which progresses towards malignancy (10).

An additional application of ctDNA's critical ability to monitor treatment and tumor staging throughout the entire course of cancer is in patients with CRC. In CRC, tumor staging follows immediately after cancer diagnosis. After surgical removal, the final stage of the tumor can then be determined. Tumor burden and residual disease continues to be the most important prognostic factors in patients with CRC. However, there is a lack of modalities for effectively monitoring response to drug treatment. Detection of ctDNA could be a potential indicator of residual cancer after resection. A study from Lecomte et al. focused on KRAS mutations in patients with CRC and demonstrated that patients with no detectable ctDNA with KRAS mutations had 100% 2-year survival rate (11). Lecomte et al. therefore suggests the prognostic value for ctDNA markers, and that the presence of such may be used to identify patients at risk for recurrence. A study from Diehl et al. confirms this, finding that patients who had detectable

ctDNA after surgery relapsed within the year (12). High concentrations of cfDNA with mutant KRAS were strong indicators of a dismal outcome in patients with metastatic CRC. Liquid biopsy can also be used to monitor response to therapy, specifically those that are known to develop resistance mechanisms. CRC tumors are sensitive to EGFR inhibition and most, if not all, patients develop tolerance within months. This mechanism is mediated by repeated postoperative tumor biopsies, and can be circumvented with the clinical use of liquid biopsy (13).

Unmet Clinical Needs: *Thyroid Cancer*

Liquid biopsy can also be applied to monitor indolent forms of thyroid cancers including thyroid nodules, which helps prevent overtreatment of patients through a blood draw that is already typical of thyroid patients. Overtreatment, typically through the use of radioactive iodine without evaluation of histological features, can lead to postoperative complications (14). High levels of ctDNA has been established to associate with thyroid cancer diagnosis and prognosis, specifically through the localization of oncogenes. In thyroid cancer cells, the capability to metabolize iodine is often lost due to hypermethylation of SLC5A8 and SLC26A4, which is associated with the $BRAF^{V600E}$ mutation. This $BRAF^{V600E}$mutation therefore renders radioactive iodine as inapplicable. The $BRAF^{V600E}$ mutation is the most frequent genetic occurrence underlying the development of papillary thyroid carcinoma (14). BRAF provides instructions for making a protein that's part of the RAS/MAPK signaling pathway, which regulates growth and proliferation of cells. Point mutations in BRAF have been found in nearly half of papillary thyroid cancers (15). Zane et. al has shown that cfDNA can be measured to discriminate healthy individuals from cancer patients, by using the hypermethylated genes as a biomarker for

diagnosis (14).A 2017 study by Khatami was additionally able to distinguish between benign and malignant tumors via the $BRAF^{V600E}$ mutation. When considering the value of a ctDNA blood draw, many patients with current thyroid imbalances undergo routine blood work, which already involves drawing blood 4 times a year. Underlying thyroid conditions may play a role in increased cancer mortality (16). Therefore, utilizing ctDNA within patients suffering from thyroid conditions would greatly add value to their overall health monitoring, while minimally changing their routine of a blood draw. One plausible use of ctDNA for BRAF mutation detection could be for an early screening tool. The ability to reliably diagnose cancerous thyroid nodules would add great value to the patient experience while bypassing fine needle biopsies. US-FNAB (ultrasound guided fine needle biopsy) carry a huge cost, and requires continuous sampling to central repository (9). This would fulfill a great clinical need by saving payers expenses, since blood drawing remains routine and regular; providing both the opportunity for diagnosis and surveillance.

Key Performance Metrics of ctDNA

Obtaining an acceptable level of clinical sensitivity and specificity remains an obstacle, frequently encountered by researchers. In order for users to confidently and comfortably use this novel technology in a clinical setting, it must be precise, accurate, and consistent. Additionally, there is a tradeoff between sensitivity and specificity, wherein a percentage or two of sensitivity may be sacrificed to have a higher specificity and vice versa. Along with determining a device's sensitivity and specificity, one can then use the number of true positives and false positives to calculate the positive predictive value (PPV). The PPV describes the accuracy of the device, meaning that when there is a positive result, there is a certain level of confidence in the fact that

it is a true positive result. Alternatively, the negative predictive value (NPV) is a similar concept as PPV but is the confidence that the negative reading is a true negative.

One of the key players in this challenge for obtaining desired levels of clinical sensitivity and specificity is that many researchers are focusing on ctDNA's clinical use in early screening methods (3).When screening patients, you are primarily concerned with the specificity of your diagnostic and reducing the number of false positive results obtained. This is because there are further severe implications regarding unnecessary costs, procedures, risks and negative consequences to one's quality of life should they obtain a false positive reading. While implications of receiving a false negative are not ideal, such as delaying the diagnosis and treatment, these are less detrimental than a patient receiving a false positive. The specificity refers to the number of true negative diagnostic calls divided by the total number of healthy patients.

While screening does represent the unmet clinical need in both ovarian and thyroid cancers, the ability to obtain detectable levels of ctDNA for thyroid cancer is a huge challenge in and of itself (17). Moreover, while ovarian cancer is more readily detectable than thyroid cancer in the general sense, focusing on screening for ovarian cancer comes with its own challenges. The incidence of ovarian cancer, most recently measured in 2014, is about 11.8 in 100,000, ultimately making up 0.1% (5). Figure 2 below illustrates this idea of needing a specificity of

99.99% in order to add clinical value to screening methods

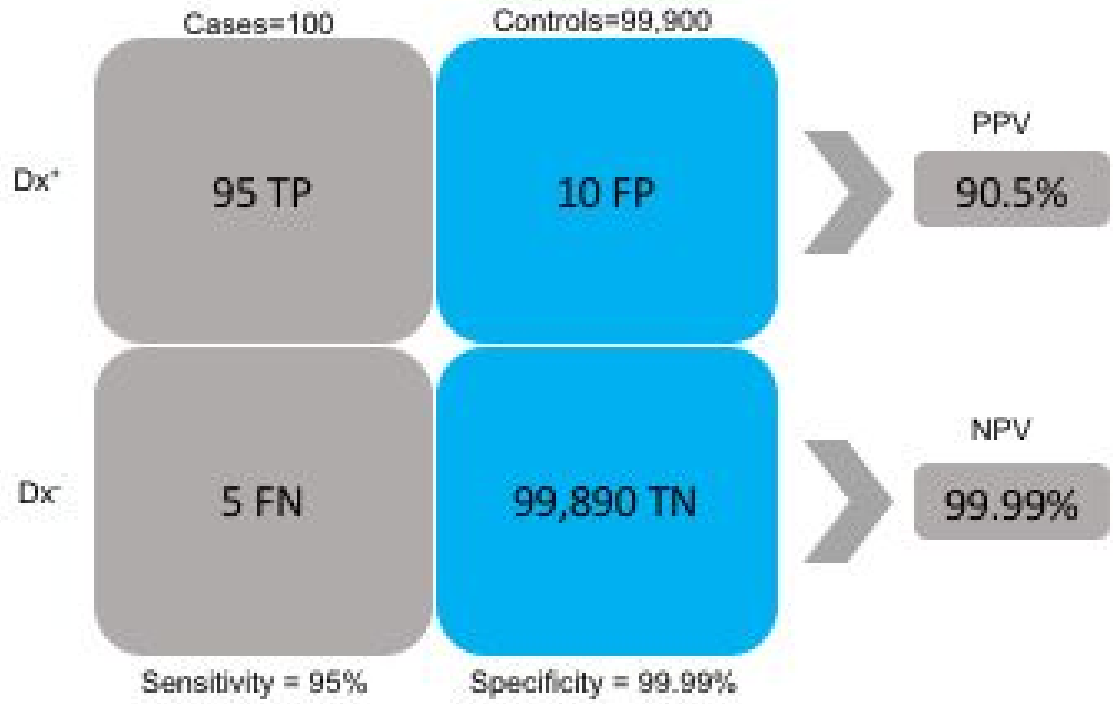


Figure 2: In the diagram, the number of cases represents the number of individuals who have the disease, and the number of controls is the number of healthy patients. (TP = true positives, TN = true negative, FP = false positives, FN = false negative)

Ultimately, such a high specificity would allow for only 10 false positives in a group of 100,00 people screened. However, even reducing this specificity to a level of 97% would produce 3,000 false positive patients that would end up with the burden of unnecessary costs and treatments.

The sensitivity of an instrument refers to the number of true positive diagnostic calls divided by the number of total diseased patients. This sensitivity allows for the number of false negatives to be determined, and for areas such as monitoring you want to focus on reducing the

number of false negatives as best you can. With monitoring, these patients are already undergoing treatment and or procedures to help get rid of their cancer. Therefore, receiving a false negative in a monitoring situation has more of a detrimental effect on the patient than a false positive would. A false negative would cause the patient to go home without receiving further treatment which could then set them back in their progression toward remission, whereas a false positive would simply mean going through more treatment which is seen as less of an issue seeing as they are already in a treatment routine. Furthermore, in regard to CRC, in later stages, the survival rates drop significantly, meaning that a false negative result in monitoring CRC patients could end up fatal. In patients with CRC who receive an intended curative treatment, around 40% experience a recurrence of their disease (18). To add clinical value, a monitoring test for CRC could sacrifice specificity slightly, compared to the high level needed for screening, to increase sensitivity to an acceptable level to reduce the number of false

positives which is shown in Figure 3.

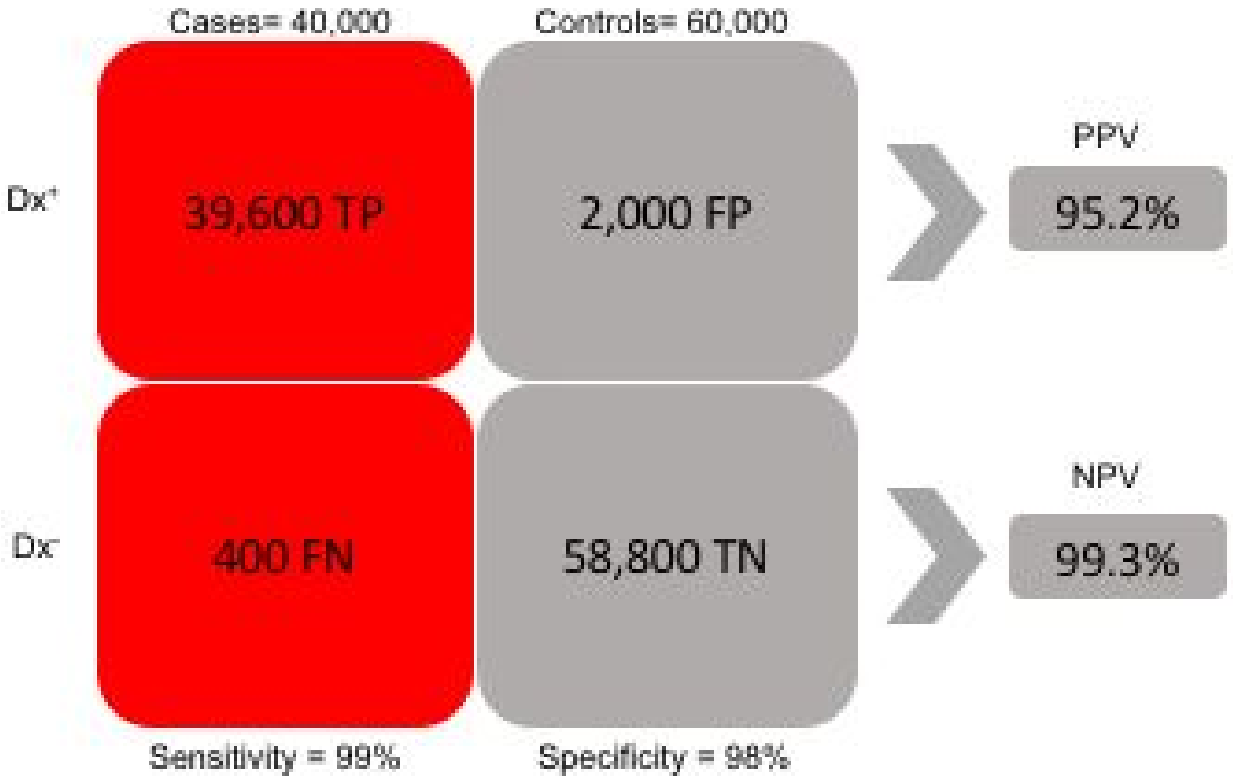


Figure 3: In the diagram, the number of cases represents the number of individuals who have the disease, and the number of controls are the number of healthy patients. (TP = true positives, TN = true negative, FP = false positives, FN = false negative)

ctDNA Implications

Exemplifying one clinical application, it is confirmed by qPCR analysis that concentrations of ctDNA fragments within a liquid biopsy have a positive relationship with the relevant tumor weight (8). This relationship suggests that elevated cfDNA levels detected in the blood can be independent prognostic determinants of death in ovarian cancer patients (19). The expected median level for preoperative cfDNA for ovarian cancer lies at 10,113 GE/ml; benign growths can be expected to cause levels around 2,365 GE/ml, and a healthy individual falls

around 1,219 GE/ml (19). Further assessments utilizing TAm-Seq technology identified mutations in the TP53 tumor suppressor gene amongst extracted cfDNA samples from 46 patients with advanced stages of ovarian cancer (19). The assessments identified TP53 mutations with high sensitivity and specificity values over 97%, some of which contained extremely low mutated allele frequencies within the analyzed sample (19).

Part of the issue in obtaining high sensitivity and specificity values relates to the availability of ctDNA — ctDNA levels obtained from a blood draw remain extremely low, especially in lower stages of cancer and higher in later cancer stages. The prevalence of ctDNA in liquid biopsy samples across cancer stages has been analyzed and quantified as shown in Figure 4 (17).

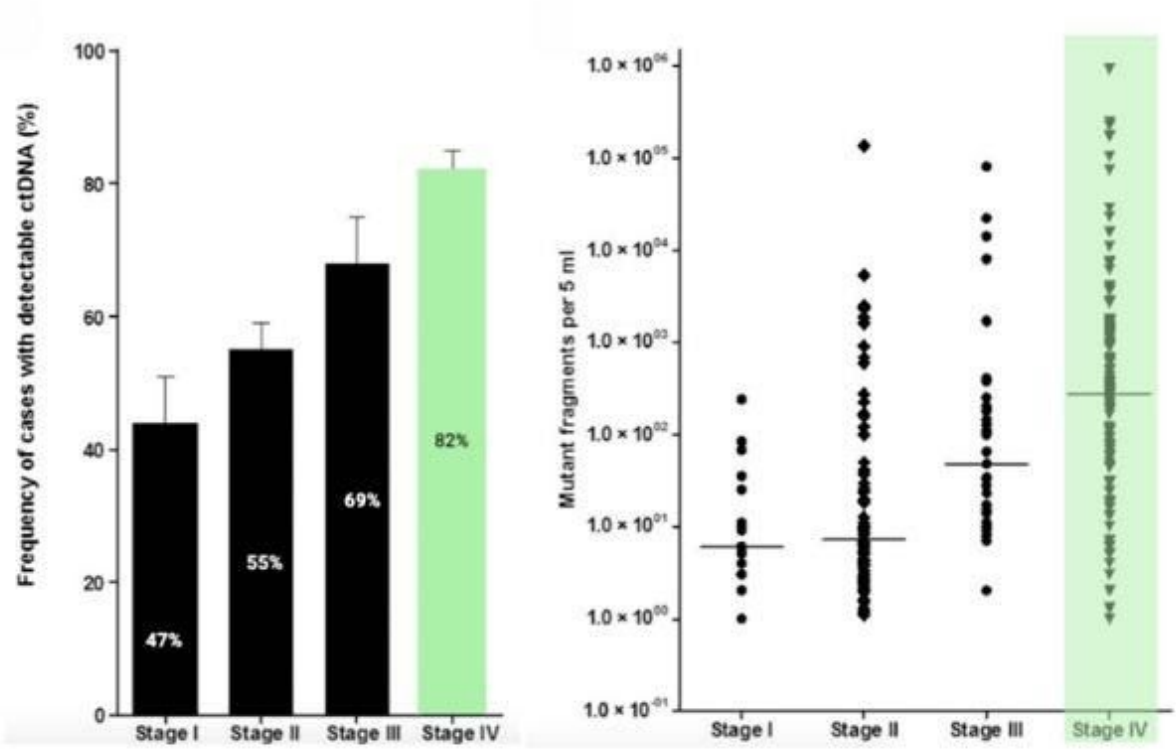


Figure 4: (A) the y-axis measures the frequency of cases for which patients had detectable levels of stages I, II, III, and IV as indicated across x-axis. Ultimately this depicts that later stages of cancer have a higher likelihood of detecting ctDNA, and indicating that liquid biopsies would be better implemented in monitoring cancer treatment responses and surgical resections in later stages. (B) The higher concentration of mutant fragments per 5 ml, as shown on the y-axis, in stage IV explains why later stages are more easily detected than stages I, II, and III, respectively, on the x-axis. These two graphs show the value of focusing on utilizing ctDNA in advanced stages, rather early screening, seeing as it may influence the reliability and practicality of using liquid biopsies in a clinical setting for the treatment of cancer. Figure adapted from Bettegowda et al.

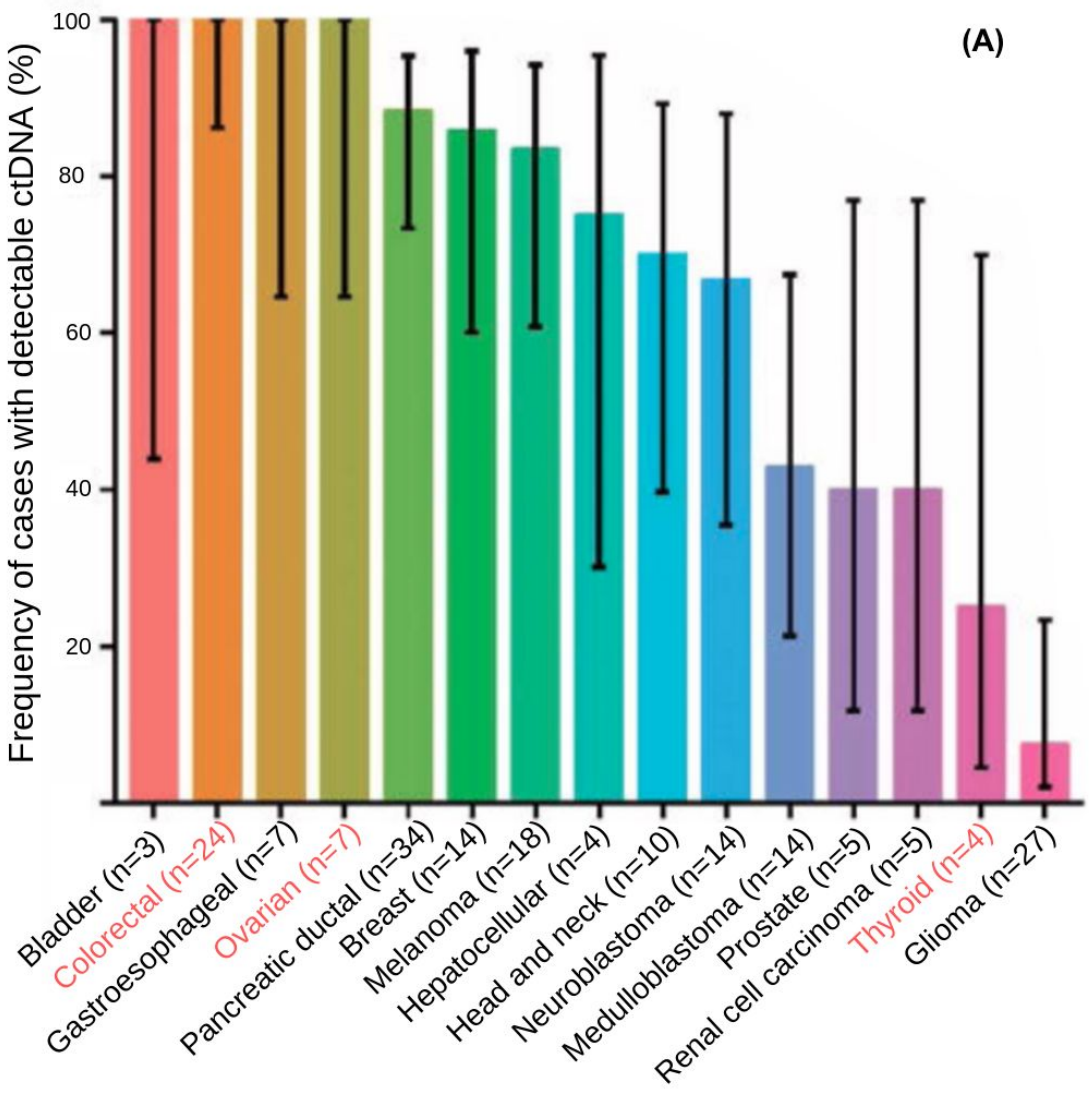
The implementation ctDNA liquid biopsies in advanced stages have increased reliability, and detectability of metastatic cancers significantly increases compared to localized, early stage cancers (17). Furthermore, the clinical application of colorectal cancer in ctDNA liquid biopsies involves the ability to monitor the disease during and after treatment (20). Specifically, recent

research found that the use of ctDNA to detect residual disease, via the presence or absence of KRAS mutation after tumor resection, was a reliable predictor for tumor recurrence, without the invasive nature of a tissue biopsy or even a colonoscopy (20).

This issue of low ctDNA concentration in blood draw samples reveals itself again, and most prevalently when attempting clinical application of ctDNA in thyroid cancer liquid biopsies. The nature of heterogeneity in these tumors presents a challenge of comprehensive determination of what genetic alterations led to the incidence of cancer. There have been experimental methods developed capable of efficiently identifying the BRAF V600e mutation in ctDNA. BRAF mutations, a strong link to thyroid cancers, have been used in thyroid oncological diagnosis (3). In addition, research has shown that detection of BRAF V600e holds clinical potential to: 1. compare preoperative and postoperative cancer presence and 2. Differentiate between benign and malignant nodule growths (19). Preoperative initial detection however, was only possible in 9 out of 61 thyroid cancer patients, and other studies have not been able to reliably reach detectability frequencies above 50% (15). Bettagowda et al. reports that while ctDNA was detectable in over 75% of patients with advanced ovarian and gastrointestinal cancers, less than 50% of patients with advanced thyroid cancer were detected (21) In order to obtain and evaluate ctDNA levels from these cancers, they report the frequencies of mutant templates per millimeter of each case. The lack of reliable detection is due to the low concentration of thyroid mutant fragments. For thyroid cancers, a ctDNA panel for the diagnosis of thyroid malignant tumors was compared with pathologic tissue examination, via fine-needle aspiration biopsy (22). The study reports a sensitivity of 7.7% and a specificity of 95.35%; these values were not sensitive enough to be comparable to the FNAB methodology (22). Therefore,

thyroid cancer continues to rely on the gold standard of fine-needle aspiration biopsy for the reliable detection of thyroid nodules.

Furthermore, it is important to note that the concentrations of ctDNA need to be high enough to detect with instrumentation during the development of assay; this benchmark is also known as the limit of detection (LOD). The LOD, however, must be lower than the concentration of ctDNA present in the blood in order to reduce the rates of false negatives. Clinically, this metric will aid in fewer cases being dismissed by other cancer monitoring techniques.


(A)
Frequency of cases with detectable ctDNA (%)
100
80
60
40
20
Bladder (n=3)
Colorectal (n=24)
Gastroesophageal (n=7)
Ovarian (n=7)
Pancreatic ductal (n=34)
Breast (n=14)
Melanoma (n=18)
Hepatocellular (n=4)
Head and neck (n=10)
Neuroblastoma (n=14)
Medulloblastoma (n=14)
Prostate (n=5)
Renal cell carcinoma (n=5)
Thyroid (n=4)
Glioma (n=27)

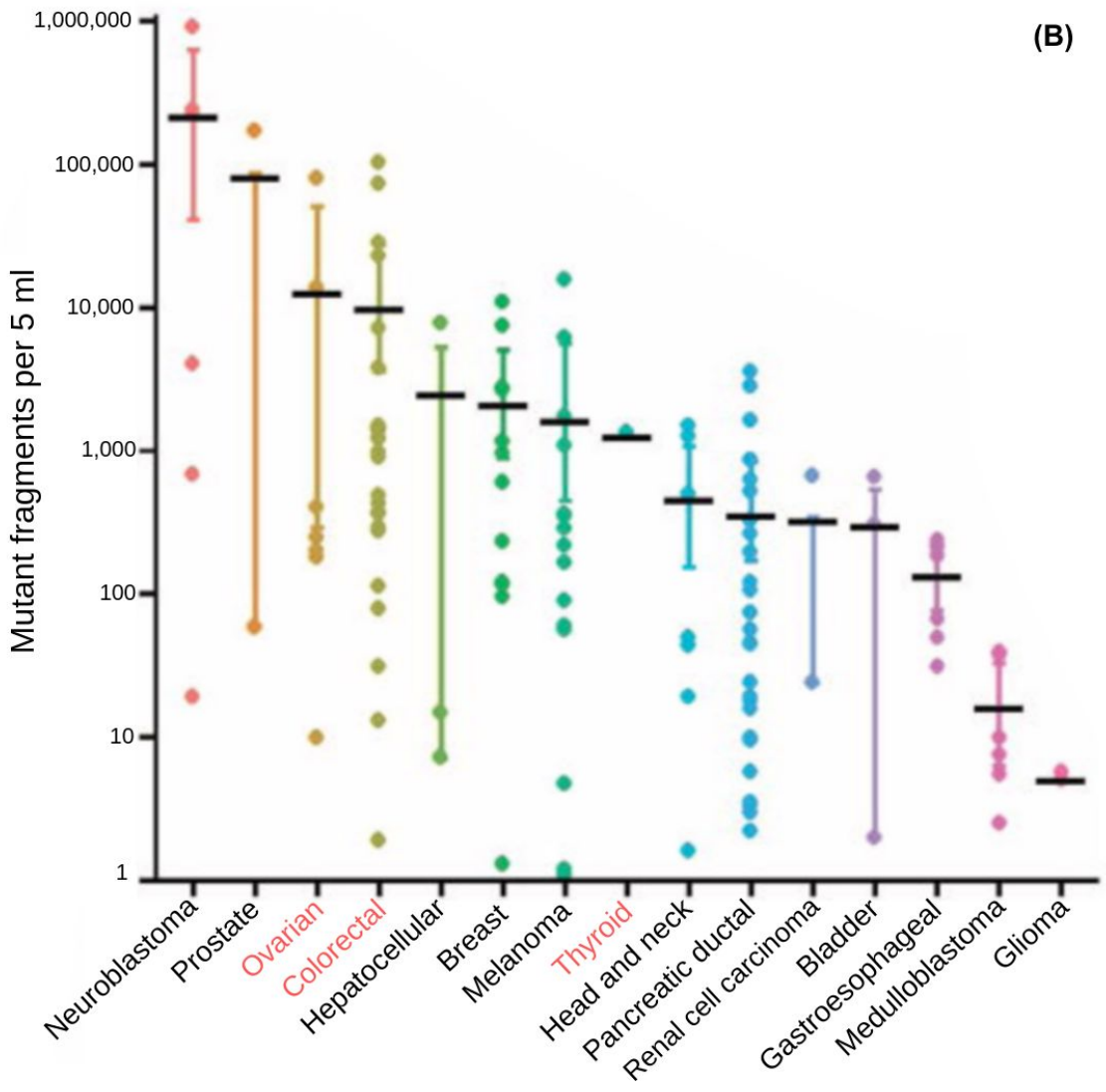


Figure 5: (A) The x-axis describes 14 different cancer types tested for their relative frequencies for which patients had detectable level of ctDNA as shown on the y-axis. (B) This graph lists the same 14 cancer types along the x-axis and their relative concentrations of ctDNA needed for detectability as shown on the y-axis. These two graphs illustrate the relationship between cancer type, and the ability to detect ctDNA in a patient and the increased concentration mutant fragments per five ml higher. Figure adapted from from Bettegowda et al.

Figure 5A displays the relationship between the concentration of ctDNA in the blood and the frequency of detectability in different cancer types. The two graphs suggest that the higher

concentration of ctDNA within the blood sample correlates with the higher frequency of detectability in cancers, such as ovarian and colorectal. As seen in Figure 5B, the concentration of ctDNA of thyroid cancer is rather low thus making frequency of detectability also low; therefore, future monitoring with ctDNA of thyroid cancer is highly unlikely for our diagnostic device. For colorectal and ovarian cancer, the concentrations of ctDNA are within the detectable limits for assay.

Conclusion

There are several unmet clinical needs for each cancer. Ovarian and thyroid cancer lack reliable screening methods for purposes of diagnostics and treatment selection. Liquid biopsy can fulfill this void by providing a minimally invasive method of diagnosing cancer as well as identifying key genetic alterations in oncogenic drivers and tumor suppressor genes which can help determine what treatment is necessary. In colorectal cancer, repeated sampling with current methods such as tissue biopsy can be detrimental to treatment efficacy. By analyzing ctDNA via liquid biopsy, the detrimental effects can be entirely circumvented in addition to providing a reliable method of treatment monitoring.

Cancers in later stages, have higher levels of ctDNA concentration, therefore the implementation of monitoring a cancer with a high frequency of detectability, such as ovarian or colorectal cancer, would be the most viable choice for an IVD ctDNA liquid biopsy. Because the concentration of ctDNA is significantly lower in thyroid cancer than the other two cancers, it is a cancer that will not be further investigated for a cancer in vitro diagnostic.

In this report, between the clinical background, assay, and regulatory teams, the amount

of ctDNA are represented as two different metrics: the concentration (mutant fragments per 5 ml) and mutant allele frequency (%). For future methodology, these values should be stated in equivalent metrics.

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