Chapter

Diagnosis of Non-Small Cell Lung Cancer via Liquid Biopsy Highlighting a *Fluorescence-in-situ-Hybridization* Circulating Tumor Cell Approach

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Abstract

Lung cancer (LC), is the most common and lethal cancer worldwide. It affects both sexes and in its early stages is clinically silent, until it reaches a more advanced stage, when it becomes highly incurable. In order to improve the high mortality associated with LC there has been an urgent need for screening high risk patients by low dose CT scan (LDCT) for the early detection of small resectable malignant tumors. However, while highly sensitive to detect small lung nodules, LDCT is non-specific, resulting in a compelling need for a complementary diagnostic tool. For example, a non-invasive blood test or liquid biopsy, (LB), could prove quite useful to confirm a diagnosis of malignancy prior to definitive therapy. With the advent of LB becoming increasingly clinically accepted in the diagnosis and management of LC, there has been an explosion of publications highlighting new technologies for the isolation of and detection of circulating tumor cells (CTCs) and cell free tumor DNA (cfDNA). The enormous potential for LB to play an important role in the diagnosis and management of LC to obtain valuable diagnostic information via an approach that may yield equivalent information to a surgical biopsy, regarding the presence of cancer and its molecular landscape is described.

Keywords: Circulating Tumor Cells (CTCs), Cell Free Tumor DNA (ctDNA), *Fluorescence in situ hybridization (FISH)*, Multiplex FISH, Cytogenetically Abnormal Cells (CACs), Liquid biopsy (LB), Lung Cancer (LC), Low Dose CT Scan (LDCT), Artificial intelligence (AI), PD-L1, *ALK*

1. Introduction

Until recently, the clinical application of CTCs had been largely confined to an FDA approved test, CellSearch®, for testing patients with advanced breast, colorectal and prostate cancer, which relies on immuno-magnetic capture of circulating cells expressing EpCAM. However, this test has not been proved to be optimal for sensitive recovery of CTCs in early stage LC. Currently, in order to make real-time decisions on how to manage LC, there are several new and emerging

label-free technologies for detecting CTCs which are more sensitive than the FDA approved test. While each platform differs in the methods that are employed for CTC enrichment and capture, all aim to accurately detect and enumerate CTCs [1]. In this chapter we present an overview of the current applications of LB, including both CTCs and cell free DNA (cfDNA) or circulating tumor DNA (ctDNA) for the detection, diagnosis and treatment of LC from early to advanced stages. We highlight the use of *fluorescence in situ hybridization* (FISH) to detect CTCs, in order to use these as adjunctive biomarkers, in conjunction with indeterminate nodules detected by LDCT scan, as a confirmatory test for early LC.

2. Incidence of lung cancer

Lung cancer (LC) is the leading cause of cancer incidence and mortality globally, with an estimation of 2.09 million new cases and 1.76 million deaths in 2018 [2]. GLOBOCAN Data shows that in industrialized nations, there is no substantial difference in LC deaths in males due to high cigarette consumption rates, but that there is a higher mortality rate in females. In developing countries, LC remains the second highest cancer-related mortality for women, behind breast cancer [3]. The LC incidence rate of women ranks from the highest in Northern America (30.7 per 100,000) and Western Europe (25.7 per 100,000) to the lowest in Western Africa (1.1 per 100,000). Even though women in China have a low prevalence of tobacco use, because of indoor pollution and occupational exposure [2, 4, 5], the incidence of LC in Chinese women also remains high (22.8 per 100,000) [2].

Because early-stage LC cases are asymptomatic, the majority of the patients are diagnosed with advanced disease [6]. The survival rate of stage I LC at 10 years is 92% [7], but the five-year survival rate of advanced LC with distant metastases is only 5% [8]; thus, early detection is critical in reducing lung cancer mortality rate.

3. Screening by LDCT

Lung cancer screening had become a controversial topic since the late 1990s, due to the fact that the risks, effectiveness, and procedures of screening, including the Early Lung Cancer Action Program and screening programs in Japan [9–11], were not verified. In 2002, a randomized trial, the National Lung Cancer Screening Trial (NLST) was initiated in the United States. The primary study goal was to compare the lung-cancer mortality rate between a large cohort of subjects screened for LC by conventional chest radiography versus low-dose computed tomography (LDCT). Follow up data until 2010 indicated that screening with LDCT can significantly reduce the death rate from LC, compared with the radiography group, and that, for the high-risk population [12] the lung cancer mortality rate was reduced by 20% in the LDCT group. The 10-year follow-up result of the Dutch Belgian Randomized Lung Cancer Screening trial (NELSON) that started in 2003 also successfully demonstrated a 26% mortality reduction of the high-risk population in the LDCT screening group, compared with the usual care group without screening [13]. These two studies have become the pivotal studies in LC screening history that have linked the utility of LDCT to reduced LC mortality amongst high-risk populations [14]. A growing list of organizations has established guidelines for LC screening with LDCT based on the evidence showed by the NLST and NELSON [15–17].

However, the benefits of LC screening with LDCT have been diminished by the high false-positive rate, as only 3.6% of the participants with positive LDCT screening results were diagnosed with lung cancer in NLST [12]. The substantial application of LDCT to lung cancer has resulted in a dramatic increase in pulmonary nodule

detection in adults at high risk, without a corresponding rise in lung cancer incidence [18]. About 80% of the patients with positive LDCT results are classified as intermediate risk of LC, thus requiring follow-up to rule out malignancy [19]. For patients in this category, most will be required to undergo invasive biopsy to further evaluate the risk of malignancy. Also, more than one-third of these patients will not be diagnosed with LC, subjecting them to potential biopsy-related complications such as pneumothorax and hemorrhage [20]. For patients who are at low-risk, repeated LDCT scans are required, which may be potentially harmful due to frequent radiation exposure [21]. In the case of ground glass nodules [GGNs], which may have an unpredictable clinical course, current diagnostic methods including biopsy and positron emission tomography (PET) are insufficient to differentiate malignant from benign nodules [22, 23]. Consequently, there is an urgent need for a non-invasive diagnostic tool such as a blood test or liquid biopsy (LB) that can evaluate the malignancy of pulmonary nodules in individuals with positive LDCT screening results (**Figure 1a** and **b**) by demonstrating the presence of circulating tumor cells (CTCs) in the blood.

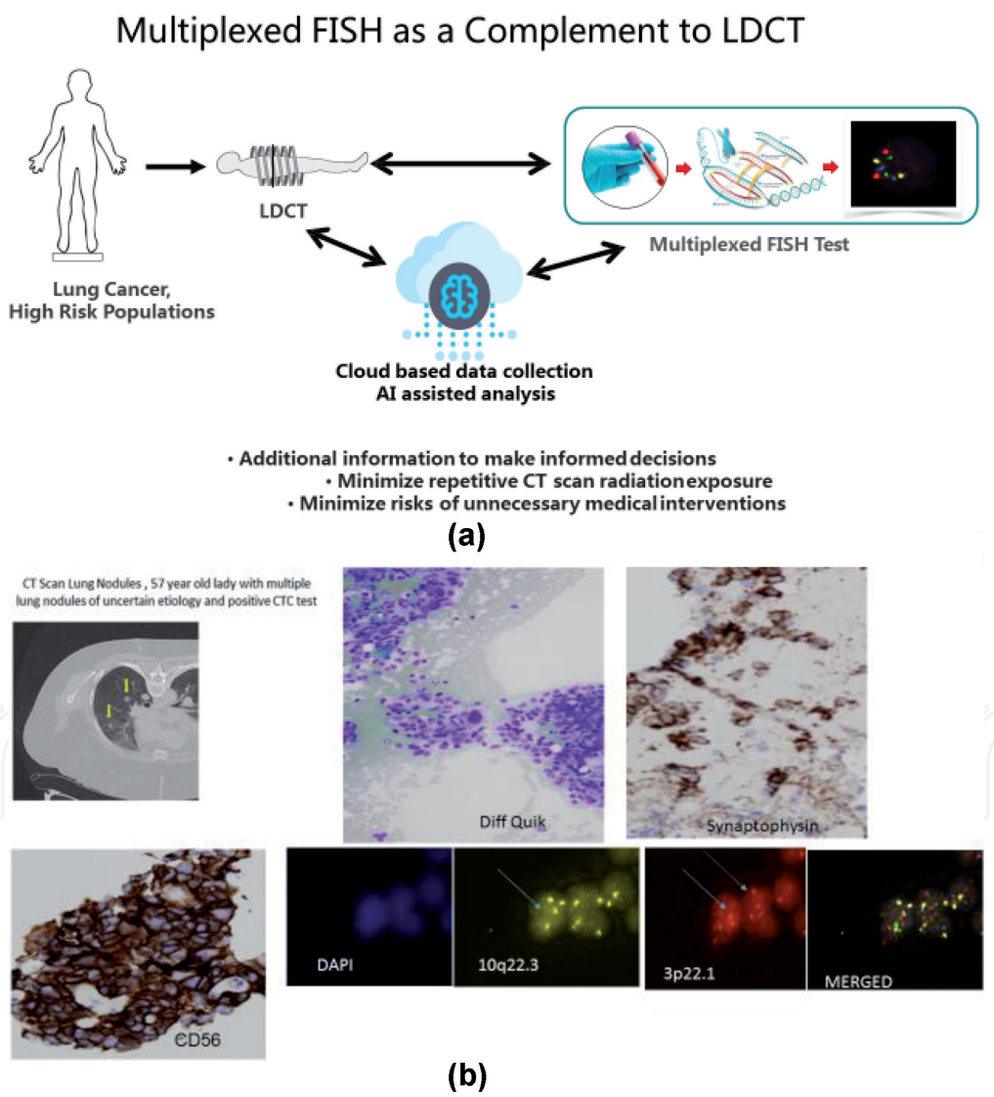

Figure 1.
(a) Schematic diagram demonstrating a hypothetical risk/benefit for lung cancer individual, undergoing LDCT scan with discovery of indeterminate lung nodule and complementary multiplexed FISH test performed on peripheral blood mononuclear cell fraction, to confirm presence of CTCs. (b) Real life example of a 55 year old lady with multiple nodules of uncertain etiology, and a fungal infection such as histoplasmosis was suspected. Multiplex 4 color FISH CTC assay performed on peripheral blood liquid biopsy showed more than 8 CTCs. Figure demonstrates CTCs stained with DAPI, and polysomy for 3p22.1 (three red signals) and polysomy 10q22.3 (3 gold signals) in same cells (merged images). Subsequent fine needle aspiration and cell block showed a well differentiated neuroendocrine tumor (diff Quik), which was positive with synaptophysin and CD56. [24].

4. Components of liquid biopsy

Liquid biopsies (LB) comprise circulating tumor cells (CTCs) from the cellular fraction of blood, and circulating tumor DNA (ctDNA), derived from the plasma fraction of blood (**Figure 2**). ctDNA originates directly from the tumor or CTCs, that are thought to release ctDNA via apoptosis and necrosis from dying cells, or active release from viable tumor cells. Both fractions have been shown to have potential for detecting, monitoring, and treating a variety of different cancers across all stages of disease. The term LB is not just confined to the use of tumor derived material from the blood stream and may also be applied to other biofluids such as urine, saliva, cerebro-spinal fluid, pleural fluid or bile from cancer patients, however for the purpose of this review, LB refers to the blood stream. Use of LB obviates the need for invasive tissue biopsies, which are frequently from inaccessible organ sites, and usually require the use of anesthesia. Complications are not unusual, and may include hemorrhage and infection, while for lung biopsies, pneumothorax is not uncommon. LB is an easy to use approach, as a simple blood draw, requiring only 10 ml of blood, may reveal circulating tumor cells (CTCs), or cancer specific mutations or aberrant methylation patterns [26] in the ctDNA portion of the plasma, that are consistent with malignancy [27, 28].

LB may be used in order to diagnose early LC and can be easily repeated over time to detect relapse of cancer or minimal residual disease (MRD) following surgery, or to monitor a patient's response to various chemo- biological or immune checkpoint therapies. The constant replenishment of CTCs and cell free ctDNA from the primary tumor and/or metastatic sites, enables LB to detect and monitor the development of new clones of CTCs that express different mutations, as compared to an original tissue biopsy or a preceding LB, which may have arisen as a response to a targeted therapy. The rate -limiting factor for the widespread use of LB especially in early-stage LC, has been the scarcity of recovery of CTCs and ctDNA. In addition, due to the wide range of different methodologies for detecting and capturing CTCs, as well as the lack of standardization and clinical validation of different platforms, it is difficult to know which is the optimal platform to choose [1].

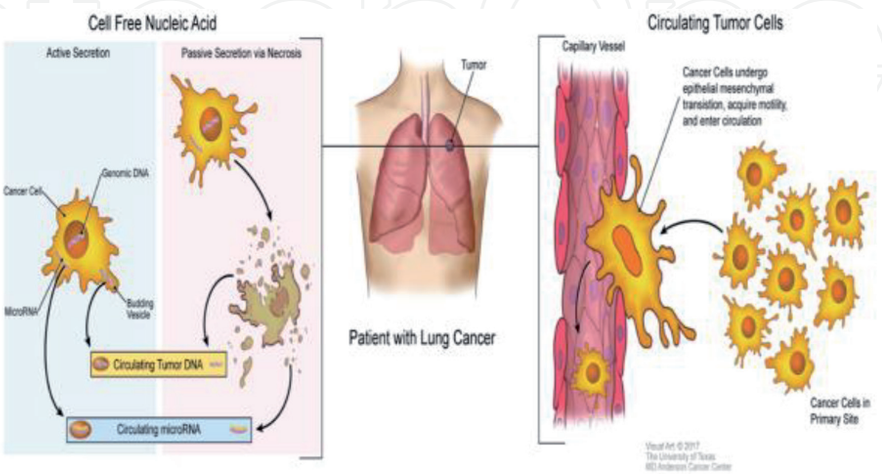

Figure 2.
Mechanism of extravasation of CTCs and circulating tumor DNA from primary lung carcinoma into the blood stream. The left-hand panel shows the source of circulating tumor DNA derived from necrosis and apoptosis of CTCs. The right-hand panel shows the mechanism of extravasation into the blood stream via EMT [25].

5. Scenarios in which LB may be indicated preferentially over tissue biopsy

First, as a complementary test in the face of abnormal images acquired by LDCT screening for lung cancer, where in order to determine the etiology of an indeterminate pulmonary nodule, a simple blood draw demonstrating the unequivocal presence of circulating tumor cells (CTCs) may be useful as a decision-making tool for the further clinical management of the patient. In this situation, tissue biopsy such as fine needle aspiration (FNA), needle biopsy or endoscopic ultra-sound guided biopsy, may be performed, however, occasionally may be both difficult and dangerous to the patient due to the small size and location of the mass and may be non-diagnostic because of the inability to procure representative tissue for pathological assessment. A positive CTC test (**Figure 1**) will lead to the procurement of an excisional surgical biopsy for standard pathological examination with curative intent or in other cases, patients may be candidates for stereotaxic radiation therapy. A negative CTC test will require follow up by LDCT, until the clinicians have determined that the nodule is stable and benign. However, in this scenario the patient will have been spared an invasive procedure for a non-malignant lesion.

Until now, current methods for detection and quantitation of CTCs have been time consuming and complex and require expensive instrumentation as well as a great deal of expertise available only at limited sites. As a result, large scale clinical trials involving thousands of patients at high risk for LC, evaluating the accuracy of screening for CTCs have not been possible. As an example, a large prospective multi-institutional study was performed using filtration of blood samples (ISET) to detect CTCs in COPD patients at high risk to develop lung cancer [29, 30]. Unfortunately, this study failed to confirm the initial promise of accurate early LC detection, due to difficulties in scaling up such technologies at multiple different sites [30]. Therefore, there is an urgent need for the establishment of platforms that can isolate CTCs from patients with early LC that are capable of producing reliable and reproducible results that are comparable amongst different populations.

Second, LB may be used as a minimally invasive, fairly rapid way, to obtain information on actionable mutations in order to deliver targeted therapies, especially in the case of advanced malignancy, where obtaining a tissue biopsy would be difficult. For advanced stage NSCLC, international guidelines have been developed by different pathology, molecular and oncology organizations, including amongst others the International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP), the National Comprehensive Cancer Network (NCCN) and ASCO regarding a minimum panel of genes that should be tested to inform treatment decisions [27].

The recent introduction of comprehensive genomic profiling by NGS using ctDNA from LB in patients with advanced stage cancers, including NSCLC, has revolutionized the ability of oncologists to treat actionable mutations in this population in real time and sequentially, without resorting to invasive tissue biopsies, which frequently could not be performed due to lack of tissue or the poor state of health of the patient. Thus, in many cases, the convenience and quick turn-around time of LB may have significantly prolonged the overall survival of patients in this category, who were discovered to have developed actionable mutations following first and second -line therapies with conventional chemotherapy regimens or biological agents [27, 31].

Third, the ultimate aim or "the holy grail" of LB, is to prescreen at risk populations for the development of potentially lethal malignancies such as LC, in order to monitor these patients and institute rapid treatment if necessary.

6. Pathogenesis of CTCs

Cancers develop in epithelial cells as a result of chronic exposure to inflammation or carcinogens, such as tobacco smoke or air pollution. In the lungs, exposed tissues, such as vulnerable epithelial cells in the upper and lower bronchial tracts, may manifest both dysplastic epithelial changes as well as concomitant molecular abnormalities, resulting in a "field -cancerization" effect (**Figure 3**). In these areas, certain cells may undergo unregulated proliferation due to the acquisition of tumor-suppressor genes and oncogenes as well as methylation of tumor suppressor genes. Other factors, including increased glucose uptake, angiogenesis, an altered tumor microenvironment (TME) and a cell's ability to avoid immune surveillance via masking of checkpoint inhibitors such as PD-L1, may allow invasion of these genetically and phenotypically abnormal cells into the blood stream where they present as circulating tumor or CTCs. One of the hallmarks of CTCs is genetic heterogeneity and genomic or chromosomal instability (CIN) [33]. CIN includes microsatellite instability (MSI), chromosome structural variations such as deletions, duplications and translocations, as well as chromosome number. Aneuploidy, due to errors in chromosomal segregation, is a consequence of CIN and is implicated in tumorigenesis as evidenced by the increased rate of malignancies found in patients with global or mosaic aneuploidies. The knowledge that aneuploidy is a *sine qua non* or essential element of a malignant cell forms the basis of certain LB tests that rely on the demonstration of aneuploidy to detect CTCs or CACs (cytogenetically abnormal cells [24, 25, 33, 34]. Genetic mutations arising in CTCs can be characterized by polymerase chain reaction (PCR) or next generation sequencing (NGS) on a single cell basis as well as by *fluorescence–in-situ-hybridization* (FISH) and immunohistochemistry. [23, 25, 35–37].

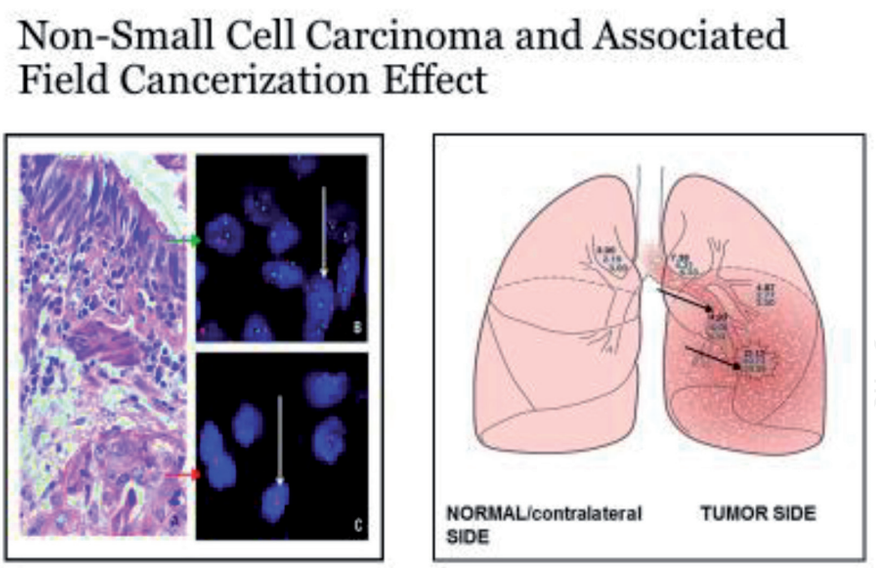

Figure 3.
Left hand panel: A. section of histologically normal ciliated bronchial epithelial cells, overlying adenocarcinoma of bronchial origin. B. Microdissection of bronchial epithelial cells adjacent to NSCLC and hybridized by FISH showed deletion of gene for surfactant protein a (SP-A) in adjacent bronchial epithelial cells, with fewer green signals (SP-A) versus red signals (centromeric 10). C. Tumor cells (white arrow) show 3 signals of centromeric 10 versus 2 signals of 10q22.3 (SP-A) (green) consistent with deletion of 10q22.3 (SP-A). (adapted from Jiang et al. [32]). Right hand panel: Composite diagram of 110 cases of NSCLC demonstrating field cancerization effect of left lung following hybridization of lung sections from tumor, bronchus adjacent to tumor, proximal bronchus on side of tumor, and normal lung on contralateral side for 3 different DNA FISH probes at 3p, and 10q) expressed as percentage deletion. The highest percentages of deletion occur in the main tumor mass, however there is evidence of increased deletion of 3p and 10q throughout the lung parenchyma and in the proximal ipsilateral main bronchus (shaded areas), compared to the contralateral side. (SPORE grant, lung cancer, the University of Texas, M.D. Anderson cancer center).

Before entering the blood stream, CTCs are required to undergo epithelial mesenchymal transition (EMT) in order to traverse the endothelial lining of small blood vessels and capillaries [37]. The CTCs have a much larger diameter than the diameter of a capillary and need to become deformable. This process requires CTC transformation involving micro- RNAs that can interfere with the translation of proteins by messenger RNA and facilitate the adhesion of the CTCs to the endothelium and subsequent migration into the blood stream [38]. The number of CTCs are extremely rare and are estimated at one CTC per $1x10^{-6}$ -10^{-7}. The half- life of CTCs in the blood is very short and estimated to be less than 2.5 hours [39].

7. Circulating tumor DNA (ctDNA) and cell free tumor DNA (cfDNA)

Studies have shown that tumor-specific biomarkers exist in the blood. These biomarkers represent tumor-derived elements from cancer cells undergoing apoptosis and death while traversing the blood stream. These biomarkers include circulating tumor DNA (ctDNA) which are fragments of DNA derived from

Comparison	**CTCs**	**cfDNA**
Origin	Intact cells	Necrotic apoptotic cells, exosomes
Definition	Tumor cells derived from primary and metastases	Fragmented DNA in circulation, includes DNA from normal dying cells, ctDNA, cfNucleic acids
Capture and Analysis Methods	Enrichment via gradient density, immunomagnetic or microfluidic based via antigen dependent methods, filtration(ISET), CellSearch detection, FISH for chromosomal abnormalities or translocations and fusions *(ALK,ROS1, MET)*	Enrichment: plasma collection Detection: PCR- or sequencing based, droplet pcr, digital pcr, Next Generation Sequencing (NGS)
Advantages	Extensive downstream analysis (DNA, RNA, protein) Cell culture via capture of viable single cells for *in vitro* or *in vivo* animal studies; NGS sequencing for mutations or copy number variations, FDA approved for prognosis and detection in stage IV breast, Colorectal and prostate cancers, Cell Search™ System. OncoDx iFISH for ARv7 Possibility to monitor expression of checkpoint inhibitors, PD-L1, PD-1 to guide immunotherapy Slide based methods such as for FISH (ref Katz), or IHC (Epic Science) can be archived	Easy to isolate from whole blood Long term storage for subsequent analysis High sensitivity read out Clinically validated for EGFR mutations in NSCLC, comprehensive genomic profiling for additional genetic mutations (Guardant 360, Foundation Medicine for comprehensive genomic profiling and tumor mutation burden (TMB), and microsatellite instability).
Disadvantages	Low cell numbers Labor intensive as both detection and enrichment steps may require highly sensitive and often expensive instrumentation Lack of uniformity of platforms to capture CTCs	Not sensitive enough for detection of early stage cancers Limited downstream analysis (DNA only) regarding cell culture, PDl1 assessment Only feasible in a high tumor burden setting

Modified from [43].

Table 1.
Comparison of circulating tumor cells (CTCs) and cell free DNA (cfDNA).

malignant cells which reside in a background of cell-free DNA (cfDNA). The DNA describes DNA that is freely circulating in the blood stream, but is not necessarily of tumor origin; cell free nucleic acid (cfNAs)includes DNA and RNA derived from cfDNA, cell free RNA (cfRNA),miRNA and exosomes [25]. Circulating tumor DNA fragments (ctDNA) result from activation of nucleases in apoptotic cells and increase in response to rapid cell turnover. The burden of ctDNA is proportional to the total tumor burden throughout the body as well as the metabolic tumor volume as measured by Positron Emission Tomography (PET-CT) [40]. ctDNA is cleared by the kidneys, liver and the spleen [41] and is easier to enrich from whole blood than CTCs, but until recently, before the advent of NGS for LB, its widespread use was limited by the need-to-know which mutations to target by PCR [42]. A comparison of CTCs versus ctDNA is presented in **Table 1**.

8. EGFR mutations detected by LB

In 2016, the U.S. Food and Drug Administration (FDA) approved the first test in LB for ctDNA, the Cobas® test (Roche, USA), which was a companion diagnostic test for the use of targeted therapy, for detecting the presence of common EGFR mutations (exon 19 deletions and the L858R point mutation), which are discovered in up to 16% of Western patients and in 50% of Asian patients with NSCLC [28]. The use of tyrosine kinase inhibitors (TKIs) in NSCLC, such as a first line TKI, like Erlotinib, is guided by the presence of alterations in EGFR with notably better response of patients harboring EGFR exon 19 deletions compared to point mutations in EGFR exon 21, whereas patients who have developed the T790M mutation after receiving first and second generation TKI's will have an improved PFS response to the third-generation EGFR TKI, Osimertinib. [44]. Resistance to TKI's may be intrinsic or acquired, with the latter occurring as an acquisition of an additional genetic mutation to a target therapy such as EGFR or through secondary mutations such as gene amplifications in other genes such as Her2neu or MET, or changes in tumor histology [28]. Thus, one disadvantage of LB compared to tissue biopsy for monitoring advanced disease, is the inability to detect transformation of NSCLC to a small cell lung cancer phenotype, which would necessitate different chemotherapy that would include etoposide and a platinum drug such as carboplatin or even the addition of an immunotherapy drug such as Atezolizumab (Tecentriq) that targets PD-L1 [28].

9. LB for detection of actionable mutations in addition to EGFR

By using a comprehensive multi-genome test panel, as opposed to a single targeted PCR test, a LB test may reveal several different mutations that may be amenable to targeted therapies. Serial blood monitoring may in addition, reveal newer actionable mutations. The use of NGS to look for actionable genes or biomarkers in formalin fixed paraffin embedded tissue sections in cancer patients with advanced disease, has been successfully applied in order to institute targeted therapies, and has resulted in improved clinical outcomes [45]. However, it has been shown that "undergenotyping" or incomplete testing for all guideline recommended biomarkers continues to be a challenge in the treatment of patients with metastatic NSCLC [27].

Studies have also shown that the results of mutational profiles from LB ctDNA in advanced or metastatic NSCLC can be very similar to that obtained in FFPE tissue from primary tumor or metastatic sites [27]. A large prospective study

of comprehensive ctDNA genotype analysis (Guardant360®) in patients with metastatic NSCLC compared to standard-of-care physician requested tissue genotyping, demonstrated that guide-line recommended biomarkers were significantly more likely to be discovered using the ctDNA LB test compared to tissue genotyping [27]. There are eight guideline recommendedbiomarkers and include *EGFR* mutations, *ALK* fusions, *ROS* fusions, *BRAF* V600E mutations, *RET* fusions, *MET* amplification and *MET* exon 14 skipping variants, and *ERBB2 (HER2)* mutations. There was >98% concordance for FDA –approved therapy targets (*EGFR, ALK, ROS1*, and *BRAF*) between tissue and cfDNA. In addition, there was a faster mean turnaround time in obtaining results of cfDNA compared to tissue (9 versus 15 days). Significantly, addition of the LB test in addition to tissue genotyping, increased the detection of actionable mutations, including those with negative, or not assessed or insufficient tissue results.

There are differences between the mutational profiles in ever-smoker versus never smokers in NSCLC, as well as differences in demographically different populations. As an example, 93% of 904 never smokers with lung adenocarcinomas in East-Asian populations using surgically resected frozen tumor tissue were shown to harbor an actionable mutation that could be exploited as a therapeutic target as compared to 31.2% of 1770 patients (779 current or former smokers) with NSCLC [46]. In this latter study, comprising 2674 patients, the incidence of METex 14 skipping was 1.3% in NSCLC and 1.9% in non-smokers with adenocarcinoma. By comparison, a NGS LB study of 6,034 Western patients with advanced NSCLC, reported METex14 skipping in 3.6% of all patients, demonstrating that this actionable mutation can be successfully detected by LB, with a genomic profile very similar to the aforementioned data obtained on tissue biopsy [47].

In another NGS study a hybrid-capture based 508-gene panel (Oseq-NT) was used, that included 119 patients with advanced EGFR –TKI-naïve NSCLC and 15 EGFR –TKI-resistant patients. In this study, somatic cfDNA mutations by NGS, were detected in 82.8% of patients. Actionable genetic mutations were detected as 27.7%, predominantly EGFR mutations, including the EGFR T790M mutation as well as BRAF mutations, MET mutation and gene fusions for EML4-ALK and KIF5B-RET [48]. In August 2020, the first NGS companion diagnostic test, the Guardant360® CDx test, that used LB to identify patients with specific types of mutations of EGFR in metastatic NSCLC was granted FDA approved [49]. This test uses a more sensitive and specific digital sequencing method compared to standard NGS assays, in 20 ml of blood, combined with high throughput tumor profiling or large panel genetic sequencing to simultaneously detect mutations in 55 tumor genes.

At the time of this writing, the FDA approval is only valid for targeted therapy in relation to EGFR. If other somatic mutations are detected by this EGFR assay, patients may then be referred to appropriate clinical trials where suitable targeted therapies are being used. There are 73 genes listed on the Guardant Health website for point mutations, indels, amplifications and fusions. These include amongst other genes, *ALK, BRAF, TP53, MET, NOTCH1, EGFR, ERBB2(Her2), CDK6, FGFR1, NTRK.* For a full description the reader is referred to the website (http://www.guardanthealth.com/). A second similar test called Foundation One Liquid CDx®, (Roche, Switzerland), received expanded approval in late October 2020 by the FDA for additional targeted drugs, known as companion diagnostics [50]. This test covers single gene alterations in more than 300 cancer -related genes, as well as multi-gene signatures such as micro-satellite instability and tumor mutational burden (TMB). TMB may be used as a predictive biomarker for delivering immune check point inhibitors and refers to the totality of somatic, and coding base substitutions or mutations, and short insertions or deletions per tumor genome, which

may result in high numbers of tumor neo-antigens, and increase the likelihood of immune recognition by the immune system.

10. Clonal hematopoiesis

The development of somatic mutations in DNA as a result of the aging process can affect certain stem cells most commonly in blood and bone marrow, and less frequently in other tissues, such as the skin, colon and esophagus. In the blood, random somatic mutations in genes (*DNMT3A, TET2,* and *ASXL1*) involved in epigenetic regulation may confer relative "fitness" on certain hematopoietic stem cells, which permits unregulated proliferation of a process known as clonal hematopoiesis (CH). This results in clonal expansion of these cells [51, 52]. CH is highly prevalent in the elderly, with between 10 and 20% of individuals over the age of seventy, harboring a clone of appreciable size. Because break- down of peripheral blood cells, including CH, comprises a large component of cfDNA, CH, which may also contain somatic DNA mutations, may be a source of "biological background noise" that can lead to false positive plasma genotyping. This has been reported in patients with advanced EGFR-mutant NSCLC where mutations in *KRAS, JAK2 V617F* and *TP53* were detected and confirmed as derived from CH and not tumor [53]. To overcome the possibility of false-positive genotyping due to CH in patients with NSCLC, paired peripheral blood cell and plasma genotyping may need to be performed, so that inappropriate therapy can be avoided.

11. CfDNA for early detection of cancer

In spite of the spectacular success in applying precision therapy via LB to patients with advanced NSCLC, in early stage LC, the presence of very low amounts of mutated tumor DNA fragments in plasma, makes it difficult to be able to detect actionable genetic mutations in the pool of cell-free DNA (cfDNA). In 2019, the FDA granted break-through Device Designation to Cancer SEEK™, which was developed for early detection of eight common-cancer types, and combines multiplexed PCR detection of over 1000 mutations identified from numerous cancer samples in cfDNA, together with a panel of validated serum protein biomarkers [54, 55] including cancer-antigen 125(CA-125), carcinoembryonic antigen (CEA), cancer antigen 19–9(CA 19–9), prolactin(PRL), hepatocyte growth factor (HGF), osteopontin (OPN), myeloperoxidase (MPO) and tissue inhibitor of metallo-proteinases 1(TIMP-1) The median sensitivity to detect the different types of cancers was 70%, ranging from 98% in ovarian cancers to 33% in breast and lung cancers [54]. While this assay has very high specificity, it has low sensitivity to detect early stage 1 lung and breast cancers. In addition, because the assay is limited in its capacity to determine in which organ the cancer is present, it may be necessary to institute additional expensive screening tests such as LDCT scan, or other endoscopic or ultrasound tests, in order to discover the organ of origin of the cancer [56] which may call into question, the actual clinical value and expense of this screening test.

In recent years, large multi-center prospective clinical screening trials involving thousands of patients using cfDNA have been conducted, such as the Circulating Cell Free Genome Atlas (CCGA) study to determine if genome-wide cfDNA sequencing in conjunction with machine learning can accurately detect and determine the tissue of origin of a large number of cancer types [54] for early cancer screening purposes. An off-shoot of this study, whole genome bisulfite screening (WGBS) has examined methylation patterns of cell-free DNA fragments using a

vast targeted methylation panel based on the The Cancer Genome Atlas (TCGA) in a large variety of cancer patients versus non-cancer controls and was able to identify with high specificity, but with lower sensitivity, especially for the less advanced stage cancers, the presence of cancer as well as the tissue of origin (GRAIL) several years in advance of the manifestation of the tumor [55–57]. Studies are in progress employing computational biology and machine learning in prospective studies in large cohorts of healthy individuals for early detection of clinically actionable information from vast amounts of cell free nucleic acids (cfNAs) both DNA and RNA, released into the blood stream through high intensity sequencing. The goal is to discover unique genetic signatures indicative of early cancer.

12. Composition of blood cells and numbers of CTCs

CTCs are extremely rare events in the peripheral blood stream, with actual numbers depending on the platform used to evaluate the numbers of CTCs. For example, using a label-dependent method that relied on immuno-magnetic beads conjugated to an antibody to EpCAM, (CellSearch®) between 2 CTCs/ml of blood for early stage breast cancer, to >5 CTCs/ml of blood, could be detected in patients with advanced stage breast cancer [58, 59]. These rare cells are surrounded by up to several hundred million lymphocytes and neutrophils per ml of blood. On the other hand, using a label-free method to enumerate cytogenetically abnormal cells (CACs) by 2-color FISH, patients with NSCLC of all stages had significantly higher numbers of CACs than did controls. Depending on the DNA probe used, mean numbers of CACs ranged from 7.23 ± 1.32/μl for deletions of surfactant protein A gene at 10q22.3 to 45.52 ± 7.49/μl for deletions of EIF1B, eukaryotic translation initiation factor, a gene located on 3p22.1 [34]. The numbers of CACs detected for patients with NSCLC were far higher than the CTCs reported in NSCLC for the Cell Search Instrument [34] and could be accounted for by the definition of CACs as a single deletion of a genetic probe compared to the internal control DNA probe, as well as the label-free method of enumeration, in which all CACs irrespective of immunophenotype, and including cancer stem cells, malignant EMT cells and malignant epithelial cells, were counted.

In early stage LC, the vast majority of CACs are CK -/CD45- /EpCAM- and may express EMT or stem cell markers [25] hence the discrepancy between the FISH method and the Cell Search method [34]. In addition to single CTCs, clusters of cells may break off from the primary tumor and travel in clusters through the blood stream. CTC clusters may form "tumor micro-emboli "(TMI) consisting of up to 50 cells (**Figure 7**), that may demonstrate more aggressive properties than single CTCs, as they may be surrounded platelets, lymphocytes, neutrophils [60], similar to the cellular components that comprise the microenvironment seen in tumors, which may be protect TMI from destruction while circulating in the peripheral blood. TMI together with CTCs have also been detected in LB from patients with early breast cancer.

13. Concept of lineage plasticity

For malignant epithelial cells to metastasize, it is postulated that they need to adopt an epithelial to mesenchymal transition (EMT) phenotype and undergo lineage plasticity by changing their genotypic and phenotypic characteristics. During the shift from an epithelial to a mesenchymal state, the adhesion molecules expressed by the cell are modified, allowing it to adopt a migratory and invasive behavior. EMT is induced by specific transcription factors such as Snail, Zeb and Twist, and miRNA's which

together with epigenetic and post translational regulators, mediate the process of EMT. EMT is involved in wound healing, embryogenesis and cancer metastases. Most importantly, EMT has been shown to trigger the dissociation of cancer cells from the primary epithelial tumor mass and enable these cells to disseminate as CTCs into the blood stream. In a label- free study of peripheral blood mononuclear cells (PBMCs), it was demonstrated by FISH that there were higher numbers of CACs in patients with lung cancer and breast cancer across all stages than had previously been reported by other methods [25, 59, 61, 62]. This included label-dependent bead-based antibody capture systems for EpCAM, in which captured cells, that are CK+/ CD45- and stain for DAPI are defined as CTCs [58]. Notably, the FISH assay identified far higher numbers of CACs in patients in both early and advanced NSCLC compared to the low numbers of CTCs reported by EpCAM immune-antibody-cell capture methods including the CTC –chip [63]. It was also demonstrated by IHC for stem cell markers (ALDH1), mesenchymal markers (SNAIL) and CK combined with FISH, using a method known as FICTION, [64] that the CACs that were previously identified based solely on genetic abnormalities [34] actually represented diverse cohorts of pluri-potential CTCs including malignant stem cells and cells which had undergone EMT with loss of epithelial markers [25]. Further evidence for lineage plasticity and phenotypic switching was obtained by serial monitoring of blood for CTCs both before, and at several time points after, resection of LC [25]. In early stage LC the vast majority of CACs are negative for epithelial and lymphoid markers (CK-/CD45-), most likely representing EMT cells and stem cells. At later time points or as LC becomes more advanced, more CACs that are CD45+/CK+ or CK+/ CD45- are identified [25]. The not infrequent finding of genetically abnormal circulating cells co-expressing CD45+/CK+, or CK -/CD45- contradicts the classic definition of a CTC as defined by the FDA approved CellSearch® test [58]. This observation may account for the higher sensitivity of an antigen-independent gene-based test. It has also been shown that only a minority of CTCs with stem cell properties are able to survive and initiate metastases [65].

Figures 4 and **5** depict a 43-year-old patient with stage IB, poorly differentiated squamous carcinoma, showed CACs of all lineages(lineage plasticity). a CD45–/ CK+ cell showing 3 red (10q22.3) and 3 green (Cep10) signals. b CD45+/CK– cell with 2 green (Cep10) and 3 red (10q22.3) signals. c CD45–/ALDH1+ stem cell with

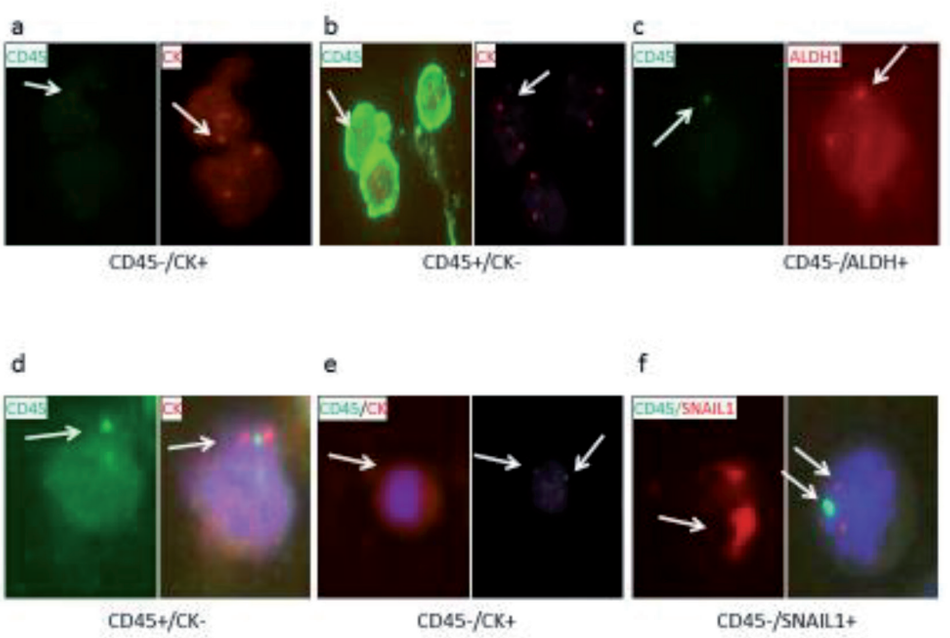

Figure 4.
Demonstrating lineage plasticity in CACs (by combined immunofluorescence and FISH (FICTION)).

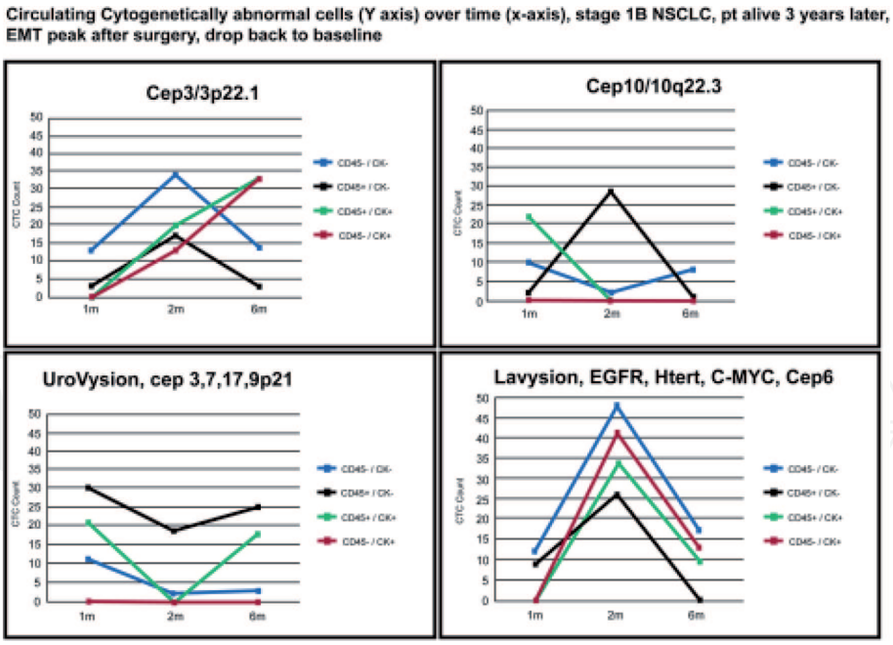

Figure 5.
Histogram of CACs from the same patient as in ***Figure 4****. FICTION method for CD45 and CK, combined with DNA probes for Cep3/3p22.1, Cep10/10q22.3, UroVysion Cep3,7,17, 9p21.3, and LAVysion, EGFR, TERT, C-MYC, and Cep6,showed increase of double-negative CTCs at 2 months in Cep3/3p22.1 abnormal cells and increase of CD45+/CK–CTCs in Cep10/10q22.3 abnormal cells, and increase of all CTC lineages in EGFR, C-MYC, and Cep6 abnormal cells at 2 months, followed by a marked decrease in all CTCs at 6 months [25]. Note that "CAC peaks" as shown in 3/4 of the above diagrams,occurred at two months post surgery, and returned to baseline at 6 months in patients with a good prognosis.*

2 red (10q22.3) and 1 green (Cep10) signal. d CD45+/CK– cell with 2 red (10q22.3) and 1 green (Cep10) signal. e CD45–/CK+ cell with 2 red (10q22.3) and 1 green (Cep10) signal. f EMT cell with 2 red (10q22.3) and 1 green (Cep10) signal. White arrows indicate the location of FISH signals [25]. **Figure 5** is derived from the same patient and demonstrates CACs from baseline before surgery to 6 months following surgery, with a return of CACs to baseline, following a peak in CACs observed at 2 months post surgery (**Figures 4**).

The significance of the EMT phenotype in initiating metastases was demonstrated by studies of CTC derived xenografts (CDX) from patients with advanced NSCLC, which demonstrated a mesenchymal phenotype [28, 66].

14. Methods for isolation of CTCs from blood

Different platforms have been developed to isolate CTCs. These can be divided into affinity, label or antigen dependent methods or affinity- or antigen-independent or label-free methods (**Figure 6**).

Affinity or label dependent devices include the CellSearch® System and the Mag –sweeper, as well as the CTC chip, because all rely on magnetic particles, beads, or posts, coated with antibodies to EpCAM that capture CTCs secondary to the expression of EpCAM on their surface membranes (**Figure 6a,b**).

Label free methods can isolate CTCs based on their physical properties and include:

a. CTC separation via density gradient centrifugation, which enables enrichment of CTCs [67] followed by *fluorescence in situ hybridization* (FISH) to identify cytogenetically abnormal cells (Sanmed Multiplex 4 color FISH test) (**Figure 6e**) [24] or immunocytochemistry for different biomarkers expressed on cancer cells such as Her2neu, estrogen receptors (ER) or cytokeratin's.

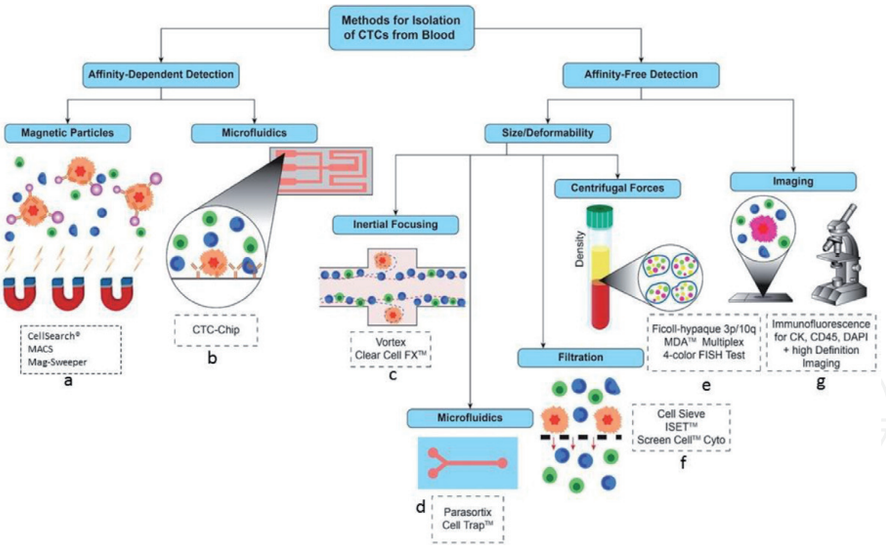

Figure 6.
Diagram depicting methods for isolation and detection of CTCs using either a) affinity –dependent detection devices usually employing magnetic particles, beads or posts coated with EpCAM such as a) CellSearch®, MACS or mag-sweeper or b) CTC chip or B) affinity-free detection devices relying on physical properties of CTCs such as larger size or deformability compared to other blood cells using c) inertial focusing or d) trapping of cells when passed through a Parasortix® filtration cassette or e) enriched on a Ficoll–Hypaque gradient due to specific gravity and centrifugal forces and characterized based on genetic abnormalities by FISH such as the Sanmed™ multiplex 4-color FISH test or f) trapped on filtration membranes that only permit passage of white blood cells with a pore size smaller than CTCs such as cell-sieve, ISET™, screen cell™ Cyto and g) total cell capture coupled with red blood cell lysis and immunocytochemistry or FISH together with high resolution imaging for cell morphology for detection of CTCs, such as the Epic or Tethis SBS platforms.

b. Microfluidic devices that use physical properties of the larger less deformable CTCs that allows inertial focusing to detect CTCs (Vortex Clear Cell FX™) (**Figure 6c**) and include a micro cavity array system that traps CTCs into 10,000 cavities arranged in a 100x100 array with each cavity fabricated to have a diameter of 8–9 μm [68].

c. A microfluidic device that traps cells (Parsortix™ Cell Trap) (**Figure 6d**) [1].

d. Various filtration devices that use membranes or screens with small pores that allow the WBCs to flow through but trap single CTCs and clusters of CTCs based on the larger size of the CTCs on the surface (Cell Sieve, ISET™, Screen Cell™Cyto) (**Figure 6f**) [1].

e. Imaging methods that promote total tumor cells capture by minimal sample manipulation examining all nucleated cells in the blood by immunofluorescence (IF) for different antigens and tumor markers such as CK, ER, AR, Her2, or CD45 or FISH for aneuploidy and high-definition imaging for cell morphology (such as the EPIC™ test or Tethis SBS Platform) (**Figure 6g**) [1, 69, 70].

15. Antigen dependent devices or methods

15.1 Immunomagnetic devices

The CellSearch® (Menarini-Silicon Biosystems, San Diego, CA) method relies on ferrofluid based immunomagnetic separation of EpCAM expression to isolate epithelial cells [58, 59, 71] which are confirmed as CTCs by staining

positive for high expression of cytokeratin's CK8, 18 and 19, and absence of expression of CD45, a lymphoid marker. Thereafter the cell, is stained with DAPI (4, 6-diaminidino-2-phenylidole) a nuclear stain. The requirements tor this test are 7.5 ml of whole blood, collected in special CellSave tubes (**Figure 1**). Most CTCs will go through epithelial–mesenchymal transition (EMT) when they extravasate into the bloodstream, resulting in a loss or down regulation of EpCAM expression and are therefore poorly detected by this isolation method [60, 62, 71]. Loss of EpCAM is particularly notable in early stage NSCLC. In spite of the loss of epithelial marker expression in the CTC population, CellSearch® is the only CTC test that was approved in 2004 by the US Food & Drug Administration for clinical use for patients with metastatic breast, colorectal and prostate cancer, as well as prediction of survival in advanced NSCLC [58]. Since then, this test has been used in many studies with reliable and reproducible results [72–74]. CellSearch® has shown prognostic significance in detecting CTCs in most breast cancer subtypes, with a cut-off of $\leq$2 being a marker for long time survival [75] while $\geq$5 CTCs was associated with a decrease in 5 year survival. This device in its conventional set up, has drawbacks regarding sensitivity for capturing the whole dynamic range of plasticity that CTCs may demonstrate as it may not detect many cells that have lost or down regulated their EpCAM expression. Thus, numerous studies of solid tumors have reported zero or only 1–2 CTCs that can be recovered by the Cell Search instrument as currently configured [59, 71]. There are also certain solid tumors such as NSCLC, pancreatic cancer and triple –negative breast cancer, where the predominant component of CTCs are of the EMT type and hence, would not be detected by CellSearch® [25, 59].

15.2 Microfluidic chips

Microfluidic chips allow for cells to be captured, immobilized and then washed out, after which they can be subjected to molecular assays. Blood flows through 78,000 micro posts placed at very narrow intervals, forcing cells to move along narrow channels and enhancing their opportunities for contact with posts coated with EpCAM (**Figure 6b**), thus CTCs expressing EpCAM, become immobilized and attach to the walls of the chip resulting in the negative depletion of white blood cells (WBCs) which lack expression of EpCAM. Other chips may use antibodies such as anti-CD45 or anti CD66 for negative depletion resulting in retention of WBCs and elution of CTCs. The advantages of the CTC-chip compared to the CellSearch® instrument is the higher yield of CTC capture (median 50 CTCs per milliliter), as well as on-chip lysis which permits extraction of DNA and RNA for molecular analysis [63].

15.3 Bead based subtraction-enrichment strategies

Positive immunomagnetic bead-based CTC enrichment methods may rely on epithelial antigens such as EpCAM for capture and/or intra-cellular tumor cell antigens such as cytokeratin for detection, however, CTCs undergoing epithelial-mesenchymal transition, may be missed [76–79]. To avoid this failure, negative selection approaches exist for unbiased CTC enrichment [80]. Negative immunomagnetic selection uses a cocktail of antibodies against hematopoietic antigens such as CD2, CD14, CD16, CD19, CD45, CD61, CD66b and Glycophorin A, to enrich for CTCs, by removing contaminating white blood cells and platelets. An example of such an assay is the RosetteSep™ (STEMCELL Technologies). Antibody labeled WBCs can also be removed by AutoMACS Separator (Miltenyi Biotec). A major disadvantage of negative selection approaches is the lower CTC purity as compared

to the positive selection approaches; however negative selection approaches show promise for identifying more CTCs for downstream analyses. Both epithelial and mesenchymal cancer cells could be enriched from patient samples [76, 79–82].

15.4 Magsweeper

MagSweeper technology is an automated immunomagnetic cell separator that uses a magnetic arm to collect cells coated with anti-EpCAM antibodies [79]. This EpCAM based isolation method can capture high-purity cells from metastatic cancer patients, but adsorption of background cells to the capturing device or the entrapment within the large magnetic particles used for labeling rare cells in large volume could lead to nonspecific contamination. However, MagSweeper is not commercially available, which might require further analysis to validate the effectiveness of the test [83].

15.5 CellCollector

The CellCollector is a modification of a medical device for use in vivo. It uses a wire with an antibody against EpCAM that is attached to the surface and is inserted through a cannula straight into patients' bloodstream and left exposed to a high volume of blood for 30 minutes to collect CTCs [84]. This device has been used successfully ex vivo to quantitate the number of CTCs in 15ml of blood from patients with prostate cancer [85]. In spite of the complicated in vivo application procedure, Luecke et al. [86] reported that the CellCollector can capture a higher volume of CTCs compared with the CellSearch method (73% vs. 29%) in 62 lung cancer patients. Further studies with larger samples will be required to demonstrate the efficiency of this technology.

16. Antigen independent platforms

16.1 Enrichment free platforms or "No Cell Left Behind"

Following red cell lysis of a whole blood sample, CTCs are captured by analyzing all nucleated cells present so that the final result is fully representative of the entire cell population in the blood sample except for the red blood cells. (Epic Science, San Diego, CA) [69, 87]. This test attempts to identify CTCs defined as: a) CK+/CD45- with abnormal morphology; b) CK - /CD45- with abnormal morphology, which may be cancer stem cells or cells undergoing EMT; c) Apoptotic CTCs, which are the abnormal cells described in a and b, but with nuclear fragmentation and d) CTC clusters, 2 or more individual cells bound together. Cells are stained with a cocktail of cytokeratin, CD45 and DAPI and then analyzed at high resolution by digital pathology methods for numerous nuclear, nucleolar and cytoplasmic features. Machine learning algorithms then quantify CTC subtypes into different categories. Cells of interest are confirmed by a trained operator as to whether they represent CTCs. This platform is also used for nuclear localization of AR-V7 in CTCs from patients with metastatic prostate cancer, which if positive, is indicative of resistance to androgen –targeted therapy, suggesting alternative therapies such as chemotherapy or other therapies [87].

A similar approach has been used for detection of CTCs and in early breast cancer using of IF for estrogen receptors and CK/and or Her2 and morphology in bright field. In combination with proprietary slides to enhance cell retention (Tethis,

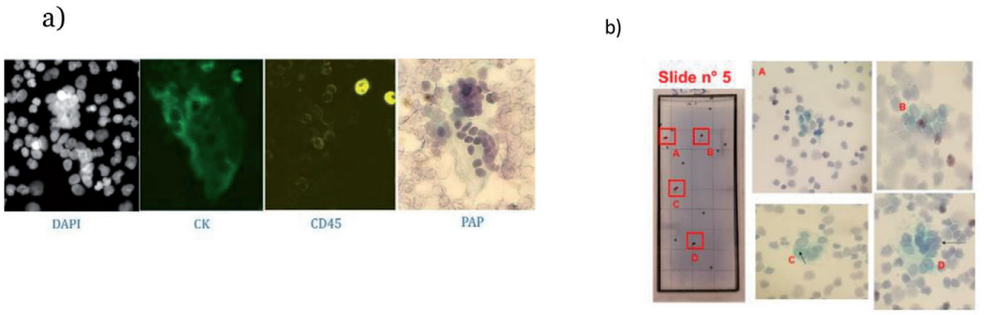

Figure 7.
CTC clusters in early breast cancer (a) 40x magnification of a CTC cluster stained with DAPI, and immunostained with CK and CD45, and stained with Papanicolau; (b) bright field analysis of a whole slide showing different CTC clusters (Papanicolau staining).

Milan, Italy), this method allows gently and quick white blood cell adhesion as a monolayer with no selection, avoiding shear stress or manipulation associated with enrichment methods and thereafter examining the whole repertoire of nucleated cells [69] (**Figure 7**) thus leaving the architecture of CTCs in the peripheral blood intact. This method, which has been automated and standardized in its pre-analytical phase of sample preparation on slides, has shown high sensitivity and specificity in single CTCs and also CTCs cluster detection in a pilot study of early breast cancer [69]: identification of CTC clusters in early breast cancer is a novel finding that will deserve further confirmation in larger clinical trials. The presence of CTC clusters in metastatic settings has been clearly associated with a more aggressive tumor phenotype [70]: identification of such biomarkers in early settings could open new perspectives for the evaluation of their prognostic relevance and consequent therapeutic decision in early breast cancer.

16.2 *Fluorescence-in-situ hybridization* or FISH-based assays

A novel way to look at the genotype of individual cells is to perform interphase FISH (iFISH) using DNA probes that may be localized to locus specific, centromeric or telomeric sites on the chromosomes. iFISH can identify if cells are diploid (normal) or aneuploid (malignant) based on the gains and/or loss of chromosomes. Panels of probes may be custom made and designed specifically to detect certain types of cancers, such as the Sanmed ™ test for LC, in which cDNA subtraction hybridization using DNA extracted from resected NSCLC specimens versus normal lung tissue was used to discover universally deleted genes [88]. The latest automated fluorescence technology using pseudo -confocal microscopy, permits up to 6 different DNA probes to be quantitated simultaneously in a single nucleus using different color fluorescent tags [89] thus allowing an opportunity for up to 6 different genetic markers to be analyzed on a per cell basis (Bioview, Rehovoth, Is.) and creating opportunities to devise novel biomarkers customized to different subtypes of cancers.

To maximize the enumeration of CTCs by FISH, a gradient centrifugation process is used, which causes the neutrophils and RBCs to precipitate at the bottom of the tube, while cells with abundant cytoplasm, such as CTCs, peripheral blood mononuclear cells (PBMCs) including monocytes and lymphocytes, band at the buffy coat due to the effect of specific gravity [24, 25, 67] (**Figure 8**). For chromosomal abnormality enumeration, thousands of purified cells from the buffy coat are subjected to iFISH, with multiple DNA probes labeled with different fluorescent tags [24] in order to identify nuclei containing gains or polysomies, and/or deletions of different targeted genes. These cells are also known as cytogenetically abnormal cells or CACs. Preparations are scanned on an automated fluorescent microscope

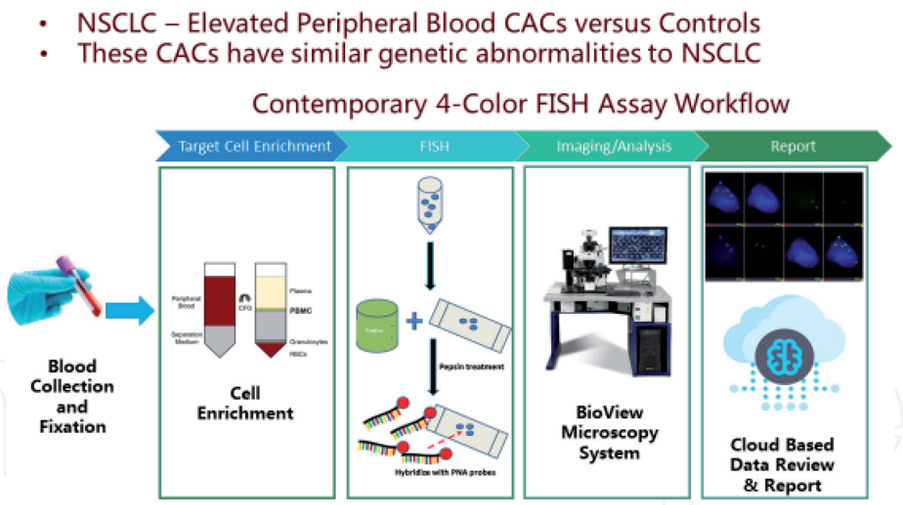

Figure 8.
The workflow of CAC enumeration, from left to right, collection of 10 ml of peripheral blood, enrichment of peripheral blood mononuclear cells, hybridization of FISH probes, fluorescence image acquisition and analysis followed by cloud based data review and report [90].

with multiple filter wheels of different wavelengths to detect different color signals (e.g., Bioview Duet Instrument Rehovoth™, Il). This instrument can be programmed to count cells of a certain size and to exclude cells the size of a lymphocyte or smaller cells. At the end of each scan, a pie chart is produced, according to a predetermined classification of signals. Digitization of cells subjected to FISH, can be performed fairly rapidly, however manual evaluation by a qualified technologist, of genetically abnormal cells, is mandatory, but can be time-consuming. Using strict criteria, only cells with intact nuclei that demonstrate good hybridization signals of all probes are analyzed (**Figure 8**).

In a 4-color FISH test such as the Sanmed™ test, the goal of the analysis is to find unequivocal aneuploid CTCs as defined by polysomy of 2 or > signals of different nucleic acid probes per nucleus. This criterion is the same one that is recommended for a similar 4-color FISH test, the Urovysion™ FISH test (Vysis, Abbott Labs, Chicago, Il) for the diagnosis of urothelial carcinoma in urine specimens in patients to rule out bladder cancer [91]. A threshold for calling a specimen positive or negative is established based on the lowest number of CTCs present in cancer patients with histologically confirmed primary cancers as compared to the highest number of CTCs present in a matched control population. The optimal threshold is the one that most accurately predicts the presence of cancer [24].

Recently, Katz et al. [24] used a 4-color FISH assay to evaluate cytogenetic abnormalities of 3p22.1 and 10q22.3 in 207 patients, including 100 control subjects, who were at risk of developing NSCLC, based on risk factors for LC as well as suspicious LDCT findings, using ≥3 CTCs as a threshold for malignancy, and successfully identified patients at stage I and II NSCLC with a high degree of accuracy (**Figure 9**, **Table 2**).

CTCs were identified as a complete cell with a nucleus larger than a lymphocyte nucleus that contained polysomy of at least 2 of 4 FISH probes per nucleus. Strikingly, the accuracy of this method to detect early-stage LC was significantly higher than other published EpCAM based technologies, most likely due to the high sensitivity of the 4-color probe cocktail that is used to detect cytogenetically abnormal circulating cells and is not dependent on EpCAM expression [24, 71, 95].

An environmental study that employed the 4-color FISH assay showed an 89.47% sensitivity and an 85.00% specificity to detect LC in 89 Chinese bus drivers who had indeterminate lung nodules on LDCT following chronic and consistent exposure to occupational pollutants [92]. Similarly, Liu et al. in a prospective

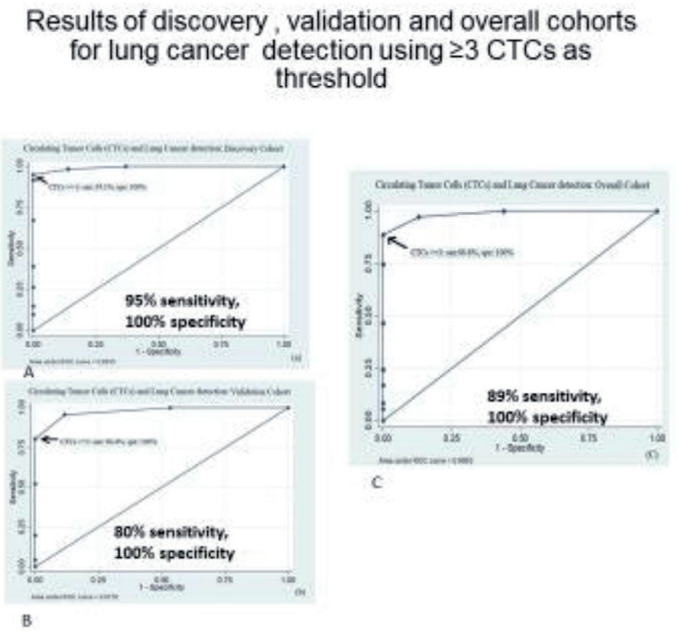

Figure 9.
High degree of sensitivity and specificity demonstrated in 3 separate cohorts comprising patients with predominantly early stage cancer and controls using a threshold of ≥3 CTCs to determine the presence of malignancy, A) discovery cohort, first 118 B) validation cohort, subsequent 89 and C) overall cohort, 207 patients (107 patients and 100 controls) [24].

Population	Type of nodules	Lung cancer stages	Sensitivity	Specificity	References
Chinese Bus Drivers, n = 89	Non-specific	Stage I	89.47%	85%	[92]
Chinese, n = 339	Solid, mGGN and PGGN	StageI	Solid nodules 63.0% mGGN 73.0% pGGN 66.7%	Non-specific	[90]
Chinese, n = 125	5–10 millimeters	StageI	70.4%	86.4%	[93]
Chinese, n = 534	Non-specific	Non-specific	Non-specific	Non-specific	[94]
Americans, n = 207, includes 100 control subjects (white = 72%, African Americans = 8%, Hispanic =1%, Asian = 19%)	Non-specific	Stage I = 55, stage II = 10, stage III = 18, stage IV = 17 Stage not ascertained = 7	88.8%	100%	[24]

Abbreviations: mGGNs, mixed ground glass nodules, pGGNs, mixed ground-glass nodules.

Table 2.
Performance of 4 color FISH test in LDCT detected lung nodules for identification of lung cancer in different populations [24, 90, 92–94].

case–control study involving 339 participants, indicated that the 4 color FISH test yielded 67.2 sensitivity% and 80.8% specificity in stage I NSCLC patients, including those with solid nodules (38.7%), mixed ground-glass nodules (mGGn) (31.9%)

and pure ground-glass nodules (pGGn) (28.4%) [90] detected by LDCT. In addition, in this study the discriminatory capability between CACs and traditional tumor serum biomarkers such as CEA, TPSA, NSE, CA19–9 and CYFRA21-1 was compared to the results of the 4 color FISH assay and showed that the sensitivity of the CAC assay was significantly higher for small nodules and ground glass nodules when compared to the serum biomarkers [90].

In a different study, 125 individuals with newly discovered pulmonary nodules 5–10 millimeters in diameter by LDCT, underwent LB for the 4 color FISH test prior to surgery, followed by histopathological examination of the resected nodules. Here, in spite of the extremely small size of the nodules, the FISH test demonstrated a 70.4% sensitivity and an 86.4% specificity for the diagnosis of LC [93]. The advantages of using this assay were demonstrated in yet another study that collected lung LDCT images of 534 patients with pulmonary nodules and invited experienced physician to score the patients' lung cancer risk and to compare the risk score to that

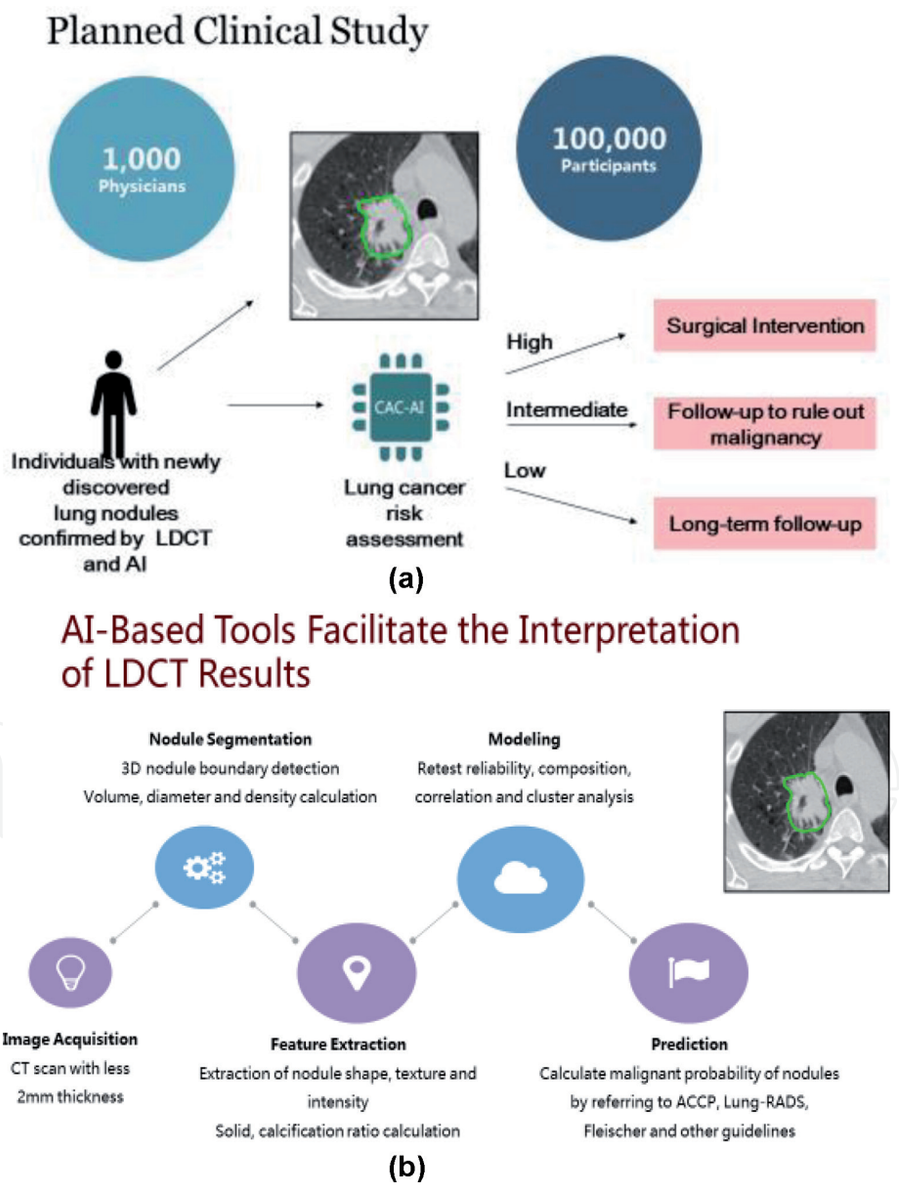

Figure 10.
(a) Overview of proposed clinical study to test robustness of AI assisted LDCT of lung nodules combined with evaluation of Sanmed test by AI in order to evaluate whether a pulmonary nodule is low, intermediate or high risk for cancer, with follow up recommendations. (b) Overview of AI based tools, include nodule segmentation, feature extraction, modeling and prediction based on established radiology guidelines, such as lung-RADS and the Fleischner society, in order to improve and standardize interpretation of lung nodules. Bueno et al. [96].

calculated by Artificial Intelligence with deep learning. In all 11 cases where both physicians and AI incorrectly predicted the lung cancer risk factor, the results of the 4- color FISH test was consistent with the results of the histopathological examinations [94]. When LDCT image analysis is insufficient to make a clinical decision, the 4 -color FISH test may be a valuable complementary tool for individuals with indeterminate LDCT results.

Based on initial studies, it appears that the multiplex–FISH LB assay is robust and has the potential to be scaled up for widespread use. Reproducible results with an acceptable degree of clinical utility were obtained on numerous blood samples from different geographic locations, in which pre-analytical values were kept constant, regarding the volume of blood (10 mL collected in K2EDTA vacuum tubes), as well as methods of fixation and stabilization of blood samples for up to 96 hours at room temperature, before being processed in a centralized certified laboratory according to standard operating procedures. The multiplex- FISH CTC assay is currently being tested in a prospective study comprising large cohorts of at-risk subjects, in combination with computerized scanning of LDCT detected indeterminate lung nodules in order to confirm that the FISH assay, in conjunction with the artificial intelligence (AI) interpretation of the lung nodule may have an important and synergistic role to play in early LC detection (**Figure 10a** and **b**).

16.3 FISH quantitation by artificial intelligence (AI)

A drawback of the different FISH assays has been the length of time taken to manually evaluate and accurately enumerate fluorescent signals due to overlapping cells and/or other technical difficulties such as splitting of signals, resulting in inaccurate counting and overcalling of CTCs and distinguishing CTCs from debris and leukocytes [94, 97]. However, the development of machine learning algorithms in implementing the CTC counting procedure has been able to make the process far more efficient and accurate. Machine learning(ML) algorithms that function in advancing cell image analysis can automatically input layers with a geometric relationship, as well as precisely capture the rows and columns of images; thus ML can rapidly recognize CTCs with extra intra-nuclear structures compared to normal cells, in order to reduce the artificial errors and improve the precision of

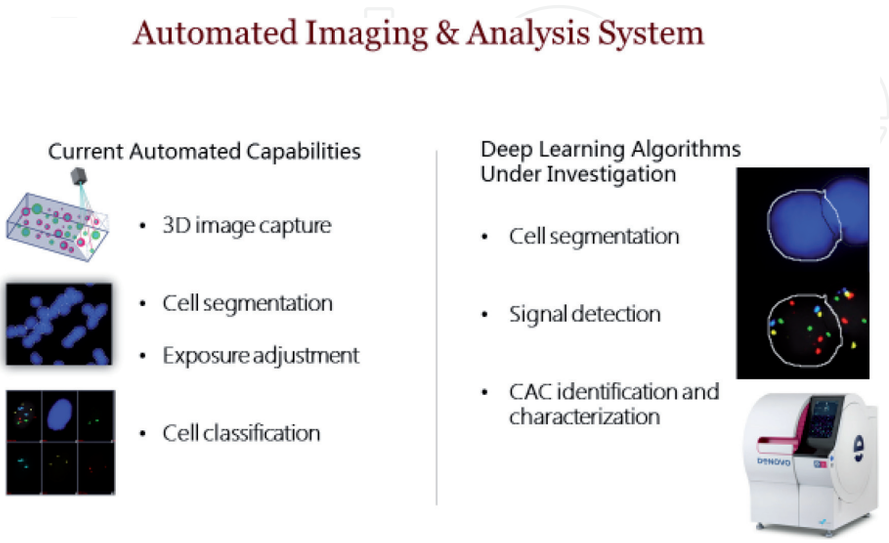

Figure 11.
Diagram of instrumentation (De novo®, BioView, Rehovoth, is) currently in use (left hand panel) for automated imaging comprising 3D image capture, cell segmentation, exposure adjustment and cell classification. Right hand panel depicts deep learning algorithms to improve classification of cells including cell segmentation, signal detection and CAC identification and characterization.

CTC identification [98, 99]. For example, the BioView platform automates image collection and is able to utilize an algorithm for identifying CTCs according to cell size, cell shape, nucleus to cytoplasm ratios, and occurrence of biomarkers identified by target features, and can automatically select CTCS from other cells in peripheral blood in a timely manner and has been widely applied in many FISH based CTC enrichment processes [100, 101] (**Figure 11**). In another AI study involving analysis of the 4-color FISH LB assay, FISH probes were segmented using 3D-Unets, which enabled a significant reduction in false –positive enumeration of polysomies obtained during traditional computer vision microscopy, while retaining all verified CTCs, greatly improving the efficiency of the scoring pathologists and the accuracy of the test [102].

16.4 Filtration methods

16.4.1 Filtration devices

CTCs may be isolated by the size of epithelial cells (ISET) (Rarecells Diagnostics, Paris, France) [103, 104] via a blood filtration approach which enriches 10 ml of peripheral blood collected in buffered EDTA and kept at room temperature. The membrane used is made of polycarbonate and allows cells <8 microns to pass through, while the larger epithelial and mononuclear white blood cells remain on top (**Figure 6f**). Half of the membrane can be used for morphology via a May Grunewald Giemsa stain, and the second half can be used for immunocytochemistry using a pan-cytokeratin antibody and an anti-vimentin antibody applied to the filters. Malignant cells are identified cytologically according to usual characteristic nuclear and cytoplasmic features. Another similar platform is the CellSieve method that uses microfilters and a pressure monitored filtration pump from Creative MicroTech, Inc., Rockville, MD (**Figure 12**). Another filtration device similar to that has enhanced cell recovery for in vivo quantitation of rare CTCs via multiphoton intravital flow cytometry [105]. Successful size- based isolation of CTCs has been described using a microcavity array system that traps CTCs into 10,000 cavities arranged in a 100x100 array with each cavity fabricated to have a diameter of 8–9 μm [68]. This method was shown to be superior to CellSearch in detecting CTCs from patients with NSCLC and small cell lung cancer. Filtration devices have been successfully and

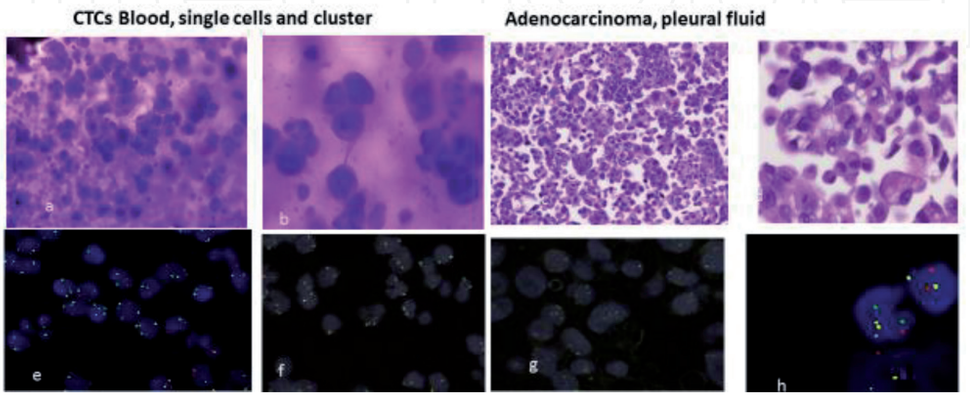

Figure 12.
Filter preparation, screen cell, of CTCs derived from a patient with stage IV adenocarcinoma of lung showing numerous CTCs, both single and in clusters a) diff- Quik 20X b) diff -Quik 40X, c) same patient, clusters of adenocarcinoma, morphologically resembling CTCs, in pleural fluid, hematoxylin and eosin 10X, d) 40X, e) CTCs multiplex 4 color FISH (3, 3p, 10, 10q) demonstrating aneuploidy 400X, f) EML-ALK translocation, CTCs X400, g)pleural fluid X400, h) multiplex 4 color FISH (3, 3p, 10, 10q), pleural fluid X600.

extensively used [103], to detect CTCs, both for screening for lung cancer in high risk COPD patients, as well as in patients with established diagnosis of LC [71]. In a large study of patients with and without COPD, investigators noted that 5/168 patients with COPD or 3%, all of whom had negative spiral CT scans, manifested CTCs one to 4 years before the appearance of indeterminate lung nodules. All nodules proved to be early stage lung cancer on surgical resection [104]. The CTCs were isolated by the ISET filtration method and were stained with both epithelial and mesenchymal markers. In a side-by-side comparison of 40 patients with advanced NSCLC, predominantly stage IV, using the filtration-based size exclusion technology ISET (Rarecell Diagnostics), investigators detected higher numbers of CTCs including epithelial marker- negative cells in 32/40 or 80% of patients as compared to CellSearch where only 9/40 or 23% of patients were found to have CTCs In addition circulating tumor microemboli (CTM) were detected by filtration but were not detectable by CellSearch [71]. Immunohistochemistry stains on cells isolated by ISET showed variable expression of EGFR, CK and Ki67, however EpCAM expression was not detected. Despite the initial promising results of ISET capability for identifying high risk individuals that could develop LC, in a recent multicenter study, CTC detection using ISET was shown not to be suitable for lung cancer screening. In this study, factors limiting the widespread use of ISET for screening for LC, included pre-analytical factors, such as use of different blood collection tubes such as EDTA (ethylenediaminetetra-acetic acid) or Streck BCT (Streck, Omaha Nebraska) tubes, and imprecise standardization of the filtration method, by different operators [30]. It was subsequently concluded that in order to define a robust CTC test, suitable for real world consumption, large multi-center trials with large numbers of patients, using uniform pre-analytical conditions and identical technical analysis is essential [30].

One notable disadvantage of the filtration methods is that there exist subpopulations of CTCs whose size is smaller or in the same size range as WBCs (around 5 microns) and therefore would be eliminated during the filtration process. This feature may contribute to a lower sensitivity of CTC detection compared to other methods for enrichment of CTCs that do not rely on filtration [81].

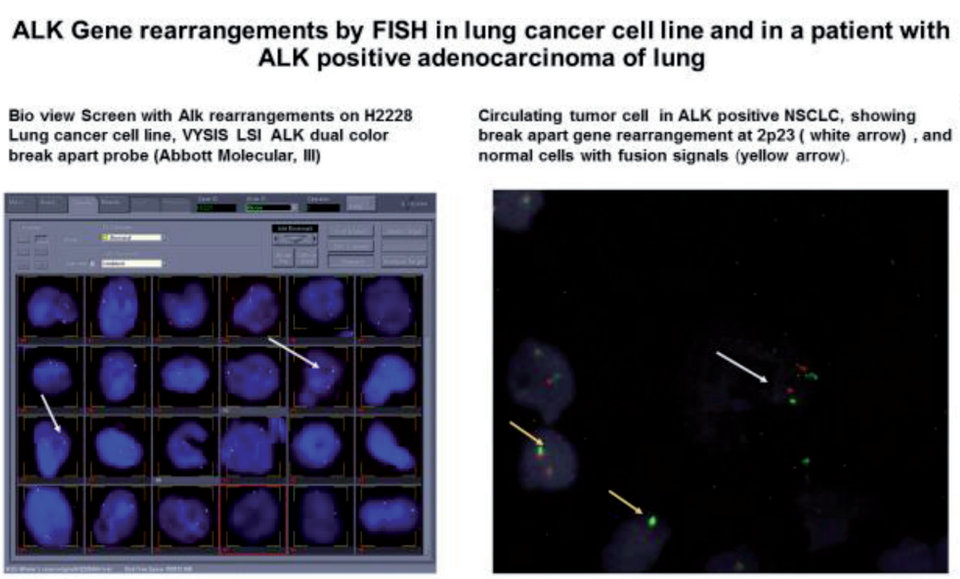

Figure 13.
Left hand panel, showing ALK gene rearrangements (white arrows) in H2228 lung cancer cell line, Vysis ALK break apart FISH probe kit (Abbott, molecular diagnostics, Des Plaines, IL). Right hand panel showing CTCs demonstrating ALK break apart gene rearrangements (white arrow) in CTC and normal cells with fusion signal seen as closely opposed green and red signals (yellow arrows), BioView duet™, scanning system, Rehovot, is.

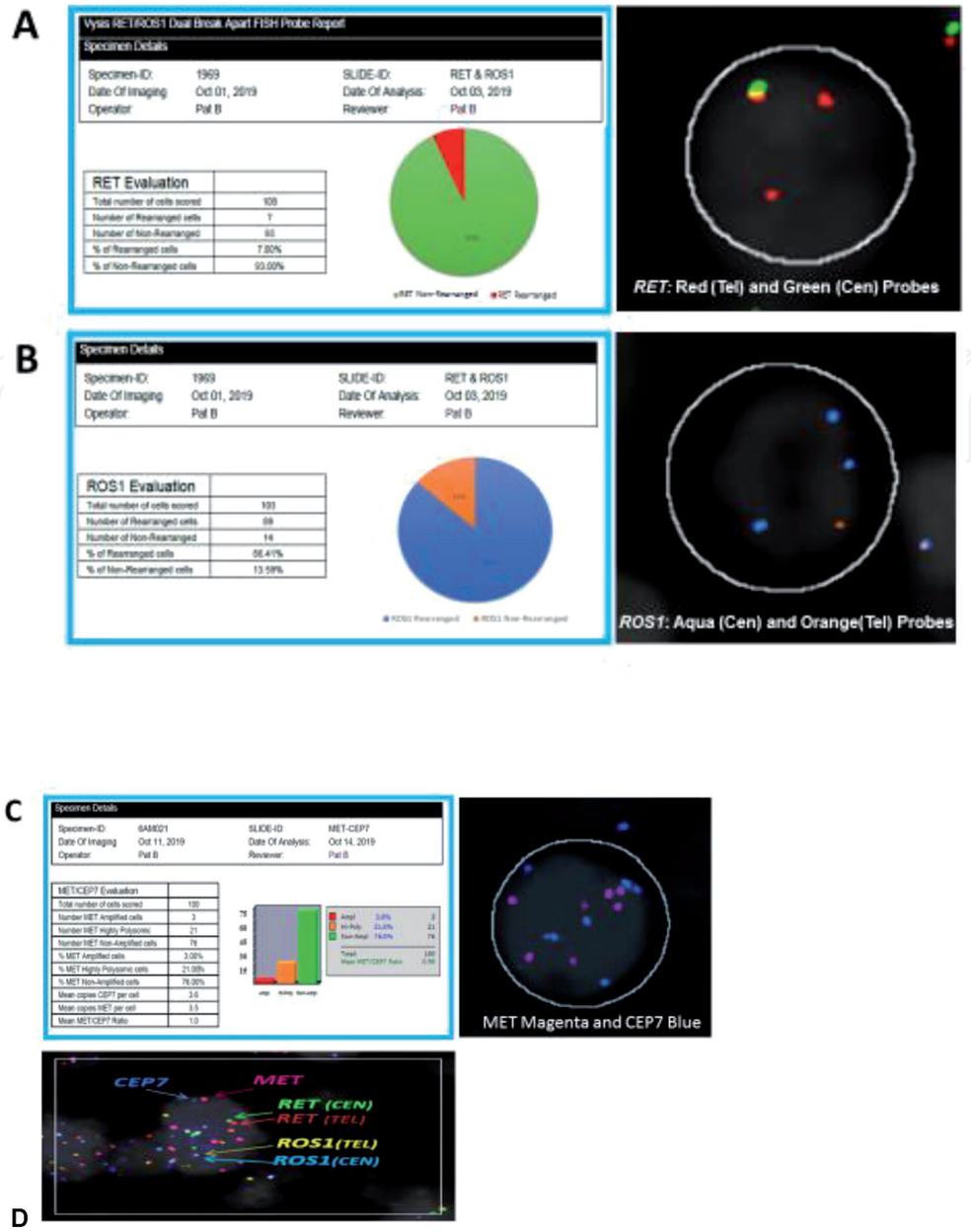

Figure 14.
(A) Multiplex fast FISH assay for detecting ROS1, RET and MET aberrations in FFPE non-small cell lung cancer specimens using BioView Duet™ scanning system for automated slide imaging and digital analysis of 6-color probe assay. Examples of case reports and representative images of cells positive for RET, ROS1 and MET aberration a) RET break apart probes labeled with Spectrum Green (5′ RET Cen) and Spectrum Red (3′ RET Tel) and captured with corresponding filters. b) ROS1 break apart probes labeled with SpectrumAqua (3′ ROS1 Cen) and SpectrumGold (5′ ROS1 Tel, pseudocolored in Orange) and captured with appropriate filters rearrangement probe. c) MET gene copy number probe labeled with Alexa 647 (pseudocolored in Magenta) and centromeric probe CEP7 labeled with Alexa 750 (Blue) and captured with appropriate filters. d) Combined image of the multiplex FISH assay with probe mix contained 6 differentially labeled fluorescent probes: 3′ ROS1, 5′ ROS1, 3′ RET, 5′ RET, MET and CEP7. (B) Figures supplied courtesy of Dr. Irina Sokolova, Tatyana Grushko and Katerina Pestova, Abbott, Molecular Diagnostics, Des Plains, IL [89].

16.5 Specific chromosomal abnormalities to detect CTCs

A method known as FICTION which combines IFISH and IHC may be used to simultaneously analyze the genotype and phenotype of a single CTC and can be used to study phenotypic and genotypic changes in the same cell [25, 64] The anaplastic lymphoma kinase (*ALK)* fusion gene is a driver gene for non-small cell lung cancer (NSCLC) [106]. *ALK*-positive NSCLC has been considered as a molecular subtype of NSCLC that provides unique clinicopathological characteristics of cancer diagnosis and prognosis [106, 107]. Initially, researchers found

that the *EML4-ALK* fusion gene exists in NSCLC patients by PCR and proteomics methods. However, due to the variation of fusion partners of ALK fusion genes, as well as the possibilities of unknown fusion partners, the FISH method with specific probes is an accepted and essential FDA approved companion diagnostic method performed on FFPE sections of NSCLC [108]. Currently, FISH has been considered as the gold standard of detecting ALK rearrangements [107, 109] using the Abbot Vysis *ALK* Break Apart FISH Probe Kit. The kit is designed with the 3′ and 5′ probes labeled by red and green signals; once abnormalities of *ALK* have occurred, deletion or splitting of the signals will be detected [110]. The limitation of the FISH based *ALK* test includes high cost and the utilization of specialized equipment [110, 111]. Studies have demonstrated CTCs containing the *ALK-EMLK1* gene rearrangements in patients with NSCLC [108–112] (see **Figure 13**) as well as in *ex vivo* cultures of CTCs from patients with NSCLC [113].

An enriched cell preparation can be used to detect oncogene fusions due to chromosomal translocations or inversions in lung cancer such as *ALK-EML1, ROS-1* or amplifications of oncogenes such as *Her2neu* or *EGFR*. Recently a novel FISH assay was described that can simultaneously detect *ROS1, RET*, and *MET* chromosomal aberrations in cells of NSCLC on FFPE tissue. This assay has the potential to be used on CTCs [89] (**Figure 14a–d**).

17. PDL-1/PD-1 on CTCs

In recent years the development of tumor immunotherapy drugs, especially immune checkpoint inhibitors (ICIs) targeting the programmed death protein (PD-1)/ programmed death ligand 1(PD-L1) has changed the paradigm in the treatment of malignant tumors and has shown superiority in terms of therapeutic effect and quality of life compared with traditional chemotherapy

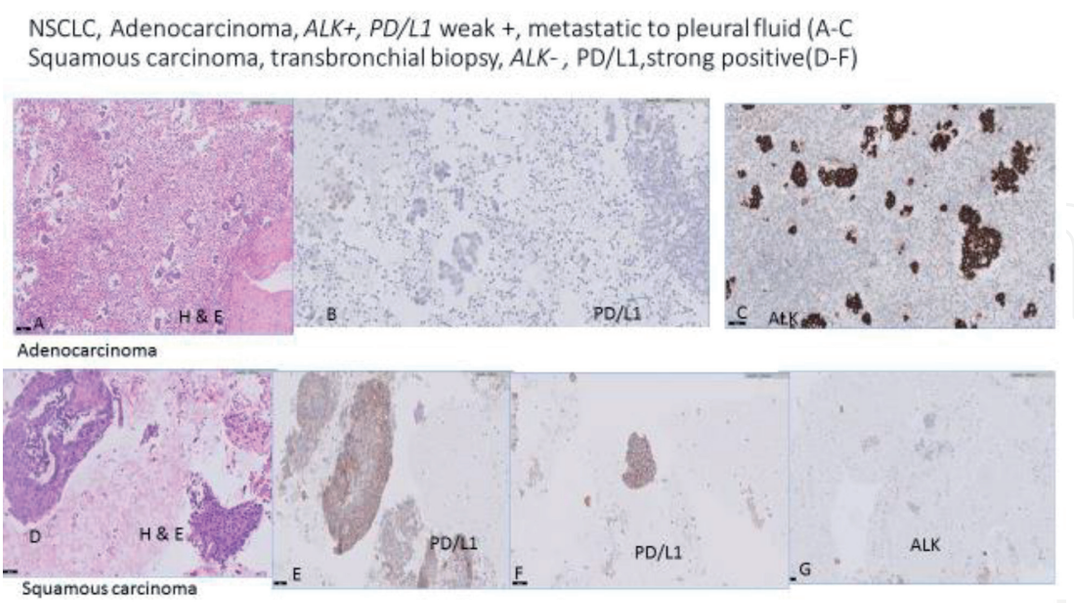

Figure 15.
Top panel: Example of metastatic adenocarcinoma of lung in pleural fluid in a patient with advanced lung cancer, A) papillary and acinar fragments of adenocarcinoma (H&EX20); B) PD/L1 IHC clone 22c3(pharm dx, Dako, CA) showed weak membranous expression, overall score < 50%; C) ALK IHC, clone D5F3 showed strong expression consistent with a positive test. Bottom panel: D) squamous carcinoma lung, moderately differentiated, transbronchial biopsy, H&E X40, E) and F) PD/L1 IHC22C3 shows strong positive membranous staining in >90% of malignant cells, G) Alk IHC, clone D5F3, showed negative expression consistent with a negative result.

[114–118]. Single-agent immune checkpoint inhibitors are now standard of care for advanced non-small cell lung cancer (NSCLC), and emerging data show that combining these agents with established chemotherapy further improves progression free survival and overall survival. The Phase III KEYNOTE-189 and IMPower-130 trial showed significantly improved survival using this strategy for non-squamous NSCLC, and the phase III KEYNOTE-407 trial showed similar results in squamous disease [119].

PD-L1 is a type 1 transmembrane protein with an extra-cellular N-terminal domain which inhibits or blocks the immune response by interacting with the PD-1 receptor which is expressed on activated T- and B-cells, and macrophages. Anti-PD-L1/PD-1 antibodies can reactive the immune system to eradicate tumors by blocking checkpoint proteins from binding with their partner proteins. PD-L1 expression may inform the use of checkpoint inhibitor combination therapy, while overall tumor mutation burden is also an emerging biomarker for ICIs. Antibodies that have FDA approval for NSCLC are two that block PD-1, namely Nivolumab (Opdivo, Bristol-Myers Squibb) approved for third line approval and Pembrolizumab (Keytruda, MSD SHARP and DOHME GMBH), which has approval for first- and second line-treatment, and one antibody blocking PD-L1, namely Atezolizumab (Tecentriq, Roche) for third line–treatment settings.

Immunohistochemistry (IHC) on tumor tissue, using the recommended FDA approved IHC-companion diagnostic for PD-L1, Ventana PD-L1 SP142 assay (Ventana Medical Systems, Tucson, AZ, USA) and PD-L1 IHC 22C3 PharmDx (Dako, North America, Santa Barbara, CA, USA) is the gold standard (**Figure 15**) and is widely adopted in PD-L1 detection.

17.1 Limitations of PD-1/PD-L1 inhibitors

1. Before or throughout therapy with PD-1/PD-L1 inhibitors, variations of PD-L1 expression in cancer cells might occur, causing different sensitivity to PD-1/PD-L1 blockade, which might be missed by a single biopsy.

2. In the tissue microenvironment, cells like lymphocytes and macrophages, also express PD-L1.

3. Patients who receive immunotherapy are often in an advanced stage when tumors develop and evolve, and the PD-L1 expression in the primary tumor may have also changed dynamically. Some of the patients at these stages might not be able to tolerate the side effects caused by invasive tissue biopsy [114–117].

CTCs originate from different tumor sites and thus, better reflect the tumor heterogeneity compared to tissue biopsies. They could therefore be potentially useful as a non-invasive method to detect PD-L1 expression in NSCLC patients [114–120]. In 2016, Schehr et al. [121] reported the finding of PD-L1-positive (PD-L1+) CTCs in NSCLC via in-house immunomagnetic enrichment system. However, other than cells expressing EpCAM, the study also mentioned the co-isolation of CD11b+, CD45-low, and cytokeratin-positive (CK+)- cells that expressed PD-L1, that could be mislabeled as CTCs, thus stressing the importance of proper identification of CTCs to avoid false positive events. Recently, Wang et al. [122] mentioned the dynamic changes of PD-L1 expression by CTCs 13 non-metastatic NSCLC patients. CTCs were in all 13 samples, PD-L1+ CTCs were detected in 66.7% of the sample. A recent study from 106 NSCLC patients, showed a 93% concordance between PD-L1

status in CTCs and tumor tissue, indicating the potential of a CTC test in determining response to ICIs [120].

Another recent study used a novel SE-iFISH (subtraction enrichment interphase FISH) strategy to examine the presence of PD-L1 on aneuploid CTCs and aneuploid circulating tumor endothelial cells (CTECs) to evaluate if their presence could be used as a surrogate biomarker to evaluate the efficiency of second-line anti-PD-1 (Nivolumab) immunotherapy. This study demonstrated that significant numbers of PD-L1+ aneuploid CTCs and CTECs could be detected in histopathologic hPD-L1- patients. In contrast to decreased PD-L1+ CTCs, the number of multiploid PD-L1+ CTECs (≥tetrasomy 8) undergoing post-therapeutic karyotype shifting increased in patients along with tumor progression following anti-PD-1 treatment and was associated with a significantly shorter PFS compared to those without PD-L1+ CTECs [123].

Many issues of PD-1/PD-L1 expression still need to be validated, including ensuring that the clones of antibody cocktail used for staining are standardized and equivalent in performance to the antibody clones included in the IHC kits that have received regulatory approval as companion diagnostics, as well as the threshold used to call a test PD-L1 positive, and whether the effect of the therapeutic use of anti-PDL1 antibodies interferes with the binding of diagnostic PD-L1 antibodies on CTCs [124]. Currently there are over 400 listed clinical trials for LC patients using ICI's alone or in combination with traditional chemotherapies [124]. Thus, if the CTC test, can be standardized, it will be of tremendous value as a complementary diagnostic tool for real time monitoring of PD-L1 expression for advanced lung cancer patients.

18. Conclusion

LB has evolved as a transformative technology for cancer diagnosis. Enormous strides have been made in recent years by the scientific and oncology communities to expand upon the tremendous value contained in a LB specimen. These readily obtained samples provide a real time window into the presence of cancer cells, the molecular evolution of the underlying tumor and its metastatic cascade and represent a far more feasible method for the longitudinal monitoring of the cancer patients as compared to direct tissue biopsy. Currently CTCs and ctDNA may be used in screening for early stage LC, as a diagnostic test to discriminate between a benign or malignant nodule on LDCT scan, as a decision –making tool or companion diagnostic for instituting targeted therapy for different genetic mutations, for detecting the presence of minimum- residual disease and as a monitoring tool for detecting response to immune-check point inhibitors. There are however still discrepancies in how to harness the power of CTCs, especially in the area immune-check point inhibitors, where standardization of CTC capture and companion PD-L1 antibodies, together with inter-laboratory standardization in interpretation of these tests, will be mandatory. Another key objective of future research will be the ability to establish mouse models from CTCs to monitor the epigenetic, and genetic profiling, functional and signaling pathways of malignant cells in response to different therapies. In order for LB for LC to become well accepted as a reliable source for actionable therapy large scale studies involving consortia of academic institutions and public/private partnerships are needed to establish reliable platforms for capturing and detecting CTCs and ctDNA that validate pre-existing discovery studies. Notwithstanding these caveats, it is apparent that LB is becoming an indispensable weapon in the battle against cancer.

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Conflict of interest

Dr. Ruth Katz is a consultant for Sanmed Bio, and on the scientific board of Lung life AI, Dr. Xin Ye and Ms. Xiaozheng Yang are employed by Sanmed Bio, Zhuhai, China. Dr. Katz is also an inventor of the Mutiplex LB FISH Test, licensed by MD Anderson Cancer Center to Lunglife AI, and sublicensed to Sanmed Biotech Ltd., Zhuhai, China as the Sanmed test for CACs. Dr. Katz was an employee of MD Anderson Cancer center from 1976 to 2018, during which time she developed the Mutiplex LB FISH Test. Dr. Katz and MD Anderson Cancer center, may in the future, be beneficiaries of royalties from this test,

She holds the following patents:
UTMDACC, Katz RL, Feng J: Detection and diagnosis of smoking-related cancers, United States, 12/761,134/UTSC: 658USC2, 4/15/2010, issued.

- UTMDACC, Katz RL: Circulating tumor and tumor stem cell.

  Detection using genomic specific probes, United States, UTFC.

  P1234WO, 12/10/2015, pending.

  Chinese Application No. 201580075104.1 based on PCT/US2015/065057 and U.S. Provisional Application No. 62/090,167; Entitled "CIRCULATING TUMOR AND TUMOR STEM CELL DETECTION USING GENOMIC SPECIFIC PROBES" by Ruth Katz.

  In the Name of Board of Regents, The University of Texas System
  Our Ref. UTFC.P1234CN; Your Ref. MDA14–035.

- The four-color FISH test described within this chapter is licensed to LunglifeAI (Los Angeles, CA, USA) and sublicensed to San Med Bio (Zhuhai, China) and Livzhon Pharma, China.

Dr. Roberta Carbone is a Tethis employee and holds equity and/or stock options: she holds a patent on the SBS technology (Method for immobilizing biological samples for analytical and diagnostic purposes, WO2019021150A1).

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