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De novo liquid biopsy and radio genomic diagnostic approach with combined deep learning artificial neural networks for NSCLC

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Abstract

Each year, the mortality rate and incidence of non-small cell lung cancer (NSCLC) are dramatically increasing. The introduction of liquid biopsy in the clinical practice of NSCLC has completely revolutionized the approach to such neoplasm since is generally detected through complex and invasive procedures and unfortunately at advanced stages. The importance and innovation of liquid biopsy are linked with the possibility of cancer detection at every stage, adjuvant treatment, resistance genotyping, systematic initiation of treatment, minimal residual disease, early detection of relapse, and screening of NSCLC. Circulating tumor DNA (ctDNA) is now emerging as a non-invasive biomarker that will help to track tumor burden and allow the monitoring of cancer genome in blood across several malignancies. Recently, the combination of liquid biopsy and radiomics seems to deliver an efficient way to study cancer evolution over time providing an important support tool to daily clinical practice. CT (Computed Tomography) images are of particular importance in this context because they convey functional and anatomical information, respectively. Machine learning provides a variety of approaches for dealing with this potentially high-dimensional challenge. In particular, we used Enet neural network for image assessment. This study represents an interesting attempt to explore the usefulness of liquid biopsy, radiomics, and deep learning in the NSCLC clinical routine. We studied a NSCLC patient cohort from the first access to our department to follow-up. Our results showed a promising correlation between the ctDNA quantity and radiomic features evaluated by automated computed tomography according to RECIST criteria with the Enet deep learning method, which allowed us to define more accurately progression-free survival (PFS) and overall survival (OS) of patients during the course of cancer history. Therefore, the above mentioned diagnostic tools including the combination of liquid biopsy, radiomics, and deep learning tools collectively can represent a very robust and new approach in the monitoring and management of NSCLC.

CHAPTER 1

Background, Rationale, and Objectives

NSCLC Epidemiology/Pathology/stages/treatment by stages

Cancer is the biggest cause of mortality that places a burden on governments and is one of the world's major health challenges. According to 2016 cancer incidence statistics provided by the National Program of Cancer Registries, Surveillance, Epidemiology, and End Results Program, and the North American Association of Central Cancer Registries, it is the second-highest cause of death in the United States. According to the latest current estimates for 2020, around 1,806,590 new cases of cancer were reported in the United States, with 606,520 individuals dying. Until 1991, cancer deaths were growing, but after 2017, they began to reduce rapidly. Approximately 2.9 million fewer cases than the peak period predicted cases were recorded, indicating a 29 percent decrease in the total cancer mortality rate. This rate of cancer drop remained in four major cancer types: breast, prostate, colorectal, and lung cancer. Nonetheless, the fall in colorectal and breast cancer in females slowed from 2008 to 2017, while reductions in prostate cancer were reversed . However, cancer prevalence increased from 2008 to 2013, rising from 3% to 5% in males and 2% to 4% in women. Cancer mortality decreased by 2.2 percent between 2016 and 2017. However, lung cancer did not exhibit a substantial decline in 2017 when compared to colorectal, breast, brain, and prostate cancer combined. The overall number of cancer patients diagnosed is around 1,806,590, which equates to almost 4,950 new cases per day. In 2014, there were around 228,820 new lung cancer

patients. However, the number of fatalities from lung cancer has been documented as 135,720. Lung cancer is the second most frequent illness in both men and women, accounting for 75-80 percent of fatalities. Lung cancer has been identified as one of the most difficult cancers to treat, accounting for over 1.38 million deaths worldwide (1). Lung cancer has been recognized as a deadly pandemic driven by a variety of known and unknown factors . The most prevalent disease diagnosed, according to the GLOBOCAN 2018 report, is lung cancer, which is commonly recorded expressly in men. The two major histological categories are small-cell carcinomas and non-small-cell carcinomas. It is also known that the presence of additional stains on tiny samples used for diagnostic reasons discriminates between adenocarcinomas and squamous cell carcinoma conceivable . Similarly, anaplastic lymphoma kinase, ROS1 and epidermal growth factor receptor predictive analyses are essential for patients with advanced non-squamous cell non-small cell lung cancer (NSCLC). Furthermore, programmed death ligand-1 immunohistochemistry is employed for biomarkers in negative NSCLCs. Nonetheless, repeated phase III studies demonstrated that therapy is guided by histology and also gives molecular profiling advice (2).

Non-Small Cell Carcinoma (NSCLC)

Non-small-cell lung carcinoma is the most common, accounting for over 75% of all lung malignancies. Chest radiography was performed on high-risk lung cancer patients. Furthermore, positron emission tomography and potentially computed tomography are conducted if an alternative diagnosis cannot be determined. If the suspicion of lung cancer is greater, a diagnostic assessment is also conducted, with three primary processes performed concurrently, including tissue diagnosis, staging, and ultimately the functional evaluation, which not only influences the prognosis determination but also the treatment planning. Molecular changes that cause carcinogenesis and driver mutations are also prevalent in NSCLC (3). Furthermore, the European Society of Medical Oncology recommendations indicated that, except for SqCC patients, the recommended therapy for these kinds of malignancies is molecular testing . The ROS1 rearrangement or anaplastic lymphoma kinase (ALK), oral tyrosine kinase inhibitors (TKIs) treatment is primarily used in patients with epidermal growth factor receptor (EGFR) mutations, and these treatments are also approved as preferred treatments by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (4). In addition to normal chemotherapy, the ALK inhibitors ceritinib and crizotinib are used in the advanced stages of NSCLC to assist in ALK testing. Patients in advanced stages should therefore be prioritized for testing for the existence of molecular abnormalities. Furthermore, various targeted medications for the changes and the driver genes,

including BRAF , HER2, NTRK1 , MET , and RET are discovered. Lung cancer pathology has therefore developed into a multistep technique that begins with immunohistochemistry (IHC) and morphology to discriminate between malignant and benign forms, primary to metastatic and other types. Molecular characterization is also utilized for similar goals (5). There have been specified mutations found in the epidermal growth factor receptor (EGFR) gene in patients with non-small cell lung cancer that are capable of predicting responsiveness to EGFR tyrosine kinase inhibitors (TKIs). International recommendations urge using molecular testing treatments for patients with advanced non-squamous NSCLC . Nonetheless, obtaining a tissue sample is a somewhat difficult process when it comes to the detection and/or diagnosis of non-small cell lung cancer. In contrast, liquid biopsy enables the evaluation of individuals with NSCLC for EGFR-based targeted treatment. This is often accomplished by assessing circulating-free tumor DNA (cfDNA) in peripheral blood samples, which may primarily serve to replace tissue biopsy swiftly and effectively (6). This method of obtaining liquid biopsy samples is more convenient and less intrusive, with as many repeats as feasible. This non-invasive approach is highly recommended for monitoring genetic changes at the molecular level and for selective molecular screening in patients with NSCLC. However, multiple previous studies show improved harmony between testing using tissue samples and plasma samples. In this context, the US Food and Drug Administration (FDA) just authorized the first liquid biopsy test in 2017 to assess driver gene alterations using cfDNA for patients with NSCLC. Since it has been observed that around 80% of lung cancer instances are non-small cell lung cancer (NSCLC), with squamous, adenocarcinomas, and big cell lung cancer also being present. Only a few unclear recommendations exist for the effective care of NSCLC, and a collaborative effort by worldwide platforms is necessary based on patient characteristics, disease severity and extent, and the most acceptable predictive and prognostic markers. Recently, the advent and use of immune checkpoint inhibitors (ICIs) in combination with several other targeted treatment techniques have transformed the entire therapeutic landscape in patients with non-small cell lung cancer (NSCLC). According to studies, about half of all NSCLC patients die within a year (49 percent). Furthermore, the 5-year survival percentage in individuals diagnosed with NSCLC in the early stages of lung cancer has been assessed to be 56%. Nonetheless, only 16% of patients are lucky enough to be diagnosed with NSCLC at these early stages, i.e., Stage I and Stage II . In light of this backdrop, the discovery of advanced diagnostic methodologies for the prompt detection of NSCLC is highly desirable, hence providing medical practitioners with an effective strategy for the proper therapeutic care of the illness, i.e., NSCLC. Currently, the diagnosis of NSCLC is predicated on low-dose CT scans

(7). NSCLC screening has mostly been done using radiological methods, particularly in high-risk groups. As a result, full recovery in these individuals may only be achieved by prompt diagnosis to assist following surgical operations for NSCLC therapy. In this respect, fresh and creative diagnostic procedures that may also serve as an alternative measure to the currently existing chest imaging method for timely screening and early identification of NSCLC, such as the use of Liquid Biopsy, must be brought into clinical usage. The use of liquid biopsy for early identification of NSCLC seems to be extremely promising and realistically accessible, with the potential to transform current diagnostic and screening procedures relevant to cancer, especially for NSCLC, with improved adherence (7). The preceding context, principally examines and delineates the function of liquid biopsy in NSCLC in connection with its analytes, applications, components, and other diagnostic procedures. This will pave the path for researchers to determine the most effective diagnostic and/or treatment technique for eliminating non-small cell lung cancer illness globally. The summary results emphasizing the essential aspects of NSCLC in combination with genetic and molecular variables were previously hinted at in the preceding parts of the present review work. However, the thorough clarification of the usage of liquid biopsy as one of the most suitable yet convenient detection techniques, ctDNA analysis methodologies, and the analytes, components, and applications of liquid biopsy are described in the following parts of this study. The most relevant information on this subject was compiled by a thorough literature analysis of prior NSCLC-related studies published between 2000 and 2020.

Immunotherapy

This increase in mortality is primarily due to a lack of screening methodologies that allow for timely disease diagnosis followed by appropriate therapeutic management via surgery, chemotherapy, radiotherapy, hormonal therapy, and, more recently, synthetic lethality and/or immunotherapy. Immunotherapy-based cancer care has been noted to be constantly changing for a broad range of malignancies. For example, the findings of the Keynote-89 and Keynote-024 studies demonstrated impacts on overall survival in metastatic non-small-cell lung cancer (NSCLC). Furthermore, it is useful in evaluating as well as establishing predictive and/or prognostic biomarkers. Immune checkpoint inhibitors (ICIs) with anti-cancerous activity, used alone or in combination, have transformed the concept of clinical and therapeutic management for a variety of cancers, including breast, bladder, colorectal, cervical, gastric (8), liver cancer, kidney, head and neck squamous cell carcinoma (HNSCC) melanoma , small-cell lung cancer (SCLC). Currently, the only predictive biomarker used as a diagnostic test for

developing first-line immunotherapy is immune-histochemical evaluation of PD-L1 tissue expression. When utilized in pathology, however, it may expose a variety of biological and technological constraints. Furthermore, due to its variable expression inter-tumor and intra-tumor, tissue-based expression analysis of PD-L1 (both in immune cells and tumor) does not enable sufficient evaluation, complicating the process of optimal treatment management or monitoring in cancer patients. As a result, for tiny tissue samples such as those of bronchial origin, analyzing a tissue-specific PD-L1 expression profile may not be typical of the overall tumor (9). In recent years, liquid biopsy (LB) has received a lot of interest when it comes to developing predictive and prognostic biomarkers for various diseases, particularly tumors of the thoracic cavity. LB is a very convincing approach because it is least invasive or nearly non-invasive, provides increased cost-effectiveness, and provides pathologists and physicians with timely information, thus guiding sufficiently during decision-making processes for strategically devising therapies for patients diagnosed with any type of cancer. Most notably, LB may help reduce worry about illness recurrence, disease-free survival, disease relapse, and resistance to prescribed medicines during treatment follow-up (10). In light of the preceding section's context, the current review has been planned to discuss the utility of liquid biopsies in the field of immune-oncology, specifically in an attempt to learn more about response prediction, disease relapse, or studying adverse effects in patients receiving immune checkpoint inhibitor therapy. Furthermore, this study focuses on the components of liquid biopsy that may be used in a variety of ways to uncover new indicators of illness prognosis and prediction in immuno-oncology.

Cancer Immunotherapy

Immunotherapy has been a significant goal in cancer treatment in recent years, and evidence suggests that combining immunotherapy with traditional therapies such as surgery, radiation, and chemotherapy might boost patient survival rates more effectively. Effective immunotherapeutic techniques need the detection of diagnostic, predictive, prognostic, and therapeutic procedures. The procedures employed in the clinic for guiding immunotherapies, such as tissue biopsy and imaging, are still not 100 percent efficient owing to disadvantages such as sensitivity and accuracy. Traditional tissue biopsy, for example, cannot always be performed regularly owing to its invasive nature. Furthermore, the information gained from a single biopsy provides just a limited picture of a tumor and so fails to reflect tumor heterogeneity . In particular, in recent years, a contemporary diagnostic approach is known as

"liquid biopsy" has received a lot of attention.
Liquid Biopsy's Utility in Cancer Immunotherapy/ Immuno-Oncology
In contrast to the currently common clinical care and patient therapeutic management standards (which include surgical resection, radiation, and/or chemotherapy), cancer immunotherapy has lately been found to bring improvements in the survival and quality of life of cancer patients (11).

The usefulness of Liquid Biopsy in Cancer Immunotherapy/ Immuno-Oncology

In contrast to the currently common clinical care and patient therapeutic management standards (which include surgical resection, radiation, and/or chemotherapy), cancer immunotherapy has lately been found to bring improvements in the survival and quality of life of cancer patients (11). As a result, immunotherapy plays an important role in considering novel interventions for the therapeutic care of cancer patients, beginning with the stage of cancer progression and metastasis and progressing to the point of cancer management through the use of adjuvant and non-adjuvant therapies, thus transforming and revolutionizing the horizon of therapeutic care strategies for almost all cancer types. Immuno-oncology also considers immune surveillance, which is intended to eliminate tumor cells through innate immunity pathways. Recently, T cell immunological checkpoints such as CTLA-4 and PD-1 have been identified to advance the usefulness of cancer immunology in the present day (81). While it is true that immune system activation is necessary for gaining control over tumor growth, it may also result in the creation of auto-immune responses. This was largely due to the discovery of monoclonal antibodies specifically created against CTLA-4 and PD-1 (inhibitory immunological checkpoints), which resulted in unexpected anticancer effects as a result of increased immune system activation at different stages of the immune cycle. Quite thoroughly, the use of immunotherapeutic techniques in oncology needs the creation, validation, and regular use of highly specific but sensitive biomarkers (prognostic as well as predictive) for the clinical treatment of cancer patients. Currently, immune-histochemical analysis of anti-PD-L1 on tissue biopsy/sample has been performed and validated through the use of prescribed diagnostic methods to devise and plan the first line of immunotherapy for adequate management of late clinical-stage and/or metastatic cancer patients , particularly non-small-cell lung cancer (12). Nonetheless, present biomarker detection technologies must be improved for better and more prompt diagnosis, as well as for developing therapy regimens for cancer patients worldwide. In this context, the use of liquid biopsy (LB) might be a viable source for providing an additional benefit by adding value to the previously existing technique

of PD-L1 IHC. Contextually, the current review aims to highlight how liquid biopsy can be used in the field of immune-oncology by demonstrating its robustness and soundness in predicting relapse, immune responses, and/or adverse events in cancer patients who have received immunotherapy that includes anti-PD-1/PD-L1 and CTLA-4 (the immune checkpoint inhibitors-ICIs), as well as a detailed discussion of the liquid biopsy components that can be used clinically.

Radiological Assessment (RECIST)

Tumor burden must be measured in phase II and III oncology studies to determine the therapeutic effect of anti-cancer drugs. The Response Evaluation Criteria in Solid Tumors (RECIST) guideline has been widely utilized in cancer studies since its establishment in 2000 and modification in 2009 (13) for the systematic evaluation of tumor burden. Cross-sectional imaging techniques, mostly computed tomography (CT), are used to measure and total the unidimensional longest diameters of target lesions to capture tumor loads (13). The RECIST criteria are based on repeated tumor burden evaluations (13), and response classification is extremely accurate. When observed tumor burdens are near the 20% cut-off value for progressing illness (i.e., 10–30%), the influence of measurement variability on response categorization may be overestimated (14). RECIST 1.1 lowered the number of target lesions, as did the techniques for measuring lymph node size; clarified disease progression, which needs at least a 20% rise and a 5-mm absolute increase in the size of target lesions; and included FDG PET in the identification of new lesions. Some of the changes, such as needing a 5 mm expansion of the targeted lesions in addition to a 20% increase, were intended to give more clinically meaningful outcome evaluation in genomically defined patient groups treated with effective molecular targeting medications. Radiologically, the change from standard cytotoxic chemotherapy to immunosuppressive treatment coupled with targeted therapy is also difficult to detect. The RECIST 1.1 (Response Evaluation Criteria in Solid Tumors, Version 1.1) criteria are commonly used to evaluate tumor response in solid tumors (14). In a few studies, RECIST 1.1, which is based on unidimensional diameter, has been found to underestimate tumor response in the evaluation of anti-angiogenic treatment (15). To circumvent the limitations of RECIST 1.1, the CHOI and modified Choi (mChoi) criteria were created and have been employed in a range of solid tumors, including gastrointestinal stromal tumors and metastatic renal cell carcinoma, employing target therapy. The inclusion of tumor attenuation data was predicated on the notion that if the targeted treatment was successful, antiangiogenic medicines

would result in reduced blood supply, resulting in less tumoral enhancement. According to multiple studies, Choi/mChoi has a stronger relationship with clinical outcomes than RECIST 1.1. (16). The RECIST collaboration advises using specialized oncology software tools to assist simplify and hence standardize target lesion size measurements to decrease between-reader variability caused by these variables (17). RECIST, on the other hand, requires readers to choose target lesions whether or not they use these software solutions. Depending on the sort of response categorization technique used, readers must base their response analysis on a more or less small sample of the total tumor burden (i.e., the maximum number of reportable targeted lesions). All existing response assessment techniques operate on the implicit premise that changes in the size of these targeted lesions are adequately representative of changes in the total tumor burden. Under the World Health Organization, reviewers were allowed to pick and measure as many target lesions as they deemed appropriate; under the first version of RECIST (RECIST 1.0), readers may select up to 10 target lesions, with a maximum of five per metastatic organ. In the updated version of RECIST (RECIST 1.1), the total number of reportable target lesions was lowered from 10 to five, and the maximum number of lesions per metastatic organ region was reduced from five to two. Two large-scale database studies using current response classifications to predict how many target lesions are required to produce a response classification concordant with RECIST 1.0 or the World Health Organization's response classifications supported the conclusion (18). In 2009, the immune-related response criteria were suggested, which are based on WHO standards and include the collection of bidimensional measurements of target lesions. The inclusion of assessments of various target lesions (each one must be at least 55 mm in diameter; a total of 10 visceral lesions, five new lesions per organ, and five new cutaneous lesions) in disease evaluations was the most important alteration. In 2013, researchers produced an updated irRC that was based on the original RECIST (102) and used unidimensional data. Due to the need to standardize and verify response criteria, the RECIST working group intended to create a database of data from immune-therapeutic studies to test and assess RECIST 1.1 and make adjustments if required. Most studies including these medications have employed RECIST 1.1 to identify main and secondary efficacy-based endpoints, with irRC or their modified definition of RECIST reserved for exploratory endpoints uncovered during the design and early collecting of the immunotherapeutic repository (19).

Liquid Biopsy

Liquid biopsy is used to track tumor changes by administering a simple blood test to cancer patients, allowing tumor cells and nucleic acid fragments to be detected. The major components of liquid biopsy are RNA or DNA fragments that are extracted from tumors and circulate freely in the circulation as ctRNAs and ctDNAs. These tumor cells are known as circulating tumor cells (CTCs), which circulate in the blood and interact with exosomes to be exploited for cancer surveillance and early diagnosis, including lung cancer. The epigenetic and genetic changes prevalent in lung cancer that are important for tumor invasion and progression may be the tumorigenesis drivers. These changes, whether genetic or epigenetic, might be readily discovered via liquid biopsy. ALK, HER2 , EGFR , FGFR , MET, ROS1, KRAS and RET (20) are some of the mutations utilized in lung cancer to determine and discover therapy methods with early diagnosis. The genome sequencing methods and subsequent developments operate as a collective ray of hope, with liquid biopsy being mostly employed in cancer investigations. The liquid biopsy is intended to evaluate biomarkers found in patients' bodily fluids, which might include urine, pericardial effusion, blood, or cerebrospinal fluid. Pathologist Thomas Ashworth was the first to identify the existence of circulating tumor cells (CTCs) in the circulation, but his study received little attention (21). However, this was recently recognized as a significant discovery that may be employed as a novel and non-invasive source of a tumor gene. This finding was unlike past ways of liquid biopsy since surgery is not necessary for this process, which is less invasive than others. It is also less expensive than others. The present argument focuses on the relevance of this procedure, which either supports the conventional theme of liquid biopsy or replaces it with cancer diagnostic treatment (22). This has grown in relevance since it not only identifies cancer but also tracks current mutations and tumor cells in the blood. Multiple sampling during this therapy assists in the prediction of tumor recurrence as well as the determination of the appropriateness and efficiency of cancer treatment. The Food and Drug Administration (FDA) has also authorized and validated liquid biopsy, deeming it to be a superior and more favorable cancer prognostic procedure. Liquid biopsy diagnosis has shown to be quite useful for a wide range of malignancies, including breast cancer, colon cancer, liver cancer, prostate cancer, melanoma, gastrointestinal cancer (23), and others (18, 24). Its use in the screening, detection and diagnosis of lung cancer has lately grabbed the attention of oncologists due to its low invasiveness while providing increased convenience (25). To be more specific, the parts that

follow will provide light on the use of liquid biopsy in the diagnosis and clinical therapy of non-small cell lung cancer (NSCLC).

Liquid Biopsy in Non-Small Cell Lung Carcinoma

Liquid biopsy is a genuine but most suitable way of identifying epigenetic and genetic modifications in blood samples utilizing non-invasive equipment. Because this technique is crucial for lung cancer prediction, diagnosis, and tailored medication, the molecular features of NSCLC might be evaluated for this purpose. Several notable techniques must be identified for the early diagnosis of mutations and cancer. Many benefits are being explored about liquid biopsy, which is favored over fine-needle aspiration biopsy for cancer detection. Aside from ctDNA mutations, ctDNA methylation, also known as methylscape, is a notable discovery for the diagnosis of non-small cell lung cancer. This may also be utilized as a dominating result for usage as a biomarker for non-small cell lung cancer. Early detection is typically possible with the use of imaging tools for diagnosis in high-risk patients, which includes low-dose computed tomography (LDCT) (26). A nationwide lung cancer study found that individuals identified using LDCT had a 20% lower mortality rate when compared to those who were screened for NSCLC using chest radiography (13). Furthermore, another experiment known as the NELSON trial revealed that LDCT scanning reduces mortality rates in men and women by 26% and 61%, respectively. LDCT does, however, have certain limitations in terms of its use as a screening tool for early NSCLC identification in both men and women (27). These unfavorable outcomes mostly include erroneous positive or false negative test findings, increased exposure to potentially harmful radiation, and over-diagnosis . Based on the evidence presented so far, it can be concluded that the identification of early-stage NSCLC and related malignancies still needs the use of tissue specimens for diagnosis. Along these lines, significant interventional progress has been made in recent years, with obtaining a lung lesion tissue sample regarded as reasonably easy, safer, quicker, and more precise (28). This technique is quickly evolving and finding usefulness not only for NSCLC diagnosis but also for clarifying NSCLC stages and developing optimal regimens for NSCLC treatment. Biopsy-based diagnosis is significantly less intrusive and provides adequate information for individualized medicine in NSCLC treatment, which is then followed by elaborative molecular analysis. Blood specimen analysis, also known as liquid biopsy, has been extensively employed by researchers all over the globe to provide early and easy NSCLC screening (29).

Components of Liquid Biopsy

Liquid biopsy is a groundbreaking technology for cancer detection that isolates circulatory tumor cells, exosomes, and circulatory tumor DNA from an unexpected and distinct viewpoint relevant to early lung cancer diagnosis. New approaches, like next-generation sequencing technologies, contribute to breaking through technical boundaries and broadening liquid biopsy applications. A cancer diagnosis is achievable using patient data acquired from liquid biopsy screening, as well as predicting and estimating the response to a potential treatment approach. Importantly, identifying the target gene alterations responsible for disorders such as cancer makes therapy options simpler and more suitable. Furthermore, they might assist in the discovery of secondary resistance, which will aid in the diagnosis of cancer progression (30). Figure 1 depicts the most important circulatory components of the liquid biopsy that may be considered for gaining a sufficient but hopeful image for non-small cell lung cancer (NSCLC) screening and diagnosis:

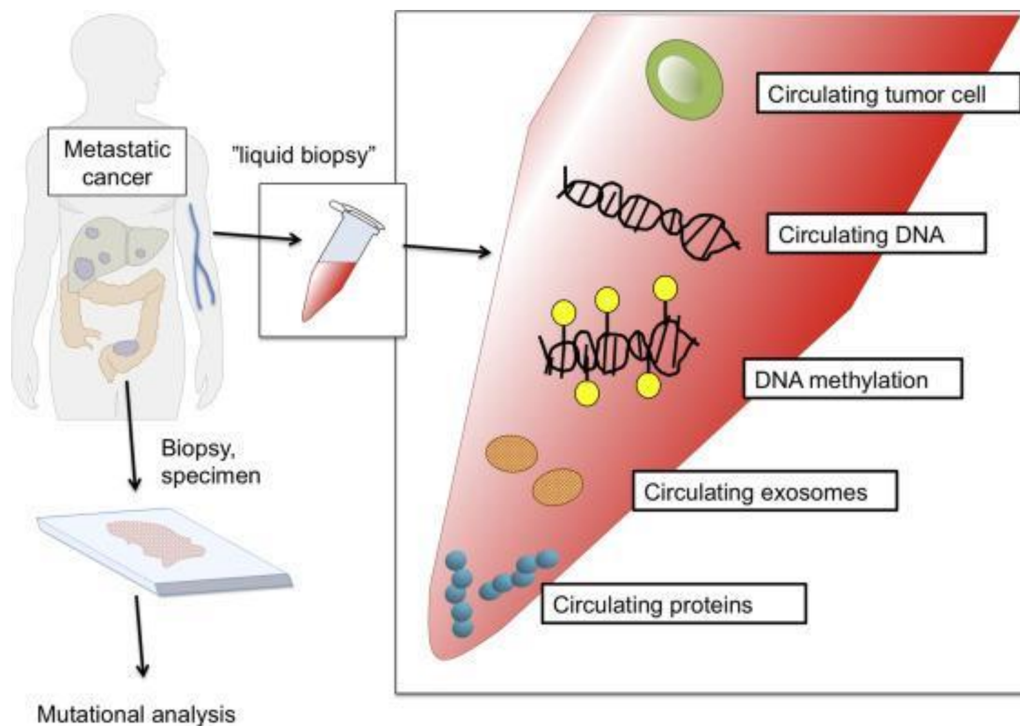

Figure 1. Liquid biopsy with circulating DNA and biomarkers (Adapted from (31)

Applications, components, and special considerations of liquid biopsy in Non-Small Cell Lung cancer patients

Because lung cancer is identified via difficult procedures in late stages and is invasive, the notion of liquid biopsy is entirely important in the inquiry (32). While liquid biopsies have

non-invasive approaches for detecting genomic changes and identifying and testing targeted treatments. Furthermore, the biopsy tracks therapy response and evaluates its efficacy and accuracy. Important aspects of liquid biopsy in lung cancer include diagnosis at all stages, adjuvant therapy, and resistance genotyping, systematic treatment start, minimum residual treatment, early recurrence detection, and screenings for effective NSCLC management. Circulating tumor cells (CTCs), cell-free circulating DNA (cfDNA) (Circulating Tumour DNA (ctDNA)), cell-free circulating free RNA (cfRNA) (Circulating Tumour Messenger RNA, Circulating Tumour MicroRNAs), Exosomes, micro vesicles, and platelets are all components of liquid biopsy (Figure 2). Furthermore, indicate that these components are detectable in bodily fluids and that the combined impact of all biomarkers is more specific than any one biomarker alone (33). According to Pantel and Alix-Panabières (2010), miRNAs may be discovered in saliva samples, and CTCs are present in the bloodstream, are separated from the main tumor, and can be employed in cancer clinical investigation and diagnosis.Furthermore, the CSF is acquired by an invasive process. Following that, we will shed light on the ideal and most relevant biomarkers that may be made accessible and exploited for liquid biopsy in NSCLC, to study their functional implications and involvement in early and timely NSCLC diagnosis, molecular screening, and patient prognosis. Not only that but liquid biopsy samples have been widely studied to understand drug responses and the extent of drug resistance in NSCLC patients by estimating the presence and/or absence of tumor-derived circulating DNA material (ctDNA) and circulatory tumor cells (CTCs) in liquid biopsy obtained from NSCLC lung lesions. Liquid biopsy sampling has the benefit of enhanced tumor heterogeneity (both geographic and temporal) and gives a more full molecular image of the tumor landscape, i.e., it can appropriately guide cancer metastatic areas as well (34).

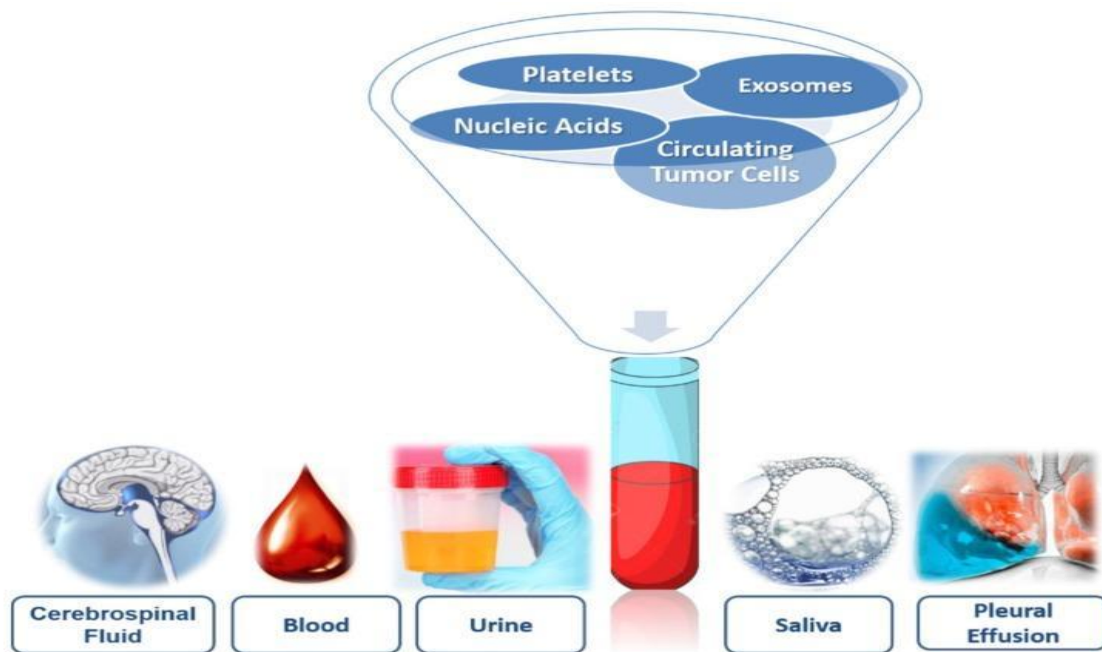

Figure 2. Components of liquid biopsy

Analytes of liquid biopsy in NSCLC

The analytical approaches are used for circulating cell-free DNA and analyte detection that is released by cancer and is referred to as liquid biopsies in clinical trials. These analytes are the body fluids having tumor-derived products present in the blood and were presented by Pantel and Alix-Panabieres. The liquid biopsy analytes contain not only the circulating tumor cells (CTCs) but also the circulating cell-free tumor DNA (ctDNA), which are also of considerable importance and are among the most highly investigated and useful analytes. Other analytes may include tumor-educated platelets, miRNAs, tumor-derived exosomes, and long non-coding RNAs (14). A detailed explanation on account of all these aforementioned analytes of the liquid biopsy specimen that can serve as be employed for NSCLC diagnosis as well as its management has been furnished in the following part of the present review paper. (Figure 3)

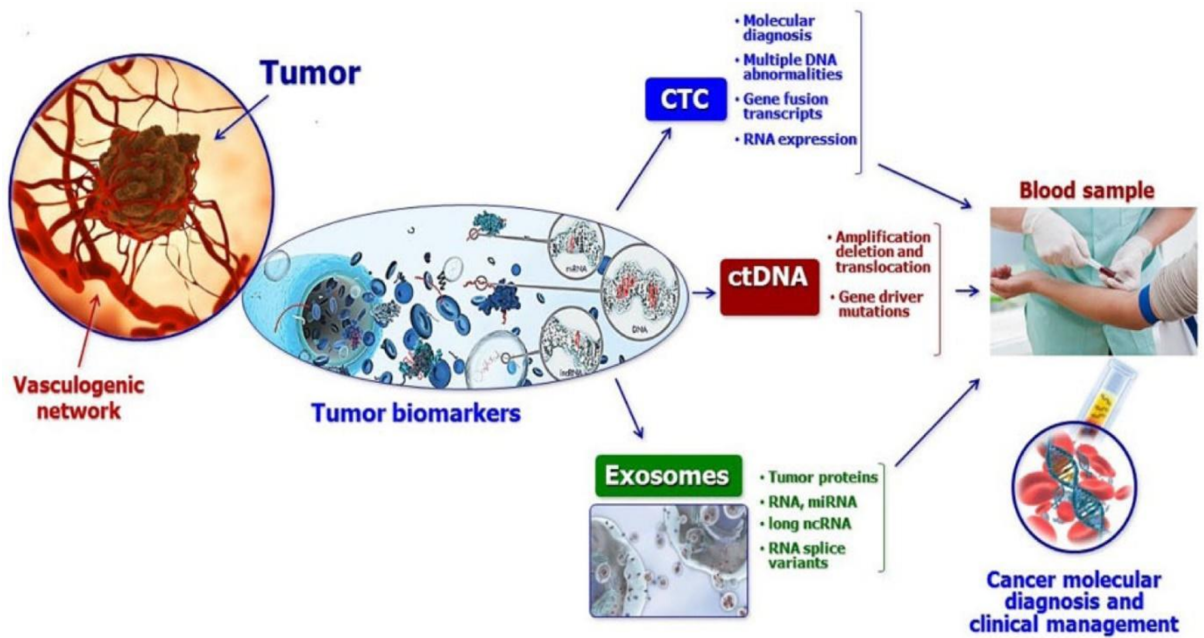

Figure 3. NSCLC Tumor biomarkers (30)

Circulating Tumor Cells

The metastatic sites and the primary tumor exfoliate the present vital cells whenever they enter the blood through circulation and are coagulated with white blood cells and red blood cells. They are known as the circulating tumor cells (CTCs) (Figure 3). These cells are effectively used in the treatment of cancer and are used for patients' regular monitoring with standard protocols (35). Thus, it controls the spread, progression, and metastasis of tumors. In the cancer study, an interesting investigation could also be initiated in the domain of circulating cell-free DNA (cfDNA) and circulating tumor cells (CTCs). These CTCs' detection is already popularized in the liquid biopsy domain with tumor surrogate samples. As discussed earlier, they appear as the marker for the detection of cancer even before the visual appearance of a tumor, including melanomas (36). The short rate of survival in metastatic patients with lung cancer is detected through the circulating tumor cells. The specificity and sensitivity are combined with the detection of CTC, which is itself a filthy challenge. The invasion process initiation is detected by oncologists through only a few CTCs in blood up to 10ml, depicting the mixing of CTCS with about 50 billion erythrocytes and 100 million leukocytes. One of the concerns remains the specificity that is essential in the present context. Poor therapeutical and clinical choices with quality negatively affected and life expectancy in cancer patients are generated through non-tumor cell wrong identification. This is a significant and crucial step that needs specificity. For the last many years, the CTC assays availability commercially has

gained wide importance. The detection of ccfDNA is difficult and has been considered a biomarker source since the discovery of epigenetic changes. The sensitivity and specificity factors in the ccfDNA concentration measurement of healthy controls remain challenging. Higher specificity could be achieved through ccfDNA identification from cancer patients' samples. Moreover, this is possible by identifying the genetic variants with mutations reported in lung cancer (37). The sampling of tumor tissues is difficult in patients that have cancer at an advanced level or if it has metastasized as the condition could worsen by preventing the surgery. Also, with metastasis, there are some risks associated. The non-invasive biomarkers will be developed through the CTC/ccfDNA and are of notable importance to oncologists. The formation of metastasis is linked to the clustering of CTCs as circulating tumor microemboli (CTM) that spread into the bloodstream (38). Even if they reside in the same patient, these CTCs are heterogeneous, and their characterization is critical for determining metastatic and invasive potential. Solid tumors are treated in a challenging phase as there are prominent chances of metastasis. Hence, an efficient and appropriate detection method is needed. Intervention efforts are affected by the tumor spread that also makes detection difficult and even impossible by the known techniques. CTCs could be discussed that are of significant importance as they

Circulating Tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) is the essential form of detective source that is helpful in the early diagnosis of cancer as it identifies gene mutations responsible for cancers. The plasma ctDNA mutations identified are isolated from the peripheral blood. Mostly, KRAS, TP53, EGFR, and PIK3CA are frequently reported. Circulating tumor DNA analysis is the detection method by analyzing the free-roaming DNA in the bloodstream. That wanderer DNA is the consequence of the dying cancerous cell. Through this DNA, the genetic makeup of the cancerous cell can be analyzed (39). Circulating tumor DNA (ctDNA) is emerging now as a noninvasive biomarker that will help to track tumor burden and allow monitoring of the cancer genome in the blood across several malignancies. ctDNA analysis with the help of liquid biopsy is the latest technique that is recently gaining increased attention in the management of early diagnosis of cancer. cDNA sequencing was reported in various cancer cell types. It has numerous applications in disease monitoring and detection of minimal residual disease (40). the pattern of lung cancer has been having a rapid growth rate and is highly metastatic with early hematogenous spread. In that case, ctDNA may be readily detectable in lung cancer patients. In addition, since ctDNA sequencing is noninvasive and "real-time", it could be an

ideal tool for investigating genomic evolution in the future, in particular during the treatment process. ctDNA analyzed the reoccurrence earlier than imaging in 72% of patients, with an average time of around 6 months. This case study has opened a new window of opportunities to treat patients while tumor burden and heterogeneity are at their lowest time of detection (41). ctDNA analysis for the identification of genetic determinants for targeted therapy has been widely implemented in lung cancer management. Allele-specific polymerase chain reaction, commonly known as PCR, is now used for the detection of epidermal growth factor receptor (EGFR) mutations in ctDNA for patients with lung cancer . Another study that studied ctDNA proposed the need for more trials for ctDNA-guided adjuvant therapy that demonstrates improved outcomes. In the diagnosis of lung cancer, it can be managed through trials of EGFR inhibitors or immune checkpoint inhibitors. Even in a false-positive result, the ctDNA assay predicting relapse in patients having no signs of residual disease has not been established robustly in any studies to date. In short, subclinical lung cancer detection is possible by identifying the common mutations that occur in ctDNA, which has high specificity but low sensitivity (42). Increasing the number of targeted mutations in the assay could potentially improve the sensitivity of the process but could suffer from cost-effectiveness issues.

1. Circulatory Tumor microRNAs

Circulating miRNAs have particular significance for clinical and expression analysis in lung cancer. For instance, it was reported that exosomal miR-126 is present in non-small cell lung cancer as a circulating biomarker. In the advanced stages of NSCLC, miR-126 is downregulated and inhibits the growth of cells and the loss of NSCLC malignancy. Hayashita et al. (2005) elucidated that miRNAs can also act as oncogenic drivers in lung cancer, such as the miR-17-92 cluster members, including “miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92-1” that are overexpressed, therefore, acting as potential oncogenes that enhance the development of tumors in NSCLC. Like many other effective factors, miRNAs are the biomarkers that can be used for the early diagnosis and detection of lung cancer (NSCLC) as the dysregulation of miRNAs results in cancer.Lung cancer development is enhanced through the Dicer1 deletion that represents a defect in one of the steps of miRNA biogenesis (43). examined the solution hybridization detection method and ELISA is accurate for the detection of miRNA but not effective in early-stage detection of NSCLC. Thus, biomarker-testing through biosensors is highly recommended for detecting non-invasive biomarkers. Moreover, the RT/PCR methods are frequently used for validating the results and to find out the differential expression of genes and microRNAs (.

The positive response of therapy towards patients depends on effective monitoring and early diagnosis and results in cancer's successful treatment. Continuous monitoring is not possible through the traditional tools as they lack the specificity and sensitivity for the evaluation of cancer development as early as possible. Liquid biopsy has overcome all these barriers as it is a very cost-effective method as well as being invasive with its specificity and sensitivity. Other than the most discussed circulating tumor DNA and circulating tumor cells, another biomarker that is potential and is promising has been introduced recently. This biomarker is known as microRNAs (miRNAs), which is also an essential part of liquid biopsy. In this regard, the expression profiling of circulatory miRNA-21 and miRNA-23a has been reported to be employed as molecular signatures or plausible biomarkers for the timely detection of non-small cell lung carcinoma (NSCLC) (44). Pertinent to miRNA analysis in Non-Small Cell Lung Carcinoma (NSCLC), these circulating miRNAs diagnose, detect, and monitor the response of patients to their targeted therapy with the key features. This new type of liquid biopsy is significant as it is painless and invasive, and its sampling procedure is also very quick . The localization of the tumor is not mandatory, and the associated complications are also prevalent but low risk. This technique is essential for detecting the differences between control and cancer samples that were not equally possible through other traditional approaches. Another important reason that makes miRNAs biomarkers is the formalin-fixed paraffin-embedded (FFPE) materials' routine preparation and the biofluids caused stability of miRNAs. These circulator miRNAs have also been shown to serve as biomarkers concerning personalized medication in patients with NSCLC. Furthermore, these circulating miRNAs are used in the treatment of NSCLC (45). in assessing radiation-driven cardiac toxicity in patients diagnosed Besides these advantages of miRNAs, their quantification is not an easy option and requires the inclusion of technological aspects. Other challenges might include cellular contamination, inadequate standardization of methods, analysis of data, interpretation, and normalization, limited amounts of the analyte, and pre-analytical variables (1988). The identification of miRNA biomarkers is another main focus of liquid biopsy that is important as it helps distinguish between the diseased samples and predict accurately the therapeutic response of patients. Furthermore, it predicts the patients' chances of relapse. The differently expressed miRNAs are selected for this particular purpose as those with greater differences could not be that optimal. Multivariate modeling is also used for this purpose, which includes unsupervised learning methods such as self-organizing maps, hierarchical clustering, canonical correlation analysis, and hierarchical clustering as unsupervised pattern recognition

methods. Similarly, classification and regression trees, artificial intelligence, partial least-squares discriminant analysis, linear discriminant analysis, and deep learning supervised classification (46).

2. Tumour-Derived Exosomes

For the early detection of lung cancer, Tumor-Derived Exosomes (TEX) is another essential concept affecting the antitumor immune responses as well as the tumor microenvironment and are known for their immunological activity that generates anti-inflammatory responses (47). This TEXs help in understanding the progression and metastasis of the tumor (48).Cancer-derived exosomal TRIM59, which is essential in regulating the NLRP3 for the promotion of NSCLC progression. Thus, lung cancer-derived exosomes hold great potential as biomarkers for the clinical diagnosis of lung cancer (Figure 4).

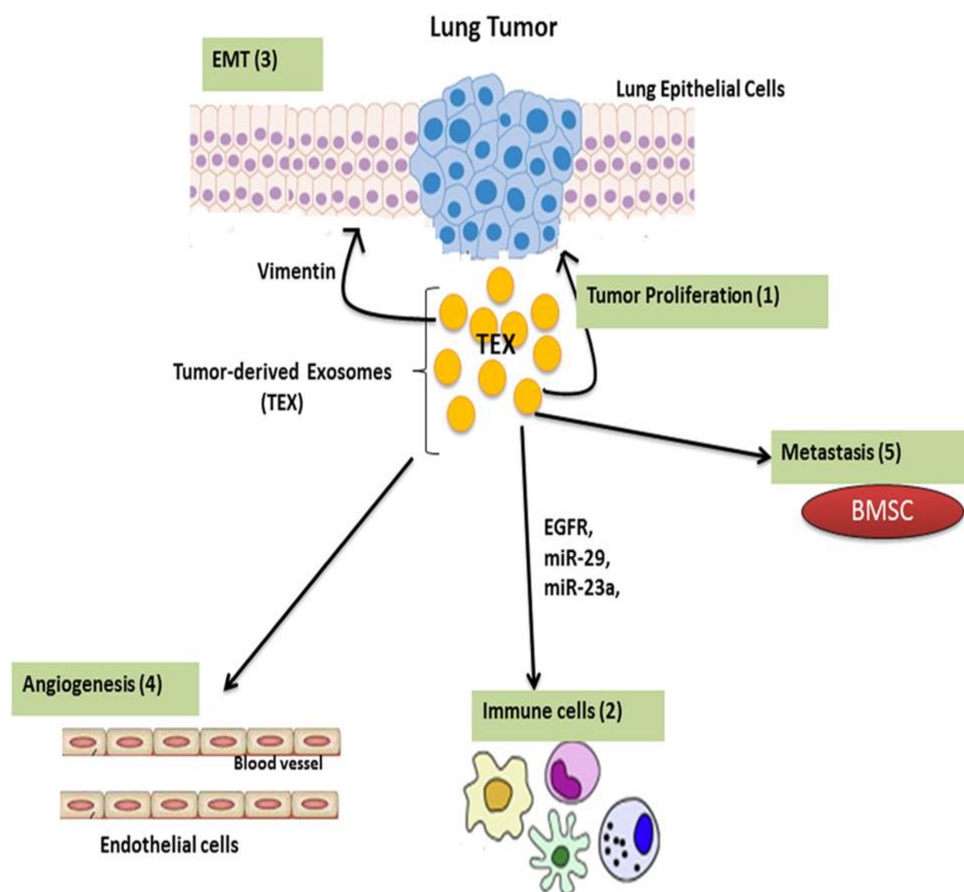

Figure 4. Lung tumor-derived exosome (TEX) functions Ref. (48)

The Radiomics

Radiomics is an innovative and promising biomedical approach that aims to provide quantitative information from medical images. This information is used as biomarkers by relating it to physiological and medical results of interest to identify any possible association between the extracted characteristics and the patient's prognosis (or other clinical outcomes). The aim is to identify customized predictive and/or prognostic models to support medical decision-making. Radiomics is of fundamental importance when it comes to Computed Tomography (CT), Magnetic Resonance (MRI), or Positron Emission Tomography (PET) studies. Indeed, the complex medical images obtained from this type of study contain a large amount of quantitative information that cannot be qualitatively appreciated by the human eye. Specifically, hundreds of quantitative features can be extracted from each tumor from CT, MR, or PET images. The process of extracting the diagnostic, prognostic, and predictive models is divided into four main phases: 1. Image acquisition and reconstruction 2. Image segmentation 3. Feature extraction 4. Creation of the informatics/statistical selection model Reduction and classification and prediction. Whatever the type of images involved, the segmentation of the tumor, the extraction and selection of features, and the classification model are the most important building blocks of a radiomics analysis (115). These data, extrapolated in very large numbers by dedicated calculation tools, require the use of advanced techniques, such as artificial intelligence methods for the management of so-called "big data", for their manipulation and analysis. This enormous wealth of numerical data, which would not be able to be processed through simple visual observation, defines many characteristics of the tumor and the surrounding environment, relating, for example, to shape, volume, and tissue structure. With these techniques, it is possible to study the possible correlation between the data obtained from the images and the molecular and genomic characteristics of the tumor, with the final aim of extracting directly from the images indications of the aggressiveness of the disease, on the most suitable therapies and response. Although radiomics can be considered a natural extension of computer-aided diagnosis and detection (CAD) systems, they are very different from these. CAD systems are usually used for the identification or diagnosis of disease .CAD systems have achieved, for example, important successes in the imaging of breast cancer. Unlike CAD systems, which are designed to give a single answer (for example, the presence or absence of a lesion), radiomics is a process used to extract numerous parameters from digital images, schedule these data in shared databases, and then

extract data for hypothesis generation, tests, or both. Since radiomics is designed to extract the maximum amount of information from standard images, the creation of databases that combine this vast amount of radiomics data (and ideally other complementary data) from millions of patients is desirable. While radiomics can be used for a variety of conditions, it is best developed in the oncology field, thanks to supporting from the National Cancer Institute (NCI), Quantitative Imaging Network (QIN), and other initiatives from the NCI Cancer Imaging Program. Quantitative image parameters based on intensity, shape, size or volume, and texture analysis provide information on the phenotype of the tumor and the microenvironment that is different from that provided by clinical reports, laboratory test results, or genomic or proteomic assays. These parameters, along with other data, can be correlated with clinical outcome data and used to support clinical decisions. Radiomics appears to provide an almost limitless supply of imaging biomarkers that could aid in the identification, diagnosis, prognosis, prediction of response to treatment, and monitoring of disease status. For a clinical radiologist, radiomics has the potential to help diagnose both common and rare cancers. The visualization of tumor heterogeneity could play a critical role in establishing the aggressiveness and prognosis of the tumor. The development of radiomics tools can help in carrying out clinical work and radiologists can play a central role in the continuous updating of databases that will be used as support for future decisions. It is desirable that in the future, the interpretation of radiological images will be discussed using radiomics, building an unprecedented data resource that will help the discovery of new correlations. Radiomics could allow the characterization of patients and their diseases through new genomic applications and innovative phenotyping methods. We believe radiomics is rapidly advancing beyond a niche research area and is emerging as a translational technology. So, it is essential to start establishing the cornerstones for the extraction, analysis, and presentation of data.

Aim Of The Study

This work aims to compare the diameter and volume of lung tumors with the cfDNA values. The Efficient Neural Network (ENet) convolution neural network was used to automatically figure out the size of the tumor.

CHAPTER 2

Materials/Patients and Methods

Our retrospective study is the result of the collaboration between the Institute of Diagnostic Imaging and the Institute of Medical Oncology of the A.O.U.P. “Paolo Giaccone” in Palermo. Our study was okayed by the University of Palermo's ethics committee, which did not ask for informed consent.

Patients’ selection and plasma collection

Patients enrolled in the present study were recruited at the Medical Oncology Department of the University General Hospital "Paolo Giaccone" in Palermo (Table 1). The study was conducted on a double cohort of patients all diagnosed with NSCLC. Patient’ sample collection was performed according to national legislation concerning ethical requirements. Written informed consent was obtained from all study participants, and the samples were analyzed anonymously. All control patients were enrolled after the exclusion of any pathological conditions. Blood samples were collected in 3 vacutainer tubes containing EDTA and processed within 2 hours of withdrawal to avoid the release of DNA from nucleated blood cells. The plasma was carefully separated from the cell fraction through two successive centrifugations: the first, performed at low speed (1200 g x 10 minutes at 4°C) to avoid cell lysis, leads to the separation of the plasma from the corpuscular part of the blood; the second one, at 3000 g x 10 minutes at 4°C, is generally performed to eliminate residual cellular debris and red blood cells from the plasma. After these two centrifugations, the plasma was transferred into new tubes and, after being properly coded, it was stored at -80°C until further molecular analysis.

Quantification and extraction of cfDNA

The extraction of cfDNA from plasma was carried out with the QIAmp Circulating Nucleic Acid (Qiagen) kit, starting with 3 mL of plasma and adding Qiagen proteinase K and ACL buffer in sequence. After 30' of incubation at 60 ° C, we continued with the addition of buffer ACB, and the sample was loaded into a vacuum pump system for transfer to absorption columns. After two further washing steps with buffer ACW1, ACW2, and ethanol and DNA elution, the samples kept in special tubes were stored at -20 °C for subsequent amplification. The extracted circulating DNA was finally quantified through the Qubit 3.0 Fluorometer, an instrument that, based on a standard curve, can return a precise concentration of cfDNA expressed in ng/ml can be seen below in figure 12.

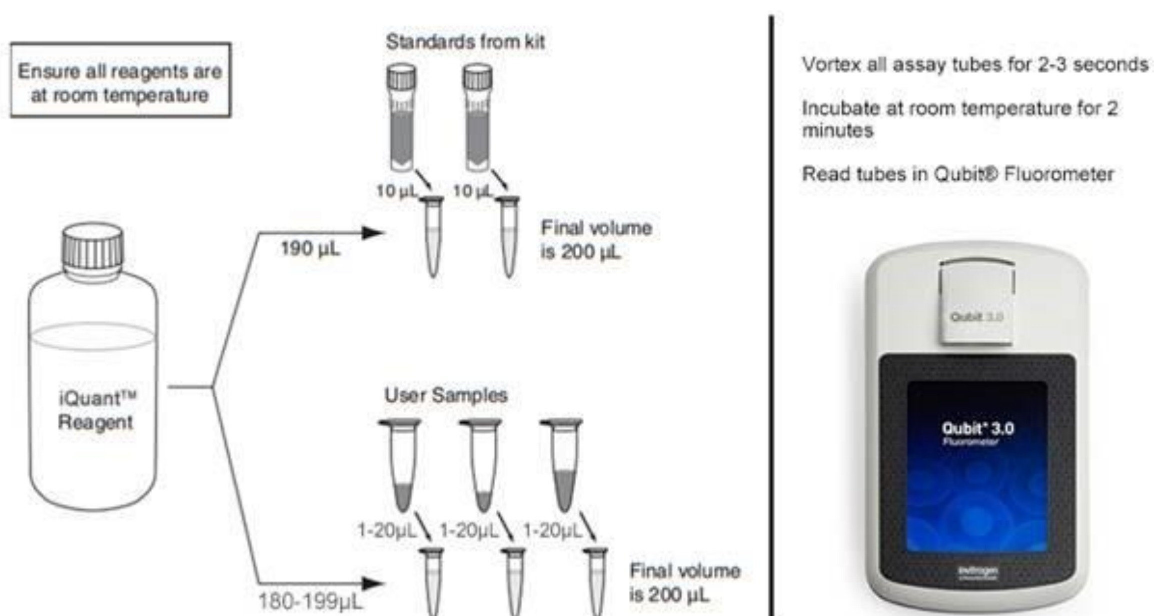

Figure 12: Quantification of cfDNA

Clinical Data

Number of patients	**16**
Males	13
Females	3
Mean age (years) ± SD	67.5 ± 7.6
Adenocarcinomas	14
Squamous cell carcinoma	2

Tab. N 1: *characteristics of the patients*

Computed Tomography

All patients included in the study underwent CT of the chest and abdomen before sampling the cfDNA with acquisition before and after administration of intravenous contrast medium.

CT scans were performed at our Institute using a 16-layer scanner (General Electric BrightSpeed, Milwaukee, WI, USA).

The scan parameters (summarized in table no. 3) were: current intensity 40-80 mAs (thin subject-obese subject); beam energy 100-140 kV (thin person-obese person); rotation time between 0.5-1 seconds; collimation 1.25 mm; layer thickness 1.25 mm; interval 0.625 mm; field of view of 35x35 cm or 40x40 cm (thin subject-obese subject); 512x512 pixel matrix; bone reconstruction algorithms for the evaluation of the lung parenchyma and standards for the study of the mediastinum.

Scans after administration of contrast medium were performed by injecting 110 mL of iodized contrast (400 mg / mL Iomeprolo, Iomeron 400, Bracco Imaging, Milan, Italy) at a flow of 3 mL/sec, followed by an infusion of 20 mL of saline via pump injector (Ulrich CT Plus 150, Ulrich Medical, Ulm, Germany).

All CT acquisitions were performed with patients in the spine position, in inspiratory apnea, and with the arms behind the head. The protocol provided for the following acquisitions:

- Basal: Chest + complete abdomen

- Arterial (40'): Chest

- Portal (70'): complete abdomen

- Late (3'): complete abdomen (only in doubtful cases)

Intensità di corrente	40-80 mAs
Energia del fascio radiante	100-140 kV
Tempo di rotazione	0.5 – 1 s
Collimazione	1.25 mm
Spessore di strato	1.25 mm

Intervallo	0.625 mm
FOV	350 x 350 mm
Matrice pixel	512 x 512
Algoritmo di ricostruzione	Bone/standard

Tab. N 2: scan parameters

The maximum dimensions were measured for each neoplasm, reported in centimeters.

Image Registration

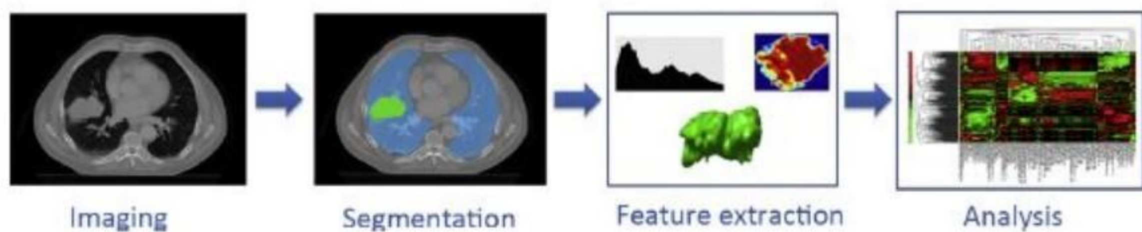

Figure # 05 Work flow of image analysis

Automatic segmentation and ENet network

Automatic segmentation was performed using the ENet convolution neural network, already validated in numerous studies (49, 50, 51). E-Net is a new convolution neural network introduced in 2016. The architectural model used to create this network is the bottleneck "bottleneck". ENet uses different types of convolutions to build an encoder/decoder-style image segmentation network (49). Figure 2b depicts the bottleneck model. ENet is based on residual network construction blocks, with each block consisting of three convolutional layers. These are a 1x1 projection, a regular convolutional layer, and a 1x1 expansion with batch normalization. ENet possesses asymmetric convolutions distinguished by separable convolutions with 5 x 1 sequences as well as 1 x 5 convolutions. The equivalent 5x5 convolution contains 25 parameters. To minimize network size, asymmetric convolution contains just 10 parameters. Finally, there is ENet, which utilizes a single beginning block as well as numerous versions of the bottleneck layer. The bottleneck layer is used to drive the network to learn the most important elements in the input data and, as a result, to disregard the unnecessary sections. A batch normalization level and PReLU56-57 are also added between all the convolutions. In addition to using several variations of this bottleneck pattern, ENet also

uses a single starting block, as shown in Figure 2a (52). The complete architecture of ENet is described in table 4 (52). Stage 0 is the initial block used to perform early sampling; stages 1-3 form the encoder; and stages 4-5 represent the decoder.

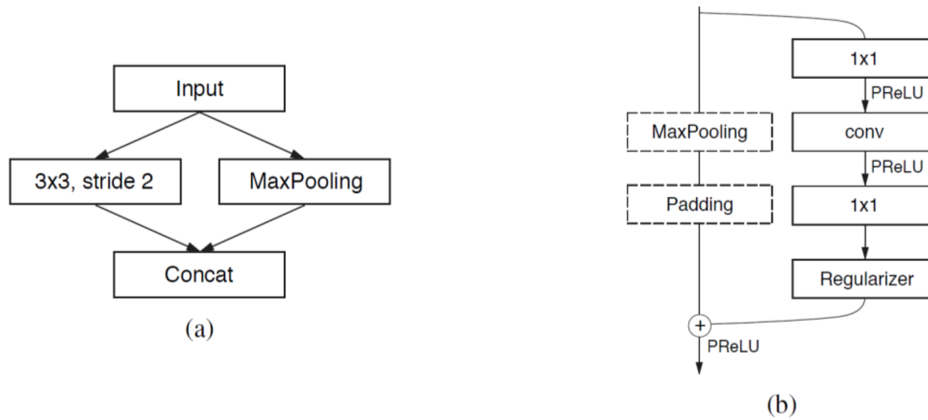

Fig. N 6: ENet architecture; (a) initial block (b) bottleneck model.

Name	Type	Stage	Output Size
initial	fig 2a	stage0	16x256x256
bottleneck1.0	Down-sampling	stage1	64x128x128
4 x bottleneck1.x		stage1	64x128x128
bottleneck2.0	Down-sampling	stage2	128x64x64
bottleneck2.1		stage2	128x64x64
bottleneck2.2	dilated 2	stage2	128x64x64
bottleneck2.3	asymmetric 5	stage2	128x64x64
bottleneck2.4	dilated 4	stage2	128x64x64
bottleneck2.5		stage2	128x64x64
bottleneck2.6	dilated 8	stage2	128x64x64
bottleneck2.7	asymmetric 5	stage2	128x64x64
bottleneck2.8	dilated 16	stage2	128x64x64
Repeat stage 2, without bottleneck2.0		stage3	
bottleneck4.0	Up-sampling	stage4	64x128x128
bottleneck4.1		stage4	64x128x128
bottleneck4.2		stage4	64x128x128
bottleneck5.0	Up-sampling	stage5	16x256x256
bottleneck5.1		stage5	16x256x256
fullconv		Final output	Cx512x512

Tab. 3 Complete architecture of the ENet model.

Statistical Analysis

To evaluate the relationship between the quantitative variables, we calculated the Kaplan-Meier survival analysis in SPSS updated software. This statistical test was non-parametric and was done on the patients because of the time frame they survived and their response to the therapy they received. This specifically includes the progression-free survival rate betterment or worst happening with the help of the diameter and volume of the lesions solely and in comparison with cell-free DNA amount individually and delta cell-free DNA, fully explained in the result section.

CHAPTER 3

Results

Among most of the patients, 14 patients had histologically confirmed adenocarcinoma and the remaining 02 patients had squamous cell carcinoma. Over time, half of the patients died of disease progression.

In figure 11, a comparative analysis of the cfDNA concentration can be seen from baseline to follow-up. There is a clear difference in cfDNA concentration during the course of the disease.

In figure no.07 below Ct images of automated segmentation done with help of Enet artificial.

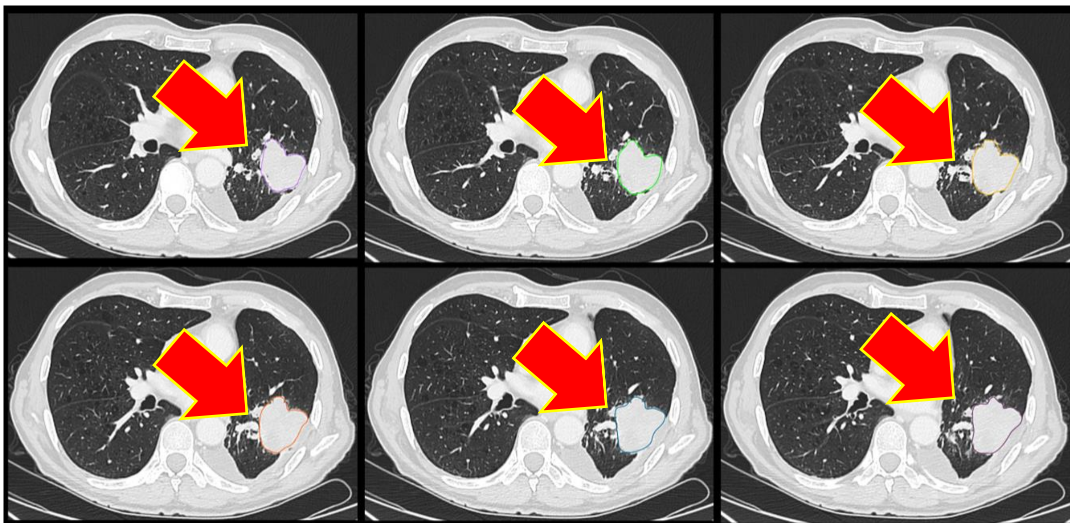

Fig. N 7: example of automatic segmentation of an NSCLC.

The tumor can be seen in the colored bordered lines around it, with an indication of a red arrow in each frame. All of the patients in the study had undergone computed tomography at their initial check-up. The diameter of the tumor in centimeters and volume of the tumor in

millimeter cubes were calculated, as well as the amount of cell-free DNA (cfDNA) of each patient in ng/µl, as shown in Table 04 (in appendix). RECIST criteria for measurement of tumor volume and diameter were followed. The same procedure was done on the follow-up and measured all the required parameters again, including ct images, tumor diameter, and volume according to the same RECIST criteria with quantification of cell-free DNA (cfDNA) as mentioned in table number 05 (in the appendix). All of the patients mentioned above received various types of systemic therapies based on the tumor stage and disease progression as shown in table 06 (in appendix). The disease's progression was observed and calculated and is mentioned in the same table number 06 (In appendix) in terms of progression-free survival, and overall survival. A clear difference in size can be seen in figures number 09 and 10 (in the appendix), after a comparative analysis of the first and second examination observations of the volume and diameter of the tumor due to the effect of systemic therapies.

Survival analysis

Analyzing the available data from the cfDNA quantification and computed tomography automated segmentation via deep learning, through Enet artificial neural network, according to tumor diameter variation (delta_DTmax), we noticed that a decrease in diameter is associated with a greater median progression-free survival. Contrariwise, an increase in diameter is associated with a worse PFS, even if results did not reach statistically significance probably due to the small sample size.(figure 08-A1) (p-value 0.7). According to cfDNA variation (delta_cfDNA), as shown in the Kaplan-Meier graph, an increase in circulating nucleic acids corresponds to a decrease in progression-free survival; PFS is greater as cell-free DNA amount drops out in plasma. This can be seen with the blue and green curves in (figures 08-A2) (p-value 0.1).

Furthermore, looking at the tumor volume variation (delta_volume), a greater progression-free survival is associated with a decrease of the volume of lesion as well as a worse PFS is linked with a significant increase of the volume of lesion (figure 08-A3) (p-value 0.03). Interestingly, while combining the variables linked to the size of the tumor (delta_diameter and delta_volume) with delta_cfDNA, our data showed that whenever delta_diameter/delta_volume increase along with delta_cfDNA (increase-increase) we noticed a decrease in progression-free survival. On the contrary, whenever delta_diameter/delta_volume decrease along with delta_cfDNA (decrease-decrease) a greater progression-free survival is observed (figure 8-A4) (figure 8-A5) (p-value 0,7 and 0.03

respectively). Unfortunately, any combination increase-decrease and decrease-increase has been studied due to the small cohort under evaluation.

Dynamics during time

Interpreting available data, in contingency table number 07 (In appendix) the diameter of lesion, evaluated following the RECIST criteria and according to systemic treatment, we observed that if the delta_diameter decreases, the disease control rate (DCR) will increase to 81.1% and if the diameter of lesion increases, the DCR will decrease to 40%, as mentioned in table number 06 (In appendix) (K concordance value 0.418, p-value 0.09). In contingency table number 8 (in appendix), we conclude that an increase of delta_cell-free DNA results in a decrease of disease control rate (42.9%) and vice versa (88.9%), with overall 68.8% DCR (K concordance value 0.476, p-value 0.049).

Furthermore, if we observe the volume of the lesion in the contingency table number 09 (in appendix), a decrease in the delta_volume, when measured according to RECIST criteria, corresponds to an increase in disease control rate in almost 100% of NSCLC patients during the course of therapy (K concordance value 0.846, p-value 0.001). Contingency table number 10 (in appendix) is a tabular representation of the statistical power deriving from the comparison of two variables, delta_cell-free DNA and delta_diameter. If both parameters, the diameter of the lesion and quantified cell-free DNA decrease, the disease control rate will be 100% and vice versa for the increasing values (K concordance value 0.737, p-value 0.016). The contingency table number 11 (in appendix) is the tabular representation of delta _cell-free DNA and delta_volume according to the RECIST criteria of measurement in automated segmentation with the help of the deep learning algorithm ANN-Enet, a decrease in both parameters (volume of lesion and cfDNA) resulted in an increase in the disease control rate, according to systemic therapies, of 100%. (K concordance value 0.800, p-value 0.005).

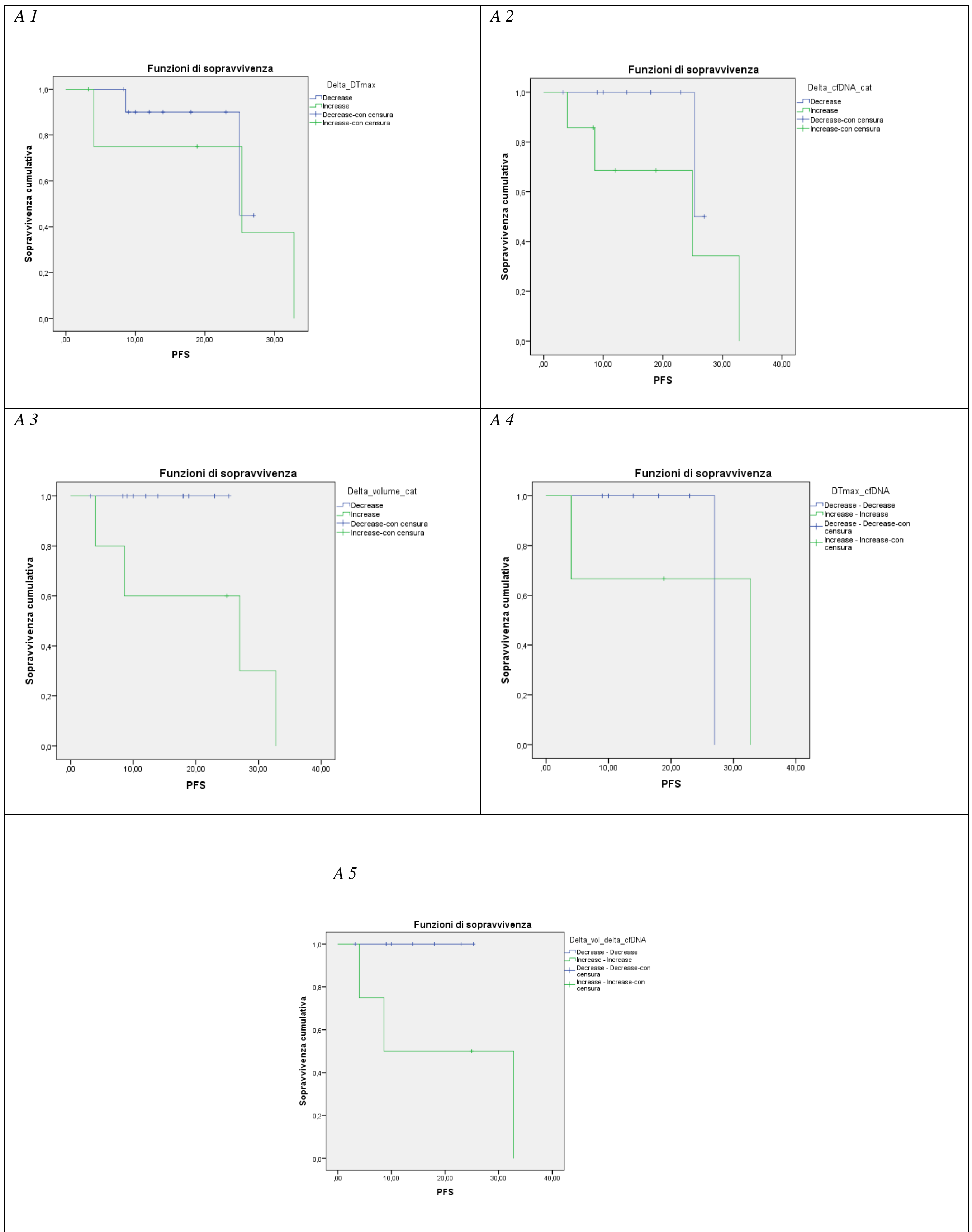

Figure No 08-A1: Graphical representation of diameter of lesions with progression-free survival rate, **A2:** Graphical representation of cell-free DNA with progression-free survival rate, **A3:** Graphical representation of Volume of lesions with progression-free survival rate, **A4:** Graphical representation of max of cell-free DNA and diameter of lesions with progression-free survival rate, **A5:** Graphical representation of Delta max of cell-free DNA and delta volume of lesions with progression-free survival rate.

CHAPTER 4

Discussion and conclusions

Lung cancer is a tumor that affects the whole globe with its profound lethal consequences, as well as economically and socially disturbing nations as a harmful health concern. Each year, the rate of mortality and incidence of this illness rises, with non-small cell lung cancer being the most frequent (NSCLC). According to the information given so far, there exist ethnic and racial inequalities in the incidence of lung cancer. There is growing evidence that mutations affecting KRAS, c-Myc, EGFR genes, and others are the primary causes of lung cancer. The biggest difficulty with lung cancer prevalence is that it is only detected in late stages, making treatment and diagnosis even more critical. As a result, there is an urgent need to find and develop effective tests and medicines for the early identification of lung cancer. Other treatments and approaches include the discovery of biological biomarkers, chemotherapy, adjuvant and radical therapy, nano-particle delivery systems, risk factor reduction such as smoking, which is a serious issue. For patients who were unable to have surgical resection due to severe problems, lung illnesses, or other causes, liquid biopsy is critical and highly recommended.

The liquid biopsy approach aid in the decision-making process for NSCLC patients' diagnosis and therapy. The liquid biopsy has numerous advantages over the invasive tissue biopsy; it is less expensive, less invasive, painless, and does not need surgery. The liquid biopsy approach distinguishes between control and tumor samples, identifies mutations, and genetic and epigenetic alterations, and gives other important benefits If a biopsy is not an option for a patient's choosing, ctDNA/cfDNA screening is performed. Furthermore, miRNAs, CTCs, TEPs, and exosomes might be employed as biomarkers. These biomarkers have yet to be used

in therapeutic settings. Other screening programs to evaluate the circulating tumor-derived biomarkers are being investigated. Aside from obvious disadvantages, each measure presented in the paper offers prospective qualities for lung cancer early detection. As a result, a liquid biopsy may become an important tool in cancer care in the future. However, clinical studies are required to validate tissue tumor biopsies. This might be a good future trend for physicians since it will greatly improve the odds of early cancer identification and diagnosis.

The interactions between three emerging oncology diagnostic technologies that have the potential to impact routine clinical practice were investigated in this study: plasma cfDNA measurements as a highly sensitive and specific tool for detecting tumor mutations; radiomics as a method to quantitatively measure intra-tumoral and inter-tumoral spatial heterogeneity on routine clinical imaging such as CT; and artificial neural networks as a predictive analytical tool. Patients with NSCLC were chosen for the study due to the disease's high mutational burden, the spatial and temporal variation in response to standard of care therapy seen in this patient group, and the disease's heterogeneous imaging features, both in terms of the wide range of sites to which it metastasizes and the variation of imaging features seen(53). Our data show that several radiomic parameters and total cfDNA have exceptionally strong correlations. Several studies suggest that most of the impact is due to the underlying relationship between cfDNA and lesion volume, which is an important problem for future research in this area. We studied the additional influence of radiomic features in association with cfDNA levels in the monitoring and managing of the disease. The interesting results here presented helped to speculate that radiomic features, in the form of a PCA signature, showed a significant connection with cfDNA levels using several statistical approaches. The radiomic signal that best-predicted cfDNA was created using a weighted sum of numerous distinct imaging properties. Despite past efforts (54, 55) to give histological relevance to these values, the biological correlates of most radiomic features and their tissue meaning remain unknown.

The more diverse a tumor, the more probable it has regions of growth and cell death, both of which contribute to total cfDNA levels. The additional radiomic elements in the generated signature show biological heterogeneity inside the tumor and indicate the spatial link between tumor voxels using more conceptually sophisticated mathematical methodologies. Because no biopsy samples from the lesions chosen for examination were available, extrapolating the characteristics of these samples to the CT imaging in this investigation proved problematic. Radiomic characteristics and tumor sidedness will need to be combined with tissue from image-guided biopsies in the future.

When multiple biopsies are impractical in the context of longitudinal tumor development monitoring, radiomic analysis may supplement invasive biopsies by elucidating the biological implications of particular textural aspects.

Most of the therapies made for the treatment of cancer rely on the pathways involved in the release of cfDNA, which includes the tumor development and necrosis. From the radiomic differences of the CT scan, it reveals there was lot of non specific morphological and functional abnormalities from patient to patient. Drug-specific therapy depends on the different stages of the cancer patient going through.

All participants in the trial provided two cfDNA samples at 6-month interval. All patients got a routine chest CT the day following cfDNA dosage. The neoplasms varied in size from 8.3 cm to 1.5 cm. The three-dimensional volumes ranged from 114200 mm^3 to 608 mm^3. The cfDNA concentrations varied from 0.2 ng/ml to 2.6 ng/ml.

Interestingly, we have noticed an inversily relationship between the delta diameter and the delta volume in several patients. In patients #7 and #15, the delta diameter is positive, while the delta volume is negative. In both cases, this information is linked to an increase in the maximal diameter of the lesion, which is accompanied by a three-dimensional volumetric reduction.This analysis validates the supremacy of 3D imaging technique of the automatic segmentation we used, in comparison to 2D image analysis where measurement of the tumor diameter could be an artifact of tumor growth. The maximum diameter of the lesion remains unchanged in the patient #08, but the delta volume increases dramatically with the increase of cell free DNA as well.

Moreover, the concordance between delta diameter and delta cfDNA is 0.476. This data proves a relationship between the dynamics of these two variables in our patients' cohort.(<0.05, see table n.8). The percentage change in CT's major diameter increases in accordance with the percentage change in cfDNA. This implies that as the breadth of the lesion increases, so does the cfDNA. Delta volume and delta cfDNA have a strong concordance (K concordance test 0.8, see table n.11), a p-value of 0.005 describes a statistically significant relationship between the two variables (p.<0.05). These data reveals that the two variables have statistically significant association.

Furthermore, patient #9 have negative delta cfDNA with an increase in both delta diameter and delta volume, while patients # 6 and #7 have a negative delta volume with an increase in both delta diameter and delta cfDNA. The patient #5 and #9 in our study were the only ones who

got immunotherapy as first-line treatment. Immunotherapy, as is well known, generates a pseudoprogression of the lesions sustained by a temporary lymphocyte infarction in the early phases of treatment (64,65,66). This scenario might have resulted in a decrease in cfDNA as a result of treatment, as well as a pseudo-rise in delta diameter and delta volume. Patients # 6 and #7, on the other hand, had a decrease in three-dimensional volume with an increase in both the larger diameter of the lesion and the cfDNA. These individuals were the only ones to acquire an extrathoracic spread of the disease, which, when present, results in a rise in cfDNA (67,68). CT is the usual approach for evaluating response to systemic therapy in the majority of metastatic cancers. A new study, however, reveals that cfDNA may have a role in predicting treatment response (55,56,57,58). Changes in cfDNA have been shown to provide earlier markers of response to therapy than morphological changes in tumor size (59,60), and if individual mutations are unique to an individual metastasis, tracking the progression of individual metastases over time may be possible, similar to the lesion-specific information provided by non-invasive imaging of metastasis during treatment (61,62). cfDNA levels at baseline and early follow-up may predict disease progression in a recent trial of metastatic melanoma patients treated with checkpoint inhibitors (63). Another recent research corroborated our results that automated tumor volumetric calculations of RPS using deep learning networks and CT images are reliable techniques. ENet had the best performance for automated segmentation, with a VD of 2.1 percent between automatic and manual segmentation. Automated and user-independent segmentation was much quicker than human segmentation for RPS volumetric analysis. Manual segmentation took 3887 seconds on average, however, automated segmentation took just a few seconds. This suggests that automated segmentation has a good chance of success in clinical practice, where time constraints may restrict the processes used (69,70).

There are some limitations to our research that should be highlighted. We did not account for the various tissue components of lesions. Depending on the histological subtype of the tumor, such as adenocarcinoma or squamous cell carcinoma, NSCLC is usually quite heterogeneous, with various tissue components such as a cellular tumor, macroscopic fat, necrosis, and cystic change. These several components have varying densities on CT scans, which may influence automatic segmentation performance. A larger sample size should be investigated to confirm our purpose.

Appendix:

Table No 04: Insight of NSCLC patients' at baseline

Patient name	Tumor size (cm)	Tumor Volume (mm^3)	CfDNA value (ng / µl)
#1	6.5cm	90000	0.927
#2	4.2cm	33720	0.45
#3	8.3cm	29830	0.84
#4	5.2cm	114200	0.457
#5	1.9cm	4034	0.4
#6	3.03cm	5210	0.28
#7	3.3cm	10870	0.2
#8	5.6cm	4414	0.476
#9	1.8cm	4758	0.526
#10	4.1cm	30650	0.413
#11	1.13cm	934	0.597
#12	2.53cm	7975	0.434
#13	4.23cm	37810	1.5
#14	3.9cm	10320	0.527
#15	5.73cm	60280	0.793
#16	4.2cm	13330	0.472

Table No 05: Insight of NSCLC patients' at follow-up

Patient name	Tumor size (cm)	Tumor Volume (mm^3)	CfDNA value (ng / µl)
#1	5.16cm	113100	1.02
#2	3.1cm	18370	0.267
#3	3.4cm	55830	0.543
#4	5.9cm	5669	1.1
#5	1.5 cm	4032	0.309
#6	2.4cm	5647	0.419
#7	1.96cm	5716	0.657
#8	6cm	20190	0.634
#9	4.4cm	4758	0.585
#10	4.3cm	26480	0.321
#11	2.3cm	608	0.568
#12	2.16cm	7185	0.374
#13	4.8cm	47490	2.6
#14	3.2cm	8401	0.74
#15	4cm	17050	0.437
#16	3.3cm	5640	0.34

Table No 06: Patient response to therapy

Patient name	*Therapy*	*vRECIST*	*Risposta RECIST 1.1*	*PFS (m)*	*OS(m)*
#1	Durvalumab	PD	PD (oligo-PD)	8.6	21.17
#2	Osimertinib	PR	PR	17.97	17.97
#3	Osimertinib	PD	PR	27	27
#4	Carbo-Pembrolizumab	PR	SD	18.87	21.47
#5	Pembrolizumab	CR	CR	13.97	13.97
#6	Osimertinib	SD	PD (oligo-PD)	24.97	32.63
#7	Carbo-Pembrolizumab	PR	PR	8.33	37.1
#8	Alectinib	PD	PD	32.8	32.8
#9	Carbo-	SD	PD	25.3	30.27
#10	Carbo-Gem	SD	PR	3.23	21.4
#11	Carbo+Pemetrexed+ Pembrolizumab	PR	PR	9	9
#12	Alectinib	SD	PR	10	10
#13	Pembrolizumab	PD	PD	4	4
#14	Carbo-Pembrolizumab	SD	PR	12	12
#15	Carbo-Pemetrexed	PR	PR	23	23
#16	Carbo+Pemetrexed+ Pembrolizumab	PR	PR	18	22
PD=Progressive Response, CR=Complete Response, PR=Partial Response, SD=Stable Disease					

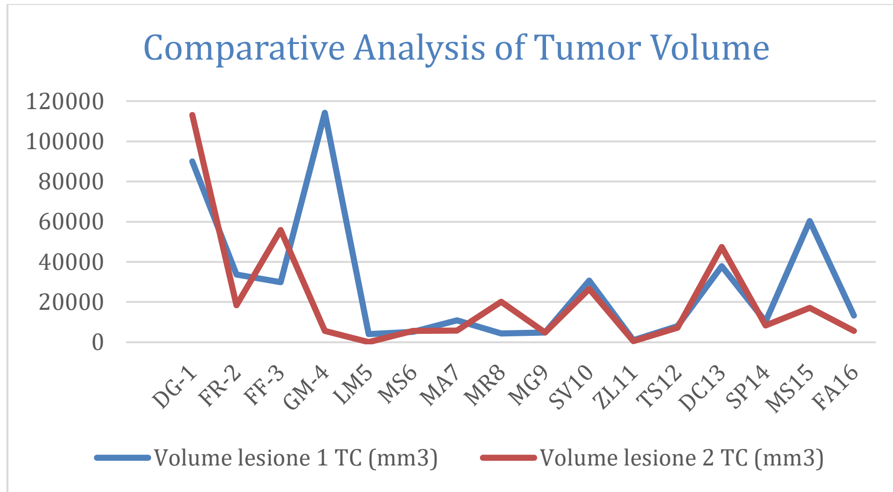

Figure No 09: First and second examination tumor volume analysis

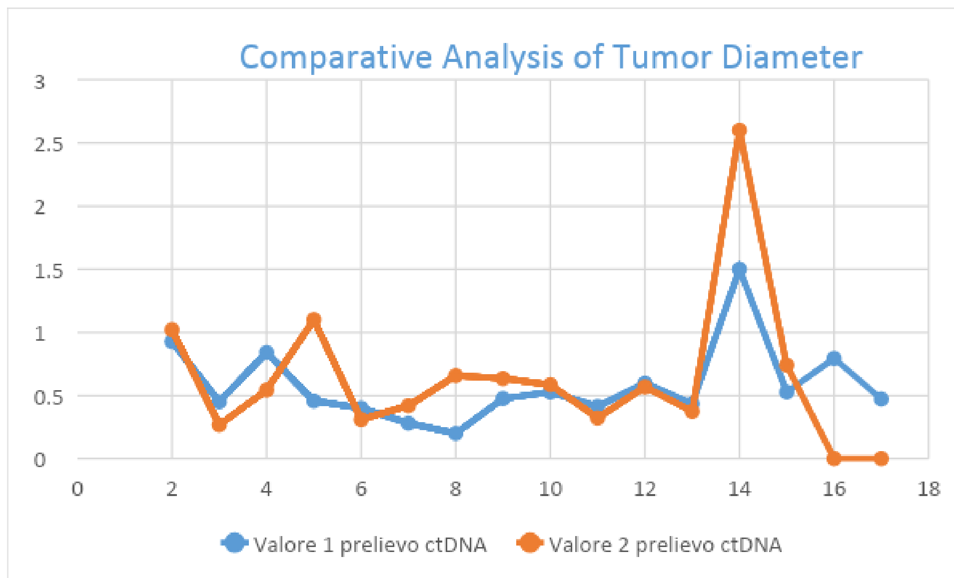

Figure No 10: First and second examination tumor diameter analysis

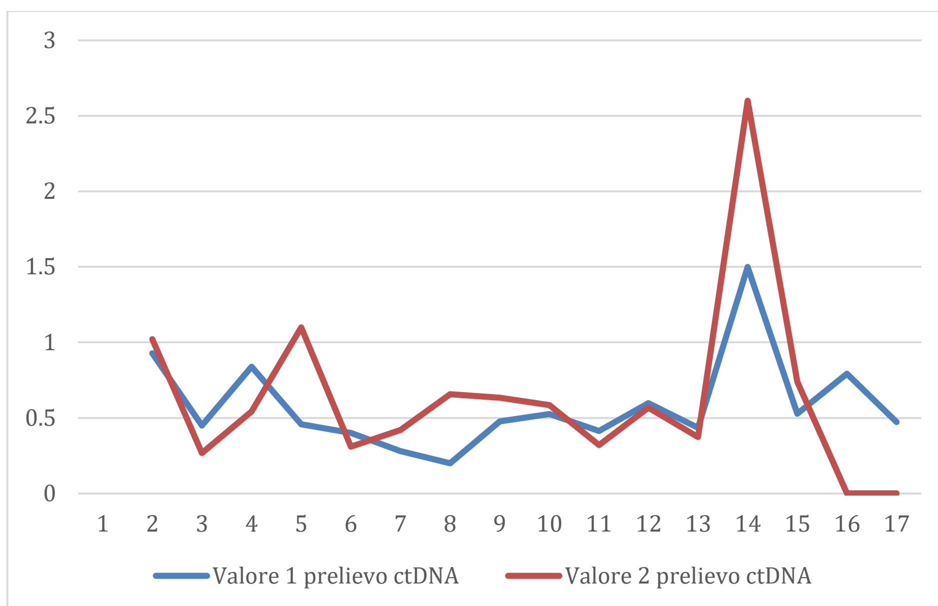

Figure No 11: CfDNA cocncentration from baseline to follow-up

Table 07: Crosstabulation of delta_diameter according to RECIST criteria

Crosstabulation Delta_DTmax_cat * RECIST_cat

			RECIST_cat		Total
			DCR	Not response	
Delta_DTmax_cat	Decrease	Count	9	2	11
		% in Delta_DTmax_cat	81.8%	18.2%	100.0%
	Increase	Count	2	3	5
		% in Delta_DTmax_cat	40.0%	60.0%	100.0%
Total		Count	11	5	16
		% in Delta_DTmax_cat	68.8%	31.3%	100.0%

Symmetrical measurements

		Value	Std error. asymptotic [a]	Approx. T [b]	Approx. Sign.
Measure of agreement	Kappa	, 418	, 244	1.673	, 094
No. of valid cases		16			

Contingency Table 08 : Crosstabulation of delta_cfDNA according to RECIST criteria

Crosstabulation Delta_cfDNA_cat * RECIST_cat

			RECIST_cat		Total
			DCR	Not response	
Delta_cfDNA_cat	Decrease	Count	8	1	9
		% in Delta_cfDNA_cat	88.9%	11.1%	100.0%
	Increase	Count	3	4	7
		% in Delta_cfDNA_cat	42.9%	57.1%	100.0%
Total		Count	11	5	16
		% in Delta_cfDNA_cat	68.8%	31.3%	100.0%

Symmetrical measurements

		Value	Std error. asymptotic [a]	Approx. T [b]	Approx. Sign.
Measure of agreement	Kappa	, 475	, 218	1,971	, 049
No. of valid cases		16			

Contingency Table 09: Crosstabulation of delta_volume according to RECIST criteria

Cross tabulation Delta_volume_cat * RECIST_cat_vol

			RECIST_cat_vol		Total
			DCR	Not response	
Delta_volume_cat	Decrease	Count	11	0	11
		% in Delta_volume_cat	100.0%	0.0%	100.0%
	Increase	Count	1	4	5
		% in Delta_volume_cat	20.0%	80.0%	100.0%
Total		Count	12	4	16
		% in Delta_volume_cat	75.0%	25.0%	100.0%

Symmetrical measurements

		Value	Std error. asymptotic [a]	Approx. T [b]	Approx. Sign.
Measure of agreement	Kappa	, 846	, 147	3.425	, 001
No. of valid cases		16			

Contingency Table 10: Crosstabulation of delta_diameter and cfDNA according to RECIST criteria

Crosstabulation DTmax_cfDNA * RECIST_cat

			RECIST_cat		Total
			DCR	Not response	
DTmax_cfDNA	Decrease - Decrease	Count	7	0	7
		% in DTmax_cfDNA	100.0%	0.0%	100.0%
	Increase - Increase	Count	1	2	3
		% in DTmax_cfDNA	33.3%	66.7%	100.0%
Total		Count	8	2	10
		% in DTmax_cfDNA	80.0%	20.0%	100.0%

Symmetrical measurements

		Value	Std error. asymptotic [a]	Approx. T [b]	Approx. Sign.
Measure of agreement	Kappa	, 737	, 241	2.415	, 016
No. of valid cases		10			

Contingency Table 11: Crosstabulation of volume and cfDNA according to RECIST criteria

Cross tabulation Volume_cfDNA * RECIST_cat_vol

			RECIST_cat_vol		Total
			DCR	Not response	
Volume_cfDNA	Decrease - Decrease	Count	8	0	8
		% in Volume_cfDNA	100.0%	0.0%	100.0%
	Increase - Increase	Count	1	3	4
		% in Volume_cfDNA	25.0%	75.0%	100.0%
Total		Count	9	3	12
		% in Volume_cfDNA	75.0%	25.0%	100.0%

Symmetrical measurements

		Value	Std error. asymptotic [a]	Approx. T [b]	Approx. Sign.
Measure of agreement	Kappa	, 800	, 188	2.828	, 005
No. of valid cases		12			

to. Do not assume null hypotheses.

b. We are using the asymptotic standard error assuming the null hypothesis.

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